

Characterization of Amyloidogenic Proteins using NMR Spectroscopy

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Luis Tobias Gardon
aus München

Düsseldorf, November 2025

aus dem Institut für Physikalische Biologie
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichtersteller:

1. Prof. Dr. Henrike Heise

2. Dr. Nils-Alexander Lakomek

Tag der mündlichen Prüfung: 28.05.2026

EIDESSTATTLICHE ERKLÄRUNG

Ich versichere an Eides Statt, dass diese Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Arbeit wurde bisher keiner Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht. Ich habe bisher keinen erfolglosen Promotionsversuch unternommen.

Düsseldorf, am 19.11.2025

Luis Gardon *Luis Gardon*

“Buy the ticket, take the ride.”

~ Hunter S. Thompson

Summary

This thesis focusses on the use of nuclear magnetic resonance (NMR) spectroscopy to study amyloidogenic systems and to explore the molecular transitions from monomeric proteins to aggregated fibrils. Amyloid formation is associated with numerous neurodegenerative diseases, yet many mechanistic aspects, such as the influence of aggregation conditions, remain poorly understood. NMR provides unique atomic-level insights into both structure and dynamics, making it a powerful and versatile tool for investigating these questions. This work highlights the broad applicability of different NMR techniques for studying aggregation processes and structural states across amyloidogenic systems.

To investigate how solution conditions and sequence modifications influence fibril structure, we focused on two related systems: LS-shaped Amyloid- β (A β) (1-42) fibrils and pyroglutamylated (pE) A β (3-42) fibrils. In the first study, we demonstrated that LS-shaped A β (1-42) fibrils were robust across a wide range of pH values (pH 2-7). Solid-state NMR revealed that the β -sheet core and rigid N-terminus remain conserved, with pH-dependent changes at the C-terminus. Specifically, we observed a rearrangement of the K28-D1 salt bridge at low pH into K28-A42 interaction at neutral pH in line with molecular dynamics (MD) simulations.

In a second study, we characterized fibrils formed by pEA β (3-42) carrying an N-terminal pyroglutamate residue at position 3. These pEA β (3-42) fibrils were grown under the same conditions as the LS-shaped A β (1-42) fibrils and showed a largely preserved overall fold, including β -sheet regions and the central turn region around V24. However, the loss of D1 eliminates the K28–D1 salt bridge and chemical shift data suggest a possible rearrangement to a K28–A42 interaction. Interestingly, glycine residues G37 and G38, which are exposed due to the truncated N-terminus, showed significant chemical shift changes and act as reporters of this conformational adaptation.

To gain deeper insight into the mechanism of amyloid fibril formation, we investigated the PI3K-SH3 domain, a well-established model system for folding and fibrillation, using a combination of solution-state and solid-state NMR spectroscopy in a third study. We followed the seeded aggregation of PI3K-SH3 by recording insensitive nuclei enhanced by polarization transfer (INEPT)- and cross-polarization (CP)-based experiments

following the conversion from monomers to fibrils. Notably, despite the differing degrees of agitation across the techniques, the observed aggregation kinetics under seeded conditions were consistent, pointing to fibril elongation as the primary mechanism of growth rather than nucleation.

In a fourth study we employed dynamic nuclear polarization (DNP)-enhanced NMR spectroscopy to probe the conformational behavior of selectively labeled isoleucine residues in folded, unfolded and fibrillar states of various proteins. These experiments demonstrated that isoleucine side chain residues can be reporters of conformation states for low populated or flexible regions and highlight the potential of DNP for broader applications to other amino acid side chains of biomolecular systems.

We further employed solvent paramagnetic relaxation enhancement (PRE) using Dotarem to assess surface accessibility in native, fibrillar, and unfolded forms of PI3K-SH3. This approach provided a simple yet effective way to map solvent exposure across conformational states.

This thesis also includes the characterization of PI3K-SH3 in its native folded form at neutral pH, an acidic low-pH condition revealing pH-dependent folding behavior, and its fibrillar state. Using solid-state NMR and atomic force microscopy (AFM), we compared fibrillar polymorphism and provide supporting protocols and methodological details, including a custom pulse sequence.

Altogether, this work demonstrates how a combination of advanced NMR methods can be employed to characterize structure, dynamics, and aggregation kinetics of amyloidogenic proteins. The strategies employed here are applicable to a broad range of systems and contribute to a deeper molecular understanding of amyloid formation.

Zusammenfassung

Diese Arbeit konzentriert sich auf die Verwendung der Kernspinresonanzspektroskopie (NMR) zur Untersuchung amyloidogener Systeme und zur Erforschung der molekularen Übergänge von monomeren Proteinen zu aggregierten Fibrillen. Die Amyloidbildung steht im Zusammenhang mit zahlreichen neurodegenerativen Erkrankungen, doch viele mechanistische Aspekte, wie beispielsweise der Einfluss der Aggregationsbedingungen, sind noch weitgehend unerforscht. Die NMR bietet einzigartige Einblicke auf atomarer Ebene sowohl in die Struktur als auch in die Dynamik und ist damit ein leistungsfähiges und vielseitiges Werkzeug zur Untersuchung dieser Fragen. Diese Arbeit unterstreicht die breite Anwendbarkeit verschiedener NMR-Techniken zur Untersuchung von Aggregationsprozessen und Strukturzuständen in amyloidogenen Systemen.

Um zu untersuchen, wie Lösungsbedingungen und Sequenzmodifikationen die Fibrillenstruktur beeinflussen, haben wir uns auf zwei verwandte Systeme konzentriert: LS-förmige Amyloid- β ($A\beta$) (1-42) Fibrillen und pyroglutamylierte (pE) $A\beta$ (3-42) Fibrillen. In der ersten Studie haben wir gezeigt, dass LS-förmige $A\beta$ (1-42) Fibrillen über einen weiten pH-Bereich (pH 2-7) robust sind. Festkörper-NMR zeigte, dass der β -Faltblattkern und der rigide N-Terminus konserviert bleiben, während am C-Terminus pH-abhängige Veränderungen auftreten. Insbesondere beobachteten wir eine Umordnung der K28-D1-Salzbrücke bei niedrigem pH-Wert zu einer K28-A42-Wechselwirkung bei neutralem pH-Wert, was mit Molekulardynamik-Simulationen (MD) übereinstimmt.

In einer zweiten Studie haben wir Fibrillen charakterisiert, die aus pEA β (3-42) gebildet wurden, das einen N-terminalen Pyroglutamatrest an Position 3 trägt. Diese pEA β (3-42) Fibrillen wurden unter den gleichen Bedingungen wie die LS-förmigen $A\beta$ (1-42) Fibrillen hergestellt und zeigten, dass die Faltung weitgehend konserviert ist, einschließlich der β -Faltblattbereiche und des zentralen Turn-Bereichs um V24. Das Fehlen von D1 eliminiert jedoch die K28-D1-Salzbrücke und die Analyse der chemischen Verschiebungen deuten auf eine mögliche Umordnung zu einer K28-A42-Wechselwirkung hin. Interessanterweise zeigten die Glycinreste G37 und G38, die aufgrund des abgeschnittenen N-Terminus exponiert sind, signifikante Änderungen der chemischen Verschiebungen und fungieren als Reporter dieser Konformationsänderung.

Um tiefere Einblicke in den Mechanismus der Amyloidfibrillenbildung zu gewinnen, untersuchten wir in einer dritten Studie die PI3K-SH3-Domäne, ein etabliertes Modellsystem für Faltung und Fibrillierung, unter Verwendung einer Kombination aus Lösungs- und Festkörper-NMR-Spektroskopie. Wir verfolgten die geseedete Aggregation von PI3K-SH3, indem wir Experimente auf Basis von INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) und CP (Cross-Polarization) durchführten, um die Umwandlung von Monomeren zu Fibrillen zu verfolgen. Bemerkenswert ist, dass trotz der unterschiedlichen Agitationsgrade der verschiedenen Techniken die beobachtete Aggregationskinetik unter geseedeten Bedingungen konsistent war, was darauf hindeutet, dass Fibrillen-Elongation der primäre Wachstumsmechanismus ist und nicht die Bildung von Nuclei.

In einer vierten Studie verwendeten wir *dynamic nuclear polarization* (DNP) verstärkte NMR-Spektroskopie, um das Konformationsverhalten selektiv markierter Isoleucinreste in gefalteten, entfalteten und fibrillären Zuständen verschiedener Proteine zu untersuchen. Diese Experimente zeigten, dass Isoleucin-Seitenkettenreste als Indikatoren für Konformationszustände in wenig populierte oder flexiblen Bereichen dienen können, und unterstreichen das Potenzial von DNP für breitere Anwendungen auf andere Aminosäureseitenketten biomolekularer Systeme.

Wir verwendeten außerdem die paramagnetische Relaxationsverstärkung (PRE) mit Dotarem, um die Oberflächenzugänglichkeit in nativen, fibrillären und ungefalteten Formen von PI3K-SH3 zu untersuchen. Dieser Ansatz bot eine einfache, aber effektive Möglichkeit, die Lösungsmittelexposition über Konformationszustände hinweg abzubilden.

Diese Arbeit umfasst auch die Charakterisierung von PI3K-SH3 in seiner nativen gefalteten Form bei neutralem pH-Wert, einer sauren Umgebung mit niedrigem pH-Wert, die ein pH-abhängiges Faltungsverhalten zeigt, und seinem fibrillären Zustand. Mithilfe von Festkörper-NMR und Rasterkraftmikroskopie (AFM) haben wir den fibrillären Polymorphismus verglichen und unterstützende Protokolle und methodische Details bereitgestellt, darunter eine benutzerdefinierte Pulssequenz.

Insgesamt zeigt diese Arbeit, wie eine Kombination fortschrittlicher NMR-Methoden eingesetzt werden kann, um die Struktur, Dynamik und Aggregationskinetik von amyloidogenen Proteinen zu charakterisieren. Die hier angewandten Strategien sind auf

eine Vielzahl von Systemen anwendbar und tragen zu einem tieferen molekularen Verständnis der Amyloidbildung bei.

Table of Contents

Summary.....	V
Zusammenfassung	VII
Table of Contents.....	X
List of Abbreviations	XII
List of Figures.....	XIV
1. Introduction	1
1.1. Neurodegenerative diseases and amyloidogenic proteins	2
1.1.1 Amyloid- β (A β) (1-42)	4
1.1.2 PI3K-SH3 domain as an amyloid model system	11
1.2. Nuclear magnetic resonance (NMR) spectroscopy	14
1.2.1 Solution-state NMR spectroscopy: Heteronuclear single quantum coherence (HSQC).....	14
1.2.2 Solid-state NMR spectroscopy: Basics and cross-polarization	16
1.2.3 Application of NMR in amyloid research	20
1.3. Scope of this work	24
2. Publications	26
2.1. Publications directly related to this dissertation	28
2.1.1 Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy	28
2.1.2 Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques	29
2.1.3 Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-Shaped A β (1–42) Amyloid Fibrils	30
2.1.4 Isoleucine Side Chains as Reporters of Conformational Freedom in Protein Folding Studied by DNP-Enhanced NMR.....	31

2.1.5	Surface Mapping of Bovine PI3K-SH3 Domain using Gadoteric Acid for Solvent PRE effect	32
2.2.	Additional scientific contributions	34
2.2.1	Photocontrolled Reversible Amyloid Fibril Formation of Parathyroid Hormone-Derived Peptides	34
2.2.2	Disordered regions of inhibitor-bound α -synuclein suppress seed-induced fibril nucleation in cells.....	35
3.	Unpublished Results.....	36
3.1.	Assignment of native PI3K-SH3 by solution-state NMR	38
3.2.	Unfolding of PI3K-SH3 at low pH and high temperature.....	42
3.3.	Influence of aggregation conditions on PI3K-SH3 fibril morphology.....	45
3.4.	Assignment of a PI3K-SH3 fibril polymorph	50
3.5.	Dependence of atomic structure on fibril growth conditions	53
3.6.	Rotational-echo, double-resonance (REDOR)-spin diffusion sequence 2D detection.....	57
3.7.	PI3K-SH3 protein purification	61
4.	Discussion and Outlook	67
5.	References	77
6.	Appendix	i

List of Abbreviations

AD: Alzheimer's disease

AFM: Atomic Force Microscopy

APP: Amyloid Precursor Protein

A β : Amyloid- β

CP: Cross-Polarization

DNP: Dynamic Nuclear Polarization

EM: Electron Microscopy

FUS LC: FUsed in Sarcoma Low-Complexity domain

Gly-HCl: Glycine Hydrochloride

GST: Glutathione S-Transferase

IDP: Intrinsically Disordered Protein

INEPT: Insensitive Nuclei Enhanced by Polarization Transfer

MAS: Magic Angle Spinning

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSQC: Heteronuclear Single Quantum Coherence

MD: Molecular Dynamics:

NMR: Nuclear Magnetic Resonance

PDSD: Proton Driven Spin Diffusion

pE: Pyroglutamate

PI3K-SH3: Phosphatidyl-Inositol-3-Kinase Src-Homology 3 domain

PMSF: PhenylMethylSulfonyl Fluoride

REDOR: Rotational-Echo, DOuble-Resonance

SDS: Sodium Dodecyl Sulfate

TOCSY: TOtal Correlation SpectroscopY

List of Figures

Figure 1: Simplified schematic of amyloid fibril formation.....	3
Figure 2: APP processing pathway.	5
Figure 3: Common fibrillar A β (1–42) Polymorphs.....	8
Figure 4: Transition of N-terminal glutamate to pyroglutamate in A β	10
Figure 5: Amyloidogenic character of the PI3K-SH3 domain.	13
Figure 6: Pulse sequence of an ^1H - ^{15}N HSQC spectrum.....	15
Figure 7: Pulse sequence of a PDSD spectrum.	18
Figure 8: Pulse sequence of a NCACX/NCOCX spectrum.	19
Figure 9: Assignment of natively folded PI3K-SH3 at neutral pH.	39
Figure 10: Assignment comparison with Hsu (2013).....	41
Figure 11: Solution-dependent equilibrium between folded and unfolded populations of PI3K-SH3.	43
Figure 12: Folded and unfolded populations of PI3K-SH3 depending on pH values.....	44
Figure 13: AFM micrographs of PI3K-SH3 aggregates after 3 weeks of incubation at different pH levels and 50 °C.	46
Figure 14: AFM micrographs of PI3K-SH3 at different aggregation conditions.....	47
Figure 15: AFM micrographs of PI3K-SH3 seeds and fibrils.	49
Figure 16: Assignment of a PI3K-SH3 fibril polymorph.	51
Figure 17: 3D spectral assignment strategy for fibrillar PI3K-SH3.....	52
Figure 18: Comparison of the atomic structure of PI3K-SH3 fibrils grown at different conditions.	54
Figure 19: Atomic structure of PI3K-SH3 fibrils.	56
Figure 20: REDOR pulse sequence for ^{15}N - ^{13}C contacts with 2D ^{13}C - ^{13}C detection.	58
Figure 21: Effect of the frequency selective REDOR pulse sequence on glutamate.	59
Figure 22: Frequency selective REDOR spectra of pEA β (3-42) fibrils.....	60
Figure 23: SDS-PAGE analysis of GST- PI3K-SH3 expression.	62
Figure 24: GST affinity chromatography and SDS-PAGE analysis:.....	63
Figure 25: Thrombin cleavage of GST-PI3K-SH3.....	64
Figure 26: Size-exclusion chromatography (SEC) of cleaved GST and PI3K-SH3.	65
Figure 27: Second GST affinity chromatography.....	66

Figure 28: Structural findings for pEA β (3–42) fibrils in comparison with LS-shaped A β (1–42) fibrils adapted from ⁹⁸	69
Figure 29: The PI3K-SH3 domain: From monomers to fibrils.	75
Figure S1: ¹ H NMR spectrum of PI3K-SH3 after ~100 hours of aggregation at 25 °C.	viii
Figure S2: Optimal control cross-polarization (CP) for N-C transfer.	ix

1

Introduction

1.1. Neurodegenerative diseases and amyloidogenic proteins

A key motivation for this work lies in the relationship between amyloidogenic proteins and neurodegenerative diseases. These disorders are characterized by a progressive decline in neurological functions such as motor coordination and cognition. Age-related neurodegenerative diseases like Alzheimer's and Parkinson affect millions of people worldwide and effective cures remain elusive due to the complex pathogenesis and irreversibility of the pathological outcomes¹. Given the demographic changes, the social and economic burden of such diseases will only continue to grow, thus representing one of the major public health challenges of our time². Neurodegenerative diseases are closely associated with misfolding and aggregation of specific, so-called amyloidogenic proteins. These proteins and peptides can form highly ordered fibrillar structures known as amyloid fibrils, which play a central role in disease pathology³⁻⁵. However, amyloid fibrils are not inherently pathological; they also play roles in various functional biological processes^{6, 7}.

It is generally assumed that amyloid fibril formation might be an inherent property of many polypeptides^{8, 9}. Amyloid fibrils are highly ordered structures most commonly adopting an architecture of parallel in-register β -sheets, in which the monomeric or dimeric protein subunits stack along the fibril axis¹⁰⁻¹². Stabilizing interaction in fibrils comprise π - π stacking, electrostatic interactions and, most dominantly, hydrogen bonds between the β -strand layers and steric zipper motifs¹³. A defining structural hallmark of amyloid fibrils is the cross- β architecture, evidenced by X-ray diffraction patterns displaying 4.8 Å and ~10 Å reflections. These patterns correspond to β -strands spacing perpendicularly to the fibril axis and β -sheets running parallel or antiparallel along it, respectively^{14, 15}.

The formation of amyloid fibrils (Figure 1) follows complex aggregation pathways, often involving misfolded or intrinsically disordered proteins (IDPs) like Amyloid- β (A β) and α -synuclein related to Alzheimer's and Parkinson's disease, respectively^{16, 17}. Monomers can transiently assemble into metastable fibrillary oligomeric intermediates, which either dissociate back into monomers or convert into nuclei (i.e. protofibrils) that initiate fibril elongation¹⁸. Amyloid growth typically proceeds via primary nucleation, where monomers attach at the growing fibril ends. Such fibrils can then fragment, creating new surfaces for monomer attachment and accelerating fibril growth¹². Additionally,

secondary nucleation, where monomers bind to a second surface on a fibril, thus leading to branched fibrils which dissociate to form two new fibrils, further enhance the overall aggregation rate¹⁹.

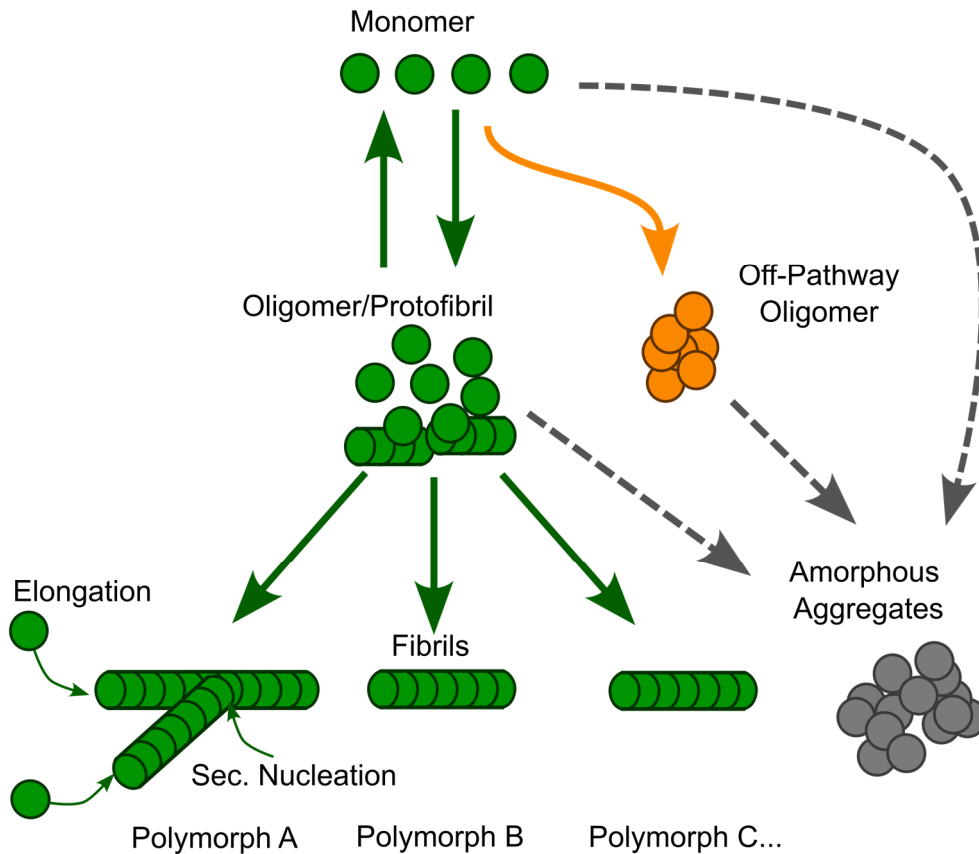


Figure 1: Simplified schematic of amyloid fibril formation. Under suitable conditions monomers assemble into oligomeric intermediates that can initiate on-pathway formation of amyloid fibrils or deviate into off-pathway aggregation. Fibrils grow via elongation by monomer addition to one end of the fibrils or by secondary nucleation where a new nucleus is formed at the surface of an existing fibril. Amorphous aggregates, composed of disordered assemblies of monomers and oligomers, represent an off-pathway route.

In many cases, amyloid fibrils consist of a rigid core surrounded by more flexible, disordered parts often referred to as “fuzzy coat”. While the structural and dynamic properties of these peripheral regions remain poorly understood, they are believed to influence fibril elongation, secondary nucleation and interaction with other proteins, RNA or membranes²⁰.

The aggregation kinetics follow a sigmoidal curve with an initial lag phase due to oligomer/fibril seed formation, followed by rapid fibril growth once nuclei have formed²¹. However, off-pathway oligomers that fail to transition into fibrils may remain stable or

1.1. Neurodegenerative diseases and amyloidogenic proteins

metastable and influence fibril formation^{22, 23}. Depending on their concentration, these off-pathway oligomers can slow down fibril growth rates by competing with monomers for aggregation pathways²². Due to their transient nature, studying on-pathway oligomers using conventional biochemical and biophysical techniques remains challenging^{24, 25}. For disease-related amyloids, such as A β peptides in Alzheimer's disease (AD), oligomeric species are of particular interest, as they are believed to be the most cytotoxic species²⁶. Large amorphous aggregates can form through disordered, off-pathway aggregation where monomers and oligomers associate in non-specific manner²⁷.

The fibril formation process and fibril polymorphism, i.e. variations in fibril structure at the molecular level, are highly dependent on external conditions, including temperature, pH, agitation, and solvent composition^{12, 28}. Moreover, just a small sequence of amino acids within the peptide sequence can act as a “facilitator” of amyloid fibril growth²⁹. A given peptide or protein can form multiple, self-propagating fibril polymorphs, each with unique stability, morphology, and/or biological impact. Polymorphism is also closely linked to disease, as different fibril structures can correlate with distinct pathological effects³⁰. Furthermore, amyloid fibrils have an inherent dynamic nature due to constant association and dissociation of monomers (molecular recycling)³¹. Small amyloid fibril fragments (seeds) can initiate new fibril growth by recruiting monomers, preserving the original fibril structure through templated elongation. In contrast, secondary nucleation involves the formation of new nuclei at the fibril surface, where solution conditions often play a more dominant role than seed structure in determining the resulting polymorph²⁸.

This structural variability poses challenges in drawing precise conclusions about amyloid formation and its biological consequences. In the following sections, we will focus on two prominent examples of amyloidogenic proteins and peptides.

1.1.1 Amyloid- β (A β) (1-42)

One of the most extensively studied amyloidogenic peptides is A β , which plays a critical role in AD. A β peptides are generated from the amyloid precursor protein (APP) whose physiological role involves the maintenance of a normal neuronal activity^{32, 33}. It is located on chromosome 21 and more abundant in brains of people with trisomy 21 (Down syndrome)^{34, 35}. Through sequential cleavage by β -secretase and γ -secretase the A β peptide is released in the extracellular matrix (Figure 2). The resulting A β peptides vary

in length, with A β (1-40) being the most abundant, while A β (1-42) is considered more aggregation-prone and toxic³⁶.

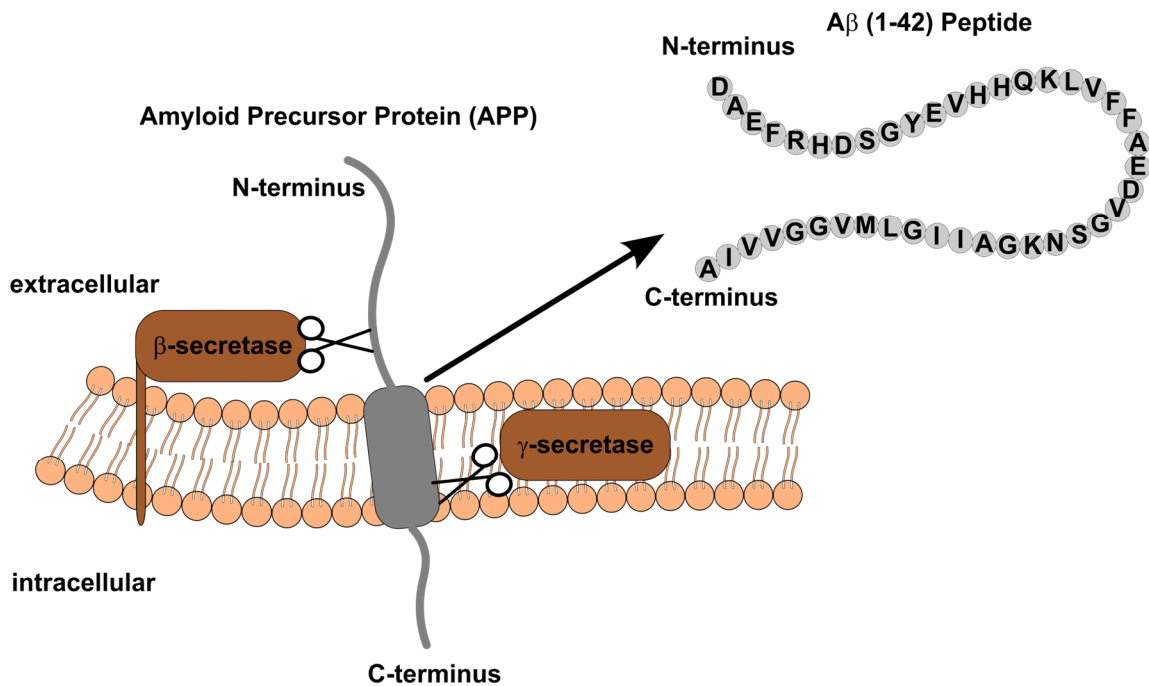


Figure 2: APP processing pathway. APP is an integral membrane protein located in the cell membrane. Sequential cleavage of the membrane-bound protease β -secretase and subsequent cleavage of the integral membrane protease γ -secretase releases the A β peptide into the extracellular matrix.

Despite extensive research, the precise mechanism by which A β aggregates and exerts toxicity, and its involvement in the AD remains unclear. Proposed mechanisms of toxicity include, amongst others, membrane disruption³⁷⁻³⁹, induction of inflammatory responses⁴⁰, and promotion of oxidative stress⁴¹. However, A β peptides have also been implicated in certain physiological roles, such as a neuroprotectant or antioxidant, suggesting a more complex function⁴². As mentioned above, A β oligomers are believed to be the most toxic species.

The A β (1-42) monomer is intrinsically disordered and lacks a well-defined structure in aqueous solution⁴³. Nonetheless, several studies report transient β -sheet-rich conformations at the C-terminus of A β (1-42)^{44, 45} and there is evidence that the monomer can adopt conformations that resemble the core structure of fibrillar A β (1-42)⁴⁶. In contrast, in fluorinated alcohols, A β (1-42) exhibits a high α -helical content⁴⁷, highlighting the conformational plasticity of this peptide under varying environmental conditions.

1.1. Neurodegenerative diseases and amyloidogenic proteins

Although transient and structural heterogeneous, A β (1-42) oligomers have been characterized through several studies. For instance, when in complex with human prion protein, A β oligomers exhibit a high β -sheet content as shown by solid-state nuclear magnetic resonance (NMR) investigations²⁵. Certain membrane associated oligomeric species, such as tetramers to octamers, contain both β -sheet-rich cores and regions of α -helical propensities⁴⁸. The transition of A β (1-42) oligomers to fibrils has also been investigated by solid-state NMR revealing a unique β -sheet arrangement of the oligomeric assemblies that could potentially be targeted by antibodies⁴⁹. Moreover, in the presence of sodium dodecyl sulfate (SDS), off-pathway A β (1-42) oligomers form, which display unique structural features and a pronounced β -strand character distinct from any other oligomer types characterized yet⁵⁰.

Tycko and colleagues made significant progress in characterizing transient oligomers by developing sophisticated freeze-trapping with a home-built device for rapid mixing and freezing⁵¹. Using this technique, they captured time-resolved data for A β (1-40) assemblies identifying interresidual contacts that begin to form after 500 milliseconds²⁴. In another study, Ishii and coworkers characterized spherical β -sheet-rich A β (1-40) oligomeric intermediates that appeared after 52 hours of incubation at 4 °C⁵². Notably, these latter two studies employed solid-phase synthesis for A β peptides to introduce site-specific isotope labeling at selected residues. This allowed a detailed analysis of specific resonances by reducing spectral overlap caused by line broadening from heterogeneous conformations. These findings are highly dependent on the specific solution conditions and environments in which the oligomers are formed.

Structural studies using solid-state NMR and cryo-electron microscopy (cryo-EM) have revealed that A β (1-42) can adopt multiple fibril polymorphs. Residue specific labeling patterns and sophisticated dipolar recoupling NMR experiments have enabled the elucidation of structural restraints revealing an S-shaped A β polymorph (Figure 3) in *in vitro* preparations⁵³⁻⁵⁵. These structures exhibit remarkable similarities, particularly in side chain packing and a flexible, disordered N-terminus.

Advances associated with the cryo-EM “resolution revolution” over the past decade have transformed structural biology, allowing high-resolution characterization of A β (1-42) fibril polymorphs. In 2017, a combined study using solid-state NMR and cryo-EM reported an LS-shaped fold for A β (1-42) fibrils (Figure 3) grown under low pH conditions, in which all residues including those at the N-terminus are part of the β -sheet

fibril core⁵⁶. Tycko and coworkers demonstrated that brain-derived seeded A β (1–42) fibrils exhibit a high degree of heterogeneity using solid-state ¹³C-¹³C fingerprint spectra⁵⁷.

Subsequent cryo-EM studies of brain-derived A β (1–42) fibrils confirmed structural polymorphisms, with all forms maintaining a characteristic S-shape fold similar to those seen *in vitro*, though side chain packing varies among them⁵⁸. Two major fibril types have been identified: Type 1 filaments, primarily found in sporadic AD, and type 2 filaments, which are more common in familial AD⁵⁸.

Notably, Tycko and colleagues have reported fibril structures that deviate significantly from the common S-shape, describing a v- and u-shape morphology⁵⁹. Additionally, recent studies have identified triple-stranded fibrils consisting of three monomers per cross-section, triggered by isomerization of L-aspartate to D-aspartate for A β (1–42) fibrils⁶⁰. Variations in the sequence by mutations as in familial AD can also lead to different polymorphs^{61, 62}.

A detailed list of A β (1–42) fibril structures with PDB codes is listed in Table 1. The polymorphic complexity of A β (1–42) fibrils, where a single peptide sequence can adopt multiple distinct, self-propagating structures, complicates efforts to define a mechanism of amyloid formation or to identify pathological relevant fibril structures.

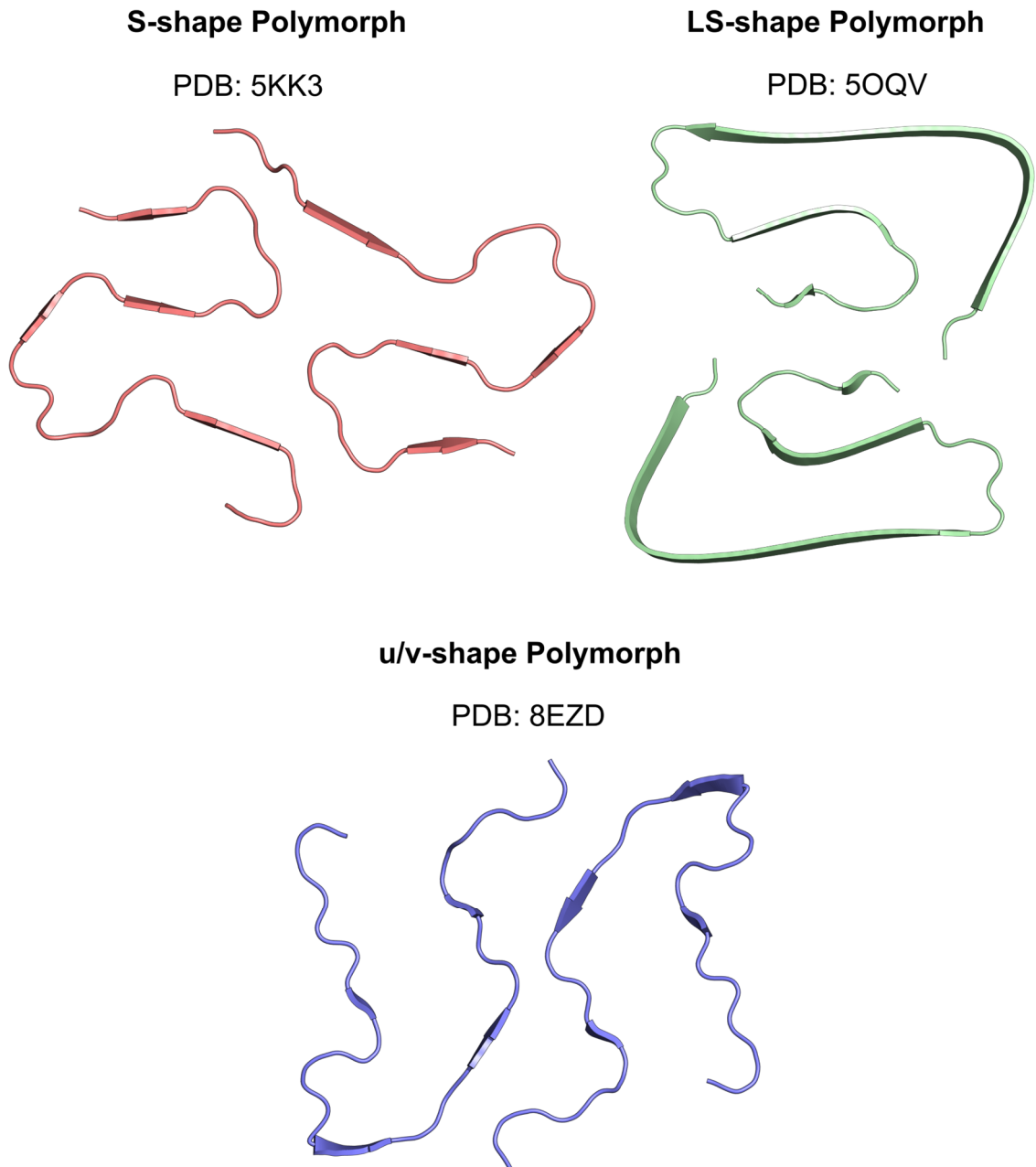


Figure 3: Common fibrillar A β (1–42) Polymorphs. Protomer interface with two monomeric subunits is shown. The S-shaped fold was the first structural motif resolved by solid-state NMR for in vitro A β (1–42)^{54, 55}. An LS-shaped polymorph, in which all residues, including the rigid N-terminus, are part of the β -sheet core, was discovered under low pH growth conditions⁵⁶. More recently, u- and v-shape polymorphs have been identified in brain-derived A β (1–42) fibrils using cryo-EM⁵⁹.

Table 1: Overview of known A β (1–42) fibril structures with corresponding literature, year of publication, method for structure determination, PDB codes, and topology shape.

Study	Year	Methods	PDB Code	Shape
Luhrs et al. ⁶³	2005	Solution-state NMR	2BEG (M35 oxidized)	u
Colvin et al. ⁵⁴	2015	Solid-state NMR	5KK3	S
Xiao et al. ⁵³	2015	Solid-state NMR	2MXU	S
Schmidt et al. ⁶⁴	2015	Cryo-EM	5AEF (low resolution)	tilde
Wälti et al. ⁵⁵	2016	Solid-state NMR	2NAO	S
Gremer et al. ⁵⁶	2017	Solid-state NMR/ Cryo-EM	5OQV	LS
Yang et al. ⁵⁸	2021	Cryo-EM	Type 1: 7Q4B, Type 2: 7Q4M	S
Lee et al. ⁵⁹	2023	Solid-state NMR/Cryo-EM	Type A: 8EZD Type B: 8EZE	u/v
Zielinski et al. ⁶²	2023	Cryo-EM	8OL2, 8OL3, 8OL5, 8OL6, 8OL7, 8OLG, 8OLN, 8OLO, 8OLQ (Sequence mutations)	LS/S
Fenandez et al. ⁶⁵	2024	Cryo-EM	8SEJ (Down syndrome)	S

Pyroglutamate A β

Truncation and post-translational modifications, which comprise pyroglutamate formation, isomerization, oxidation, nitration and glycosylation can further influence A β aggregation properties, and toxicity⁶⁶. In fact, many A β peptides found in AD patients plaques show modifications, one of the most abundant ones being pyroglutamylation of the N-terminus⁶⁷⁻⁶⁹ (Figure 4). It was first discovered in 1992 by Mori et al.⁷⁰ in AD plaques, hinting at an important role in AD.

For pyroglutamylation of the A β peptide the first two or 10 amino acids are cleaved off by proteases. These include Aminopeptidase A⁷¹(for first amino acid D1), Neprilysin⁷² (first two amino acids), meprin- β ^{73, 74} and Dipeptidylpeptidase IV⁷⁵. The N-terminal

1.1. Neurodegenerative diseases and amyloidogenic proteins

glutamate is converted to a pyroglutamate residue by glutaminyl cyclases⁷⁶, which are elevated in AD⁷⁷, and inhibitors of glutaminyl cyclases and the proteases mentioned above offer potential targets for therapeutics for AD⁷⁸. Donanemab, an antibody that selectively binds to pEA β (3–42) aggregates, demonstrated efficacy in clinical trials by reducing amyloid plaque load and slowing cognitive decline^{79, 80}. Pyroglutamate at the N-terminus is also found in other proteins, including antibodies⁸¹, where it is believed to protect against exopeptidase degradation and may contribute to structural stability⁸². Pyroglutamate at the N-terminus is also found in other proteins, including antibodies⁸¹, where it is believed to protect against exopeptidase degradation and may contribute to structural stability⁸².

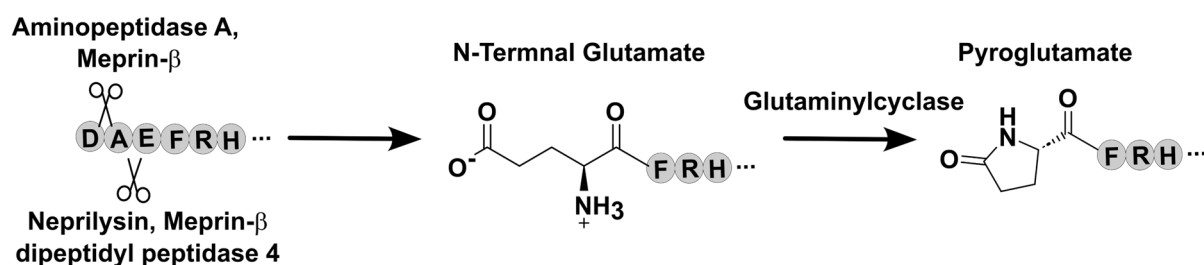


Figure 4: Transition of N-terminal glutamate to pyroglutamate in A β . After cleavage of β and γ -secretase, aminopeptidases, like Aminopeptidase A, Meprin- β , Neprilysin, or dipeptidyl, peptidase 4 can remove one or both N-terminal residues of the A β peptide. This processing leaves an exposed glutamate at the new N-terminus. The enzyme glutaminyl cyclase (QC) then catalyzes the intramolecular cyclization of this N-terminal glutamate residue to form pyroglutamate (pE) in a dehydration reaction.

Pyroglutamylated (pE) A β (3–42) exhibits increased aggregation propensity and toxicity compared to full length A β (1–42)^{83, 83}. Early studies reported no difference in toxicity of A β (1–42) and pEA β (3–42)⁸⁴, however, subsequent work, including transgenic mouse models, demonstrated that pEA β (3–42) is more toxic^{85–87}. Due to the loss of three charges, pEA β (3–42) is more hydrophobic and less soluble than unmodified A β (1–42)⁸⁸. Further, it shows a higher aggregation rate and can also serve as seed for cross seeding A β aggregation⁸⁹. Monomeric pyroglutamylated A β (3–40/42) peptides show differences in the chemical environment at N-terminal residues due to the cyclization of the N-terminal glutamate (Figure 4) which was observed using solution-state NMR^{90–92}. In addition, it was shown that pEA β (3–42) transitions into an α -helical intermediate before aggregating into fibrils⁹⁰.

Fibrillar pEA β (3–40) and pEA β (11–40) solid-state NMR results obtained by Scheidt et al. using residue selective labeling with solid-phase synthesis of the peptides revealed that

their fibril structures closely resemble those of A β (1–40) fibrils produced under comparable conditions^{93, 94}. In these fibrils grown at neutral pH values, the N-terminal region remains dynamically disordered, with the N-terminal residues up to residue 10 being unstructured⁹⁵. This suggests that the fibrillar core architecture is largely unaffected by the N-terminal pyroglutamylation at the flexible ends. Additionally, freeze-trapped oligomeric intermediates of pEA β (3–40) and pEA β (11–40) did not display major structural differences from their respective fibrillar form⁹⁶.

In atomic force microscopy (AFM) and electron microscopy (EM) images, fibrils formed by pEA β (3/11–40/42) often appear to be shorter and form in bundles when compared to A β (1–40/42) fibrils^{75, 89, 94, 97}, a finding we confirmed in our study for pEA β (3–42)⁹⁸. However, not all studies agree on these morphological differences. Scheidt et al. reported that fibrils of pEA β (3–40) formed under neutral pH conditions can adopt morphologies similar to those of A β (1–40) peptide⁹³.

At a low pH value of 2 and in presence of organic solvent, A β (1–42) fibrils adopt a structure in which the N-terminus is part of the rigid fibril core, stabilized by the salt bridge between the side chains of D1 and K28. We were particularly interested in how pyroglutamylation affects this structural motif, not only at the very N-terminal residues, but also in more distant regions of the fibril. In Section 2.1.1 we report a high-resolution solid-state NMR study of pEA β (3–42) fibrils, revealing an LS-shape fold that overall resembles the structure of A β (1–42) fibrils⁵⁶ but with notable differences at the N- and C-terminus⁹⁸. These results suggest that although the overall fibril architecture is preserved, pyroglutamylation introduces alterations at the fibril ends that may influence stability or molecular interactions in close proximity to it.

1.1.2 PI3K-SH3 domain as an amyloid model system

Phosphatidylinositol 3-kinases (PI3K) are multicomponent enzyme complexes that catalyze the phosphorylation of phosphatidylinositol, a critical step in signaling pathways involved in cell migration, proliferation and growth⁹⁹. One of its key subunits is the SH3 domain, an 84 amino acid protein module that binds proline-rich sequences, particularly PxxP motifs, where x can be any amino acid¹⁰⁰. SH3 domains mediate protein-protein interactions across a variety of cellular signaling pathways¹⁰¹. The native structure of the PI3K-SH3 domain consists of five β -strands forming a β -sandwich fold, interspersed with loop regions critical for binding and specificity^{102–104}. The RT loop

1.1. Neurodegenerative diseases and amyloidogenic proteins

(between the first and second β -strands) and the n-Src loop (between the second and third β -strands) of SH3 domains contribute to the binding of a proline-rich ligand¹⁰¹. The residues L11–D21 and D68–Y73, which are in close spatial proximity in the 3D structure, were identified as the binding interface for proline-rich peptide ligands¹⁰²⁻¹⁰⁴.

Under low pH conditions, the native fold of the PI3K-SH3 domain unfolds, depending on solution parameters such as pH and ionic strength and eventually form amyloid fibrils¹⁰⁵ (Figure 5). However, studies have also shown the presence of partially folded or differently folded populations even at low pH¹⁰⁶⁻¹⁰⁸, which may influence the formation of amyloid fibrils or promote the development of amorphous aggregates¹⁰⁷. It was shown that at pH values below 2 aggregation tends to favor rapid formation of amorphous aggregates, while between pH 2 and 3, well-defined fibrils are more likely to form¹⁰⁵. Increasing the pH after fibril growth can lead to fibril disassembly and the recovery of monomeric protein¹⁰⁹.

To date, two fibril structures of PI3K-SH3 domains have been reported at near atomic resolution^{109, 110}. In a solid-state NMR study, a structural model of PI3K-SH3 fibrils formed at room temperature and pH 2 over ~3 weeks was derived based on distance restraints using selective ¹³C labeling¹¹⁰. The analysis revealed that the secondary structure adopted in the fibrillar state is significantly different from that of the native monomeric fold. Notably, no highly flexible regions were observed in the fibrillar state, suggesting a rigid amyloid core.

Röder et al. reported a structure of seeded PI3K-SH3 fibrils grown at pH 2.5 in the presence of 10 mM glycine hydrochloride (Gly-HCl) at 50 °C, solved by cryo-EM, revealing a parallel, in-register cross- β architecture formed by homodimers with disordered N- and C-terminal regions¹⁰⁹. Two distinct polymorphs were observed which differ in fibril thickness, likely reflecting the presence of double and single filament fibrils. While the overall fold and cross- β architecture of the PI3K-SH3 fibrils are consistent between the pH 2/ RT and the pH 2.5/50 °C structures, differences in the local secondary structure were observed, particularly around residues 25 and 60. Notably, the fibril diameter observed in this study was approximately half of that reported by Zurdo et al.¹⁰⁵, suggesting a distinct fibril morphology. Mutation studies supported the structural model and identified several hydrophobic residues within the fibril core as critical determinants of fibril stability and growth. These residues appear to play a key role in stabilizing the cross- β structure and promoting fibril growth^{109, 111}.

A recurring feature across both the fibrillar and solution-state monomeric structures is the presence of a glycine-serine overhang at the N-terminus. This motif originates from the thrombin cleavage site used during recombinant protein purification and it remains in the final PI3K-SH3 protein sequence. Notably, the bovine and human PI3K-SH3 domain sequences differ at a single amino acid position: lysine (K) in bovine PI3K-SH3 is replaced by an arginine (R) in human at position 49.

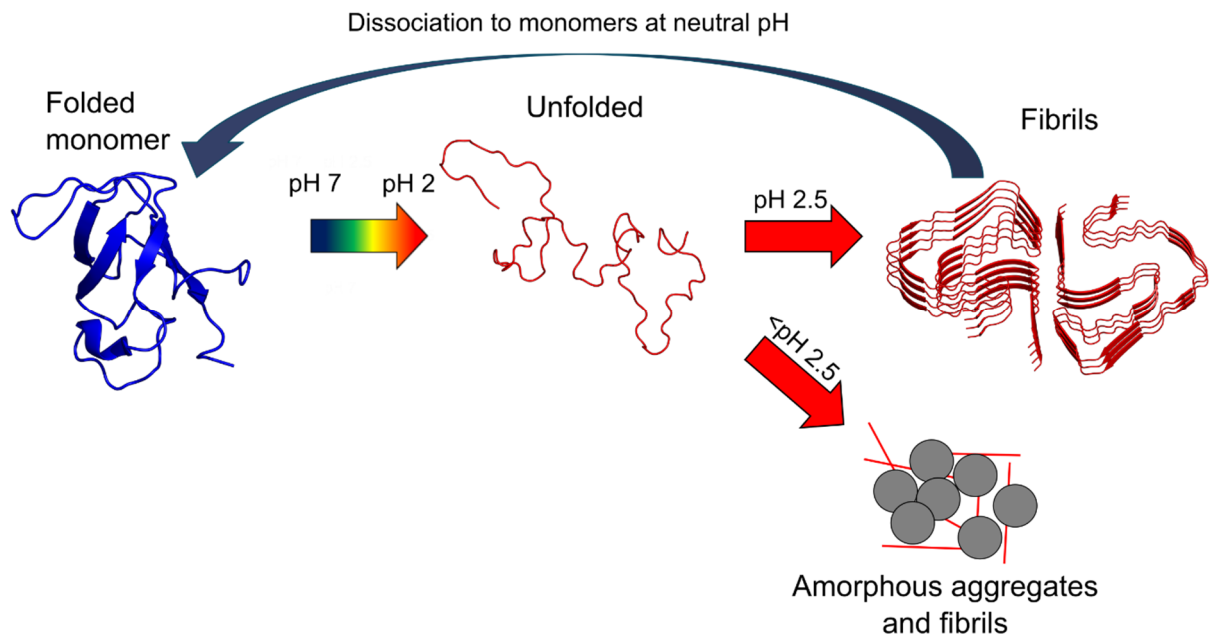


Figure 5: Amyloidogenic character of the PI3K-SH3 domain. At neutral pH, the PI3K-SH3 domain adopts a well-folded β -sandwich structure. Lowering the pH to values below 4 induces partial or complete unfolding of the monomer. Incubating the protein domain at low pH promotes the formation of amyloid fibrils. However, pH incubation below a value of 2 favors the formation of amorphous aggregates and accelerates aggregation rates. Notably, increasing the pH after fibril formation can lead to dissociation of the fibrils back into monomers.

1.2. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is a powerful technique for studying the structure and dynamics of molecules in both solution and solid states. It relies on the interaction of nuclei with non-zero spin (e.g. ^1H , ^{13}C , ^{15}N) with an external magnetic field. In an external magnetic field, nuclear spins align either parallel (low energy) or antiparallel (high energy) to the field with a slight overpopulation of parallel arrangement (approximately 1 in 10000 at room temperature). When exposed to a radiofrequency pulse at the Larmor frequency, a value dependent on the local magnetic environment, these spins can transition between states. The Larmor resonance frequency is highly sensitive to the chemical environment, particularly the electron density surrounding the nucleus, which makes NMR a highly informative tool for structural studies.

Solution-state NMR is widely used to study proteins in their soluble, dynamic states. It offers high spectral resolution due to rapid molecular tumbling, which averages out anisotropic interactions. This makes it ideal for determining structures and dynamics of small to medium-sized proteins (typically < 30 kDa). However, this limit can be pushed towards ~ 100 kDa with sophisticated methodology¹¹². In contrast, solid-state NMR is specifically suited for systems that are insoluble or lack sufficient mobility, such as amyloid fibrils, membrane proteins, or protein aggregates. Line broadening of peaks by anisotropic interactions like dipolar couplings and chemical shift anisotropy can be averaged out by magic angle spinning (MAS).

NMR has broad applications ranging from analytics in organic chemistry and metabolomics to battery research and material science. In structural biology, NMR spectroscopy is an indispensable technique for probing atomic-level structure, interactions, and molecular dynamics. In the following sections, we will discuss basic experiments including solution and solid-state experiments relevant to the study of amyloidogenic proteins.

1.2.1 Solution-state NMR spectroscopy: Heteronuclear single quantum coherence (HSQC)

A ^1H - ^{15}N HSQC experiment¹¹³ is commonly used in solution-state NMR to observe correlations between amide protons and nitrogen in a peptide backbone. The basic pulse sequence is depicted in Figure 6. In simple terms, the pulse sequence starts with an INEPT

(Insensitive Nuclei Enhanced by Polarization Transfer)¹¹⁴ block transferring the magnetization from the more sensitive ^1H spins to the less sensitive ^{15}N spins. This magnetization evolves during indirect time dimension (t_1) under the influence of the ^{15}N chemical shift, with a 180° pulse on ^1H decoupling the J-coupling during t_1 . After t_1 , the magnetization is transferred back to ^1H via reverse INEPT transfer and the signal is detected in direct dimension (t_2) after a 180° refocusing pulse. The delays in the INEPT steps are carefully matched to the scalar J-coupling (1J), which is typically around 92 Hz for backbone amide groups, corresponding to a delay of ~ 2.7 ms ($1/4J$). These delays ensure an optimal transfer of magnetization.

However, this type of coherence transfer is ineffective in solids, where rapid transverse relaxation (due to strong dipolar couplings and slow molecular tumbling) causes magnetization to decay before it can be transferred efficiently. Therefore, alternative polarization transfer methods, such as cross-polarization (CP) or dipolar recoupling techniques, are used in solid-state NMR to overcome these challenges and enable the detection of similar heteronuclear correlations.

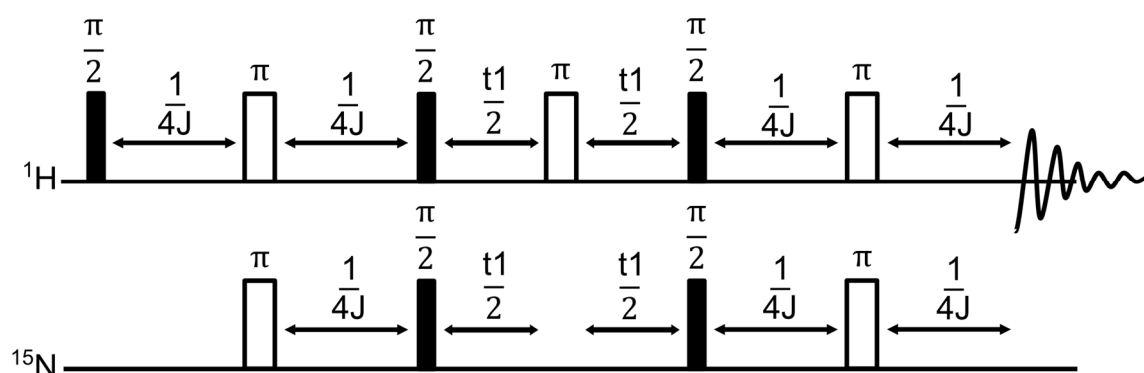


Figure 6: Pulse sequence of an ^1H - ^{15}N HSQC spectrum. 90° pulses ($\pi/2$) are represented as filled, 180° pulses (π) as hollow rectangles. In an INEPT transfer, after initial excitation with 90° pulse on ^1H , magnetization is being transferred from ^1H to ^{15}N spin via 180° and 90° on both ^1H and ^{15}N . During t_1 evolution on ^{15}N , a 180° decoupling pulse is applied on ^1H , followed by an 90° and 180° pulse to bring back the magnetization to ^1H and the FID is recorded.

The resulting 2D ^1H - ^{15}N HSQC spectrum serves as a fingerprint of the protein's backbone allowing for a quick assessment of the folding of the protein. Each cross-peak typically corresponds to one backbone amide group. Backbone amides of the N-terminal residue and some solvent-exposed residues (e.g. lysine) may be broadened or missing due to rapid proton exchange with water at physiological pH. Similarly, proline residues do not appear

1.2. Nuclear magnetic resonance (NMR) spectroscopy

in ^1H - ^{15}N HSQC spectra because the nitrogen in the peptide bond lacks a directly bonded proton. In contrast, the side chains of glutamine and asparagine often remain visible.

IDPs typically exhibit narrow dispersion in the ^1H dimension of the HSQC spectrum with most amide signals clustered between 7.5 and 8.5 ppm. This reflects their lack of stable secondary structure, leading to a similar chemical environment for many residues. In contrast, β -sheet structures tend to shift the resonances of amide protons downfield (toward higher ppm), while α -helical regions often show signals slightly upfield (closer to ~ 8.0 ppm), though the precise shifts depend on the local environment¹¹⁵.

1.2.2 Solid-state NMR spectroscopy: Basics and cross-polarization

As mentioned before, anisotropic interactions in solids are a major source of line broadening in NMR spectra. In static powder samples, where molecules are randomly oriented, these interactions lead to a distribution of resonance frequencies across the sample, resulting in inhomogeneous line broadening (powder pattern). Although this broadening can complicate spectral analysis, it also contains valuable information on the anisotropy of the underlying interactions.

The most prominent sources of anisotropy are chemical shift anisotropy (CSA) and dipolar couplings:

- CSA arises from the fact that the local electronic shielding around a nucleus is described by a tensor. This means that the observed chemical shift depends on the orientation of the shielding tensor (and therefore the molecule) relative to the external magnetic field.
- Dipolar couplings occur between nuclear spin pairs and depend on both their internuclear distance (r^{-3}) and the angle between the internuclear vector and the magnetic field.

Both interactions scale with the factor:

$$3\cos^2\theta - 1$$

Where θ is the angle between interaction tensor (dipolar vector or CSA tensor) and the external magnetic field. To average out these anisotropic interactions and narrow the lines, solid-state NMR employs MAS. In MAS, the sample is packed into a rotor and spun rapidly (typically tens of kHz) at the magic angle (54.74°) relative to the external

magnetic field. At this angle, $3\cos^2\theta-1$ becomes zero and (if the spinning is fast enough compared to the strength of the anisotropic interaction) the orientation dependence is averaged out over time, narrowing down the signal to an isotropic peak.

Magnetization transfer from highly polarized to low polarized spins in solid-state NMR is commonly achieved via cross-polarization (CP)^{116, 117}. In this process the I-spin (typically a proton) is initially excited and is subsequently held in a spin-lock condition by continuous RF irradiation. At the same time, an RF pulse is applied to the S spins (e.g. ^{13}C or ^{15}N), fulfilling the Hartmann-Hahn condition. When the Hartmann-Hahn matching condition is fulfilled during that spin-lock, that is, the nutation frequencies of both spins are equal in the rotating frame ($\omega_I^I = \omega_I^S$), efficient polarization transfer occurs via dipolar interaction. This spin-lock period is held for a defined contact time. The process can also be described using the spin temperature formalism, where the system of coupled I and S spins is treated thermodynamically: the initially highly polarized I-spin acts as a "hot reservoir" and exchanges polarization with the "colder" S-spin until an equilibrium is approached. A prerequisite is that dipolar coupling is not averaged out by movement.

A commonly used pulse sequence for ^{13}C – ^{13}C correlation spectra that uses CP transfer is Proton-Driven Spin Diffusion (PDSD)¹¹⁸. The pulse sequence is depicted in Figure 7. After CP, t_1 evolution of the S-spin occurs while the I-spins (protons) are decoupled to prevent heteronuclear dipolar interactions. A 90° pulse then stores magnetization along the z-axis. During the following mixing time, polarization is transferred between neighboring ^{13}C nuclei via spin diffusion, which is mediated by the strong dipolar coupling network of protons (i.e. through flip-flop transitions). Finally, a second 90° pulse rotates the z-magnetization of the S-spins back into the transverse plane, allowing detection of the signal under continuous proton decoupling.

1.2. Nuclear magnetic resonance (NMR) spectroscopy

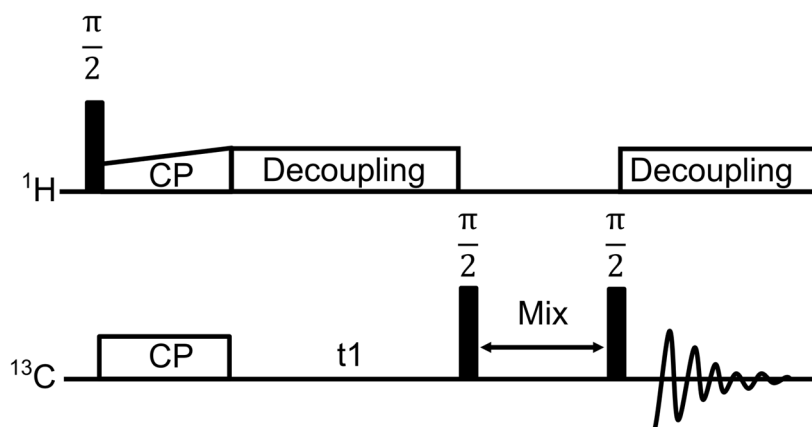


Figure 7: Pulse sequence of a PDSD spectrum. After an initial 90° pulse on ^1H spin, magnetization is transferred during CP spin lock pulses on both nuclei followed by t_1 evolution on ^{13}C . Magnetization is then stored on the z axis with a 90° pulse on ^{13}C , followed by a mixing time to enable spin diffusion. Here, magnetization is transferred to nearby ^{13}C nuclei via dipolar coupling. It is important to note that there is no decoupling on ^1H to facilitate the transfer. Next, a second 90° pulse flips magnetization back to the xy plane and the FID is recorded.

In solid-state NMR, ^{13}C - ^{13}C correlation spectra, such as PDSDs, are used as fingerprint spectra for proteins as each rigid protein shows a unique pattern of ^{13}C - ^{13}C cross correlation peaks, analogous to the ^1H - ^{15}N peaks in HSQC spectra in solution-state NMR.

Typical mixing times for hydrated or gel embedded proteins in solid-state are 20 ms – 1 s. Shorter mixing times (~ 20 – 50 ms) primarily give rise to intraresidual correlations, i.e., between nuclei within the same amino acid residue. In contrast, longer mixing times (~ 100 ms to 1 s) enable the detection of interresidual contacts, which are important for identifying long-range spatial restraints between different residues. At higher MAS rates, the efficiency of proton-driven spin diffusion is reduced due to averaging of the dipolar couplings, which are the main mechanism for polarization transfer in PDSD. To counteract this, continuous-wave (CW) irradiation or rotor-synchronized pulses are applied during the mixing period to recouple heteronuclear dipolar coupling and, thus, enhance magnetization transfer through the proton network. Common spectra variants that use such strategies include DARR (Dipolar Assisted Rotational Resonance) and CORD (Combined Rotation and Dipolar recoupling).

However, in solid-state NMR studies of proteins, especially those with long or repetitive sequences and different conformations, uniform ^{13}C , ^{15}N labeling often leads to spectral crowding which complicates spectral analysis. To address this, selective or sparse labeling techniques are frequently employed¹¹⁹. These include the use of $[1\text{-}^{13}\text{C}]$ or $[2\text{-}^{13}\text{C}]$

glycerol or glucose as carbon sources during protein expression or amino acid-type selective labeling or unlabeled. Such strategies help reduce signal overlap and facilitate more straightforward resonance assignments and structural analysis.

In ^{13}C – ^{13}C correlation spectra, characteristic chemical shifts allow identification of specific amino acid types, providing so-called "spin systems". To establish a sequential assignment of the protein backbone, heteronuclear ^{15}N – ^{13}C correlation experiments are used (Figure 8). In NCACX and NCOCX experiments¹²⁰, the magnetization is first transferred from ^1H to ^{15}N , followed by evolution during the indirect ^{15}N dimension (t_1). Subsequently, magnetization is transferred to the $^{13}\text{C}\alpha$ (in NCACX) of the same residue or ^{13}CO (in NCOCX) of the preceding residue. From there, a spin diffusion step enables further transfer to neighboring ^{13}C nuclei. These experiments can be performed in 2D or 3D formats, depending on whether the $^{13}\text{C}\alpha$ or ^{13}CO nuclei evolved during a second indirect dimension (t_2), enabling more detailed backbone assignments.

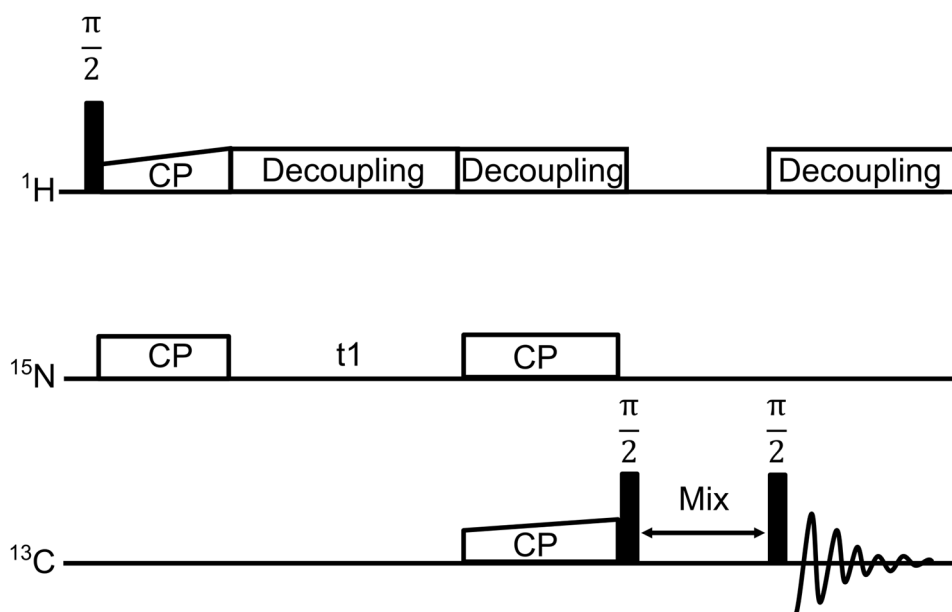


Figure 8: Pulse sequence of a NCACX/NCOCX spectrum. The initial CP transfer from ^1H to ^{15}N , followed by t_1 evolution on the ^{15}N channel, which is similar to the initial steps described for the PDSD experiment in Figure 7. Next, a second CP transfer from ^{15}N to ^{13}C is performed. In NCACX the transfer targets the intraresidual $\text{C}\alpha$, while in NCOCX it targets the carbonyl carbon (CO) of the preceding residue. A spin diffusion step, achieved through two 90° pulses, and a defined mixing time allows magnetization transfer to nearby ^{13}C nuclei.

1.2. Nuclear magnetic resonance (NMR) spectroscopy

1.2.3 Application of NMR in amyloid research

This section will focus on a summary with key findings and developments in NMR and how they can be used on amyloidogenic proteins.

Solution-state NMR is a powerful technique for investigating monomeric IDPs and peptides, which often evade structural characterization due to their flexibility and lack of stable secondary or tertiary structure. However, detailed reviews comprise methods to study them and summarize challenges and solutions for these¹²¹⁻¹²³. For example, analysis like residue specific secondary chemical shift propensities provide information on transient secondary structure propensities¹²⁴ that can be important to draw conclusions for protein-protein interaction and, therefore, the aggregation behavior of the respective IDP. Binding to larger complexes or lipid membranes increases the rotational correlation time of a protein, leading to signal attenuation or broadening in solution-state NMR. This effect can serve as an indicator of membrane interaction, as observed for some amyloidogenic proteins¹²⁵. However, more sophisticated experiments such as relaxation dispersion can provide insights into invisible intermediate states^{44, 126}, the interaction with membranes¹²⁷, and information of the residue specific dynamics of IDPs¹²⁸. Additionally, experiments based on exchange saturation transfer, where the signal of the visible state (e.g., monomer) is influenced by selective radiofrequency saturation of an invisible state (e.g., oligomer or protofibril), can yield valuable information on monomer to (proto)fibril interactions and attachment¹²⁹.

In addition, both solution and solid-state NMR based aggregation studies provide insights into fibril formation^{105, 130} or help extract information of kinetics¹⁰⁶. While solution-state methods track the decay of monomeric species, solid-state NMR detects ordered more rigid aggregates like fibrils. In solid-state NMR, the sample is subject to constant ultracentrifugation due to sample rotor spinning that may influence the aggregation behavior. This was utilized for studying early formed aggregates of amyloidogenic proteins, which tend to sediment along the rotor wall due to their increased molecular weight. These sedimented species contain rigid components that become visible to direct observation in solid-state CP NMR spectra^{131, 132}. Berkeley et al. investigated the aggregation process of the low-complexity domain of the RNA-binding protein fused in sarcoma (FUS LC) using a combination of solid-state NMR techniques including 1D CP, INEPT and direct excitation experiments over time to capture the maturation and aggregation of FUS LC¹³³. Additionally, they also obtained 2D CP and INEPT based

spectra at the end of the time course, providing insights into which regions of the aggregates remain flexible, and which become rigid. Notably, they observed a discrepancy between the decay of INEPT signals and the increase in CP signals during maturation, suggesting the existence of an intermediate species with dynamics in a regime that is invisible to both techniques¹³³. To further investigate liquid to solid phase transition preceding fibril formation, Emmanouilidis et al. studied the full length FUS protein using both solution- and solid- state NMR demonstrating that the interface of FUS condensates plays an important role in the maturation process^{134, 133}. Additionally, they also obtained 2D CP and INEPT based spectra at the end of the time course, providing insights into which regions of the aggregates remain flexible, and which become rigid. Notably, they observed a discrepancy between the decay of INEPT signals and the increase in CP signals during maturation, suggesting the existence of an intermediate species with dynamics in a regime that is “invisible” to both techniques¹³³. To further investigate liquid to solid phase transition preceding fibril formation Emmanouilidis et al. studied the full length FUS protein using both solution- and solid- state NMR demonstrating that the interface of FUS condensates plays an important role in the maturation process¹³⁴.

Solid-state NMR is particularly well suited for studying large macromolecular assemblies such as oligomers protofibrils or amyloid fibrils as discussed in recent literature¹³⁵. Amyloid fibrils, due to their size and structural homogeneity, enable the acquisition of high-resolution NMR spectra. Solid-state NMR has been extensively used to determine the 3D structures of amyloid fibrils, including A β ^{54, 55}, PI3K-SH3¹¹⁰, α -synuclein¹³⁶, and HET-s¹³⁷ through the use of long-range distance restraints often obtained by selective isotope labeling strategies. Although cryo-EM has gained popularity for rapid structure determination of fibrils, solid-state NMR remains uniquely powerful for detecting atomic-level details, especially for systems that are less amenable to cryo-EM due to flexibility, heterogeneity, or the lack of periodic twist or bundling for amyloid fibrils. In such cases or when investigating more dynamic or disordered regions of fibrils, solid-state NMR is still a valuable tool to determine structural insights, as Mammeri et al. demonstrated for tau fibrils¹³⁸. Moreover, ¹³C–¹³C correlation experiments such as PDSD (see Section 1.2.2) are well suited for distinguishing easily between different fibril polymorphs. For example, brain-derived extracts can be used to seed fibrillar samples of A β and both the linewidths and chemical shift positions in the resulting spectra can serve as indicators of structural polymorphism due to different folding of the protomers⁵⁹. Because chemical shifts are highly sensitive to local molecular environments, even small

1.2. Nuclear magnetic resonance (NMR) spectroscopy

structural variations among polymorphs can be detected¹³⁹. “Macroscopic” differences in fibril structures such as single vs double filament arrangement of the protomers are often difficult to distinguish in solid-state NMR spectra alone as the local chemical environment can remain largely unchanged. Techniques like AFM or EM are more reliable for these morphological variations (Section 3.3). A combined approach integrating solid-state NMR with imaging techniques is therefore recommended for these structural investigations.

Recent technical advances have substantially improved the resolution of both solid- and solution-state NMR. MAS rates over 100 kHz using 0.7 mm rotors or smaller allow for reduced sample amounts and provide high-resolution in ^1H - ^{15}N dimensions. This enables the acquisition of high-quality ^1H - ^{15}N fingerprint spectra and facilitates, or even enables, the resonance assignment of fibrillar samples, as demonstrated for e.g. A β (1-42) fibrils¹⁴⁰. Simultaneously, the development of stronger magnetic fields significantly improves spectral resolution in both solid- and solution-state NMR¹⁴¹.

Dynamic nuclear polarization (DNP)-enhanced solid-state NMR has emerged as a powerful tool for studying biomolecules, as highlighted in a recent review^{142, 143}. The mechanism of DNP relies on the transfer of polarization from unpaired electron spins (paramagnets), typically introduced via stable radicals, to nearby nuclear spins, greatly enhancing NMR signal sensitivity. This transfer is achieved most efficiently at low temperatures (around 100 Kelvin). By the rapid freezing of molecular conformations, DNP provides “snapshots” of conformational states allowing the detection of transient states or semi-rigid regions. These states are often invisible for conventional solid-state NMR or other structural biology techniques such as cryo-EM and X-ray crystallography^{144, 145}.

Tycko and colleagues have demonstrated this approach on A β (1-40), capturing monomeric, oligomeric, protofibrillar, and fibrillar states and detecting long-range tertiary contacts characteristic of fibrillar forms for all stages¹⁴⁶. Similarly, Bayro et al. identified interresidual contacts for PI3K-SH3 fibrils at cryogenic temperatures using DNP which were not observed in spectra recorded at room temperature¹⁴⁷. Debelouchina et al. demonstrated that DNP-enhanced NMR can be used to obtain resonance assignments for selectively labeled GNNQQNY fibrils employing ^{13}C - ^{13}C correlation spectra and ^{15}N - ^{13}C spectra using transferred-echo double resonance-based recoupling of dipolar interactions¹⁴⁸.

In our own work, we demonstrated that DNP-enhanced NMR is a valuable tool for studying side chain conformations of all states along the amyloidogenic pathway including folded monomers, unfolded intermediates, and mature fibrils¹⁴⁹. Notably, Frederick and colleagues reported in-cell DNP-enhanced solid-state NMR studies using selectively labeled α -synuclein¹⁵⁰. By probing its structural state during incorporation and aggregation within cells, they found that the N-terminal region of fibrillar α -synuclein tends to adopt partially α -helical conformation, as revealed by secondary chemical shift analysis. Radicals used for DNP-enhanced NMR can be applied not only in the bulk solvent to enhance the overall signal, but also in a targeted manner by placing labels harboring a radical in close proximity to specific sites of interest, enabling localized signal enhancement through site-directed DNP excitation^{151, 152}.

Beyond DNP, paramagnetic agents and spin labels are broadly applied in NMR to study structure, dynamics and solvent accessibility of biomolecular systems including amyloidogenic proteins^{153, 154}. Paramagnets enhance the relaxation rates of nearby nuclear spins resulting in line broadening (paramagnetic relaxation enhancement, PRE). Moreover, angle dependent effect of the paramagnet caused by magnetic susceptibility anisotropy leads to residual dipolar coupling and pseudo chemical shifts that can provide valuable information on distance or orientation of molecules. Site-directed spin labeling is widely employed to obtain distance information¹⁵⁵ and it has been used to detect protein¹⁵⁶, membrane¹⁵⁷ or ligand interaction¹⁵⁸. A commonly used biradical for DNP-enhanced NMR, TOTAPOL, has been observed to bind amyloid fibril surfaces which lead to line broadening at increased concentrations due to PRE effect¹⁵⁹. In contrast, the use of solvent PRE, where the paramagnetic agent is distributed in the solvent rather than covalently attached, has been used to probe surface accessibility, support structure determination¹⁶⁰ and investigate surfaces of intrinsically disordered proteins¹⁶¹ and amyloid fibrils¹⁶² using both solution- and solid-state NMR. Site-directed spin labeling is widely employed to obtain distance information¹⁵⁵ and it has been used to detect protein¹⁵⁶, membrane¹⁵⁷, or ligand interaction¹⁵⁸. A commonly used biradical for DNP-enhanced NMR, TOTAPOL, has been observed to bind amyloid fibril surfaces which lead to line broadening at increased concentrations due to PRE effect¹⁵⁹. In contrast, the use of solvent PRE, where the paramagnetic agent is distributed in the solvent rather than covalently attached, has been used to probe surface accessibility, support structure determination¹⁶⁰ and investigate surfaces of intrinsically disordered proteins¹⁶³ and amyloid fibrils¹⁶² using both solution- and solid-state NMR.

1.3. Scope of this work

The field of amyloid research is broad and complex. Many amyloid-forming peptides and proteins are implicated in severe neurodegenerative diseases, highlighting the urgent need for comprehensive understanding of their structural and dynamic properties. Various biophysical techniques have been applied to study their structure, folding, and aggregation. Among these, NMR spectroscopy is of particular importance due to its ability to probe these systems at atomic resolution across different states from monomers to aggregated fibrils.

Despite its strengths, NMR also presents challenges: In solid-state NMR, for example, full structural characterization of amyloid fibrils often requires sophisticated labeling strategies and the acquisition of sufficient long-range distance restraints. Spectral complexity due to polymorphism can lead to line broadening and peak doubling, and therefore complicate analysis. However, its relevance is described in Section 1.2.3 and proven by continuous work being published on amyloidogenic proteins using NMR.

In this work, we contribute to the field by developing and applying a combination of NMR-based methods tailored to amyloidogenic systems.

We investigate the stability and structural features of fibrillar A β (1-42) using solid-state NMR under varying pH conditions, probing the robustness of the LS-shaped polymorph. Combining experimental and computational methods allow us to test the influence of pH on fibril integrity and the protonation of side chains, which are crucial for understanding their behavior in binding or interaction studies.

Furthermore, we characterize the lesser studied variant of pyroglutamylated A β (pEA β (3-42)) fibrils. While this form is increasingly recognized for its role in disease, its structural properties have remained poorly characterized. Using advanced NMR strategies, we want to uncover how this modification at the N-terminus influences fibril architecture.

Using an integrative approach of solution-state NMR, MAS solid-state NMR, and high-resolution MAS (HR-MAS) we follow the seeded aggregation pathway of PI3K-SH3 from monomeric to fibrillar form and compared the kinetics.

Side chain conformations carry crucial information about protein folding and interaction. We investigate the conformational ensemble of different model proteins for folding with

DNP-enhanced NMR and achieved snapshot-like spectra of their isoleucine side chains. This approach opens the door to studying a wider variety of side chains, including functionally relevant residues such as charged or aromatic groups, which could reveal new insights into protein–protein interactions, binding interfaces, and the structural determinants of misfolding.

Further, additional data presented in this work deepen our understanding of the amyloidogenic proteins investigated and might help in future research.

2

Publications

This chapter lists all my publications that were carried out during my doctoral studies including their titles, authors, journals and dates of publication together with one manuscript currently in preparation. A brief description of my contributions to each study is given alongside the abstract of the respective article.

The chapter is divided into two parts:

1. **Publications that are directly related to the topics of this dissertation**, which are discussed in the introduction and discussion chapters)
2. **Additional scientific contributions** made during my doctoral studies.

Full journal versions of the articles that are directly related to the dissertation as well as the manuscript are included in the Appendix.

2.1. Publications directly related to this dissertation

2.1.1 Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy

Luis Gardon, Nina Becker, Lothar Gremer, Henrike Heise

Chemistry—A European Journal, Research Article Volume30, Issue10

<https://doi.org/10.1002/chem.202303007>

First published: 15 December 2023

Contribution:40%

Performed sample preparation of pyroglutamate A β according to the protocol by Gremer et al. (2017)⁵⁶, including pyroglutamate formation and fibril formation. Additionally prepared a pH adjusted sample of pyroglutamate A β at pH 6.5. Performed experiments, including solid-state NMR measurements (^{13}C – ^{13}C and ^{15}N – ^{13}C correlation spectra, rotational resonance, and water-edited spectra) as well as atomic force microscopy. Carried out spectral analysis and resonance assignment. Contributed significantly to the writing of the manuscript and preparation of the figures.

Reprint permission:

The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution"—the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned). Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content. If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted. Any translations, for which a prior translation agreement with Wiley-VCH has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley-VCH publication. The publisher has not endorsed this translation."

Abstract:

Extracellular amyloid- β (A β) plaques, primarily formed by A β (1-40) and A β (1-42) fibrils, are a hallmark of Alzheimer's disease. The A β peptide can undergo a high variety of different post-translational modifications including formation of a pyroglutamate (pGlu, pE) at N-terminal Glu3 or Glu11 of truncated A β (3-x) or A β (11-x), respectively. Here we studied structural similarities and differences between pEA β (3-42) and LS-shaped A β (1-42) fibrils grown under identical conditions (pH 2) using solid-state NMR spectroscopy. We show that the central region of pEA β (3-42) fibrils including the turn region around V24 is almost identical to A β (1-42) showing similar β -strands also at the N-terminus. The missing N-terminal residues D1-A2 along with pE3 formation in pEA β (3-42) preclude a salt bridge between K28-D1' as in A β (1-42) fibrils. G37 and G38 act as highly sensitive internal sensors for the modified N-terminus, which remains rigid over ~five pH units.

2.1.2 Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques

Luis Gardon, Nina Becker, Nick Rähse, Christoph Hölbling Athina Apostolidis, Celina M. Schulz, Kevin Bochinsky, Lothar Gremer, Henrike Heise, Nils-Alexander Lakomek

Frontiers in Molecular Biosciences, Sec. Structural Biology, Volume 10 - 2023 |

<https://doi.org/10.3389/fmolb.2023.1254721>

First published: 17 November 2023

Contribution: 30%

Purified and prepared samples of PI3K-SH3 domain for analysis. Performed a range of experiments including solution-state, HR-MAS, and solid-state NMR measurements (1D ^1H and 2D ^1H - ^{15}N HSQC spectra, as well as 1D ^{13}C CP and INEPT spectra) and atomic force microscopy. Performed data analysis, including signal integration and kinetic fitting. Contributed significantly to the writing of the manuscript and preparation of the figures.

Abstract:

2.1. Publications Directly Related to This Dissertation

Misfolding and aggregation of amyloidogenic proteins are molecular hallmarks of neurodegenerative diseases. The bovine PI3K-SH3 domain serves as a well-established model system to investigate protein aggregation mechanisms. In this study, we applied a combined NMR approach to monitor the fibril formation kinetics of low pH-denatured [^{13}C , ^{15}N] labeled PI3K-SH3 under seeded conditions. Using solution-state NMR, high-resolution magic angle spinning (HR-MAS) NMR, and solid-state NMR, we tracked the depletion of monomeric species and the growth of aggregated fibrils over time. Solution NMR enabled residue-specific analysis of monomer decay, while solid-state NMR captured both monomer decay and fibril formation within the same sample. HR-MAS NMR served as a hybrid method bridging solution and solid-state NMR. Under 1% seeding and pH 2.5, all three methods yielded comparable aggregation kinetics with mono-exponential behavior and lifetimes between 14–38 hours. Importantly, we observed that the disappearance of monomer correlated with the appearance of fibril mass. Atomic force microscopy confirms that fibrils substantially grew in length during the aggregation experiment. This argues for fibril elongation as the dominant growth mechanism in fibril mass. Thus, an integrated approach highlights the strength of complementary NMR techniques in resolving the kinetics of amyloid fibril formation and provides a robust framework for future mechanistic studies of protein aggregation.

2.1.3 Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-Shaped A β (1–42) Amyloid Fibrils

Nina Becker, Benedikt Frieg, Lothar Gremer, Tatsiana Kupreichyk, Luis Gardon, Patrick Freiburg, Philipp Neudecker, Dieter Willbold, Holger Gohlke, Henrike Heise

Journal of the American Chemical Society, Volume 145, Issue 4

<https://doi.org/10.1021/jacs.2c09231>

First published: 18 January 2023

Contribution: 10%

Performed resonance assignment of monomeric A β at pH 7. Performed NMR experiments for the pH sensing study, including INEPT based spectra of buffer and A β samples across varying pH values for the review. Designed figures for data representation,

including the Table of Contents (TOC) figure. Contributed to manuscript writing and final editing.

Abstract:

Alzheimer's disease is a neurodegenerative disorder associated with the deposition of misfolded aggregates of the amyloid- β protein ($A\beta$). $A\beta(1-42)$ is one of the most aggregation-prone components in senile plaques of AD patients. We demonstrated that relatively homogeneous $A\beta(1-42)$ fibrils with one predominant fold visible in solid-state NMR spectra can be obtained at acidic pH. The structure of these fibrils differs remarkably from some other polymorphs obtained at neutral pH. In particular, the entire N-terminal region is part of the rigid fibril core. Here, we investigate the effects of a pH shift on the stability and the fold of these fibrils at higher pH values. Fibril bundling at neutral pH values renders cryo-EM studies impractical, but solid-state NMR spectroscopy, molecular dynamics simulations, and biophysical methods provide residue-specific structural information under these conditions. The LS-fold of the $A\beta(1-42)$ fibrils does not change over the complete pH range from pH 2 to pH 7; in particular, the N-terminus remains part of the fibril core. We observe changes in the protonation state of charged residues starting from pH 5 on a residue-specific level. The deprotonation of the C-terminal carboxyl group of A42 in the intermolecular salt bridge with D1 and K28 is slow on the NMR time scale, with a local pK_a of 5.4, and local conformations of the involved residues are affected by deprotonation of A42. Thus, we demonstrate that this fibril form is stable at physiological pH values.

2.1.4 Isoleucine Side Chains as Reporters of Conformational Freedom in Protein Folding Studied by DNP-Enhanced NMR

Leonardo Levorin, Nina Becker, Boran Uluca-Yazgi, Luis Gardon, Mirko Kraus, Marc Sevenich, Athina Apostolidis, Kai Schmitz, Neomi Rüter, Irina Apanasenko, Dieter Willbold, Wolfgang Hoyer, Philipp Neudecker, Lothar Gremer, Henrike Heise

J. Am. Chem. Soc. 2025, Volume 147, Issue 18, 15867–15879

<https://doi.org/10.1021/jacs.5c04159>

First published: 26 April 2025

Contribution: 10%

2.1. Publications Directly Related to This Dissertation

Sample preparation of PI3K-SH3 in natively folded, unfolded and fibrillar form. Assisting the recording of spectra and analysis. Writing parts of original draft and contributed to manuscript proofreading and helped with figure design.

Abstract:

Conformations of protein side chains are closely linked to protein function. DNP-enhanced solid-state NMR (ssNMR), which operates at cryogenic temperatures (<110 K), can be used to freeze-trap protein conformations, including the side chains. In the present study, we employed two-dimensional DNP-enhanced ssNMR to get detailed insights into backbone and side chain conformations of isoleucine. We used different amino acid selectively labeled model proteins for intrinsically disordered proteins (IDPs), denatured and well-folded proteins, and amyloid fibrils. ^{13}C chemical shifts are closely correlated with secondary structure elements and χ_1 and χ_2 angles in isoleucine side chains. Thus, line shape analysis by integration of representative peak areas in 2D spectra provides an accurate overview of the distribution of backbone and side chain conformations. For the well-folded proteins GABARAP and bovine PI3-kinase (PI3K) SH3 domain, most Ile chemical shifts in frozen solution are well resolved and similar to those observed in solution. However, line widths of individual Ile residues are directly linked to residual mobility, and line broadening or even signal splitting appears for those Ile residues, which are not part of well-defined secondary structure elements. For unfolded PI3K SH3 and the IDP α -synuclein (α -syn), all Ile side chains have full conformational freedom, and as a consequence, inhomogeneous line broadening dominates the cryogenic spectra. Moreover, we demonstrate that conformational ensembles of proteins strongly depend on solvent and buffer conditions. This allowed different unfolded structures for chemical and acidic pH denaturation of the PI3K SH3 domain to be distinguished. In amyloid fibrils of α -syn and PI3K SH3, chemical shifts typical for β -strand like secondary structure dominate the spectra, whereas Ile residues belonging to the fuzzy coat still add the IDP-type line shapes. Hence, DNP-enhanced ssNMR is a useful tool for investigating side chain facilitated protein functions and interactions.

2.1.5 Surface Mapping of Bovine PI3K-SH3 Domain using Gadoteric Acid for Solvent PRE effect

Luis Gardon, Melinda Jaspert, Nina Becker, Leonardo Levorin, Athina Apostolidis, Philipp Neudecker, Nils-Alexander Lakomek, Lothar Gremer, Henrike Heise

In preparation

Contribution: 40%

Purification and sample preparation of PI3K-SH3 domain in natively folded, unfolded and fibrillar form. Recording solution- and solid-state NMR spectra and performed atomic force microscopy measurements. Performed data analysis, including signal integration, resonance assignment and surface plotting for native and fibrillar state of PI3K-SH3. Writing original draft and designing figures.

Abstract:

Amyloid fibril formation is related to numerous neurodegenerative diseases. PI3K-SH3 serves as a well-established model system for investigating the mechanisms underlying amyloid fibril formation. Here, we employ a combination of solution and solid-state NMR spectroscopy combined with solvent Paramagnetic Relaxation Enhancement (sPRE) using meglumin gadoteric acid (Dotarem) to map the surface of both bovine PI3K-SH3 in all stages of fibrillation. We detect a high accessibility at low concentrations of Dotarem in regions of the globular monomeric state at neutral pH corresponding to the binding site of PI3K-SH3 to proline rich sequences. In PI3K-SH3 fibrils the fibril core appears shielded from the solvent, whereas the homodimer interface remains solvent-accessible. Notably, a few residues stay shielded at elevated Dotarem concentrations in both the monomeric and fibrillar state. These results demonstrate that sPRE is a straightforward and effective method for probing protein surface accessibility. Moreover, it might present a valuable complementary approach for characterizing aggregation interfaces and surfaces of amyloidogenic proteins in different states.

2.2. Additional scientific contributions

2.2.1 Photocontrolled Reversible Amyloid Fibril Formation of Parathyroid Hormone-Derived Peptides

André Paschold, Moritz Schäffler, Xincheng Miao, Luis Gardon, Stephanie Krüger, Henrike Heise, Merle I. S. Röhr, Maria Ott, Birgit Strodel, Wolfgang H. Binder

Bioconjugate Chemistry, Volume 35, Issue 7

<https://doi.org/10.1021/acs.bioconjchem.4c00188>

First published: 12 June 2024

Contribution: 5%

Performed solid-state NMR experiments and data interpretation demonstrating the interresidual spatial proximity between the side chains of L28 and F34. Contributed to manuscript writing and designed the corresponding figures.

Abstract:

Peptide fibrillization is crucial in biological processes such as amyloid-related diseases and hormone storage, involving complex transitions between folded, unfolded, and aggregated states. We here employ light to induce reversible transitions between aggregated and nonaggregated states of a peptide, linked to the parathyroid hormone (PTH). The artificial light-switch 3-{{(4-aminomethyl)phenyl}diazanyl}benzoic acid (AMPB) is embedded into a segment of PTH, the peptide PTH25-37, to control aggregation, revealing position-dependent effects. Through *in silico* design, synthesis, and experimental validation of 11 novel PTH25-37-derived peptides, we predict and confirm the amyloid-forming capabilities of the AMPB-containing peptides. Quantum-chemical studies shed light on the photoswitching mechanism. Solid-state NMR studies suggest that β -strands are aligned parallel in fibrils of PTH25-37, while in one of the AMPB-containing peptides, β -strands are antiparallel. Simulations further highlight the significance of π - π interactions in the latter. This multifaceted approach enabled the identification of a peptide that can undergo repeated phototriggered transitions between fibrillated and defibrillated states, as demonstrated by different spectroscopic techniques. With this strategy, we unlock the potential to manipulate PTH to reversibly switch

between active and inactive aggregated states, representing the first observation of a photostimulus-responsive hormone.

2.2.2 Disordered regions of inhibitor-bound α -synuclein suppress seed-induced fibril nucleation in cells

Celina M. Schulz, Emil D. Agerschou, Luis Gardon, Miriam Alexander, Matthias Stoldt, Henrike Heise, Gültekin Tamgüney, Wolfgang Hoyer

Cell Reports Physical Science, [Volume 5, Issue 9](#),

<https://doi.org/10.1016/j.xcrp.2024.102180>

First published: 18 September 2024

Contribution: 5%

NMR analysis of the alpha synuclein in complex with AS69 leading to the identification of the binding region. Contributed to manuscript writing and designed the corresponding figures.

Abstract:

Inhibitors of amyloid fibril formation can act in diverse ways and aid in elucidating the mechanisms of protein aggregation. The engineered binding protein β -wrapin AS69 binds monomers of Parkinson-disease-associated α -synuclein (α S), yet achieves inhibition at substoichiometric concentration. The substoichiometric activity was not attributed to the binding protein per se, but to its 1:1 complex with α S, in which AS69 sequesters α S residues 30–60 into a globular protein fold, whereas other α S parts remain intrinsically disordered regions (IDRs). Here, we investigate AS69- α S fusion constructs that form the AS69: α S complex by intramolecular folding and expose different IDRs. We find that not only the globular part of the complex but also α S IDRs are critical for substoichiometric inhibition, which is achieved by interference with primary and secondary fibril nucleation. The effects *in vitro* are reproduced in cellular seeding assays, indicating that secondary nucleation drives seeding in aggregate biosensing.

3

Unpublished Results

This Chapter presents additional experiments performed by me that have not been published. These experiments were designed to complement the findings of Chapter 2. Contributions from colleagues, where applicable, are acknowledged in the individual section.

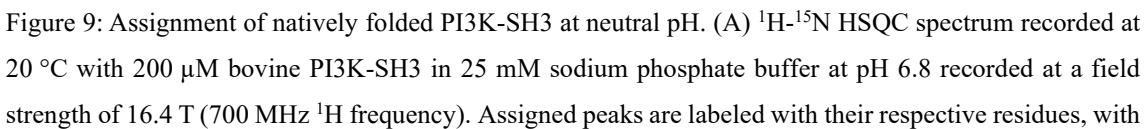
3.1. Assignment of native PI3K-SH3 by solution-state NMR

In this study, a backbone resonance assignment with partial side chain assignment of the bovine PI3K-SH3 domain in aqueous buffer at neutral pH was performed. Bovine PI3K-SH3 is known to adopt a β -sandwich structure in its native state (see Section 1.1.2). The NMR sample was prepared in a 25 mM sodium phosphate buffer at 20 °C and a pH value of 6.8. Well-dispersed peaks were observed in the ^1H - ^{15}N HSQC spectrum, indicating a globular folded protein. Resonance signals occurring >9 ppm in ^1H dimension are consistent with β -strand content (Figure 9 A).

Sequential assignment was conducted using the HNCACB, HNCOC and HNCA experiments. An exemplary excerpt of HNCACB spectra is depicted in Figure 9 B, red lines indicating the sequential link for $\text{C}\alpha$ and blue lines for $\text{C}\beta$. In these experiments, magnetization is transferred from ^1H to ^{15}N to $^{13}\text{C}\alpha$ (positive signal) and $^{13}\text{C}\beta$ (negative signal) of the same residue and to the preceding residue (with lower intensity) via J-coupling, enabling sequential assignment.

Additionally, ^{13}C - ^{13}C correlation-total correlation spectroscopy (TOCSY) spectra in solution were recorded under the same conditions for assigning ^{13}C chemical shifts of the side chain atoms. Peak doubling and shoulder signals were detected for residues near the C-terminus, most likely due to the presence of two conformations of the C-terminal proline residue (cis and trans) which was observed in ^1H - ^{15}N HSQC as well as ^{13}C - ^{13}C TOCSY spectra (Figure 9 C). The assignment data is presented in Table S1 in the appendix, and the chemical shifts are deposited in the BMRB under the accession code 53388. Chemical shift referencing was performed using DSS, setting the ^1H signal of the methyl group to 0 ppm.

Tobias Stief helped record HNCACB, HNCOC and HNCA spectra and Philipp Neudecker TOCSY spectra.



3.1. Assignment of native PI3K-SH3 by solution-state NMR

peak doubling observed for residues I82 and S83. Proline residues are not visible in the HSQC spectrum. (B) HNCACB strips of PI3K-SH3 recorded under the same conditions as in (A). Positive ($C\alpha$) contours are shown in green, while negative ($C\beta$) contours are displayed in purple. The sequential walk is indicated by red ($C\alpha$) and blue ($C\beta$) lines. (C) Excerpt of a ^{13}C - ^{13}C TOCSY correlation spectrum recorded under the same conditions as in (A) for the $C\alpha$ - $C\beta$ region of proline at a field strength of 18.8 T (800 MHz ^1H frequency).

A comparison with the resonance assignment of native bovine PI3K-SH3 by Hsu (2013)¹⁶⁴ in 50 mM potassium phosphate (pH 6.0) revealed that only a few resonances deviate by more than 1 ppm in ^{13}C or ^{15}N dimensions (Figure 10 A). This close correspondence indicates that the structure of PI3K-SH3, as represented by PDB entry 1PNJ¹⁰², which shares the same sequence and experimental conditions as Hsu's assignment, remains conserved.

The secondary structure propensities of individual amino acids in the PI3K-SH3 sequence was determined using TALOS¹⁶⁵, based on chemical shift data (Figure 10 B). Secondary chemical shift analysis indicates that the secondary structure elements of bovine PI3K-SH3 largely correspond to those observed in PDB 1PNJ¹⁰². Notably, the n-Src loop exhibits helical content, consistent with the findings of Hsu and previous studies^{102, 164, 166}.

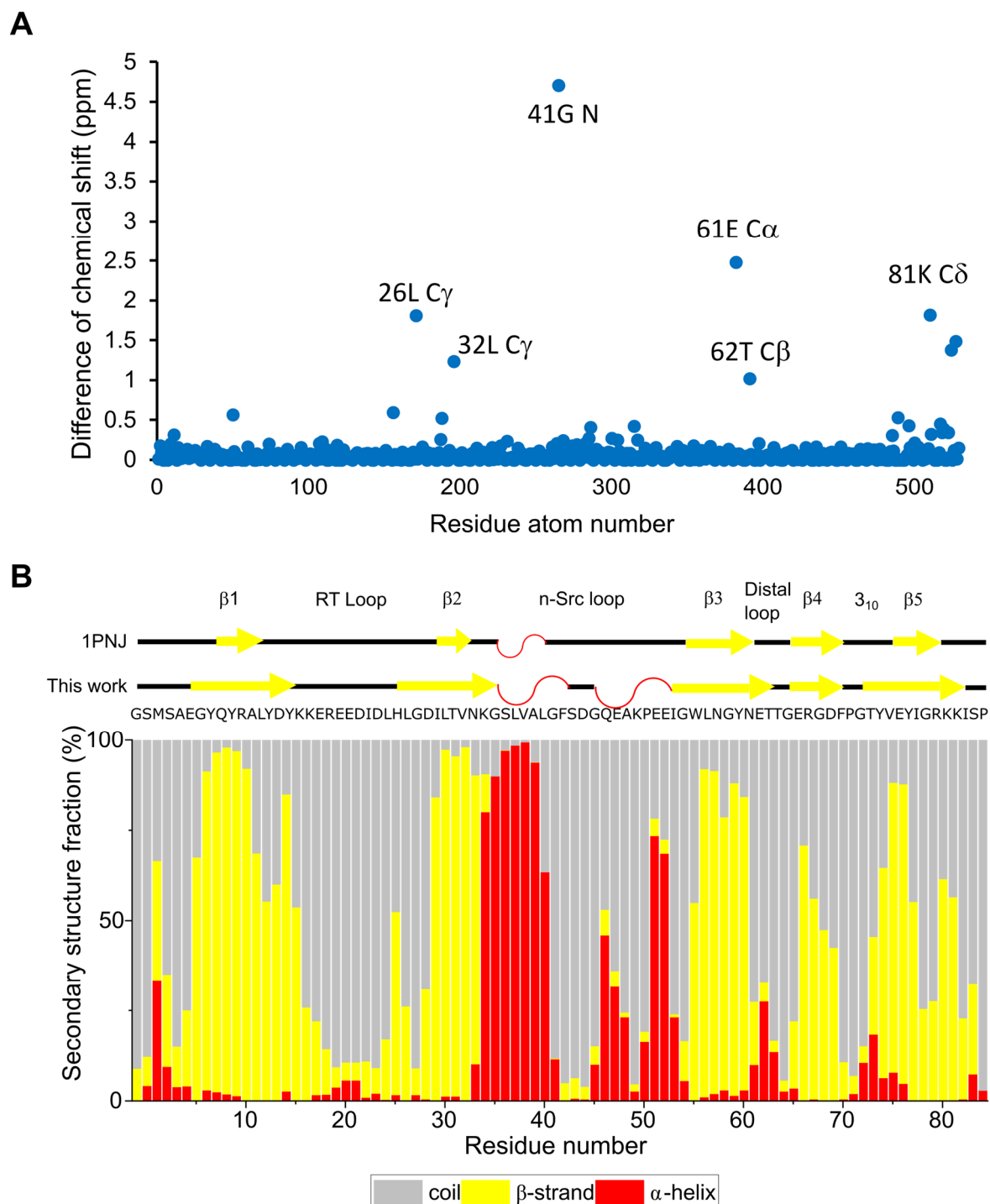


Figure 10: Assignment comparison with Hsu (2013). A) Chemical shift differences between this study and bovine PI3K-SH3 assignment by Hsu (2013). Resonances exhibiting deviations greater than 1 ppm are highlighted. B) Secondary chemical shift analysis of bovine PI3K-SH3 using TALOS. The secondary structure propensity for each residue is plotted as percentages, with coil (gray), β -strand (yellow), and α -helix (red). The primary sequence and the most likely secondary structure elements are shown above, compared to those reported by Booker et al. in the NMR structure (PDB:1PNJ)¹⁰². Characteristic β -sheet and loop elements typical of the SH3 domain family are also indicated.

3.2. Unfolding of PI3K-SH3 at low pH and high temperature

Lowering the pH causes PI3K-SH3 to unfold and eventually form amyloid fibrils^{105, 107}. However, the population of the unfolded state is influenced by solution conditions, and a folded population can persist under specific conditions¹⁰⁶. Earlier studies have shown that increasing salt concentration stabilizes more compact states¹⁰⁵.

To investigate this dependency, we systematically varied buffer concentration, temperature, and pH. In ¹H-¹⁵N HSQC spectra, a well-folded protein exhibits a well-dispersed set of peaks, whereas intrinsically disordered or unfolded proteins display a narrower spectral width. The presence of β -sheet content is typically indicated by peaks in the 8.5–9.5 ppm range. As shown in Figure 11, increasing the temperature from 10 °C to 40 °C shifts the equilibrium between folded and unfolded state of PI3K-SH3 toward a more unfolded population. Notably, this transition is reversible: lowering the temperature restores the folded state. Similarly, increasing the concentration of Gly-HCl from 10 mM to 50 mM significantly shifts the equilibrium toward the unfolded state. Decreasing the pH to 1.5 results in a similar effect, increasing the unfolded population of PI3K-SH3 (Figure 12). This shift is reflected in the ¹H-¹⁵N HSQC spectra, where the protein shows less dispersed peaks typical of unfolded states. This observation aligns with the effects of increasing temperature and higher Gly-HCl concentrations, which also promote unfolding.

Melinda Jaspert helped with the acquisition of the ¹H-¹⁵N HSQC spectra at different solution conditions.

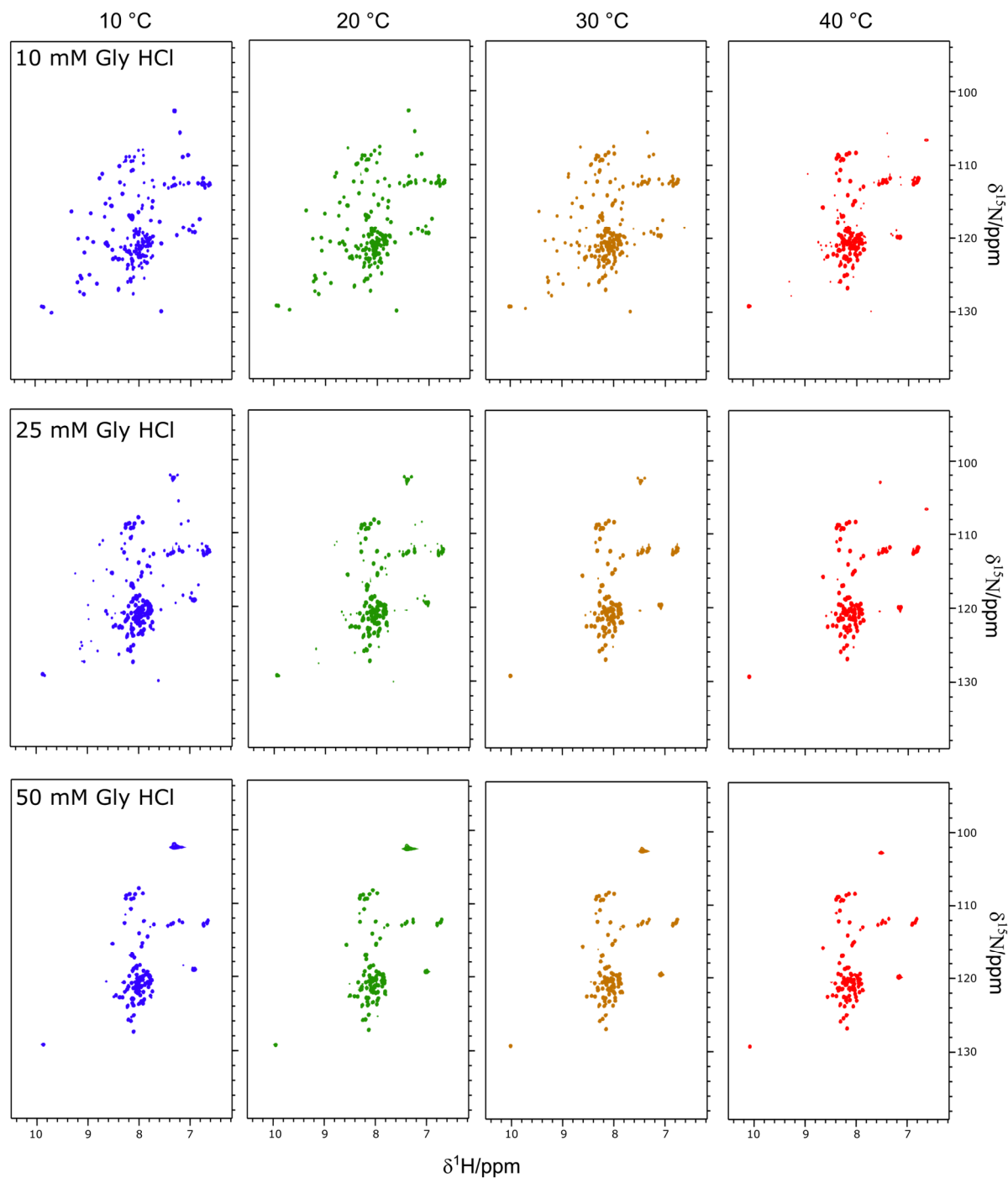


Figure 11: Solution-dependent equilibrium between folded and unfolded populations of PI3K-SH3. ^1H - ^{15}N HSQC spectra of 250 μM PI3K-SH3 are shown at varying temperatures and Gly-HCl concentrations. Spectra were recorded at 10 mM Gly-HCl (top), 25 mM Gly-HCl (middle), and 50 mM Gly-HCl (bottom) at a field strength of 16.4 T (700 MHz ^1H frequency). Temperature-dependent shifts in folding are indicated by color: blue (10 $^{\circ}\text{C}$), green (20 $^{\circ}\text{C}$), brown (30 $^{\circ}\text{C}$), and red (40 $^{\circ}\text{C}$). Increasing temperature and Gly-HCl concentration progressively shift the population toward the unfolded state.

3.2. Unfolding of PI3K-SH3 at low pH and high temperature

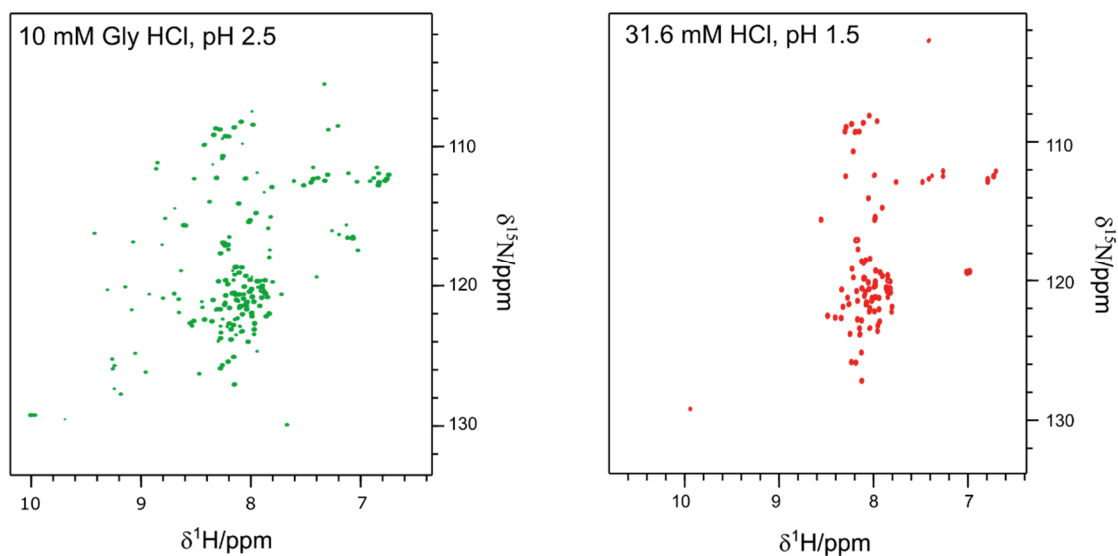


Figure 12: Folded and unfolded populations of PI3K-SH3 depending on pH values. ^1H - ^{15}N HSQC spectra of 250 μM PI3K-SH3 in 10 mM Gly-HCl at pH 2.5 (green) and pH 1.5 in aqueous HCl (red) at 20 $^\circ\text{C}$, recorded at 16.4 T (700 MHz ^1H frequency). In the red spectrum, the region from 8.5 ppm to 10 ppm is unpopulated, indicating a completely unfolded state.

3.3. Influence of aggregation conditions on PI3K-SH3 fibril morphology

As outlined in the previous section, solution conditions significantly influence the folding of the PI3K-SH3 domain. To assess their impact on the aggregation and fibril morphology, we performed AFM under varying pH conditions:

- 100 μ M of lyophilized monomeric PI3K-SH3 dissolved in 100 mM (pH 1), 31.6 mM (pH 1.5), 10 mM (pH 2), 3.2 mM (pH 2.5) and 1 mM (pH 3) of aqueous HCl and incubated for 3 weeks in 1.5 ml Eppendorf tubes at 50 °C.

AFM micrographs taken after three weeks of incubation at a pH value of 1 revealed formation of amorphous aggregates and bundled fibrils (Figure 13). At pH values of 1.5, fibrils were more prevalent alongside aggregates. The most well-defined fibrils, without amorphous aggregates, were observed for the incubation at pH values of 2 and 2.5, in agreement with previous findings in the literature¹⁰⁵. However, the number of fibrils was lower compared to those incubated at a pH value of 1.5. AFM images acquired at earlier time points (data not shown) further revealed that fibrils and aggregates begin to form within one day at pH values of 1 and 1.5 at 50 °C. Thus, decreasing the pH to values below 2 not only accelerates aggregation and fibrillation but also increases the formation of amorphous species. In contrast, at a pH value of 3, only low-density, slow forming aggregates were observed which may reflect early-stage aggregation events.

3.3. Influence of aggregation conditions on PI3K-SH3 fibril morphology

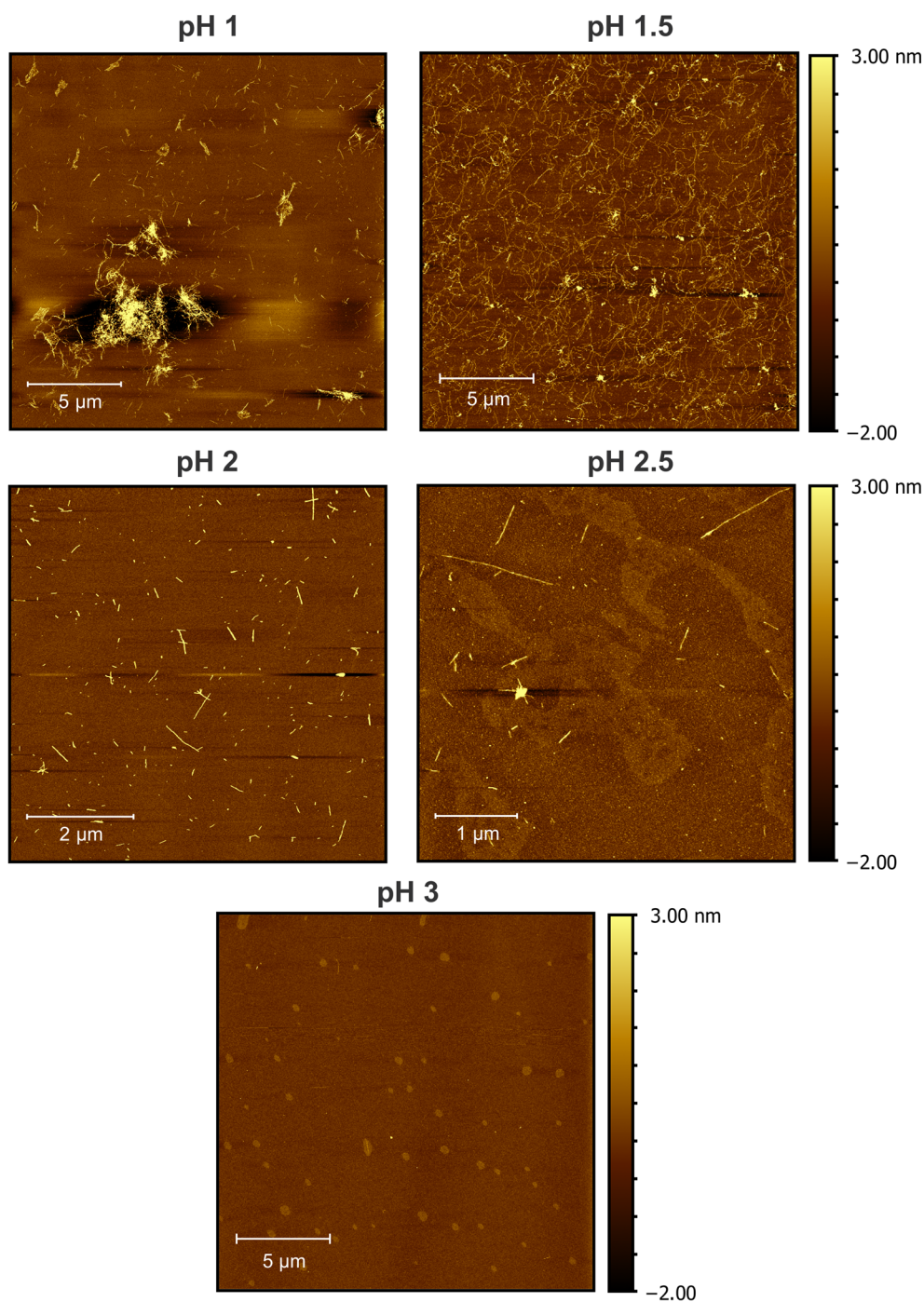


Figure 13: AFM micrographs of PI3K-SH3 aggregates after 3 weeks of incubation at different pH levels and 50 °C. Samples contain 100 μM PI3K-SH3 in aqueous HCl solutions of varying pH values, as indicated. After incubation under quiescent conditions at pH 1, clumps and amorphous aggregates dominate. At pH value of 1.5 fibrils as well as small aggregates are present. At pH 2 and pH 2.5, well-defined fibrils are visible. At pH values of 3 small condensates could be observed. These results indicate that lowering the pH accelerates aggregation while promoting the formation of amorphous aggregates.

We additionally tested the following conditions:

- **Fibrils pH 2/25°C:** 1 mM of lyophilized monomeric PI3K-SH3 dissolved in 10 mM aqueous HCl (pH 2) and incubated for 3 weeks at 25 °C and under quiescent conditions in 1.5 mL Eppendorf tubes according to Colvin et al.¹¹⁰.
- **Unseeded Fibrils pH 2.5/50°C:** 100 µM of lyophilized monomeric PI3K-SH3 dissolved in 10 mM Gly-HCl (pH 2.5) and incubated for 2 days at 50 °C without seeds and with 600 rpm shaking in 1.5 mL Eppendorf tubes.

AFM micrographs of the fibrils grown at pH 2/25 °C under quiescent conditions (Figure 14, left) revealed primarily amorphous aggregates with fibrillar structures interspersed, consistent with a less homogenous fibril formation at high protein concentrations. Unseeded fibrils grown at pH 2.5 and 50 °C in presence of 10 mM GlyHCl and shaking (Figure 14, right) appeared to be long and homogenous with only smaller aggregates visible. This confirms the results that at pH values between 2 and 3 more homogeneous fibrils form.

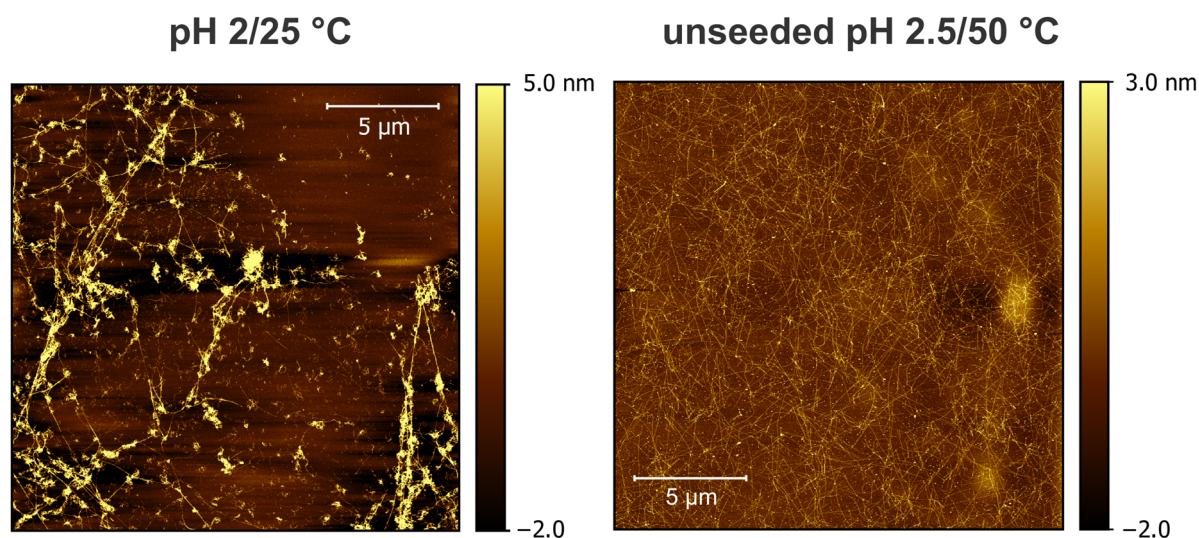


Figure 14: AFM micrographs of PI3K-SH3 at different aggregation conditions. Fibrils grown at pH 2, 1 mM PI3K-SH3 concentration and 25 °C for three weeks (left) show fibrillar structures as well as big aggregates that seem amorphous. Unseeded fibrils growth conditions at pH 2.5 in presence of 10 mM Gly-HCl and 50 °C for 2 days (right) show homogenous fibrils with smaller aggregates being visible.

3.3. Influence of aggregation conditions on PI3K-SH3 fibril morphology

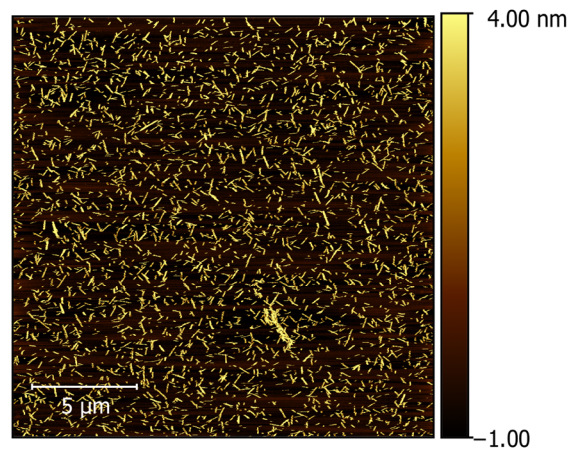
The longest, most homogeneous fibrils were obtained via seeded fibril formation of PI3K-SH3 as described by Röder et al.¹⁰⁹:

- **Seeded fibrils pH 2.5/50 °C:** 100 μ M of lyophilized monomeric PI3K-SH3 dissolved in 10 mM Gly-HCl (pH 2.5) and incubated for 1 day at 50 °C with 5% (mol/mol) preformed fibril seeds (mol equivalent to monomers) and under quiescent conditions in 1.5 mL Eppendorf tubes.

The fibril seeds were prepared by sonication, as described in Gardon et al.¹⁰⁶, resulting in fragmented fibrils that can act as nucleation sites for elongation. The resulting seeded fibrils were several microns in length and exhibited high morphological uniformity as observed by AFM (Figure 15).

PI3K-SH3 concentrations on the mica surfaces were 0.5 μ M for the fibril seeds and 2 μ M for all other samples.

Fibril seeds



Seeded fibrils

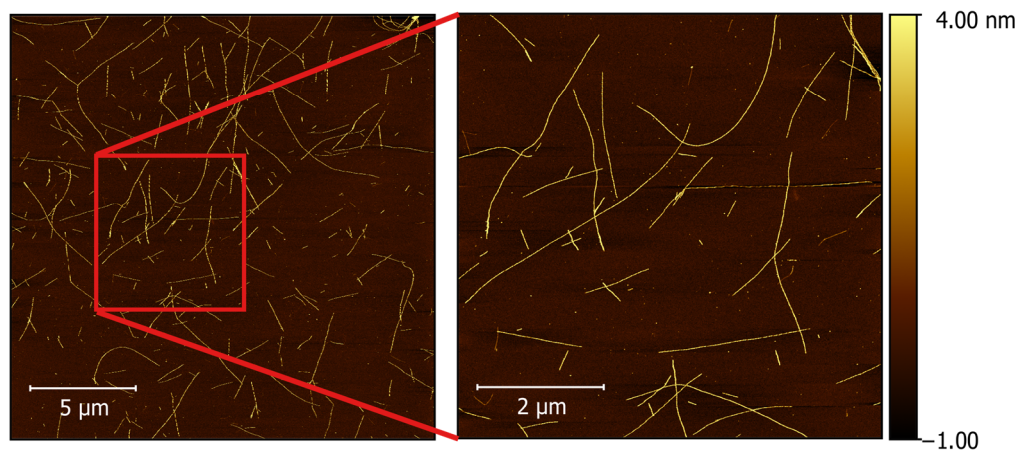


Figure 15: AFM micrographs of PI3K-SH3 seeds and fibrils. Seeds were produced by sonication of fibrils producing small fibrillar fragments. Incubation with 5% seeds (mol/mol) on 100 μM monomeric PI3K-SH3 in 10 mM Gly-HCl (pH 2.5) resulted in the formation of long, predominantly unbranched fibrils with high homogeneity. These fibrils exhibit a nanometer-scale width and extend to several microns in length.

3.4. Assignment of a PI3K-SH3 fibril polymorph

As described previously, the seeded fibril formation for PI3K-SH3 at pH value of 2.5 and 50 °C revealed the most homogeneous and well-defined amyloid fibrils, which we characterized using solid-state NMR.

For the seeded fibrils at pH 2.5/50 °C a high homogeneity is also evident from solid-state spectra as linewidths of the resonances are relatively narrow (~ 0.6 ppm for Ala residues (full width at half maximum at 11 kHz MAS and 800 MHz in PDSO spectra), allowing for the assignment of ~50% of the signals (Figure 16 A). The assigned residues are plotted on the cryo-EM structure of PI3K-SH3 fibrils¹⁰⁹ (PDB:6R4R; Figure 16 B).

For the assignment, we employed a combination of NCACX, CaNCO, NCOCA, and NCOCX spectra. An exemplary sequential walk is shown in Figure 17. The NCACX spectrum serves as a starting point, linking the N chemical shift to the C α and C γ of the same amino acid. The CaNCO spectrum connects the C α and N of residue *i* to the N of residue *i*-1, while NCOCA and NCOCX spectra correlate the N chemical shift of residue *i* with the CO and C γ of residue *i*-1. One continuous stretch of residues (Y12 to D28) could not be assigned and appears to be absent in the spectra. INEPT based experiments did not show any signals excluding highly flexible regions. ¹³C direct excitation spin diffusion 2D spectra also did not show significant differences to CP based spectra (data not shown). Additionally, several peaks remain visible in the spectra but could not be assigned unambiguously.

A significant contribution to the assignment process was made by Athina Apostolidis and Nina Becker. Nina Becker helped record NCACX, CaNCO, NCOCA, and NCOCX spectra.

3.4. Assignment of a PI3K-SH3 fibril polymorph

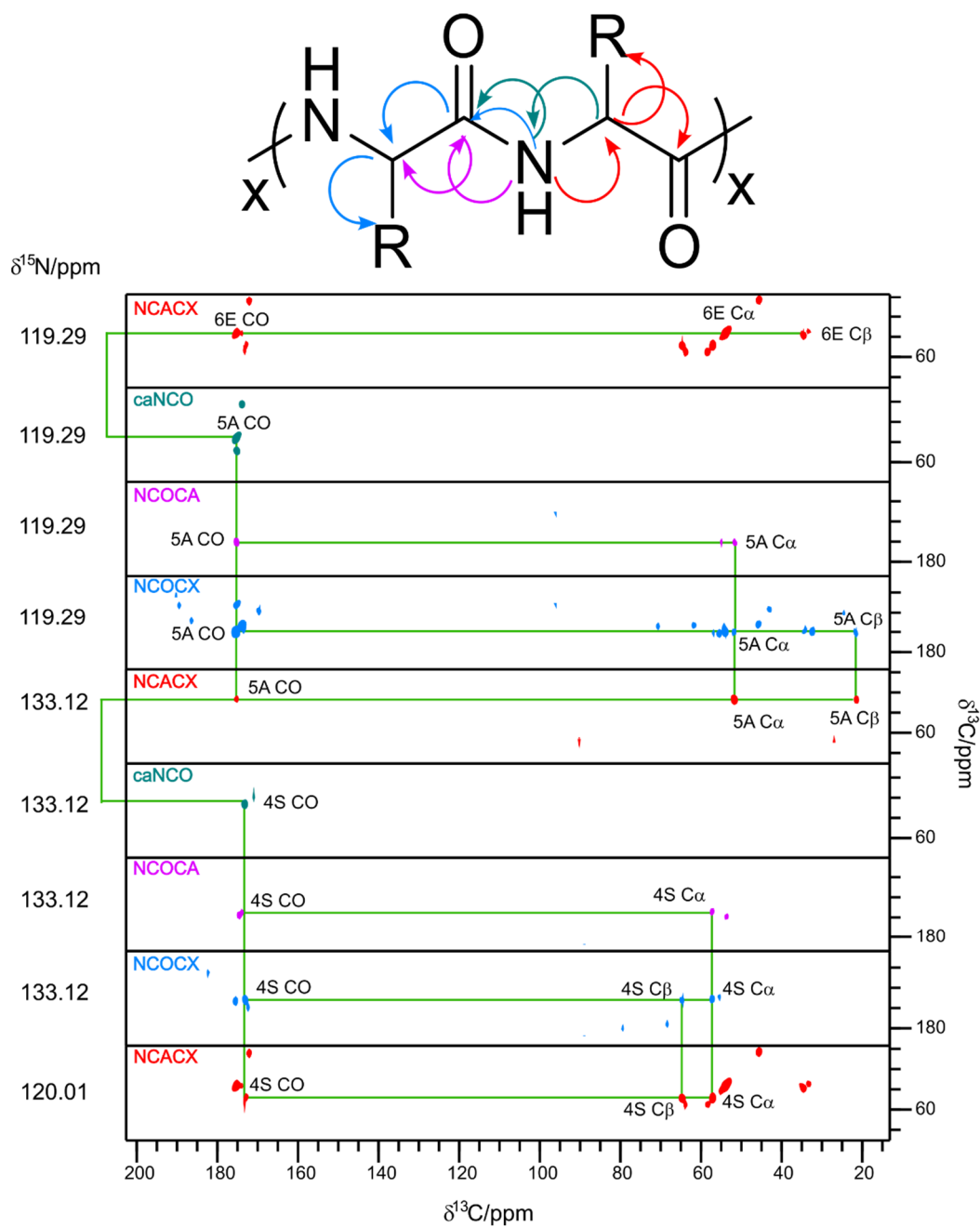


Figure 17: 3D spectral assignment strategy for fibrillar PI3K-SH3. The peptide backbone is shown with magnetization transfer pathways indicated by colored lines corresponding to different spectra: Red (NCACX) – transfer from N to C α and C χ of the same amino acid (i); Cyan (caNCO) – transfer from C α (not detected) to N of residue i and CO of residue i-1; Purple (NCOCA) – transfer from N of residue i to CO and C α of residue i-1; Blue (NCOCX) – transfer from N of residue i to CO and C χ of residue i-1. A sequential walk is represented by green lines. The spectra were recorded at a field strength of 14.1 T (600 MHz ^1H frequency)

3.5. Dependence of atomic structure on fibril growth conditions

As AFM micrographs provide only morphological information about aggregates and fibrils, we sought to understand how different fibrillation conditions alter the atomic structure of PI3K-SH3 fibrils. For this, we recorded ^{13}C - ^{13}C correlation PDSD spectra (Section 1.2.2) to obtain molecular fingerprints and compare fibrillar polymorphs present in the samples generated under different aggregation conditions. These conditions described in Section 3.4 included:

- **Fibrils pH 2/25°C:** 1 mM of lyophilized monomeric PI3K-SH3 dissolved in 10 mM aqueous HCl (pH 2) and incubated for 3 weeks at 25 °C and under quiescent conditions in 1.5 mL Eppendorf tubes according to Colvin et al.¹¹⁰.
- **Fibrils pH 1.5/50°C:** 100 μM of lyophilized monomeric PI3K-SH3 dissolved in 31.6 mM aqueous HCl (pH 1.5) and incubated for 2 weeks at 50 °C and under quiescent conditions in 1.5 mL Eppendorf tubes.
- **Unseeded fibrils pH 2.5/50°C:** 100 μM of lyophilized monomeric PI3K-SH3 dissolved in 10 mM Gly-HCl (pH 2.5) and incubated for 2 days at 50 °C without seeds and with 600 rpm shaking in 1.5 mL Eppendorf tubes.

The resulting PDSD spectra (Figure 18) show clear differences in chemical shifts compared to those from the partially assigned seeded fibril sample incubated with 10 mM Gly-HCl and 50 °C (Section 3.4, Figure 18, red). For the fibrils grown at pH 2/25 °C (Figure 18 A, black) fewer cross peaks were observed which could indicate either spectral overlap or a higher degree of structural flexibility in some regions. However, INEPT based spectra sensitive for highly flexible regions showed no signals, suggesting a lack of significantly dynamic regions. Notably, differences in the $\text{C}\alpha$ - $\text{C}\beta$ cross peak region of serine, threonine, alanine, and other areas were observed, suggesting a different local conformation of the fibrils.

The pH 1.5/50 °C fibrils exhibited even fewer detectable cross peaks (Figure 18 B, blue) and the visible signals were distinct from those of seeded pH 2.5/50°C fibrils. This further supports that different incubation conditions promote distinct fibrillar polymorphs for PI3K-SH3.

3.5. Dependence of atomic structure on fibril growth conditions

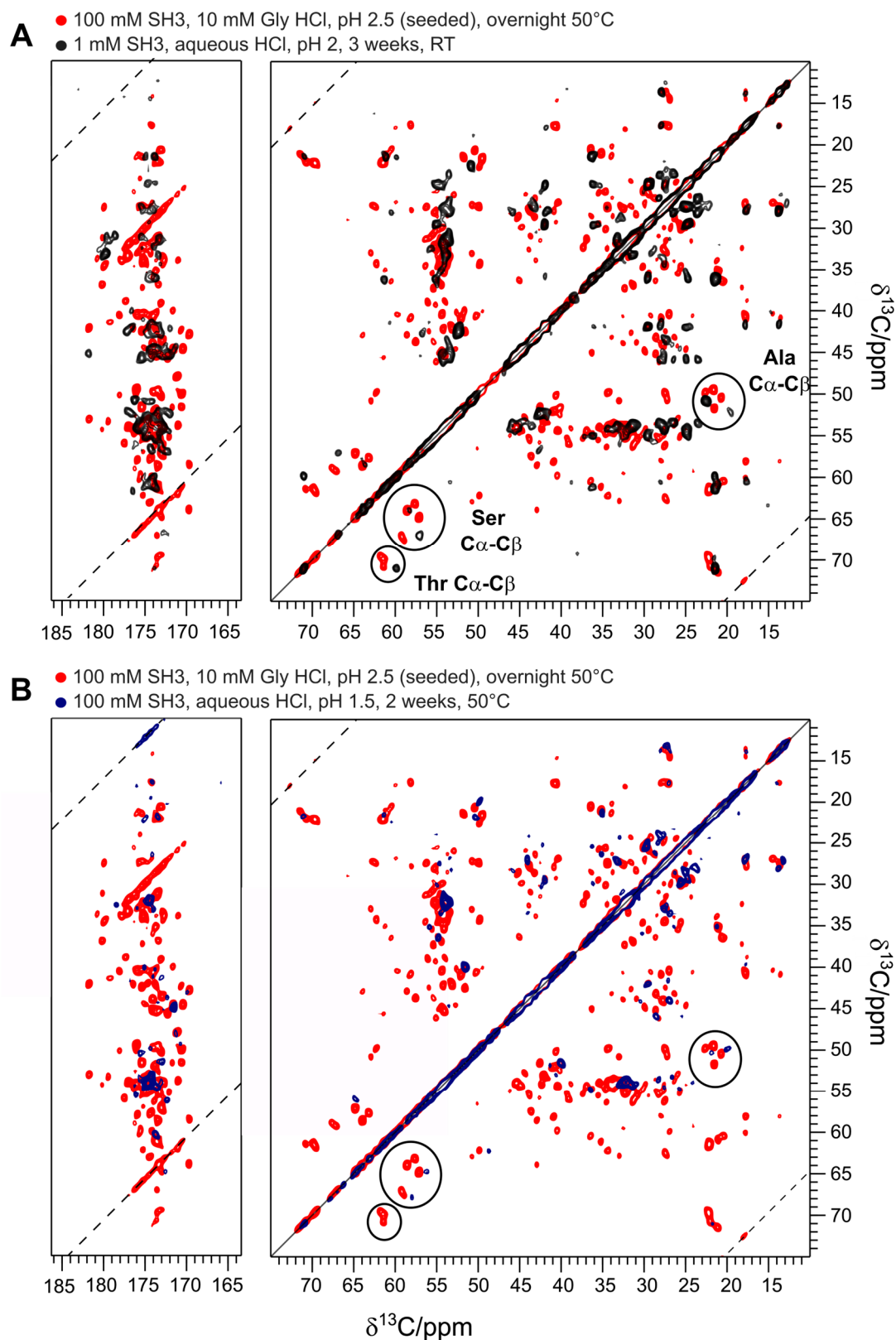


Figure 18: Comparison of the atomic structure of PI3K-SH3 fibrils grown at different conditions. A) PDSD spectra of seeded fibrils grown pH 2.5/50 °C (red, Section 3.4) and fibrils grown at pH 2/25 °C (black). B) PDSD spectra of seeded fibrils grown pH 2.5/50 °C (red, Section 3.4) and fibrils grown at pH 1.5/50 °C (blue). The spectra were recorded at a field strength of 18.8 T (800 MHz ^1H frequency) with 11 kHz MAS spinning and a 50 ms mixing time.

Upon closer investigation of the fibrils grown at pH 2/25 °C we observed notable differences in chemical shifts compared to those reported in Bayro et al.¹¹⁰, as indicated in Figure 19 A. The crosses denote previously published chemical shifts by Bayro et al. This suggests that a distinct polymorph has formed under these incubation conditions, differing from those previously described. Even more unexpectedly, the spectrum of fibrils grown unseeded under pH 2.5/50 °C shaking conditions display strikingly similar chemical shift patterns to those of the pH 2/25 °C fibrils (Figure 19 B). This indicates that structurally related polymorphs can arise despite considerable differences in incubation parameters (most notably temperature and protein concentration), highlighting the complexity of fibril formation for PI3K-SH3 fibrils.

3.5. Dependence of atomic structure on fibril growth conditions

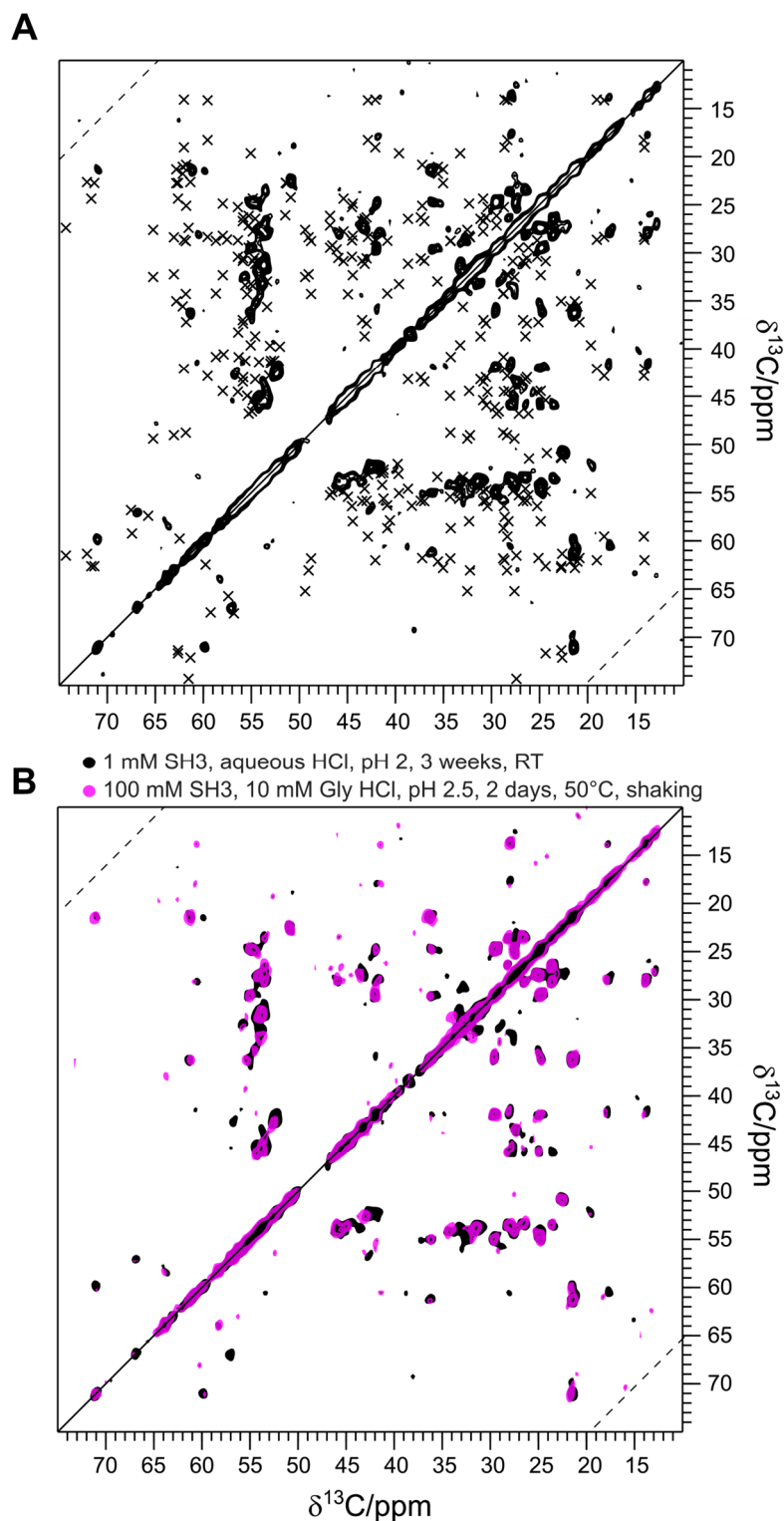


Figure 19: Atomic structure of PI3K-SH3 fibrils. A) PDSD spectrum of the aliphatic region of PI3K-SH3 fibrils grown at pH 2, following the protocol of Bayro et al.¹¹⁰ B) PDSD spectra of the aliphatic region of seeded fibrils grown pH 2/25 °C (black) and unseeded fibrils grown at pH 2.5/50 °C (pink). The spectra were recorded at a field strength of 18.8 T (800 MHz ^1H frequency) with 11 kHz MAS spinning and a 50 ms mixing time.

3.6. Rotational-echo, double-resonance (REDOR)- spin diffusion sequence 2D detection

In the revision of the paper “*Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy*”, an open question remains regarding the proximity of A42-CO and K28 N ζ in pEA β (3-42) fibrils. Based on the LS-shaped A β (1-42) structure (PDB:6R4R⁵⁶), the expected distance between K28 N ζ and A42 CO is estimated to be 5 Å, corresponding to a dipolar coupling of ~ 25 Hz, a challenging interaction to detect via NMR spectroscopy.

To probe this potential contact, we tested multiple approaches on both A β (1-42) and pEA β (3-42) fibrils. First, we recorded 2D NHHC spectra. After an initial ^1H to ^{15}N CP and t_1 evolution on ^{15}N , the magnetization was transferred back to ^1H via CP followed by ^1H - ^1H mixing to enable transfer to nearby ^1H nuclei. Subsequently, magnetization is transferred to ^{13}C via CP and detected. However, these 2D NHHC experiments did not reveal a cross-peak between A42 CO and K28 N ζ , in either LS-A β (1-42) or pEA β (3-42) fibrils. Since the NHHC experiment relies on the ^1H - ^1H mixing, it is less sensitive to dynamic protonated COOH group of A42. Therefore, the negative result of the NHHC experiment does not necessarily rule out a close proximity of K28 and A42.

As an alternative approach, we designed a frequency-selective REDOR experiment with 2D C α /CO detection (Figure 20). In a REDOR experiment¹⁶⁷, a series of rotor-synchronized 180° pulses is applied to recouple dipolar interactions between two heteronuclei, causing line broadening in the spectra. Comparing this to a reference spectrum without the REDOR element allows for distance estimation by varying the length of the REDOR element, as dipolar interaction scales with r^{-3} .

Our experiment involved an initial ^1H to ^{13}C CP step and t_1 evolution. The first 90° pulse on ^{13}C in the spin diffusion sequence element is a frequency selective sinc pulse (1 ms) with the irradiation point set to C α which reduces the spectral width in t_1 for C α . Magnetization is transferred back to transversal by the second 90° pulse with the irradiation point set to CO. Subsequently, the REDOR block is applied with two 180° pulses on ^{15}N per rotor period with irradiation point set to ^{15}N of interest. After four rotor periods, a selective 180° refocusing pulse is applied to both ^{15}N and ^{13}C (CO) followed by four additional rotor periods with REDOR recoupling.

3.6. Rotational-echo, double-resonance (REDOR)-spin diffusion sequence 2D detection

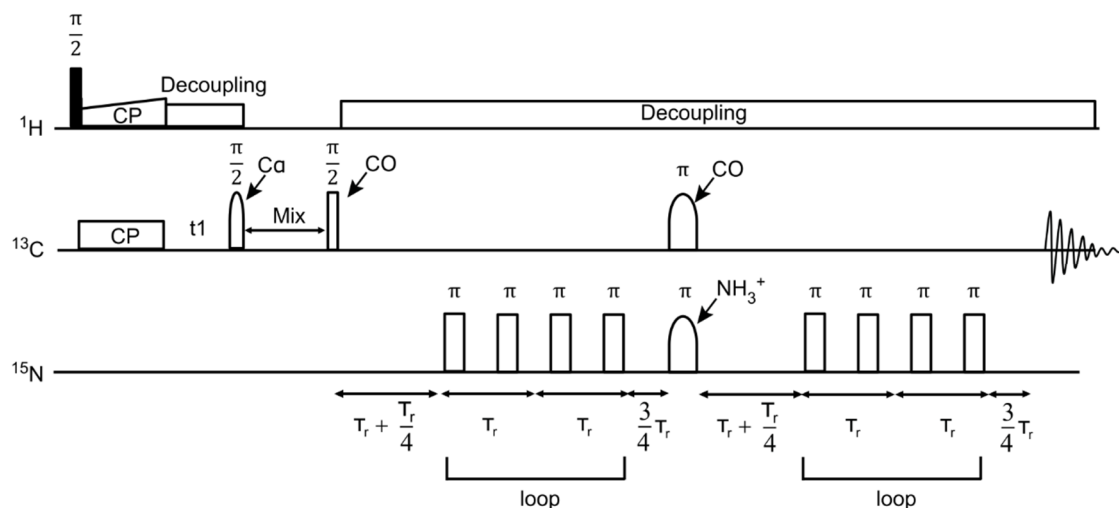


Figure 20: REDOR pulse sequence for ^{15}N - ^{13}C contacts with 2D ^{13}C - ^{13}C detection. Ramped CP is followed by t_1 evolution for resolution for $\text{C}\alpha$, which are flipped back to z axis by a selective 90° sinc pulse (1 ms) for spin diffusion. REDOR element: ^{13}C ^{15}N recoupling is achieved by two 180° hard pulses on ^{15}N per rotor period, refocusing of the magnetization in the middle of the sequence is achieved by selective gaussian shaped 180° refocusing pulses on ^{13}CO (182 μs) and $^{15}\text{NH}_3^+$ (545 μs) for REDOR-recoupling or a selective 180° pulse on ^{13}CO alone for the S_0 curve¹⁶⁸. Irradiation points were set to 51 ppm for $\text{C}\alpha$ and 179.5 ppm for CO of A42 and 40 ppm for NH_3^+ $\text{N}\zeta$ of K28. τ_r is the duration of one rotor cycle (90.9 μs at 11 kHz). Proton decoupling during t_1 evolution, the selective 90° pulse of the spin echo and the REDOR element is achieved by SPINAL-64 decoupling.

To validate this sequence, we tested it on glutamate. Figure 21 shows the effect of the REDOR recoupling compared to a reference spectrum. The 1D spectra are the first FID of a 2D experiment as proof of concept. The reference spectrum was obtained by setting the selective ^{15}N refocusing 180° pulse in the REDOR element to 0. After a REDOR recoupling time of 11.66 ms (32 loop counters), we observed a significant REDOR effect. The higher field signal corresponding to the CO group exhibits greater line broadening and signal attenuation compared to the $\text{C}\delta$ of glutamate (Figure 21). This is expected, as the amide group is in closer proximity to the CO group than to the $\text{C}\delta$, leading to a stronger dipolar interaction.

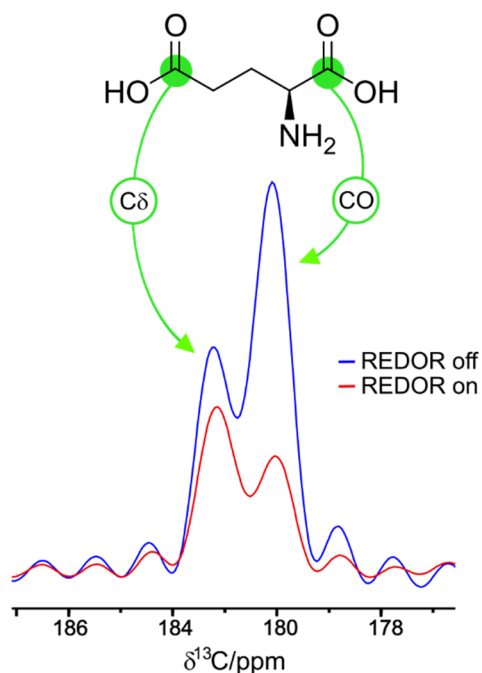


Figure 21: Effect of the frequency selective REDOR pulse sequence on glutamate. The 2D pulse sequence shown in Figure 20 was tested as 1D experiment with 10 ms mixing time in spin diffusion, 11 kHz MAS frequency and 14.1 T (600 MHz 1H Larmor frequency). Selective sinc pulse on $C\alpha$ is set to 56 ppm and for 1 ms. The REDOR cycle was set to 11.6 ms. In the reference spectrum (blue), recorded without a ^{15}N refocusing 180° pulse in the REDOR element, the signals corresponding to $C\delta$ (~183 ppm) and CO (~180 ppm) of glutamate are labeled with arrows. In the REDOR spectrum, the CO signal exhibits greater attenuation compared to $C\delta$, reflecting its closer proximity to the NH_3 group.

For $A\beta$ fibrils, however, the pulse sequence did not yield the expected results. The selective sinc pulse carrier was set to 51 ppm ($C\alpha$ of A42) and the carrier frequency was subsequently set to 179 ppm (CO of A42). While the A42 $C\alpha/CO$ cross-peak was detectable and well-resolved for short REDOR sequences (Figure 22), the T_2 relaxation of the CO signal proved too fast for a total recoupling time of 5.8 ms. Detecting a dipolar coupling of 25 Hz would have required at least 10 ms of recoupling, which was not possible under these conditions. The Bruker code for implementing the sequence in TopSpin (version 4) is given in the appendix.

3.6. Rotational-echo, double-resonance (REDOR)-spin diffusion sequence 2D detection

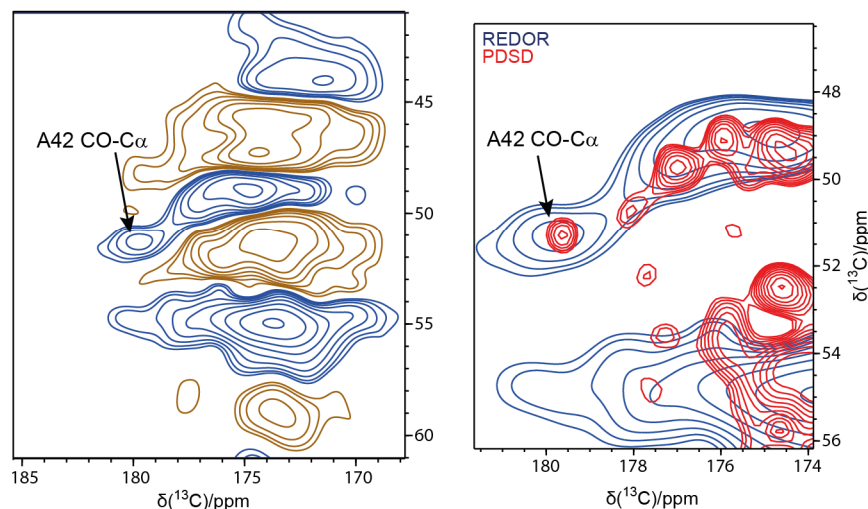


Figure 22: Frequency selective REDOR spectra of pEA β (3-42) fibrils. Left: The spectrum recorded at a field strength of 14.1T (600 MHz ^1H Larmor frequency) at a MAS of 11 kHz with 200 ms spin diffusion mixing time with positive (blue) and negative (brown) contour lines with zero REDOR cycles (but including the selective 180° pulse on ^{13}CO) show that the A42-CO-C α cross-peak is detectable (and sort of distinguishable from other signals). Phase modulation along F1 is caused by the excitation profile of the selective 90° sinc pulse. Right: Overlay with a standard PDSF spectrum recorded at a field strength of 18.4 T (800 MHz ^1H frequency) at a spinning speed of 11 kHz with 200 ms spin diffusion mixing time confirm the position of the A42-CO-C α cross-peak (right). Unfortunately, after 5 ms of REDOR recoupling time, no A42 CO-C α cross-peak was visible (data not shown).

3.7. PI3K-SH3 protein purification

The following protocol for purification of PI3K-SH3 is taken from Gardon et al.¹⁰⁶ with small additions and detailed figures for the procedure:

The bovine PI3K-SH3 domain was expressed in *Escherichia coli* BL21 (DE3) as an N-terminal glutathione S-transferase (GST)-fusion followed by a thrombin protease cleavage site using pGEX-4T (GenScript Biotech, Netherlands) as vector plasmid. The 86 amino acid sequence of the purified bovine PI3K-SH3 domain used is GS MSAEGYQYRA LYDYKKEREE DIDLHLGDIL TVNKGSLVAL GFSDGQEA KP EEIGWLNGYN ETTGERGDFP GTYVEYIGRK KISP with the N-terminal Gly-Ser residues remaining as overhang from the thrombin cleavage.

BL21 cells were grown in M9 medium uniformly ¹³C and ¹⁵N labeling with 3.2 g U-¹³C-glucose and 2 g ¹⁵N-ammonium chloride per 1 culture medium as the sole carbon and nitrogen sources) at 37 °C for ~4.5 h until an OD600 of 0.6 was reached. Protein expression was induced by the addition of 0.5 mM isopropyl β-d-1-thiogalactopyranoside. The cells were then grown overnight at 28 °C, harvested by centrifugation (5.000 x g, 15 min) and resuspended in 5–8 mL per g cell mass of lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 100 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.6 containing 5 mg/mL lysozyme). Successful induction of protein expression was confirmed via SDS-PAGE, showing a prominent band at ~35 kDa corresponding to the GST-SH3 fusion protein (Figure 23).

3.8. PI3K-SH3 protein purification

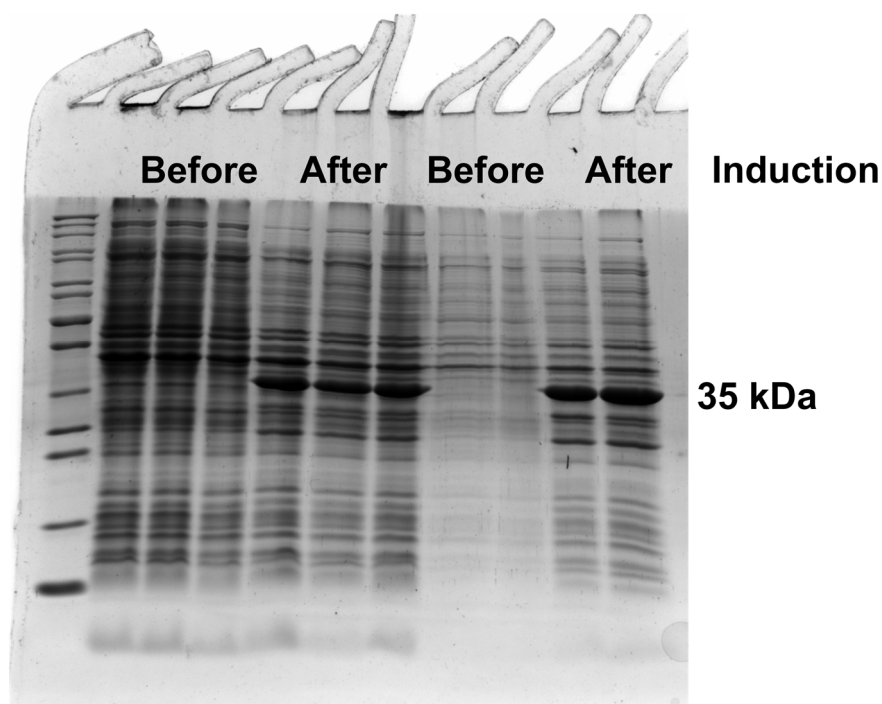


Figure 23: SDS-PAGE analysis of GST- PI3K-SH3 expression. A distinguishable band at 35 kDa appears after IPTG induction indicating successful expression of GST-PI3K-SH3 fusion protein.

After sonication with a Bandelin Sonopuls sonicator (Bandelin) using a VS 70T probe (60% amplitude, 3×5 min, 3 s “on,” 5 s “off,” ice cooling) the solution was ultracentrifuged in an Optima XPN-80 ultracentrifuge (Beckman) equipped with a 70Ti rotor at 4 °C and 42.000 rpm for 1 h. The supernatant of the ultracentrifugation was loaded on two serially connected 5 mL Protino GST/4B columns (Macherey-Nagel, Düren, Germany) equilibrated with 50 mM HEPES buffer, 100 mM NaCl, pH 7.6, and 3 mM Dithiothreitol, pH adjusted with NaOH. The protein was eluted with 20 mM reduced glutathione in 50 mM HEPES, 100 mM NaCl, 3 mM DTT, pH adjusted to a value of 7.6 with NaOH and detected via SDS-PAGE (Figure 24).

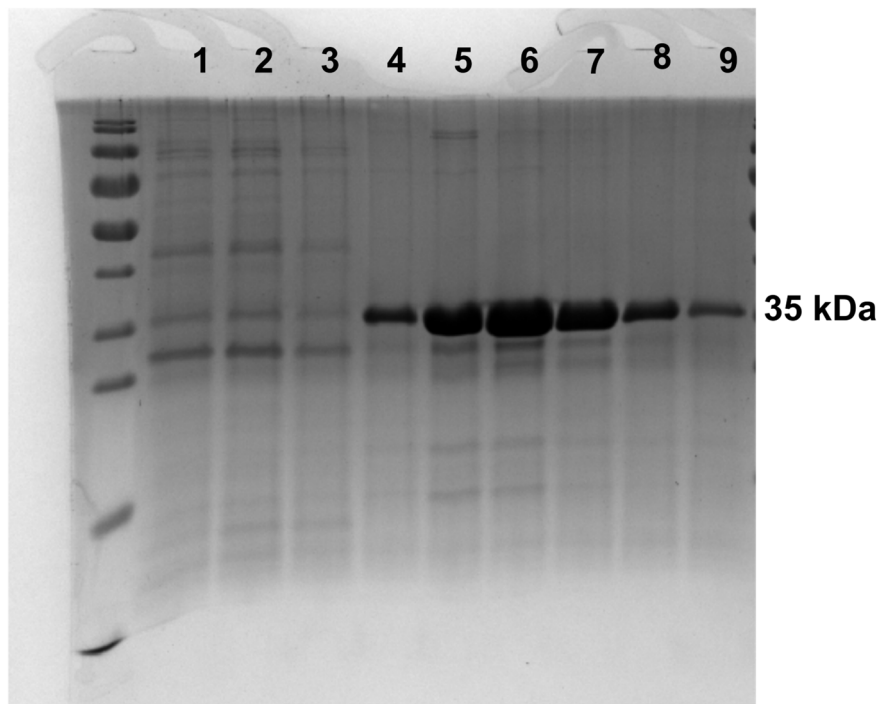
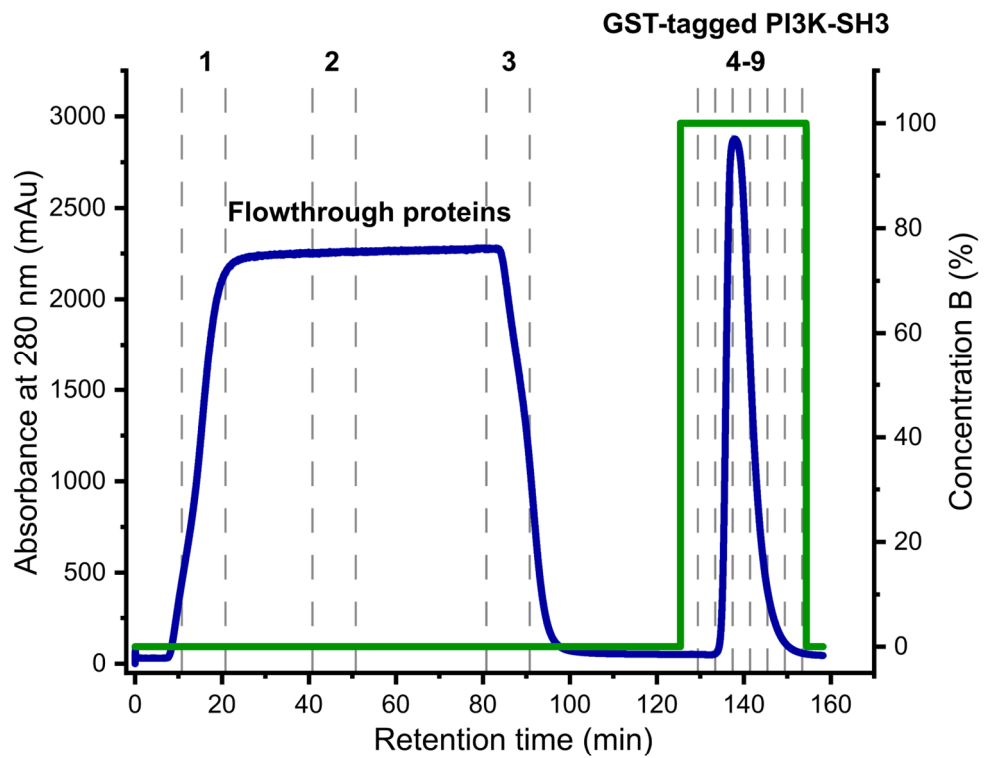


Figure 24: GST affinity chromatography and SDS-PAGE analysis. A) GST-PI3K-SH3 was purified from the lysate using GST-affinity chromatography on a GST-4B column. The fusion protein bound to the column and was eluted using glutathione containing buffer. The collection points of different fractions for SDS-PAGE analysis throughout the affinity chromatography are indicated. B) SDS-PAGE analysis of collected fractions. A distinct band at ~35 kDa corresponding to GST-PI3K-SH3 is observed in the elution fractions, while no such band is visible in the flowthrough fractions indicating successful binding to the column.

3.8. PI3K-SH3 protein purification

The fractions containing the eluted GST-PI3K-SH3 fusion protein (SDS-PAGE, Figure 24) were cleaved using 5–7.5 NIH units of thrombin (SERVA Electrophoresis GmbH, Heidelberg, Germany) per mg protein for 2 days at 4 °C under mild shaking conditions. The cleavage efficiency was assessed by SDS-PAGE analysis (Figure 25) where incubation with thrombin resulted in two distinct bands corresponding to GST (~25 kDa) and SH3 (~10 kDa) indicating successful cleavage of the fusion protein.

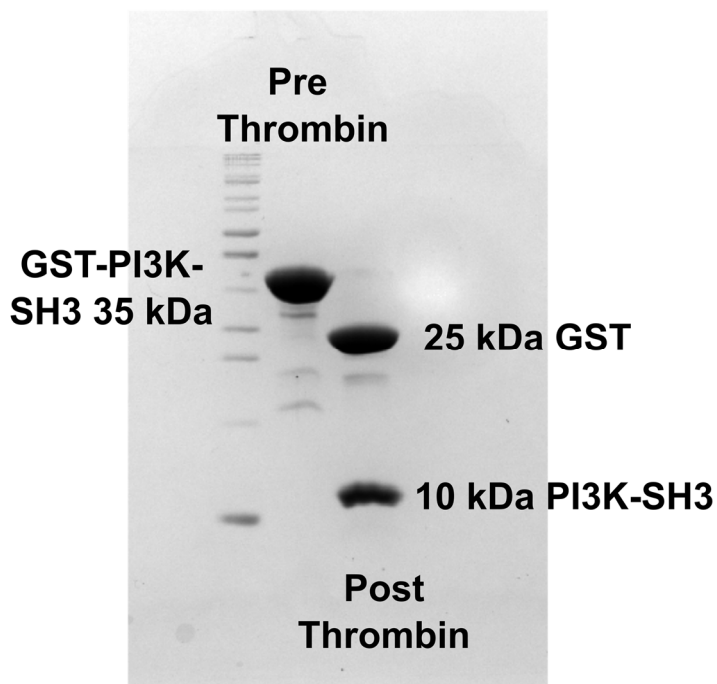


Figure 25: Thrombin cleavage of GST-PI3K-SH3. SDS-PAGE analysis revealed that the band referring to GST-PI3K-SH3 at 35 kDa is split into two bands, 25 kDa for GST and 10 kDa for PI3K-SH3 after thrombin incubation, indicating successful cleavage.

Then, the solution was loaded on a Superdex 75 HiLoad 16/600 size exclusion column (GE Healthcare Europe GmbH, Freiburg, Germany), equilibrated, and run with 5 mM ammonium acetate (pH 7.7) as a volatile buffer system, leading to the separation of the cleaved GST from PI3K-SH3 as seen in chromatogram and SDS-PAGE (Figure 26).

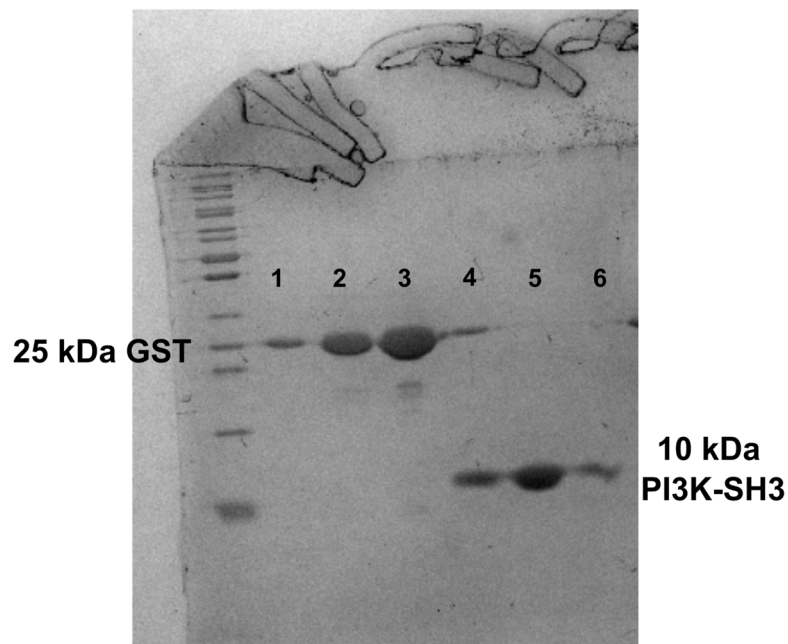
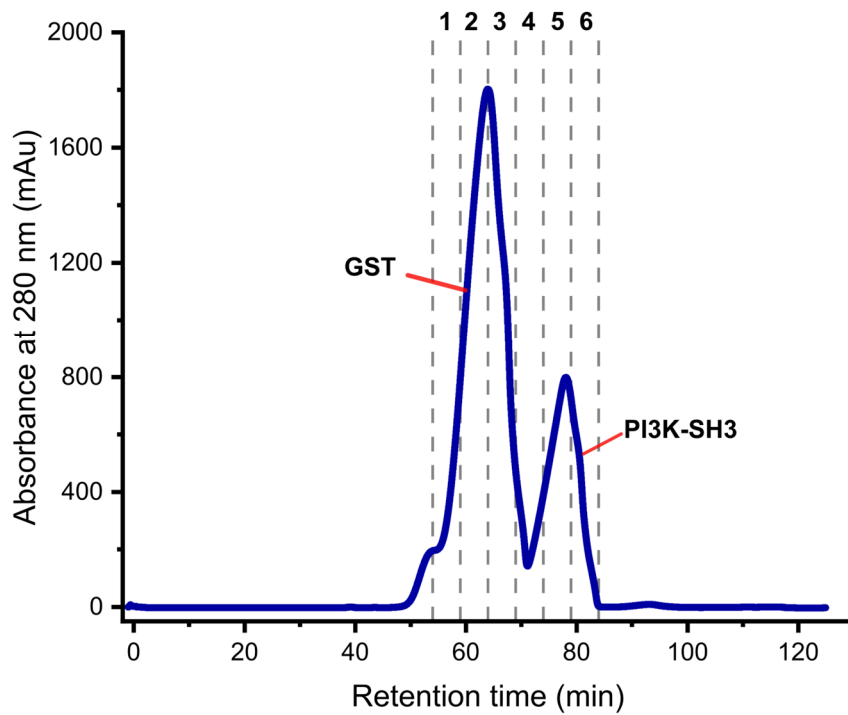


Figure 26: Size-exclusion chromatography (SEC) of cleaved GST and PI3K-SH3. A) Exemplary SEC chromatogram. Due to its higher molecular weight, GST elutes earlier than PI3K-SH3. The collection points of different fractions for SDS-PAGE analysis throughout the SEC chromatography are indicated. B) SDS-PAGE analysis of the collected SEC fractions. The first elution peak corresponds to a band at ~25 kDa (GST) while the second peak shows a band at ~10 kDa, corresponding to PI3K-SH3 domain.

3.8. PI3K-SH3 protein purification

To ensure complete removal of GST traces, fractions containing PI3K-SH3 were pooled and loaded again on two serially connected 5 mL Protino GST/4B columns (Macherey-Nagel, Düren, Germany) equilibrated with 5 mM ammonium acetate (Figure 27). The 5 mM ammonium acetate buffer was used as a volatile buffer to facilitate lyophilization. As PI3K-SH3 lacks the GST-tag after thrombin cleavage, PI3K-SH3 does not bind to the columns. The flow-through fractions containing purified bovine uniformly ^{13}C and ^{15}N labeled PI3K-SH3 were pooled, aliquoted, and freeze-dried for further usage. Analysis by SDS-PAGE and NMR suggest bovine PI3K-SH3 purities above 97%, as neither method detected impurities.

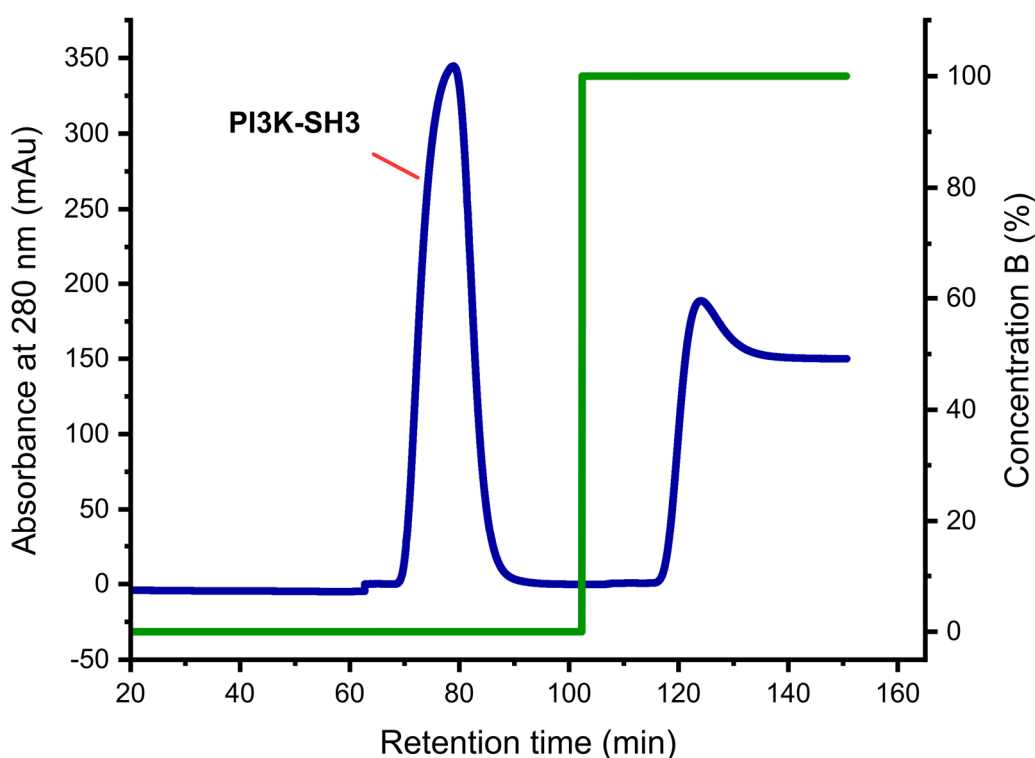


Figure 27: Second GST affinity chromatography. Residual GST protein, not fully separated by the prior SEC, is retained on the GST-affinity column, while PI3K-SH3 flows through. Elution with glutathione containing buffer elutes remaining GST.

4

Discussion and Outlook

NMR has proven to be an essential tool in the study of amyloidogenic proteins, offering atomic-level insights into their structure, dynamics, and aggregation behavior. In this work, we applied both solid-state and solution-state NMR to explore these aspects in detail.

We presented an in-depth structural investigation of a pyroglutamylated A β (3–42) fibril polymorph⁹⁸ (Figure 28), grown under the same conditions as the well-characterized LS-shaped A β (1–42) fibrils⁵⁶. While previous studies, such as those by Scheidt et al.^{93, 94}, reported only minor structural changes in pEA β variants with a flexible N-terminus, our chemical shift analysis revealed significant deviations at the N- and C-terminus. This suggests a distinct conformational influence exerted by the pyroglutamate modification despite a conserved turn region and rigid N-terminus (Figure 28). In the LS-shape A β (1–42) fibrils, however, the N-terminus is part of the fibril core and stabilized by a salt bridge between K28 and the side chain of D1 under acidic conditions (pH value of 2)^{169, 169}. Upon shifting the pH of LS-shape A β (1–42) fibrils, this interaction rearranges toward a salt bridge between K28 and the deprotonated C-terminus of A42, supported by molecular dynamics simulations. This observation highlights a unique advantage of NMR: its ability to directly probe the protonation states and rates of specific residues through their chemical shifts, not accessible by other structural techniques.

Clearly, the stabilizing interaction of K28 and D1 at pH 2 is not present in pEA β (3–42) fibrils as the first two amino acids are missing. In pEA β (3–42) fibrils, the absence of the native N-terminus leaves G37 and G38 exposed, which exhibit the most pronounced chemical shift perturbations compared to A β (1–42) fibrils at pH 2, likely reflecting solvent interactions or altered packing. However, the N-terminus in pEA β (3–42) fibrils is rigid, as pE3 shows up in dipolar based CP spectra and the N-terminal residues show high β -sheet content⁹⁸, indicating that the LS-shape is conserved. Interestingly, the C-terminal chemical shifts of pEA β (3–42) fibrils at pH 2 share notable similarities with those of A β (1–42) fibrils at pH 7¹⁶⁹, suggesting a convergence of conformational states despite different chemical environments. This could point toward a similar backbone conformation or, as a braver hypothesis, a shared salt-bridge rearrangement mechanism involving the C-terminus of A42 and K28.

These findings open exciting avenues for future experiments. For instance, cross-seeding A β (1–42) with pEA β (3–42) could reveal which structural features, particularly at the C-terminus, are conserved or propagated by pEA β (3–42) seeds. This could be

experimentally accessed by ^{13}C – ^{13}C correlation experiments with solid-state NMR. Due to their stability under variable pH conditions, these LS-shaped fibrils, whether $\text{A}\beta(1\text{--}42)$ or $\text{pEA}\beta(3\text{--}42)$ fibrils, provide a robust platform for further interaction studies. They are well suited to investigate residue-specific binding events and conformational rearrangements upon ligand or peptide binding (e.g. D-peptides¹⁷⁰), utilizing one of the key strengths of NMR spectroscopy: the ability to directly observe molecular interactions at atomic resolution^{171, 172}.

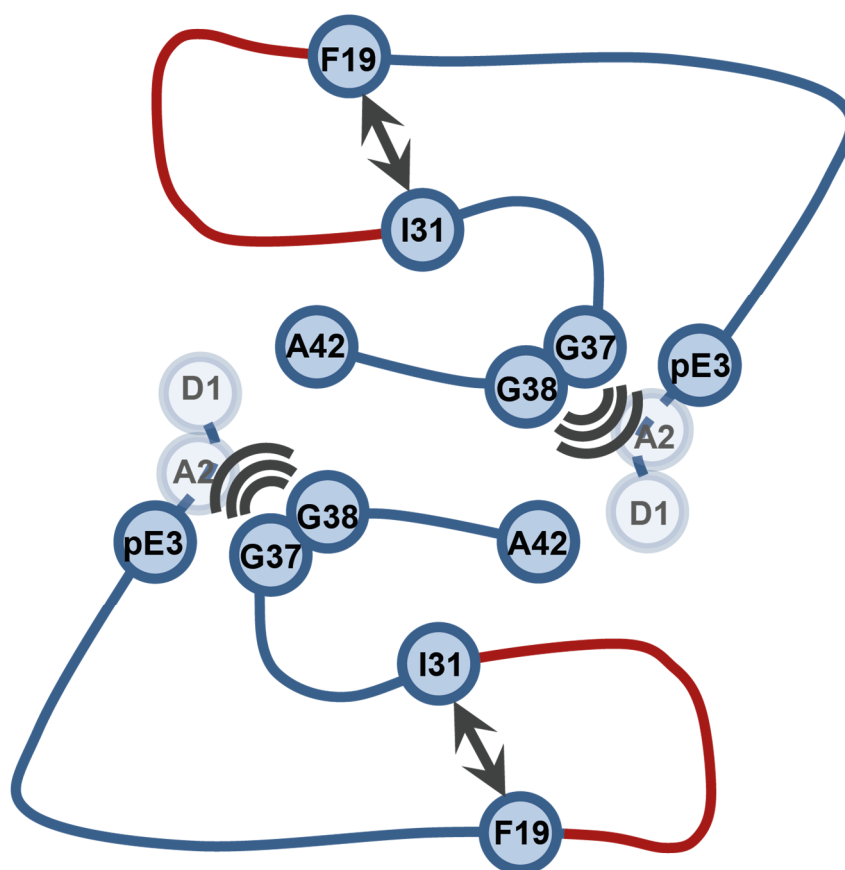


Figure 28: Structural findings for $\text{pEA}\beta(3\text{--}42)$ fibrils in comparison with LS-shaped $\text{A}\beta(1\text{--}42)$ fibrils adapted from ⁹⁸. The LS-shape with β sheet regions is conserved. The first two amino acids are absent, and the N-terminal glutamate is converted to a pyroglutamate residue (pE3). A spatial contact between F19 and I31 was observed and the turn region V24-K28 is conserved as indicated by minor chemical shift deviations. G37 and G38 act as sensors for the missing N-terminus.

In further studies, we examined the PI3K-SH3 domain across its amyloidogenic conformations, including the native, folded state, intermediate states, and fibrillar states.

We performed a backbone and side chain resonance assignments for the monomeric native state of PI3K-SH3, which are largely in agreement with the findings of Hsu et al.¹⁶⁴ (Section 3.1, Figure 10). However, in contrast to their assignment, we observed evidence

for a second conformational state at the C-terminus and nearby residues (Figure 29). While a proline cis/trans isomerization at the C-terminus could in principle explain the peak doubling for the C-terminal residues, such isomerization at this position has not been reported and is unlikely as terminal prolines typically experience high conformational freedom and lack steric constraints. Alternatively, the data may reflect conformational heterogeneity in this region. Solvent PRE studies showed the accessibility of the solvent with Dotarem as a paramagnetic probe on the native monomeric state. We observed a “hot spot” of PRE effect in a certain area, that is close to the canonical binding site of PI3K-SH3 to proline rich peptides^{102, 103} potentially indicating transient interactions with Dotarem. Alternatively, PI3K-SH3 might tend to self-assemble, protecting other regions from the solvent.

The unfolded state of PI3K-SH3 present at low pH values also displayed intriguing features (Figure 29). At pH 2.5, we observed a population of both folded and unfolded species, as indicated by a double set of peaks in ^1H - ^{15}N -HSQC spectra. The equilibrium between these two conformations appears to be sensitive to ionic strength, temperature, and pH (Figure 11 and 12), suggesting a dynamic exchange. As demonstrated in Section 3.2, lowering the pH value below 2.5, increasing the salt concentration of glycine hydrochloride, or raising the temperature shifted the equilibrium towards the unfolded state. Aggregation proceeds more rapidly under conditions that favor the unfolding suggesting that the unfolded state represents the more aggregation prone species.

A similar phenomenon was reported by Zurdo et al., who described a partially folded state at low pH values relevant for the aggregation behavior of PI3K-SH3¹⁰⁵. At pH values below 2 they observed amorphous aggregates and a faster aggregation, which we independently verified by AFM (Section 3.3). In addition, they observed that increasing salt concentration at constant low pH values also accelerated aggregation of PI3K-SH3. Interestingly, hydrodynamic radius measurements performed by Zurdo et al. revealed a more compact structure of the unfolded, state at pH values below 2.4¹⁰⁵.

Further studies could explore the influence of buffer composition and, even more promising, protein concentration on this equilibrium, as crowding effects may shift the balance between folded and unfolded states. Higher protein concentrations change the buffer salt-to-protein ratio, suggesting a pronounced observation of folded state at higher PI3K-SH3 concentrations. What remains unclear is whether the folded state undergoes conformational exchange with the unfolded state without directly contributing to

aggregation, or whether it plays a more active and complex role in the aggregation and fibrillation process. Clarifying the contributions of these conformational species in the early stages of PI3K-SH3 aggregation represents an important direction for future work.

We demonstrated that a combination of solution-state and solid-state NMR techniques can be used effectively to monitor the kinetics of protein aggregation¹⁰⁶ (Figure 29). While monomeric species with rapid tumbling are accessible via INEPT-based experiments, larger, slowly tumbling aggregates such as fibrils can be detected using CP-based methods. For the seeded aggregation of PI3K-SH3, no invisible species could be detected by NMR as the decay of INEPT signal closely mirrored the increase in CP signal. This differs from observations on FUS LC, where invisible intermediates were inferred¹³³ (Section 1.2.3). This likely reflects the fact that elongation is the primary mechanism in our seeded aggregation study and invisible species emerge more at early nucleation events when no protofibrils are formed yet. To complement the NMR data, we used atomic force microscopy (AFM) at the end of the experiments to confirm fibril formation and to exclude the presence of large amounts of amorphous aggregates that might influence kinetic interpretations.

Notably, both INEPT and CP based NMR approaches allow for two-dimensional spectra, such as HSQCs for the solution-state and PDSOs for the solid-state, to monitor aggregation progression and assess the final aggregated species¹³⁴. These 2D experiments enable measurements of residue-specific aggregation kinetics, which may provide insights into early nucleation sites, critical for understanding oligomer formation and for potential therapeutic development.

Despite its advantages in monitoring aggregation, solid-state NMR typically requires longer acquisition times for 2D experiments limiting the time resolution for tracking aggregation in real time. Moreover, sample requirements are more demanding and require relatively large quantities of sample material. This, in turn, can lead to increased molecular crowding, potentially influencing aggregation behavior and complicating direct comparison between the two experimental setups (solution-state vs solid-state NMR).

In follow-up studies we investigated the aggregation behavior of PI3K-SH3 in more detail (unpublished data). Unseeded aggregation at 20 °C and 250 μ M in solution monitored by ¹H-¹⁵N HSQC spectra showed a sigmoidal decrease of INEPT based signals intensities for monomeric PI3K-SH3. This pattern is indicative of the typical amyloid aggregation

phases (Section 1.1): an initial lag phase corresponding to the formation of oligomers, an exponential growth phase corresponding to fibril elongation through monomer addition once protofibrils are formed (potentially also involving secondary nucleation and fragmentation) and a stationary phase where monomer addition and dissociation to fibrils reach equilibrium.

DNP-enhanced solid-state NMR has proven particularly powerful for probing conformational ensembles in disordered systems. In a previous work, Uluca et al. demonstrated the applicability to use DNP-enhanced NMR using selectively C α and C β labeled valine residues, among others, for determining transient secondary structure conformations¹⁷³. For α -synuclein monomers in solution, chemical shifts matched those expected for random coil values. However, when measured under cryogenic temperatures using DNP-enhanced NMR, chemical shifts consistent with both, α -helical and β -sheet populations, were observed for these residues in different proportions. This finding indicates that residues appearing as random coil at room temperature are rapidly interchanging between α -helical and β -sheet structures, and DNP-enhanced NMR at cryogenic temperatures is capable of “trapping” and revealing their conformational states.

Building on this concept, we uniformly ¹³C and ¹⁵N labeled isoleucine residues in different proteins focusing on backbone and side chain conformations with all other amino acids in natural abundance¹⁴⁹. Our data show that isoleucine residues in well-defined secondary structure elements with restricted motion exhibit narrower lines with low chemical shift dispersion. In contrast, isoleucine residues in flexible, less ordered systems give rise to broader signals reflecting increased conformational heterogeneity.

To understand how these conformational features manifest in an aggregation-prone system, we investigated the conformational behavior of PI3K-SH3 at acidic pH using DNP-enhanced NMR. Surprisingly, at pH 2.5, the protein retained a native-like conformation as indicated by the chemical shift dispersion in DNP-enhanced NMR spectra, suggesting incomplete/insufficient unfolding. Only at pH 1 we observed broader line shapes and chemical shift dispersion indicative of an unstructured, denatured state, including α -helical states associated with backbone flexibility. Chemical denaturation with 6 M guanidine hydrochloride yielded similar broad lines but lacked the α -helical chemical shifts and lower gauche minus content, suggesting a more extended, sterically restricted ensemble.

This behavior aligns with findings by Dumarieh et al., where they analyzed α -synuclein in different conformational states using DNP for line shape analysis¹⁷⁴. In their study, α -synuclein treated with 8 M urea is pushed toward coil-like structures, yet line shapes were narrower due to the restricted conformational freedom imposed by the denaturant. As they pointed out: “counterintuitively, what is commonly considered the most “disordered” state exhibits the least conformational diversity from a statistical mechanics perspective”. Our results suggest that achieving full denaturing of PI3K-SH3 at cryogenic temperatures requires lower pH values than at room temperature. A possible explanation could be that freezing does not occur fast enough to trap all conformations that are present at room temperature equally, allowing proteins time to adopt compact stable arrangements. As shown above, in solution-state NMR (Section 3.2, Figure 11), the equilibrium of folded and unfolded states for monomeric PI3K-SH3 at low pH is temperature dependent, with low temperature stabilizing the folded conformation. Additionally, the high protein concentrations typically used for DNP samples (~1.3 mM for PI3K-SH3) might contribute to self-stabilizing effects.

We have also extended the labeling approach to leucine residues with consistent results (unpublished data). Alongside this, we additionally generated point mutations (Ile->Val) for PI3K-SH3 to unambiguously identify isoleucine residues in our spectra. Given that point mutations can dramatically impact fibril structure^{175, 176}, especially in the context of electrostatic interactions, even minor changes in solution conditions can result in distinct aggregation outcomes¹⁷⁷. These mutants will serve as a basis for future studies to examine their ability to form fibrils and may reveal novel insights into the mechanisms of fibril assembly and structural diversity, especially in the context of electrostatic interactions, even minor changes in solution conditions can result in distinct aggregation outcomes¹⁷⁷. These mutants will serve as a basis for future studies to examine their ability to form fibrils and may reveal novel insights into the mechanisms of fibril assembly and structural diversity.

Finally, to complement our findings on monomeric states and the aggregation behavior, we also characterized the fibrillar state of PI3K-SH3, produced according to the protocol of Röder et al.¹⁰⁹, seeded, with incubation at a pH 2.5 and at 50 °C (Section 3.4). We obtained well-resolved spectra with relatively high-resolution allowing assignment of 50% of the sequence with one continuous stretch covering residues Y12 to D28 remaining unassigned and, presumably, absent in the spectra. INEPT spectra, which can detect highly flexible parts of fibrils¹⁷⁸, showed no detectable signals excluding highly flexible

regions. However, a region with intermediate flexibility being invisible in CP and INEPT based spectra could explain the absence of the peaks. Spectral overlap in PI3K-SH3 fibrils limited our assignment coverage and sparse labeling alone was insufficient to resolve all ambiguities. From solvent PRE data on the fibrillar state of PI3K-SH3, we observed that the core region of one subunit of the homodimer was largely shielded from solvent, while the dimer interface remained exposed, suggesting a predominantly single filament type for the fibrils investigated, which Röder et al. identified as the minor species¹⁰⁹.

In addition, we used other protocols for fibril growth and used ^{13}C - ^{13}C correlation spectra to compare the atomic structure of the resulting fibrils (Section 3.5, Figure 29). Interestingly, the observed chemical shifts deviate from those reported by Bayro et al. for PI3K-SH3 fibrils grown under the same conditions¹¹⁰, indicating another polymorph (Figure 19 A). However, when we incubated the sample unseeded at a pH value of 2.5 in presence of glycine hydrochloride and 50 °C with shaking, we surprisingly observed a high overlap compared to the spectra of fibrils grown at pH 2 with high protein concentration (Figure 19 B). This might represent a common structural motif within the atomic structure that forms during aggregation, even under different conditions. However, these chemical shifts differ from the ones we obtained in the partial assignment of the fibril polymorph produced with seeding in presence of glycine hydrochloride at 50 °C (Section 3.4). Ongoing work is trying to assign this polymorph structure using 2D ^{13}C - ^{13}C correlation spectra as well as NCACX and NCOCX spectra. At this point, it should be noted that determining high-resolution structures of fibrils via solid-state NMR remains challenging. The technique requires careful isotope labeling strategies and sufficient long-range restraints for structure calculation.

Although Röder et al. claimed consistency between their cryo-EM structure and that of Colvin et al., our results diverge. Zurdo et al. reported PI3K-SH3 fibril diameters that differed from those reported in other studies, which were found to be pH-dependent¹⁰⁵. These observations support the presence of structural polymorphism in PI3K-SH3 fibrils, which has not been previously reported.

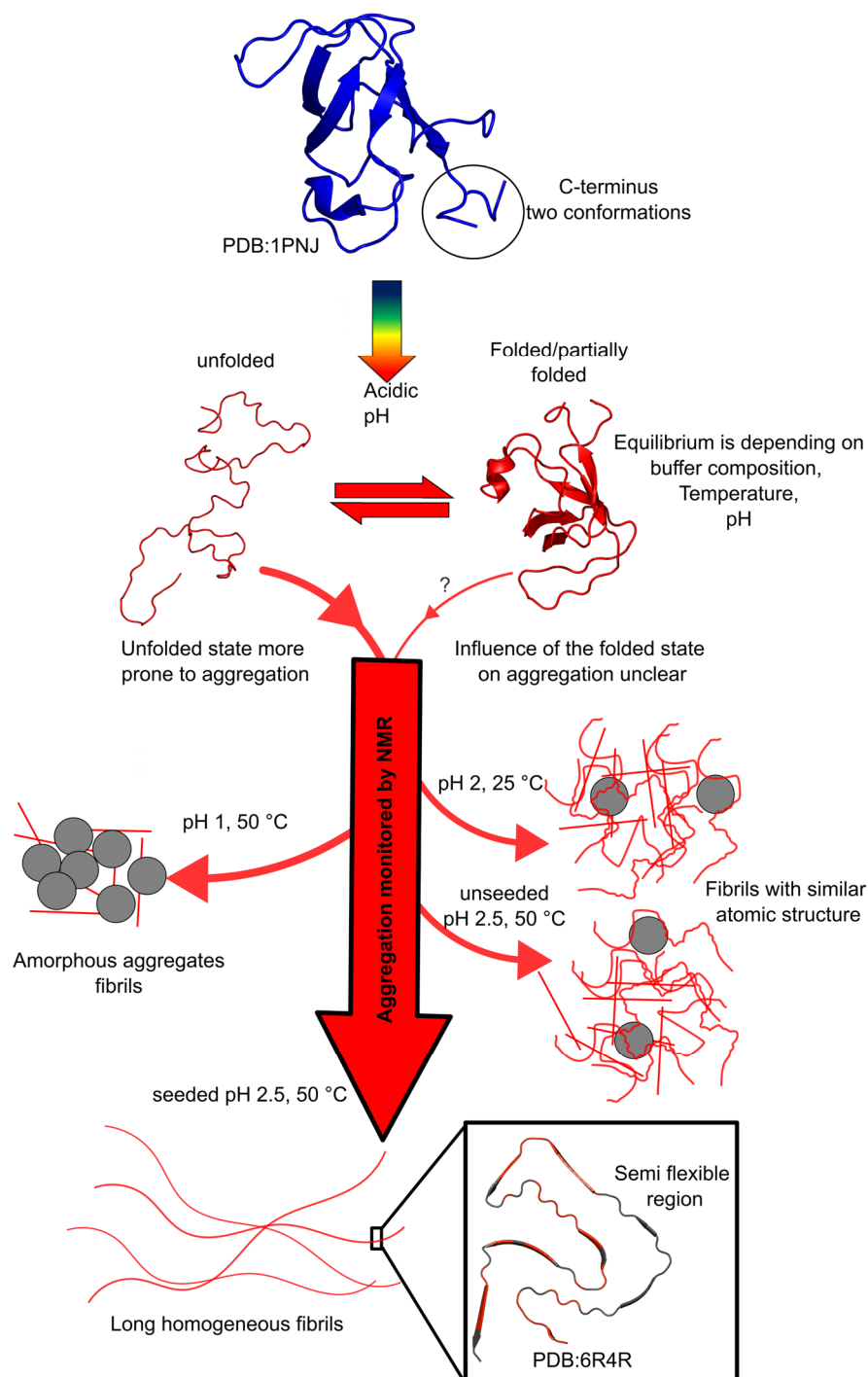


Figure 29: The PI3K-SH3 domain: From monomers to fibrils. At neutral pH values, PI3K-SH3 adopts a well-folded, β -sandwich native state with two distinct conformations observed at the C-terminus. Upon decreasing the pH to acidic pH values the protein unfolds with a folded species still present. The equilibrium between these states depends strongly on the solution conditions. Fibrillation can be monitored by NMR and is highly sensitive to the aggregation conditions. At low pH values around 1, more amorphous aggregates are formed. At pH 2/25 °C and unseeded pH 2.5 with 10 mM Gly-HCl at 50 °C fibrillar structures were observed, showing similar chemical shifts and thus, suggesting a common underlying atomic structure. The seeded incubation with pH 2.5 and 50 °C led to long homogeneous fibrils. Solid-state NMR analysis further indicated the presence of a semi flexible region within the fibril structure.

While cryo-EM has revolutionized structural biology by enabling high-resolution structures including those of amyloid fibrils and even those derived from human brain tissue, NMR remains an indispensable tool. Its strength lies in probing molecular dynamics, transient states, and site-specific interactions that are often invisible to cryo-EM. NMR can detect protonation state differences and other small variations in structure with high accuracy and linewidths provide information about the overall ensemble conformation that is present in the sample. Moreover, samples can be studied under native-like conditions, albeit under centrifugal forces present in solid-state NMR MAS rotors. MD simulations are advancing rapidly and increasingly used to complement experimental techniques; however, they are still constrained by the accuracy of available force fields and by the relatively short timescales that can be accessed computationally.

So far, no single method can fully capture the complexity of amyloid formation. NMR, despite its challenges, provides essential information. For this reason, a truly integrative approach, combining cryo-EM, NMR, computational modeling, and other biophysical methods, offers the most complete strategy for tackling the multifaceted questions surrounding amyloidogenic proteins. Such integrative approaches will not only advance our fundamental understanding of amyloidogenic proteins but also inform the development of therapeutic strategies for amyloid-related diseases.

5

References

References

- 1 Lampitey, R. N. L.; Chaulagain, B.; Trivedi, R.; Gothwal, A.; Layek, B.; Singh, J. A Review of the Common Neurodegenerative Disorders: Current Therapeutic Approaches and the Potential Role of Nanotherapeutics. *Int J Mol Sci* **2022**, *23* (3). DOI: 10.3390/ijms23031851.
- 2 2024 Alzheimer's disease facts and figures. *Alzheimers Dement* **2024**, *20* (5), 3708-3821. DOI: 10.1002/alz.13809.
- 3 Sadigh-Eteghad, S.; Sabermarouf, B.; Majdi, A.; Talebi, M.; Farhoudi, M.; Mahmoudi, J. Amyloid-beta: a crucial factor in Alzheimer's disease. *Med Princ Pract* **2015**, *24* (1), 1-10. DOI: 10.1159/000369101.
- 4 Dobson, C. M. Protein folding and misfolding. *Nature* **2003**, *426* (6968), 884-890. DOI: 10.1038/nature02261.
- 5 Breydo, L.; Wu, J. W.; Uversky, V. N. Alpha-synuclein misfolding and Parkinson's disease. *Biochim Biophys Acta* **2012**, *1822* (2), 261-285. DOI: 10.1016/j.bbadis.2011.10.002.
- 6 Otzen, D.; Riek, R. Functional Amyloids. *Cold Spring Harb Perspect Biol* **2019**, *11* (12). DOI: 10.1101/cshperspect.a033860.
- 7 Fowler, D. M.; Koulov, A. V.; Balch, W. E.; Kelly, J. W. Functional amyloid--from bacteria to humans. *Trends Biochem Sci* **2007**, *32* (5), 217-224. DOI: 10.1016/j.tibs.2007.03.003.
- 8 Dobson, C. M. The structural basis of protein folding and its links with human disease. *Philos Trans R Soc Lond B Biol Sci* **2001**, *356* (1406), 133-145. DOI: 10.1098/rstb.2000.0758.
- 9 Chiti, F.; Webster, P.; Taddei, N.; Clark, A.; Stefani, M.; Ramponi, G.; Dobson, C. M. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proc Natl Acad Sci U S A* **1999**, *96* (7), 3590-3594. DOI: 10.1073/pnas.96.7.3590.
- 10 Zanjani, A. A. H.; Reynolds, N. P.; Zhang, A.; Schilling, T.; Mezzenga, R.; Berryman, J. T. Amyloid Evolution: Antiparallel Replaced by Parallel. *Biophys J* **2020**, *118* (10), 2526-2536. DOI: 10.1016/j.bpj.2020.03.023.
- 11 Taylor, A. I. P.; Staniforth, R. A. General Principles Underpinning Amyloid Structure. *Front Neurosci* **2022**, *16*, 878869. DOI: 10.3389/fnins.2022.878869.
- 12 Willbold, D.; Strodel, B.; Schroder, G. F.; Hoyer, W.; Heise, H. Amyloid-type Protein Aggregation and Prion-like Properties of Amyloids. *Chem Rev* **2021**, *121* (13), 8285-8307. DOI: 10.1021/acs.chemrev.1c00196.
- 13 Makin, O. S.; Atkins, E.; Sikorski, P.; Johansson, J.; Serpell, L. C. Molecular basis for amyloid fibril formation and stability. *Proc Natl Acad Sci U S A* **2005**, *102* (2), 315-320. DOI: 10.1073/pnas.0406847102.
- 14 Astbury, W. T.; Dickinson, S.; Bailey, K. The X-ray interpretation of denaturation and the structure of the seed globulins. *Biochem J* **1935**, *29* (10), 2351-2360. DOI: 10.1042/bj0292351.
- 15 Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J Mol Biol* **1997**, *273* (3), 729-739. DOI: 10.1006/jmbi.1997.1348.
- 16 Iadanza, M. G.; Jackson, M. P.; Hewitt, E. W.; Ranson, N. A.; Radford, S. E. A new era for understanding amyloid structures and disease. *Nat Rev Mol Cell Biol* **2018**, *19* (12), 755-773. DOI: 10.1038/s41580-018-0060-8.
- 17 Knowles, T. P.; Vendruscolo, M.; Dobson, C. M. The amyloid state and its association with protein misfolding diseases. *Nat Rev Mol Cell Biol* **2014**, *15* (6), 384-396. DOI: 10.1038/nrm3810.
- 18 Chatani, E.; Yamamoto, N. Recent progress on understanding the mechanisms of amyloid nucleation. *Biophys Rev* **2018**, *10* (2), 527-534. DOI: 10.1007/s12551-017-0353-8.
- 19 Tornquist, M.; Michaels, T. C. T.; Sanagavarapu, K.; Yang, X.; Meisl, G.; Cohen, S. I. A.; Knowles, T. P. J.; Linse, S. Secondary nucleation in amyloid formation. *Chemical communications (Cambridge, England)* **2018**, *54* (63), 8667-8684. DOI: 10.1039/c8cc02204f.
- 20 Ulamec, S. M.; Brockwell, D. J.; Radford, S. E. Looking Beyond the Core: The Role of Flanking Regions in the Aggregation of Amyloidogenic Peptides and Proteins. *Front Neurosci* **2020**, *14*, 611285. DOI: 10.3389/fnins.2020.611285.

- 21 Kumar, E. K.; Haque, N.; Prabhu, N. P. Kinetics of protein fibril formation: Methods and mechanisms. *Int J Biol Macromol* **2017**, *100*, 3-10. DOI: 10.1016/j.ijbiomac.2016.06.052.
- 22 Hasecke, F.; Miti, T.; Perez, C.; Barton, J.; Scholzel, D.; Gremer, L.; Gruning, C. S. R.; Matthews, G.; Meisl, G.; Knowles, T. P. J.; et al. Origin of metastable oligomers and their effects on amyloid fibril self-assembly. *Chem Sci* **2018**, *9* (27), 5937-5948. DOI: 10.1039/c8sc01479e.
- 23 Dear, A. J.; Meisl, G.; Saric, A.; Michaels, T. C. T.; Kjaergaard, M.; Linse, S.; Knowles, T. P. J. Identification of on- and off-pathway oligomers in amyloid fibril formation. *Chem Sci* **2020**, *11* (24), 6236-6247. DOI: 10.1039/c9sc06501f.
- 24 Jeon, J.; Yau, W. M.; Tycko, R. Early events in amyloid-beta self-assembly probed by time-resolved solid state NMR and light scattering. *Nat Commun* **2023**, *14* (1), 2964. DOI: 10.1038/s41467-023-38494-6.
- 25 Konig, A. S.; Rosener, N. S.; Gremer, L.; Tusche, M.; Flender, D.; Reinartz, E.; Hoyer, W.; Neudecker, P.; Willbold, D.; Heise, H. Structural details of amyloid beta oligomers in complex with human prion protein as revealed by solid-state MAS NMR spectroscopy. *J Biol Chem* **2021**, *296*, 100499. DOI: 10.1016/j.jbc.2021.100499.
- 26 Cline, E. N.; Bicca, M. A.; Viola, K. L.; Klein, W. L. The Amyloid-beta Oligomer Hypothesis: Beginning of the Third Decade. *J Alzheimers Dis* **2018**, *64* (s1), S567-S610. DOI: 10.3233/JAD-179941.
- 27 Niu, Z.; Gui, X.; Feng, S.; Reif, B. Aggregation Mechanisms and Molecular Structures of Amyloid-beta in Alzheimer's Disease. *Chemistry* **2024**, *30* (48), e202400277. DOI: 10.1002/chem.202400277.
- 28 Hadi Alijanvand, S.; Peduzzo, A.; Buell, A. K. Secondary Nucleation and the Conservation of Structural Characteristics of Amyloid Fibril Strains. *Front Mol Biosci* **2021**, *8*, 669994. DOI: 10.3389/fmolb.2021.669994.
- 29 Ventura, S.; Zurdo, J.; Narayanan, S.; Parreno, M.; Mangues, R.; Reif, B.; Chiti, F.; Giannoni, E.; Dobson, C. M.; Aviles, F. X.; et al. Short amino acid stretches can mediate amyloid formation in globular proteins: the Src homology 3 (SH3) case. *Proc Natl Acad Sci U S A* **2004**, *101* (19), 7258-7263. DOI: 10.1073/pnas.0308249101.
- 30 Tycko, R. Amyloid polymorphism: structural basis and neurobiological relevance. *Neuron* **2015**, *86* (3), 632-645. DOI: 10.1016/j.neuron.2015.03.017.
- 31 Carulla, N.; Caddy, G. L.; Hall, D. R.; Zurdo, J.; Gairi, M.; Feliz, M.; Giralt, E.; Robinson, C. V.; Dobson, C. M. Molecular recycling within amyloid fibrils. *Nature* **2005**, *436* (7050), 554-558. DOI: 10.1038/nature03986.
- 32 Muller, U. C.; Deller, T.; Korte, M. Not just amyloid: physiological functions of the amyloid precursor protein family. *Nat Rev Neurosci* **2017**, *18* (5), 281-298. DOI: 10.1038/nrn.2017.29.
- 33 Bishop, G. M.; Robinson, S. R. Physiological roles of amyloid-beta and implications for its removal in Alzheimer's disease. *Drugs Aging* **2004**, *21* (10), 621-630. DOI: 10.2165/00002512-200421100-00001.
- 34 Teller, J. K.; Russo, C.; DeBusk, L. M.; Angelini, G.; Zaccheo, D.; Dagna-Bricarelli, F.; Scartezzini, P.; Bertolini, S.; Mann, D. M.; Tabaton, M.; et al. Presence of soluble amyloid beta-peptide precedes amyloid plaque formation in Down's syndrome. *Nat Med* **1996**, *2* (1), 93-95. DOI: 10.1038/nm0196-93.
- 35 Hattori, M.; Fujiyama, A.; Taylor, T. D.; Watanabe, H.; Yada, T.; Park, H. S.; Toyoda, A.; Ishii, K.; Totoki, Y.; Choi, D. K.; et al. The DNA sequence of human chromosome 21. *Nature* **2000**, *405* (6784), 311-319. DOI: 10.1038/35012518.
- 36 Kuperstein, I.; Broersen, K.; Benilova, I.; Rozenski, J.; Jonckheere, W.; Debulpaep, M.; Vandersteen, A.; Segers-Nolten, I.; Van Der Werf, K.; Subramaniam, V.; et al. Neurotoxicity of Alzheimer's disease Aβ peptides is induced by small changes in the Aβ₄₂ to Aβ₄₀ ratio. *EMBO J* **2010**, *29* (19), 3408-3420. DOI: 10.1038/emboj.2010.211.
- 37 Strodel, B.; Lee, J. W.; Whittleston, C. S.; Wales, D. J. Transmembrane structures for Alzheimer's Aβ₄₂ oligomers. *J Am Chem Soc* **2010**, *132* (38), 13300-13312. DOI: 10.1021/ja103725c.

- 38 Sciacca, M. F.; Kotler, S. A.; Brender, J. R.; Chen, J.; Lee, D. K.; Ramamoorthy, A. Two-step mechanism of membrane disruption by Abeta through membrane fragmentation and pore formation. *Biophys J* **2012**, *103* (4), 702-710. DOI: 10.1016/j.bpj.2012.06.045.
- 39 Kengwerere, M. K.; Kenyaga, J. M.; Xiao, P.; Gunaga, S. S.; Scott, F. J.; Wutoh-Hughes, X.; Wang, J.; Lum, B.; Sun, Y.; Mentink-Vigier, F.; et al. Structural convergence and membrane interactions of Abeta(1-42) along the primary nucleation process studied by solid state NMR. *Commun Chem* **2025**, *8* (1), 131. DOI: 10.1038/s42004-025-01537-8.
- 40 Zhao, X.; Sun, J.; Xiong, L.; She, L.; Li, L.; Tang, H.; Zeng, Y.; Chen, F.; Han, X.; Ye, S.; et al. beta-amyloid binds to microglia Dectin-1 to induce inflammatory response in the pathogenesis of Alzheimer's disease. *Int J Biol Sci* **2023**, *19* (10), 3249-3265. DOI: 10.7150/ijbs.81900.
- 41 Tamagno, E.; Guglielmotto, M.; Vasciaveo, V.; Tabaton, M. Oxidative Stress and Beta Amyloid in Alzheimer's Disease. Which Comes First: The Chicken or the Egg? *Antioxidants (Basel)* **2021**, *10* (9). DOI: 10.3390/antiox10091479.
- 42 Azargoonjahromi, A. The duality of amyloid-beta: its role in normal and Alzheimer's disease states. *Mol Brain* **2024**, *17* (1), 44. DOI: 10.1186/s13041-024-01118-1.
- 43 Roche, J.; Shen, Y.; Lee, J. H.; Ying, J.; Bax, A. Monomeric Abeta(1-40) and Abeta(1-42) Peptides in Solution Adopt Very Similar Ramachandran Map Distributions That Closely Resemble Random Coil. *Biochemistry* **2016**, *55* (5), 762-775. DOI: 10.1021/acs.biochem.5b01259.
- 44 Kakeshpour, T.; Ramanujam, V.; Barnes, C. A.; Shen, Y.; Ying, J.; Bax, A. A lowly populated, transient beta-sheet structure in monomeric Abeta(1-42) identified by multinuclear NMR of chemical denaturation. *Biophys Chem* **2021**, *270*, 106531. DOI: 10.1016/j.bpc.2020.106531.
- 45 Sgourakis, N. G.; Yan, Y.; McCallum, S. A.; Wang, C.; Garcia, A. E. The Alzheimer's peptides Abeta40 and 42 adopt distinct conformations in water: a combined MD / NMR study. *J Mol Biol* **2007**, *368* (5), 1448-1457. DOI: 10.1016/j.jmb.2007.02.093.
- 46 Barz, B.; Buell, A. K.; Nath, S. Compact fibril-like structure of amyloid beta-peptide (1-42) monomers. *Chemical communications (Cambridge, England)* **2021**, *57* (7), 947-950. DOI: 10.1039/d0cc06607a.
- 47 Crescenzi, O.; Tomaselli, S.; Guerrini, R.; Salvadori, S.; D'Ursi, A. M.; Temussi, P. A.; Picone, D. Solution structure of the Alzheimer amyloid beta-peptide (1-42) in an apolar microenvironment. Similarity with a virus fusion domain. *Eur J Biochem* **2002**, *269* (22), 5642-5648. DOI: 10.1046/j.1432-1033.2002.03271.x.
- 48 Ciudad, S.; Puig, E.; Botzanowski, T.; Meigooni, M.; Arango, A. S.; Do, J.; Mayzel, M.; Bayoumi, M.; Chaignepain, S.; Maglia, G.; et al. Abeta(1-42) tetramer and octamer structures reveal edge conductivity pores as a mechanism for membrane damage. *Nat Commun* **2020**, *11* (1), 3014. DOI: 10.1038/s41467-020-16566-1.
- 49 Xiao, Y.; Matsuda, I.; Inoue, M.; Sasahara, T.; Hoshi, M.; Ishii, Y. NMR-based site-resolved profiling of beta-amyloid misfolding reveals structural transitions from pathologically relevant spherical oligomer to fibril. *J Biol Chem* **2020**, *295* (2), 458-467. DOI: 10.1074/jbc.RA119.008522.
- 50 Tay, W. M.; Huang, D.; Rosenberry, T. L.; Paravastu, A. K. The Alzheimer's amyloid-beta(1-42) peptide forms off-pathway oligomers and fibrils that are distinguished structurally by intermolecular organization. *J Mol Biol* **2013**, *425* (14), 2494-2508. DOI: 10.1016/j.jmb.2013.04.003.
- 51 Jeon, J.; Thurber, K. R.; Ghirlando, R.; Yau, W. M.; Tycko, R. Application of millisecond time-resolved solid state NMR to the kinetics and mechanism of melittin self-assembly. *Proc Natl Acad Sci U S A* **2019**, *116* (34), 16717-16722. DOI: 10.1073/pnas.1908006116.
- 52 Chimon, S.; Shaibat, M. A.; Jones, C. R.; Calero, D. C.; Aizezi, B.; Ishii, Y. Evidence of fibril-like beta-sheet structures in a neurotoxic amyloid intermediate of Alzheimer's beta-amyloid. *Nat Struct Mol Biol* **2007**, *14* (12), 1157-1164. DOI: 10.1038/nsmb1345.
- 53 Xiao, Y.; Ma, B.; McElheny, D.; Parthasarathy, S.; Long, F.; Hoshi, M.; Nussinov, R.; Ishii, Y. Aβ(1–42) fibril structure illuminates self-recognition and replication of amyloid in Alzheimer's disease. *Nature Structural & Molecular Biology* **2015**, *22*, 499, Article. DOI: 10.1038/nsmb.2991.

- 54 Colvin, M. T.; Silvers, R.; Ni, Q. Z.; Can, T. V.; Sergeyev, I.; Rosay, M.; Donovan, K. J.; Michael, B.; Wall, J.; Linse, S.; et al. Atomic Resolution Structure of Monomorphic A β 42 Amyloid Fibrils. *Journal of the American Chemical Society* **2016**, *138* (30), 9663-9674. DOI: 10.1021/jacs.6b05129.
- 55 Wälti, M. A.; Ravotti, F.; Arai, H.; Glabe, C. G.; Wall, J. S.; Böckmann, A.; Güntert, P.; Meier, B. H.; Riek, R. Atomic-resolution structure of a disease-relevant A β (1-42) amyloid fibril. *Proceedings of the National Academy of Sciences of the United States of America* **2016**, *113* (34), E4976-E4984. DOI: 10.1073/pnas.1600749113.
- 56 Gremer, L.; Schölzel, D.; Schenk, C.; Reinartz, E.; Labahn, J.; Ravelli, R. B. G.; Tusche, M.; Lopez-Iglesias, C.; Hoyer, W.; Heise, H.; et al. Fibril structure of amyloid- β (1-42) by cryo-electron microscopy. *Science* **2017**, *358* (6359), 116-119. DOI: 10.1126/science.aao2825.
- 57 Qiang, W.; Yau, W. M.; Lu, J. X.; Collinge, J.; Tycko, R. Structural variation in amyloid-beta fibrils from Alzheimer's disease clinical subtypes. *Nature* **2017**, *541* (7636), 217-221. DOI: 10.1038/nature20814.
- 58 Yang, Y.; Arseni, D.; Zhang, W.; Huang, M.; Lovestam, S.; Schweighauser, M.; Kotecha, A.; Murzin, A. G.; Peak-Chew, S. Y.; Macdonald, J.; et al. Cryo-EM structures of amyloid-beta 42 filaments from human brains. *Science* **2022**, *375* (6577), 167-172. DOI: 10.1126/science.abm7285.
- 59 Lee, M.; Yau, W. M.; Louis, J. M.; Tycko, R. Structures of brain-derived 42-residue amyloid-beta fibril polymorphs with unusual molecular conformations and intermolecular interactions. *Proc Natl Acad Sci U S A* **2023**, *120* (11), e2218831120. DOI: 10.1073/pnas.2218831120.
- 60 Hsiao, L. C.; Lee, C. H.; Mazmanian, K.; Yoshida, M.; Ito, G.; Murata, T.; Utsunomiya-Tate, N.; Haino, T.; Tate, S. I.; Hsu, S. D. Impacts of D-aspartate on the Aggregation Kinetics and Structural Polymorphism of Amyloid beta Peptide 1-42. *J Mol Biol* **2025**, *437* (12), 169092. DOI: 10.1016/j.jmb.2025.169092.
- 61 Sgourakis, N. G.; Yau, W. M.; Qiang, W. Modeling an in-register, parallel "iowa" abeta fibril structure using solid-state NMR data from labeled samples with rosetta. *Structure* **2015**, *23* (1), 216-227. DOI: 10.1016/j.str.2014.10.022.
- 62 Zielinski, M.; Peralta Reyes, F. S.; Gremer, L.; Schemmert, S.; Frieg, B.; Schafer, L. U.; Willuweit, A.; Donner, L.; Elvers, M.; Nilsson, L. N. G.; et al. Cryo-EM of Abeta fibrils from mouse models find tg-APP(ArcSwe) fibrils resemble those found in patients with sporadic Alzheimer's disease. *Nat Neurosci* **2023**, *26* (12), 2073-2080. DOI: 10.1038/s41593-023-01484-4.
- 63 Luhrs, T.; Ritter, C.; Adrian, M.; Riek-Loher, D.; Bohrmann, B.; Dobeli, H.; Schubert, D.; Riek, R. 3D structure of Alzheimer's amyloid-beta(1-42) fibrils. *Proc Natl Acad Sci U S A* **2005**, *102* (48), 17342-17347. DOI: 10.1073/pnas.0506723102.
- 64 Schmidt, M.; Rohou, A.; Lasker, K.; Yadav, J. K.; Schiene-Fischer, C.; Fandrich, M.; Grigorieff, N. Peptide dimer structure in an Abeta(1-42) fibril visualized with cryo-EM. *Proc Natl Acad Sci U S A* **2015**, *112* (38), 11858-11863. DOI: 10.1073/pnas.1503455112.
- 65 Fernandez, A.; Hoq, M. R.; Hallinan, G. I.; Li, D.; Bharath, S. R.; Vago, F. S.; Zhang, X.; Ozcan, K. A.; Newell, K. L.; Garringer, H. J.; et al. Cryo-EM structures of amyloid-beta and tau filaments in Down syndrome. *Nat Struct Mol Biol* **2024**, *31* (6), 903-909. DOI: 10.1038/s41594-024-01252-3.
- 66 Kummer, M. P.; Heneka, M. T. Truncated and modified amyloid-beta species. *Alzheimers Res Ther* **2014**, *6* (3), 28. DOI: 10.1186/alzrt258.
- 67 Saido, T. C.; Iwatsubo, T.; Mann, D. M.; Shimada, H.; Ihara, Y.; Kawashima, S. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. *Neuron* **1995**, *14* (2), 457-466. DOI: 10.1016/0896-6273(95)90301-1.
- 68 Koutarapu, S.; Ge, J.; Dulewicz, M.; Srikrishna, M.; Szadziewska, A.; Wood, J.; Blennow, K.; Zetterberg, H.; Michno, W.; Ryan, N. S.; et al. Chemical imaging delineates Abeta plaque polymorphism across the Alzheimer's disease spectrum. *Nat Commun* **2025**, *16* (1), 3889. DOI: 10.1038/s41467-025-59085-7.

- 82

- 86 Alexandru, A.; Jagla, W.; Graubner, S.; Becker, A.; Bauscher, C.; Kohlmann, S.; Sedlmeier, R.; Raber, K. A.; Cynis, H.; Ronicke, R.; et al. Selective hippocampal neurodegeneration in transgenic mice expressing small amounts of truncated Abeta is induced by pyroglutamate-Abeta formation. *J Neurosci* **2011**, *31* (36), 12790-12801. DOI: 10.1523/JNEUROSCI.1794-11.2011.
- 87 Wittnam, J. L.; Portelius, E.; Zetterberg, H.; Gustavsson, M. K.; Schilling, S.; Koch, B.; Demuth, H. U.; Blennow, K.; Wirths, O.; Bayer, T. A. Pyroglutamate amyloid beta (Abeta) aggravates behavioral deficits in transgenic amyloid mouse model for Alzheimer disease. *J Biol Chem* **2012**, *287* (11), 8154-8162. DOI: 10.1074/jbc.M111.308601.
- 88 Schlenzig, D.; Manhart, S.; Cinar, Y.; Kleinschmidt, M.; Hause, G.; Willbold, D.; Funke, S. A.; Schilling, S.; Demuth, H. U. Pyroglutamate formation influences solubility and amyloidogenicity of amyloid peptides. *Biochemistry* **2009**, *48* (29), 7072-7078. DOI: 10.1021/bi900818a.
- 89 Dammers, C.; Schwarten, M.; Buell, A. K.; Willbold, D. Pyroglutamate-modified A β (3-42) affects aggregation kinetics of A β (1-42) by accelerating primary and secondary pathways. *Chemical Science* **2017**, *8* (7), 4996-5004, 10.1039/C6SC04797A. DOI: 10.1039/C6SC04797A.
- 90 Dammers, C.; Reiss, K.; Gremer, L.; Lecher, J.; Ziehm, T.; Stoldt, M.; Schwarten, M.; Willbold, D. Pyroglutamate-Modified Amyloid-beta(3-42) Shows alpha-Helical Intermediates before Amyloid Formation. *Biophys J* **2017**, *112* (8), 1621-1633. DOI: 10.1016/j.bpj.2017.03.007.
- 91 Dammers, C.; Gremer, L.; Neudecker, P.; Demuth, H. U.; Schwarten, M.; Willbold, D. Purification and Characterization of Recombinant N-Terminally Pyroglutamate-Modified Amyloid- β Variants and Structural Analysis by Solution NMR Spectroscopy. *PloS one* **2015**, *10* (10), e0139710. DOI: 10.1371/journal.pone.0139710.
- 92 Dammers, C.; Gremer, L.; Reiss, K.; Klein, A. N.; Neudecker, P.; Hartmann, R.; Sun, N.; Demuth, H. U.; Schwarten, M.; Willbold, D. Structural Analysis and Aggregation Propensity of Pyroglutamate Abeta(3-40) in Aqueous Trifluoroethanol. *PloS one* **2015**, *10* (11), e0143647. DOI: 10.1371/journal.pone.0143647.
- 93 Scheidt, H. A.; Adler, J.; Krueger, M.; Huster, D. Fibrils of Truncated Pyroglutamyl-Modified A β Peptide Exhibit a Similar Structure as Wildtype Mature A β Fibrils. *Scientific Reports* **2016**, *6* (1), 33531. DOI: 10.1038/srep33531.
- 94 Scheidt, H. A.; Adler, J.; Zeitschel, U.; Höfling, C.; Korn, A.; Krueger, M.; Roßner, S.; Huster, D. Pyroglutamate-Modified Amyloid β (11- 40) Fibrils Are More Toxic than Wildtype Fibrils but Structurally Very Similar. *Chemistry- A European Journal* **2017**, *23* (62), 15834-15838, <https://doi.org/10.1002/chem.201703909>. DOI: <https://doi.org/10.1002/chem.201703909> (accessed 2021/11/08).
- 95 Scheidt, H. A.; Morgado, I.; Rothmund, S.; Huster, D. Dynamics of amyloid beta fibrils revealed by solid-state NMR. *J Biol Chem* **2012**, *287* (3), 2017-2021. DOI: 10.1074/jbc.M111.308619.
- 96 Scheidt, H. A.; Das, A.; Korn, A.; Krueger, M.; Maiti, S.; Huster, D. Structural characteristics of oligomers formed by pyroglutamate-modified amyloid beta peptides studied by solid-state NMR. *Phys Chem Chem Phys* **2020**, *22* (29), 16887-16895. DOI: 10.1039/d0cp02307h.
- 97 Wulff, M.; Baumann, M.; Thummler, A.; Yadav, J. K.; Heinrich, L.; Knupfer, U.; Schlenzig, D.; Schierhorn, A.; Rahfeld, J. U.; Horn, U.; et al. Enhanced Fibril Fragmentation of N-Terminally Truncated and Pyroglutamyl-Modified Abeta Peptides. *Angew Chem Int Ed Engl* **2016**, *55* (16), 5081-5084. DOI: 10.1002/anie.201511099.
- 98 Gardon, L.; Becker, N.; Gremer, L.; Heise, H. Structural Impact of N-terminal Pyroglutamate in an Amyloid-beta(3-42) Fibril Probed by Solid-State NMR Spectroscopy. *Chemistry* **2024**, *30* (10), e202303007. DOI: 10.1002/chem.202303007.
- 99 Cantley, L. C. The phosphoinositide 3-kinase pathway. *Science* **2002**, *296* (5573), 1655-1657. DOI: 10.1126/science.296.5573.1655.
- 100 Ren, R.; Mayer, B. J.; Cicchetti, P.; Baltimore, D. Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **1993**, *259* (5098), 1157-1161. DOI: 10.1126/science.8438166.

References

- 101 Kurochkina, N.; Guha, U. SH3 domains: modules of protein-protein interactions. *Biophys Rev* **2013**, *5* (1), 29-39. DOI: 10.1007/s12551-012-0081-z.
- 102 Booker, G. W.; Gout, I.; Downing, A. K.; Driscoll, P. C.; Boyd, J.; Waterfield, M. D.; Campbell, I. D. Solution structure and ligand-binding site of the SH3 domain of the p85 alpha subunit of phosphatidylinositol 3-kinase. *Cell* **1993**, *73* (4), 813-822. DOI: 10.1016/0092-8674(93)90259-s.
- 103 Batra-Safferling, R.; Granzin, J.; Modder, S.; Hoffmann, S.; Willbold, D. Structural studies of the phosphatidylinositol 3-kinase (PI3K) SH3 domain in complex with a peptide ligand: role of the anchor residue in ligand binding. *Biol Chem* **2010**, *391* (1), 33-42. DOI: 10.1515/BC.2010.003.
- 104 Koyama, S.; Yu, H.; Dalgarno, D. C.; Shin, T. B.; Zydowsky, L. D.; Schreiber, S. L. Structure of the PI3K SH3 domain and analysis of the SH3 family. *Cell* **1993**, *72* (6), 945-952. DOI: 10.1016/0092-8674(93)90582-b.
- 105 Zurdo, J.; Guijarro, J. I.; Jimenez, J. L.; Saibil, H. R.; Dobson, C. M. Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain. *J Mol Biol* **2001**, *311* (2), 325-340. DOI: 10.1006/jmbi.2001.4858.
- 106 Gardon, L.; Becker, N.; Rahse, N.; Holbling, C.; Apostolidis, A.; Schulz, C. M.; Bochinsky, K.; Gremer, L.; Heise, H.; Lakomek, N. A. Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques. *Front Mol Biosci* **2023**, *10*, 1254721. DOI: 10.3389/fmolb.2023.1254721.
- 107 Guijarro, J. I.; Sunde, M.; Jones, J. A.; Campbell, I. D.; Dobson, C. M. Amyloid fibril formation by an SH3 domain. *Proc Natl Acad Sci U S A* **1998**, *95* (8), 4224-4228. DOI: 10.1073/pnas.95.8.4224.
- 108 Ahn, H. C.; Le, Y. T.; Nagchowdhuri, P. S.; Derose, E. F.; Putnam-Evans, C.; London, R. E.; Markley, J. L.; Lim, K. H. NMR characterizations of an amyloidogenic conformational ensemble of the PI3K SH3 domain. *Protein Sci* **2006**, *15* (11), 2552-2557. DOI: 10.1110/ps.062154306.
- 109 Roder, C.; Vettore, N.; Mangels, L. N.; Gremer, L.; Ravelli, R. B. G.; Willbold, D.; Hoyer, W.; Buell, A. K.; Schroder, G. F. Atomic structure of PI3-kinase SH3 amyloid fibrils by cryo-electron microscopy. *Nat Commun* **2019**, *10* (1), 3754. DOI: 10.1038/s41467-019-11320-8.
- 110 Bayro, M. J.; Maly, T.; Birkett, N. R.; Macphee, C. E.; Dobson, C. M.; Griffin, R. G. High-resolution MAS NMR analysis of PI3-SH3 amyloid fibrils: backbone conformation and implications for protofilament assembly and structure. *Biochemistry* **2010**, *49* (35), 7474-7484. DOI: 10.1021/bi100864t.
- 111 Larsen, J. A.; Barclay, A.; Vettore, N.; Klausen, L. K.; Mangels, L. N.; Coden, A.; Schmit, J. D.; Lindorff-Larsen, K.; Buell, A. K. The mechanism of amyloid fibril growth from Phi-value analysis. *Nat Chem* **2025**, *17* (3), 403-411. DOI: 10.1038/s41557-024-01712-9.
- 112 Tugarinov, V.; Muhandiram, R.; Ayed, A.; Kay, L. E. Four-dimensional NMR spectroscopy of a 723-residue protein: chemical shift assignments and secondary structure of malate synthase g. *J Am Chem Soc* **2002**, *124* (34), 10025-10035. DOI: 10.1021/ja0205636.
- 113 Bodenhausen, G.; Ruben, D. J. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chemical Physics Letters* **1980**, *69* (1), 185-189. DOI: 10.1016/0009-2614(80)80041-8.
- 114 Morris, G. A.; Freeman, R. Enhancement of nuclear magnetic resonance signals by polarization transfer. *Journal of the American Chemical Society* **1979**, *101* (3), 760-762. DOI: 10.1021/ja00497a058.
- 115 Wishart, D. S.; Sykes, B. D.; Richards, F. M. The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* **1992**, *31* (6), 1647-1651. DOI: 10.1021/bi00121a010.
- 116 Hartmann, S. R.; Hahn, E. L. Nuclear Double Resonance in the Rotating Frame. *Physical Review* **1962**, *128* (5), 2042-2053. DOI: 10.1103/PhysRev.128.2042.
- 117 Pines, A.; Gibby, M. G.; Waugh, J. S. Proton-Enhanced Nuclear Induction Spectroscopy. A Method for High Resolution NMR of Dilute Spins in Solids. *The Journal of Chemical Physics* **1972**, *56* (4), 1776-1777. DOI: 10.1063/1.1677439 (accessed 7/25/2025).

- 118 Bloembergen, N. On the interaction of nuclear spins in a crystalline lattice. *Physica* **1949**, *15* (3), 386-426. DOI: [https://doi.org/10.1016/0031-8914\(49\)90114-7](https://doi.org/10.1016/0031-8914(49)90114-7).
- 119 Lacabanne, D.; Meier, B. H.; Bockmann, A. Selective labeling and unlabeled strategies in protein solid-state NMR spectroscopy. *J Biomol NMR* **2018**, *71* (3), 141-150. DOI: 10.1007/s10858-017-0156-z.
- 120 Pauli, J.; Baldus, M.; van Rossum, B.; de Groot, H.; Oschkinat, H. Backbone and side-chain ¹³C and ¹⁵N signal assignments of the alpha-spectrin SH3 domain by magic angle spinning solid-state NMR at 17.6 Tesla. *Chembiochem* **2001**, *2* (4), 272-281. DOI: 10.1002/1439-7633(20010401)2:4<272::AID-CBIC272>3.0.CO;2-2.
- 121 Schiavina, M.; Bracaglia, L.; Bolognesi, T.; Rodella, M. A.; Tagliaferro, G.; Tino, A. S.; Pierattelli, R.; Felli, I. C. Intrinsically disordered proteins studied by NMR spectroscopy. *Journal of Magnetic Resonance Open* **2024**, *18*. DOI: 10.1016/j.jmro.2023.100143.
- 122 Munari, F.; D'Onofrio, M.; Assfalg, M. Solution NMR insights into dynamic supramolecular assemblies of disordered amyloidogenic proteins. *Arch Biochem Biophys* **2020**, *683*, 108304. DOI: 10.1016/j.abb.2020.108304.
- 123 Karamanos, T. K.; Kalverda, A. P.; Thompson, G. S.; Radford, S. E. Mechanisms of amyloid formation revealed by solution NMR. *Prog Nucl Magn Reson Spectrosc* **2015**, *88-89*, 86-104. DOI: 10.1016/j.pnmrs.2015.05.002.
- 124 Borchers, W. M.; Daughdrill, G. W. Using NMR Chemical Shifts to Determine Residue-Specific Secondary Structure Populations for Intrinsically Disordered Proteins. *Methods Enzymol* **2018**, *611*, 101-136. DOI: 10.1016/bs.mie.2018.09.011.
- 125 Viennet, T.; Wordehoff, M. M.; Uluca, B.; Poojari, C.; Shaykhalishahi, H.; Willbold, D.; Strodel, B.; Heise, H.; Buell, A. K.; Hoyer, W.; et al. Structural insights from lipid-bilayer nanodiscs link alpha-Synuclein membrane-binding modes to amyloid fibril formation. *Commun Biol* **2018**, *1*, 44. DOI: 10.1038/s42003-018-0049-z.
- 126 Carr, H. Y.; Purcell, E. M. Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments. *Physical Review* **1954**, *94* (3), 630-638. DOI: 10.1103/PhysRev.94.630.
- 127 Ulmer, T. S.; Bax, A.; Cole, N. B.; Nussbaum, R. L. Structure and dynamics of micelle-bound human alpha-synuclein. *J Biol Chem* **2005**, *280* (10), 9595-9603. DOI: 10.1074/jbc.M411805200.
- 128 Stief, T.; Vormann, K.; Lakomek, N. A. Sensitivity-enhanced NMR (¹⁵N R(1) and R(1rho) relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields. *Methods* **2024**, *223*, 1-15. DOI: 10.1016/j.ymeth.2024.01.008.
- 129 Fawzi, N. L.; Ying, J.; Ghirlando, R.; Torchia, D. A.; Clore, G. M. Atomic-resolution dynamics on the surface of amyloid-beta protofibrils probed by solution NMR. *Nature* **2011**, *480* (7376), 268-272. DOI: 10.1038/nature10577.
- 130 Fawzi, N. L.; Ying, J.; Torchia, D. A.; Clore, G. M. Kinetics of amyloid beta monomer-to-oligomer exchange by NMR relaxation. *J Am Chem Soc* **2010**, *132* (29), 9948-9951. DOI: 10.1021/ja1048253.
- 131 Bertini, I.; Gallo, G.; Korsak, M.; Luchinat, C.; Mao, J.; Ravera, E. Formation kinetics and structural features of Beta-amyloid aggregates by sedimented solute NMR. *Chembiochem* **2013**, *14* (14), 1891-1897. DOI: 10.1002/cbic.201300141.
- 132 Ravera, E. The bigger they are, the harder they fall: A topical review on sedimented solutes for solid-state NMR. *Concepts in Magnetic Resonance Part A* **2015**, *43* (6), 209-227. DOI: 10.1002/cmr.a.21318.
- 133 Berkeley, R. F.; Kashefi, M.; Debelouchina, G. T. Real-time observation of structure and dynamics during the liquid-to-solid transition of FUS LC. *Biophys J* **2021**, *120* (7), 1276-1287. DOI: 10.1016/j.bpj.2021.02.008.
- 134 Emmanouilidis, L.; Bartalucci, E.; Kan, Y.; Ijavi, M.; Perez, M. E.; Afanasyev, P.; Boehringer, D.; Zehnder, J.; Parekh, S. H.; Bonn, M.; et al. A solid beta-sheet structure is formed at the surface of FUS droplets during aging. *Nat Chem Biol* **2024**, *20* (8), 1044-1052. DOI: 10.1038/s41589-024-01573-w.
- 135 Tycko, R. The evolving role of solid state nuclear magnetic resonance methods in studies of amyloid fibrils. *Curr Opin Struct Biol* **2025**, *92*, 103043. DOI: 10.1016/j.sbi.2025.103043.

- 136 Tuttle, M. D.; Comellas, G.; Nieuwkoop, A. J.; Covell, D. J.; Berthold, D. A.; Kloepper, K. D.; Courtney, J. M.; Kim, J. K.; Barclay, A. M.; Kendall, A.; et al. Solid-state NMR structure of a pathogenic fibril of full-length human alpha-synuclein. *Nat Struct Mol Biol* **2016**, *23* (5), 409-415. DOI: 10.1038/nsmb.3194.
- 137 Van Melckebeke, H.; Wasmer, C.; Lange, A.; Ab, E.; Loquet, A.; Bockmann, A.; Meier, B. H. Atomic-resolution three-dimensional structure of HET-s(218-289) amyloid fibrils by solid-state NMR spectroscopy. *J Am Chem Soc* **2010**, *132* (39), 13765-13775. DOI: 10.1021/ja104213j.
- 138 El Mammeri, N.; Duan, P.; Dregni, A. J.; Hong, M. Amyloid fibril structures of tau: Conformational plasticity of the second microtubule-binding repeat. *Sci Adv* **2023**, *9* (28), eadh4731. DOI: 10.1126/sciadv.adh4731.
- 139 Lu, J. X.; Qiang, W.; Yau, W. M.; Schwieters, C. D.; Meredith, S. C.; Tycko, R. Molecular structure of beta-amyloid fibrils in Alzheimer's disease brain tissue. *Cell* **2013**, *154* (6), 1257-1268. DOI: 10.1016/j.cell.2013.08.035.
- 140 Bahri, S.; Silvers, R.; Michael, B.; Jaudzems, K.; Lalli, D.; Casano, G.; Ouari, O.; Lesage, A.; Pintacuda, G.; Linse, S.; et al. (1)H detection and dynamic nuclear polarization-enhanced NMR of Aβeta(1-42) fibrils. *Proc Natl Acad Sci U S A* **2022**, *119* (1). DOI: 10.1073/pnas.2114413119.
- 141 Callon, M.; Malar, A. A.; Pfister, S.; Rimal, V.; Weber, M. E.; Wiegand, T.; Zehnder, J.; Chavez, M.; Cadalbert, R.; Deb, R.; et al. Biomolecular solid-state NMR spectroscopy at 1200 MHz: the gain in resolution. *J Biomol NMR* **2021**, *75* (6-7), 255-272. DOI: 10.1007/s10858-021-00373-x.
- 142 Jaudzems, K.; Polenova, T.; Pintacuda, G.; Oschkinat, H.; Lesage, A. DNP NMR of biomolecular assemblies. *J Struct Biol* **2019**, *206* (1), 90-98. DOI: 10.1016/j.jsb.2018.09.011.
- 143 Mandala, V. S.; Hong, M. High-sensitivity protein solid-state NMR spectroscopy. *Curr Opin Struct Biol* **2019**, *58*, 183-190. DOI: 10.1016/j.sbi.2019.03.027.
- 144 Lends, A.; Birlirakis, N.; Cai, X.; Daskalov, A.; Shenoy, J.; Abdul-Shukoor, M. B.; Berbon, M.; Ferrage, F.; Liu, Y.; Loquet, A.; et al. Efficient 18.8 T MAS-DNP NMR reveals hidden side chains in amyloid fibrils. *J Biomol NMR* **2023**, *77* (3), 121-130. DOI: 10.1007/s10858-023-00416-5.
- 145 Gupta, R.; Lu, M.; Hou, G.; Caporini, M. A.; Rosay, M.; Maas, W.; Struppe, J.; Suiter, C.; Ahn, J.; Byeon, I. J.; et al. Dynamic Nuclear Polarization Enhanced MAS NMR Spectroscopy for Structural Analysis of HIV-1 Protein Assemblies. *J Phys Chem B* **2016**, *120* (2), 329-339. DOI: 10.1021/acs.jpcc.5b12134.
- 146 Potapov, A.; Yau, W. M.; Ghirlando, R.; Thurber, K. R.; Tycko, R. Successive Stages of Amyloid-beta Self-Assembly Characterized by Solid-State Nuclear Magnetic Resonance with Dynamic Nuclear Polarization. *J Am Chem Soc* **2015**, *137* (25), 8294-8307. DOI: 10.1021/jacs.5b04843.
- 147 Bayro, M. J.; Debelouchina, G. T.; Eddy, M. T.; Birkett, N. R.; MacPhee, C. E.; Rosay, M.; Maas, W. E.; Dobson, C. M.; Griffin, R. G. Intermolecular structure determination of amyloid fibrils with magic-angle spinning and dynamic nuclear polarization NMR. *J Am Chem Soc* **2011**, *133* (35), 13967-13974. DOI: 10.1021/ja203756x.
- 148 Debelouchina, G. T.; Bayro, M. J.; van der Wel, P. C.; Caporini, M. A.; Barnes, A. B.; Rosay, M.; Maas, W. E.; Griffin, R. G. Dynamic nuclear polarization-enhanced solid-state NMR spectroscopy of GNNQQNY nanocrystals and amyloid fibrils. *Phys Chem Chem Phys* **2010**, *12* (22), 5911-5919. DOI: 10.1039/c003661g.
- 149 Levorin, L.; Becker, N.; Uluca-Yazgi, B.; Gardon, L.; Kraus, M.; Sevenich, M.; Apostolidis, A.; Schmitz, K.; Ruter, N.; Apanasenko, I.; et al. Isoleucine Side Chains as Reporters of Conformational Freedom in Protein Folding Studied by DNP-Enhanced NMR. *J Am Chem Soc* **2025**, *147* (18), 15867-15879. DOI: 10.1021/jacs.5c04159.
- 150 Ansari, S.; Lagasca, D.; Dumarieh, R.; Xiao, Y.; Krishna, S.; Li, Y.; Frederick, K. K. In cell NMR reveals cells selectively amplify and structurally remodel amyloid fibrils. *bioRxiv* **2024**. DOI: 10.1101/2024.09.09.612142.

- 151 Rogawski, R.; McDermott, A. E. New NMR tools for protein structure and function: Spin tags for dynamic nuclear polarization solid state NMR. *Arch Biochem Biophys* **2017**, *628*, 102-113. DOI: 10.1016/j.abb.2017.06.010.
- 152 Viennet, T.; Viegas, A.; Kuepper, A.; Arens, S.; Gelev, V.; Petrov, O.; Grossmann, T. N.; Heise, H.; Etzkorn, M. Selective Protein Hyperpolarization in Cell Lysates Using Targeted Dynamic Nuclear Polarization. *Angew Chem Int Ed Engl* **2016**, *55* (36), 10746-10750. DOI: 10.1002/anie.201603205.
- 153 Busch, H.; Yasir Ateeque, M.; Taube, F.; Wiegand, T.; Corzilius, B.; Kunze, G. Probing Biomolecular Interactions with Paramagnetic Nuclear Magnetic Resonance Spectroscopy. *Chembiochem* **2025**, *26* (6), e202400903. DOI: 10.1002/cbic.202400903.
- 154 Lenard, A. J.; Mulder, F. A. A.; Madl, T. Solvent paramagnetic relaxation enhancement as a versatile method for studying structure and dynamics of biomolecular systems. *Prog Nucl Magn Reson Spectrosc* **2022**, *132-133*, 113-139. DOI: 10.1016/j.pnmrs.2022.09.001.
- 155 Wu, K. P.; Baum, J. Detection of transient interchain interactions in the intrinsically disordered protein alpha-synuclein by NMR paramagnetic relaxation enhancement. *J Am Chem Soc* **2010**, *132* (16), 5546-5547. DOI: 10.1021/ja9105495.
- 156 Janowska, M. K.; Wu, K. P.; Baum, J. Unveiling transient protein-protein interactions that modulate inhibition of alpha-synuclein aggregation by beta-synuclein, a pre-synaptic protein that co-localizes with alpha-synuclein. *Sci Rep* **2015**, *5*, 15164. DOI: 10.1038/srep15164.
- 157 Chiliveri, S. C.; Louis, J. M.; Ghirlando, R.; Bax, A. Transient lipid-bound states of spike protein heptad repeats provide insights into SARS-CoV-2 membrane fusion. *Sci Adv* **2021**, *7* (41), eabk2226. DOI: 10.1126/sciadv.abk2226.
- 158 Takahashi, H.; Ayala, I.; Bardet, M.; De Paepe, G.; Simorre, J. P.; Hediger, S. Solid-state NMR on bacterial cells: selective cell wall signal enhancement and resolution improvement using dynamic nuclear polarization. *J Am Chem Soc* **2013**, *135* (13), 5105-5110. DOI: 10.1021/ja312501d.
- 159 Nagaraj, M.; Franks, T. W.; Saeidpour, S.; Schubeis, T.; Oschkinat, H.; Ritter, C.; van Rossum, B. J. Surface Binding of TOTAPOL Assists Structural Investigations of Amyloid Fibrils by Dynamic Nuclear Polarization NMR Spectroscopy. *Chembiochem* **2016**, *17* (14), 1308-1311. DOI: 10.1002/cbic.201600185.
- 160 Madl, T.; Bermel, W.; Zangger, K. Use of relaxation enhancements in a paramagnetic environment for the structure determination of proteins using NMR spectroscopy. *Angew Chem Int Ed Engl* **2009**, *48* (44), 8259-8262. DOI: 10.1002/anie.200902561.
- 161 Merle, D. A.; Witternigg, A.; Tam-Amersdorfer, C.; Hartlmüller, C.; Spreitzer, E.; Schrank, E.; Wagner-Lichtenegger, S.; Werzer, O.; Zangger, K.; Kungl, A. J.; et al. Increased Aggregation Tendency of Alpha-Synuclein in a Fully Disordered Protein Complex. *J Mol Biol* **2019**, *431* (14), 2581-2598. DOI: 10.1016/j.jmb.2019.04.031.
- 162 Aucoin, D.; Xia, Y.; Theint, T.; Nadaud, P. S.; Surewicz, K.; Surewicz, W. K.; Jaroniec, C. P. Protein-solvent interfaces in human Y145Stop prion protein amyloid fibrils probed by paramagnetic solid-state NMR spectroscopy. *J Struct Biol* **2019**, *206* (1), 36-42. DOI: 10.1016/j.jsb.2018.04.002.
- 163 Oktaviani, N. A.; Risor, M. W.; Lee, Y. H.; Megens, R. P.; de Jong, D. H.; Otten, R.; Scheek, R. M.; Enghild, J. J.; Nielsen, N. C.; Ikegami, T.; et al. Optimized co-solute paramagnetic relaxation enhancement for the rapid NMR analysis of a highly fibrillogenic peptide. *J Biomol NMR* **2015**, *62* (2), 129-142. DOI: 10.1007/s10858-015-9925-8.
- 164 Hsu, S. T. NMR assignments of PI3-SH3 domain aided by protonless NMR spectroscopy. *Biomol NMR Assign* **2014**, *8* (2), 291-295. DOI: 10.1007/s12104-013-9503-5.
- 165 Shen, Y.; Bax, A. Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *Journal of Biomolecular NMR* **2013**, *56* (3), 227-241. DOI: 10.1007/s10858-013-9741-y.
- 166 Liang, J.; Chen, J. K.; Schreiber, S. T.; Clardy, J. Crystal structure of P13K SH3 domain at 20 angstroms resolution. *J Mol Biol* **1996**, *257* (3), 632-643. DOI: 10.1006/jmbi.1996.0190.
- 167 Gullion, T.; Schaefer, J. Rotational-echo double-resonance NMR. 1989. *J Magn Reson* **2011**, *213* (2), 413-417. DOI: 10.1016/j.jmr.2011.09.003.

- 168 Jaroniec, C. P.; Tounge, B. A.; Herzfeld, J.; Griffin, R. G. Frequency selective heteronuclear dipolar recoupling in rotating solids: accurate (^{13}C) - (^{15}N) distance measurements in uniformly (^{13}C) , (^{15}N) -labeled peptides. *J Am Chem Soc* **2001**, *123* (15), 3507-3519. DOI: 10.1021/ja003266e.
- 169 Becker, N.; Frieg, B.; Gremer, L.; Kupreichyk, T.; Gardon, L.; Freiburg, P.; Neudecker, P.; Willbold, D.; Gohlke, H.; Heise, H. Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-Shaped A β (1-42) Amyloid Fibrils. *J Am Chem Soc* **2023**, *145* (4), 2161-2169. DOI: 10.1021/jacs.2c09231.
- 170 Kutzsche, J.; Schemmert, S.; Bujnicki, T.; Zafiu, C.; Halbgebauer, S.; Kraemer-Schulien, V.; Pils, M.; Blomeke, L.; Post, J.; Kulawik, A.; et al. Oral treatment with the all-d-peptide RD2 enhances cognition in aged beagle dogs - A model of sporadic Alzheimer's disease. *Heliyon* **2023**, *9* (8), e18443. DOI: 10.1016/j.heliyon.2023.e18443.
- 171 Hobbs, B.; Drant, J.; Williamson, M. P. The measurement of binding affinities by NMR chemical shift perturbation. *J Biomol NMR* **2022**, *76* (4), 153-163. DOI: 10.1007/s10858-022-00402-3.
- 172 Niu, Z.; Sarkar, R.; Aichler, M.; Wester, H. J.; Yousefi, B. H.; Reif, B. Mapping the Binding Interface of PET Tracer Molecules and Alzheimer Disease Abeta Fibrils by Using MAS Solid-State NMR Spectroscopy. *Chembiochem* **2020**, *21* (17), 2495-2502. DOI: 10.1002/cbic.202000143.
- 173 Uluca, B.; Viennet, T.; Petrovic, D.; Shaykhalishahi, H.; Weirich, F.; Gonulalan, A.; Strodel, B.; Etzkorn, M.; Hoyer, W.; Heise, H. DNP-Enhanced MAS NMR: A Tool to Snapshot Conformational Ensembles of alpha-Synuclein in Different States. *Biophys J* **2018**, *114* (7), 1614-1623. DOI: 10.1016/j.bpj.2018.02.011.
- 174 Dumariéh, R.; Lagasca, D.; Krishna, S.; Kragelj, J.; Xiao, Y.; Ansari, S.; Frederick, K. K. Structural Context Modulates the Conformational Ensemble of the Intrinsically Disordered Amino Terminus of alpha-Synuclein. *J Am Chem Soc* **2025**, *147* (14), 11800-11810. DOI: 10.1021/jacs.4c15653.
- 175 Chiti, F.; Stefani, M.; Taddei, N.; Ramponi, G.; Dobson, C. M. Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* **2003**, *424* (6950), 805-808. DOI: 10.1038/nature01891.
- 176 Buell, A. K.; Tartaglia, G. G.; Birkett, N. R.; Waudby, C. A.; Vendruscolo, M.; Salvatella, X.; Welland, M. E.; Dobson, C. M.; Knowles, T. P. Position-dependent electrostatic protection against protein aggregation. *Chembiochem* **2009**, *10* (8), 1309-1312. DOI: 10.1002/cbic.200900144.
- 177 Bader, R.; Bamford, R.; Zurdo, J.; Luisi, B. F.; Dobson, C. M. Probing the mechanism of amyloidogenesis through a tandem repeat of the PI3-SH3 domain suggests a generic model for protein aggregation and fibril formation. *J Mol Biol* **2006**, *356* (1), 189-208. DOI: 10.1016/j.jmb.2005.11.034.
- 178 Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O. C.; Riedel, D.; Baldus, M. Molecular-level secondary structure, polymorphism, and dynamics of full-length alpha-synuclein fibrils studied by solid-state NMR. *Proc Natl Acad Sci U S A* **2005**, *102* (44), 15871-15876. DOI: 10.1073/pnas.0506109102.
- 179 Nielander, A. C.; McEnaney, J. M.; Schwalbe, J. A.; Baker, J. G.; Blair, S. J.; Wang, L.; Pelton, J. G.; Andersen, S. Z.; Enemark-Rasmussen, K.; Čolić, V.; et al. A Versatile Method for Ammonia Detection in a Range of Relevant Electrolytes via Direct Nuclear Magnetic Resonance Techniques. *ACS Catalysis* **2019**, *9* (7), 5797-5802. DOI: 10.1021/acscatal.9b00358.
- 180 Tosner, Z.; Brandl, M. J.; Blahut, J.; Glaser, S. J.; Reif, B. Maximizing efficiency of dipolar recoupling in solid-state NMR using optimal control sequences. *Sci Adv* **2021**, *7* (42), eabj5913. DOI: 10.1126/sciadv.abj5913.

Acknowledgement

First of all my biggest thank goes to Henrike for making it possible for me to pursue my PhD and always giving me advice, tips and tricks throughout my journey. You gave me the opportunity to truly enjoy science and the freedom to work on my own ideas.

I would also like to thank Nils for taking the second supervision and taking the time to help me whenever I needed help, and Lothar for giving me important scientific advice and showing me the tips and tricks in the wet lab. I learned a lot from you!

A very big thank you goes to my entire research group, especially my colleague Nina, who supervised me during my master's thesis and taught me the hands on working with the NMR spectrometer. We had a very successful time collaborating and publishing together, and I hope we will remain friends for many years to come. I am also grateful to my newer colleagues Leonardo, Melly, and Mirko for the fun and productive times we shared. I truly believe we made a brilliant team.

To San: thank you for having my back and always supporting me throughout this entire journey. I'm so happy I found you. *Anh yêu em!*

I also want to thank my family for the support and foundation to make it here. Especially my mother! We've had rough times, but you were always by my side and proud of me. Thank you for everything!

Last but not least a big thank you to all the friends who made this possible.

To my lifelong friends, 63 Gang, for standing by me over 25 years.

To my school friends, RMC, to get me through my life and reminding me that I can always count on you.

To my bachelors' friends, DAG, for helping me survive bachelor's, a truly fun time and a forever lasting friendship.

To my Master's friends, Filip, Sebi and Marcel for countless discussions at Botschaft and board game sessions (long time ago).

A special thanks to Toni and Laura for giving me a place where I could feel welcome and for reminding me that there's more to life than work.

And of course, Florb was also there.

6

Appendix

Appendix

Chemical shift values for monomeric PI3K-SH3 in its native state at pH 6.8 (BMRB: 53388):

Residue Number	Residue Type	C	CA	CB	CD	CD1	CD2	CE	CG	CG1	CG2	CZ	HN	N
3	M	176.3	55.7	32.8										
4	S	174.0	58.4	64.0									8.3	117.1
5	A	177.1	52.1	19.8									8.4	125.8
6	E	175.9	56.2	31.4					36.5				8.2	119.4
7	G	171.9	44.8										8.5	109.4
8	Y	174.3	56.5	41.9									9.4	121.8
9	Q	174.0	54.7	32.7					35.8				9.2	119.7
10	Y	173.4	56.5	43.5									9.3	119.7
11	R	176.9	53.1	34.5	43.4				27.1				9.2	120.5
12	A	177.8	52.9	19.2									9.2	127.8
13	L	174.9	55.1	44.0		25.6	22.3		26.7				9.3	125.8
14	Y	173.4	54.1	42.5									7.1	112.2
15	D	175.6	54.3	41.7									8.1	117.5
16	Y	173.8	57.4	42.2									8.7	121.9
17	K	174.1	53.5	32.7	42.1				24.2				7.6	129.8
18	K	176.4	57.3	33.0	29.8			42.3	23.3				8.3	123.1
19	E	176.2	56.4	32.5					36.3				9.3	123.0
20	R	176.8	54.4	31.6	43.3				27.0				8.8	122.1
21	E	178.3	59.6	29.8					36.5				8.6	120.7
22	E	176.8	57.3	29.8					36.8				8.8	115.3
23	D	175.5	53.6	42.8									8.1	119.7
24	I	174.0	59.4	41.5		15.1				24.8	19.4		8.2	111.2

25	D	177.7	54.3	42.2									8.0	115.7
26	L	176.5	53.2	45.3		24.4							9.1	116.1
27	H	173.6	52.7	29.2									8.1	121.5
28	L	178.0	57.9	42.2			25.1		27.2				8.5	124.4
29	G	174.4	45.1										9.0	115.9
30	D	174.5	55.3	41.4									8.8	123.5
31	I	174.8	57.9	38.2		10.2				26.6	17.4		8.0	120.4
32	L	176.6	53.3	45.2		25.1	25.7		27.8				9.3	125.7
33	T	175.1	62.0	70.6							24.0		9.6	117.7
34	V	174.9	60.9	36.2						22.4	20.3		10.0	131.3
35	N	176.7	54.0	39.3									9.5	126.9
36	K	178.2	60.1	33.0	29.8			42.3	25.1				9.0	127.0
37	G	176.5	46.8										9.0	104.9
38	S	175.8	61.0	63.0									7.8	117.0
39	L	179.5	57.5	41.2		25.7	23.4		27.3				7.4	121.6
40	V	180.2	65.9	32.1						22.6	21.0		8.2	120.5
41	A	178.9	54.5	18.4									7.9	122.4
42	L	177.2	55.0	42.9		25.6	22.4		26.8				7.3	116.7
43	G	174.5	45.2										7.9	106.5
44	F	175.5	57.5	39.6									7.6	119.6
45	S	174.4	57.4	65.0									8.7	117.6
46	D	176.5	56.5	40.8					180.1				8.6	122.6
47	G	176.9	45.2										9.3	116.0
48	Q	176.8	58.1	29.2	180.1				35.0				8.2	120.6
49	E	175.6	57.9	26.1					33.5				9.6	117.9

Appendix

50	A	176. 0	51.7	19.1									6.6	120. 1
51	K		53.4	32.4	29.2			42.1	24.1				7.5	118. 6
52	P	176. 9	64.8	30.7	50.9				27.8					
53	E	177. 2	58.5	27.5					35.3				10.0	119. 3
54	E	176. 9	56.7	30.2					36.9				7.6	119. 6
55	I	176. 1	62.8	38.3		13.2				27.0	17.0		7.3	120. 7
56	G	173. 8	46.2										7.7	108. 9
57	W	174. 9	57.9	30.6									8.1	124. 7
58	L	173. 7	53.4	45.2		25.7			27.8				8.3	123. 7
59	N	175. 3	53.0	42.8					178. 3				7.9	117. 3
60	G	169. 5	46.6										8.9	111. 5
61	Y	173. 7	56.0	42.0									8.8	120. 7
62	N	174. 1	52.5	39.3									9.1	126. 5
63	E	178. 9	57.9	29.3	177. 3				35.3				9.3	125. 7
64	T	176. 1	66.0	68.9							23.0		8.2	116. 1
65	T	176. 4	61.5	70.5							21.8		7.4	109. 2
66	G	174. 0	45.6										8.7	112. 5
67	E	173. 8	55.7	31.9					36.6				7.1	118. 6
68	R	175. 1	54.1	34.1	43.4				27.5			159. 7	8.7	120. 2
69	G	171. 0	45.9										8.9	111. 7
70	D	175. 9	53.6	42.2					179. 0				8.5	121. 0
71	F		54.6	39.1									8.9	113. 7
72	P	178. 6	61.6	30.8	49.9				26.9					
73	G	174. 5	46.5										7.9	112. 2
74	T	175. 7	62.7	69.0							22.9		7.3	104. 0

75	Y	175.0	58.1	39.0									7.5	119.5
76	V	173.1	58.6	36.5						21.0	19.1		7.3	108.9
77	E	175.7	53.2	33.1					35.3				8.8	115.4
78	Y	176.0	58.6	39.2									9.5	125.5
79	I		61.0	38.9		13.0				27.1	17.5		8.3	122.0
80	G		44.9											
81	R	176.8	55.0	32.1	43.3				24.0				8.3	117.1
82	K	175.2	55.8	34.5	29.6			42.2	24.7				8.3	122.7
83	K	176.2	56.3	32.9	29.1			41.9	24.8				8.6	127.4
84	I	174.9	61.0	39.1		13.8				26.6	17.9		8.4	123.7
85	S		55.8	64.6									8.3	121.3
86	P		64.9	32.2	50.6				27.3					

Chemical shift values for seeded, fibrillar PI3K-SH3 (pH 2.5):

Residue Number	Residue Type	C	CA	CB	CG	N	CD	CD1	CD2	CE	CE1	CE2	CG1	CG2	CZ
1	G	169.6	43.6												
2	S	173.3	58.6	63.9		121.2									
3	M	175.2	55.3	32.3	32.3	121.2				18.2					
4	S	173.0	57.1	64.7		119.4									
5	A	175.2	51.6	21.7		133.1									
6	E	175.3	54.2	34.6	34.7	119.4	180.6								
7	G	171.5	44.8			109.0									
8	Y	173.7	52.5	39.7		129.1									
11	R	173.5	55.6	30.9	27.7		43.8								

Appendix

12	A	173. 2	50. 3	20. 7		128. 3									
13	L	174. 5	55. 3	46. 0	28.2	124. 6		30. 9	30. 9						
31	I	175. 5	59. 9	40. 4		116. 5		14. 4					26. 9	17. 7	
32	L	175. 4	55. 9	42. 5	31.5	126. 4		27. 4	24. 3						
33	T	173. 2	61. 5	69. 6		124. 4								22. 2	
34	V	173. 9	60. 4	36. 4		124. 9							20. 5		
35	N		54. 3	36. 6		124. 6									
36	K	174. 9	55. 5	35. 5	25.9	120. 5	29.5			43. 8					
37	G		43. 8			104. 3									
38	S	174. 3	57. 5	63. 1		116. 5									
39	L	176. 2	53. 9	44. 8	27.4	122. 4		27. 2	27. 2						
40	V	173. 7	61. 2	35. 2		123. 9							21. 1		
41	A	173. 3	49. 7	21. 3		129. 4									
45	S	175. 3	58. 9	62. 6		115. 8									
48	Q	175. 7	54. 3	31. 5	33.9	126. 1	180. 1								
49	E	176. 3	54. 4	31. 3	36.9	130. 1	180. 1								
50	A	176. 1	49. 5	21. 5		130. 1									
51	K		53. 6	34. 0	26.4	121. 2	28.6			43. 2					
52	P	175. 2	56. 2	31. 5	27.4	121. 4	49.8								
53	E	171. 1	54. 1	32. 1	33.6	121. 6	180. 7								
54	E		54. 0	32. 0	33.3	121. 6									
55	I	175. 9	58. 1	40. 9		121. 0		13. 6					27. 9	17. 7	
58	L	175. 0	54. 0	42. 2	30.0	108. 7		25. 0							
59	N		54. 4	40. 4		127. 9									
60	G	171. 4	46. 0			109. 2									

61	Y		55. 3	39. 3	129. 2	128. 7						118. 9			
64	T	173. 7	61. 5	69. 9		124. 6								22. 1	
65	T	173. 7	61. 5	70. 7		124. 5								22. 1	
66	G	172. 4	45. 7			118. 8									
68	R	175. 5	54. 1	36. 3	27.9	124. 6	45.1								159. 1
69	G	173. 7	47. 5			112. 9									
72	P		62. 6	33. 0	27.2		50.7								
73	G	174. 1	45. 4			109. 2									
74	T	174. 2	62. 5	71. 6		121. 4								21. 1	
75	Y	171. 3	54. 4	44. 9	126. 5	128. 1					118. 2				159. 1
76	V	173. 5	60. 7	33. 9		128. 5							20. 9		

Quality control ^1H -NMR: solvent residuals ammonium acetate

For this work, particularly in the aggregation studies, it is crucial to use a pure and uncontaminated sample. 1D water suppressed ^1H spectra are sufficient to detect impurities. For aggregation and unfolding studies, a Gly-HCl buffer at pH 2.5 was used. Figure S1 shows a 1D ^1H spectrum recorded at the end of a 25 °C aggregation measurement with 200 μM PI3K-SH3, 2% seeds, and Gly-HCl at pH 2.5. The glycine signals from the buffer are marked in the spectrum. At low pH the glycine group exchanges sufficiently slow to be detected in the spectra. Ca bound ^1H signals are visible at around ~ 3.6 ppm. Furthermore, a second set of peaks is visible that is likely due to residuals of the lyophilization of the purified SH3 domain in 5 mM ammonium acetate with ammonium signals becoming visible at low pH. These exhibit a doublet of triplets due to ^{15}N ^{14}N coupling¹⁷⁹.

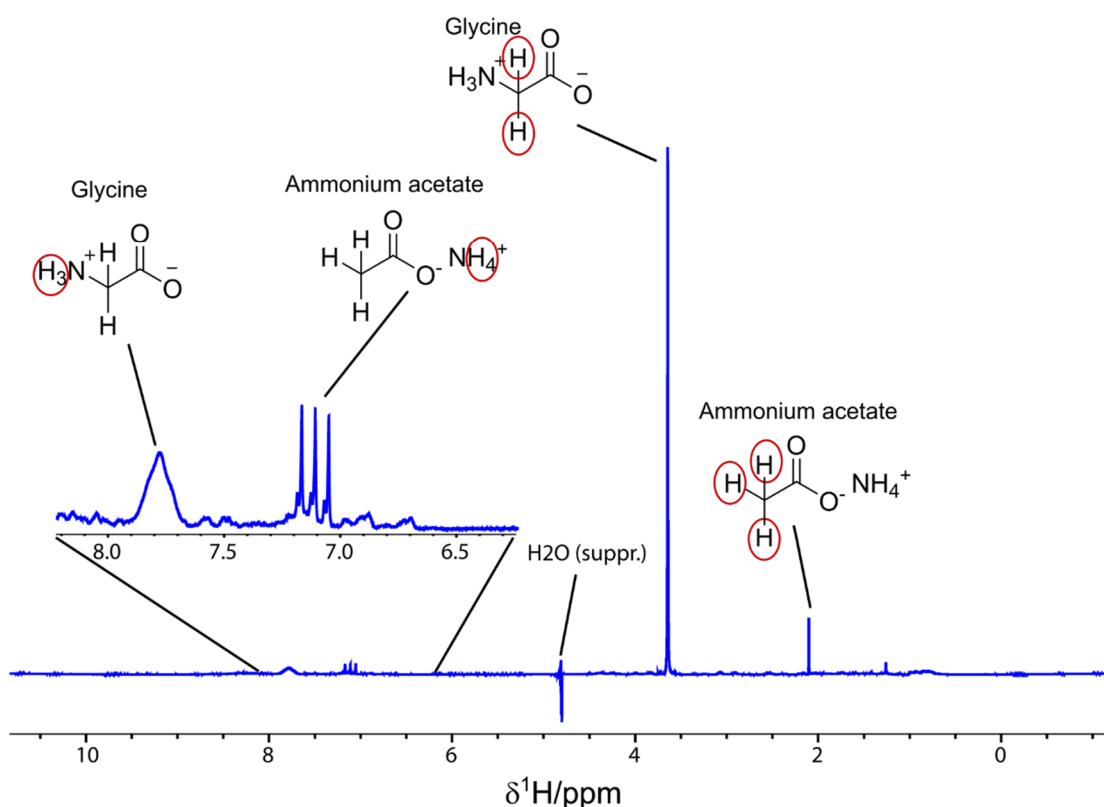


Figure 30: ^1H NMR spectrum of PI3K-SH3 after ~100 hours of aggregation at 25 °C. Sample conditions: 200 μM PI3K-SH3, 2% seeds, Gly-HCl buffer (pH 2.5). Distinct peaks corresponding to glycine and residual ammonium acetate from protein lyophilization are labeled, with their respective molecular structures shown above.

Optimal control double CP

We tested the optimal control CP method developed by Tosner et al.¹⁸⁰ as an alternative to conventional ramp or tangent-shaped pulses for N-C transfer, with the shaped pulse applied to carbon in a double CP spectrum. The contact time must be rotor-synchronized (e.g. 4615.385 μs for 13 kHz MAS spinning), as deviations lead to signal loss. To validate the method, we tested it on the tripeptide Methionine-Leucine-Phenylalanine (MLF), following the approach in Tosner's study, and obtained similar results. Optimal control CP provided a more efficient N-C transfer (Figure S2). However, in PI3K-SH3 fibrils, the improvement was less pronounced but still observable. In 2D spectra with spin diffusion, such as NCACX and NCOCX, the difference became even smaller.

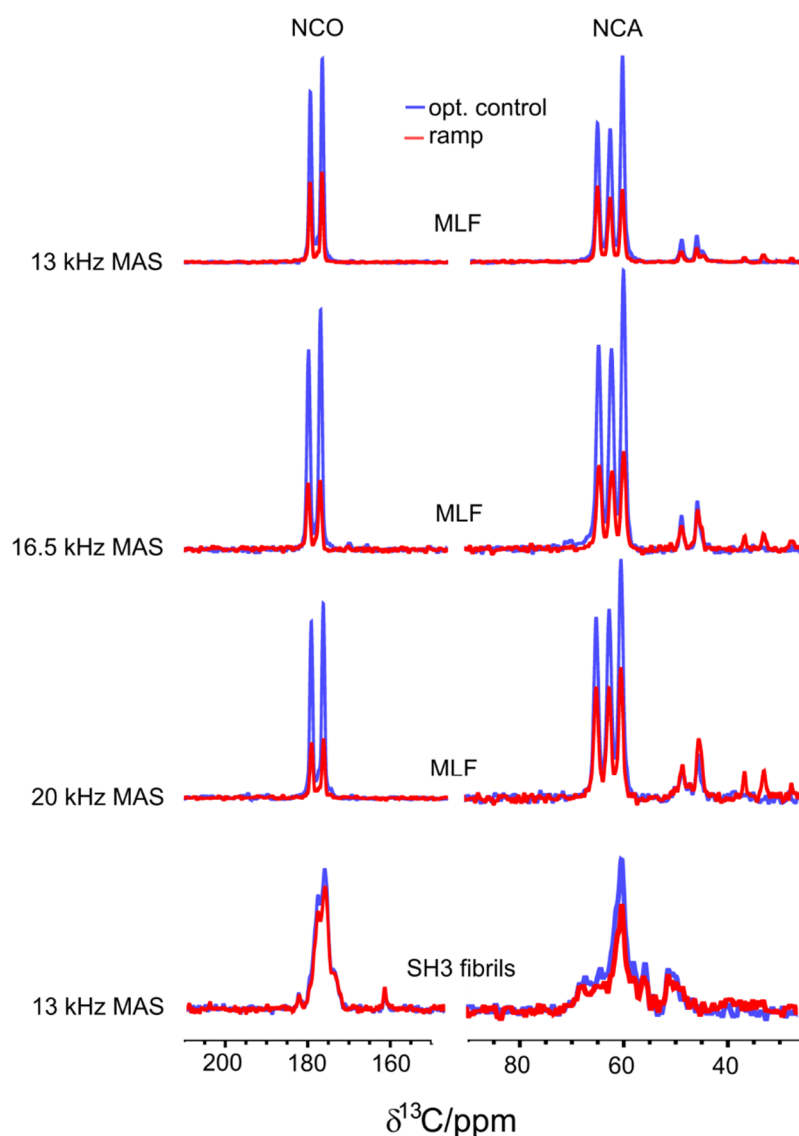


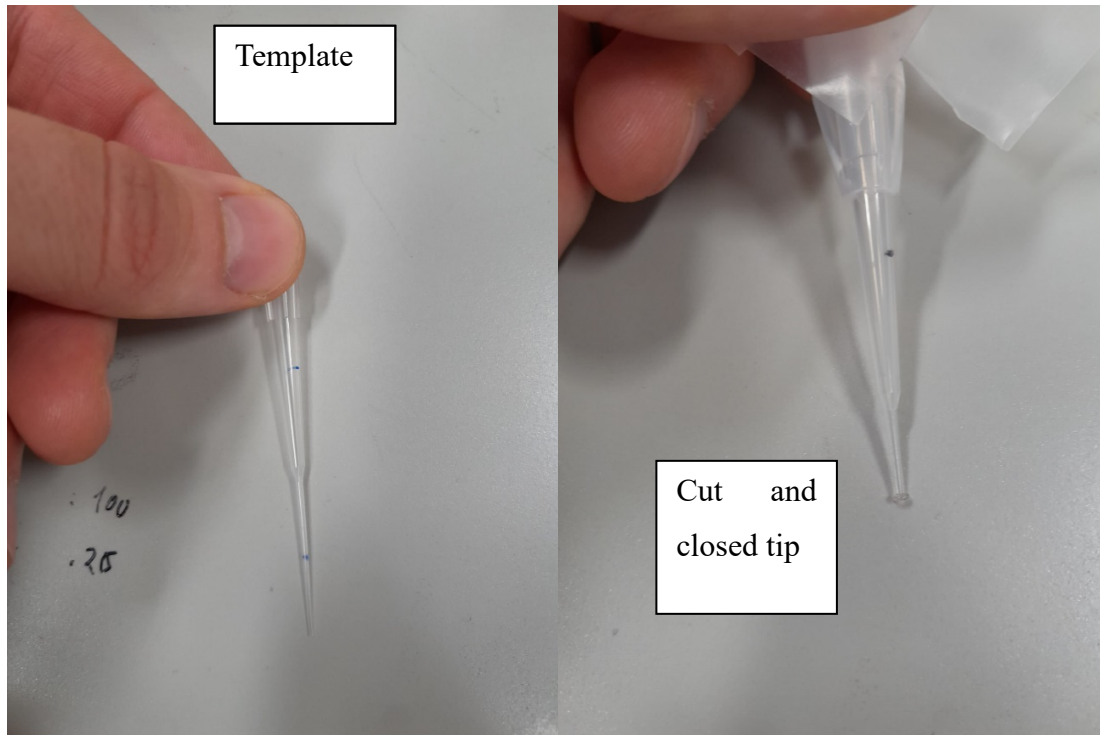
Figure 31: Optimal control cross-polarization (CP) for N-C transfer. Left: NCO spectra, right: NCA spectra of MLF and PI3K-SH3 fibrils recorded at different MAS frequencies. Blue spectra represent double CP experiments using optimal control CP, while red spectra correspond to ramped double CP.

Step by step protocol for filling 3.2 mm MAS rotors using homemade rotor filling devices (for fibrillar SH3 samples)

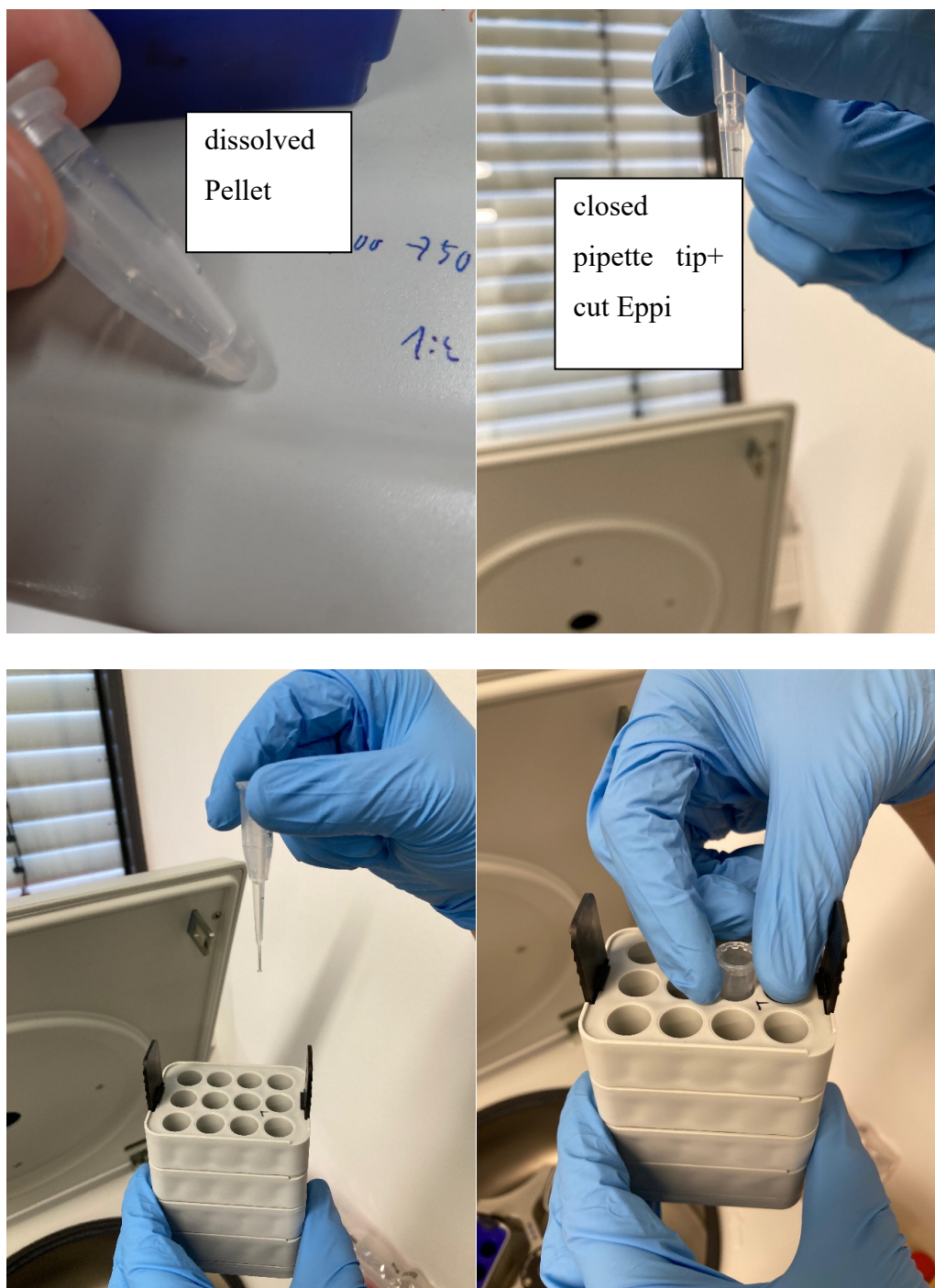
1. Transfer fibril solution (200 nmol monomer equivalent) to two centrifuge tubes for ultracentrifugation. Spin at 55000 rpm for 1 h using a TLA 55 rotor. After centrifugation determine the protein concentration in the supernatant to assess the efficiency of fibril formation, typically, 10-20% of the protein remains in the soluble fraction.

Appendix

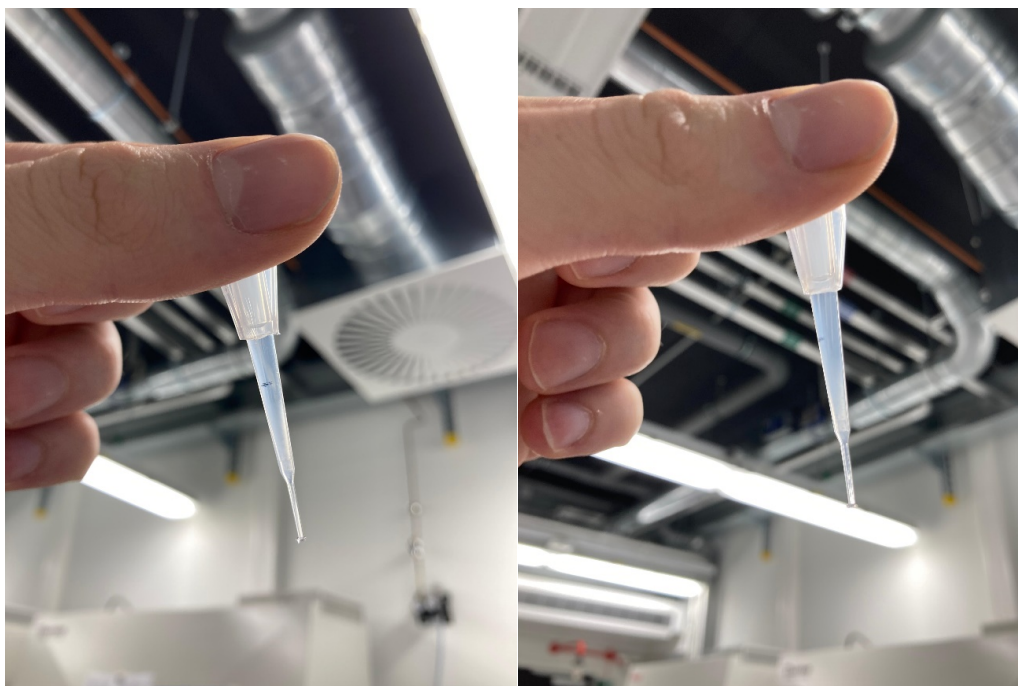
2. While centrifuging, prepare a homemade rotor filling tool. Take standard gel loader pipette tip and mark the cutting point using a marked template pipette tip to ensure it fits the rotor filling device. Seal the narrow end using a lighter.



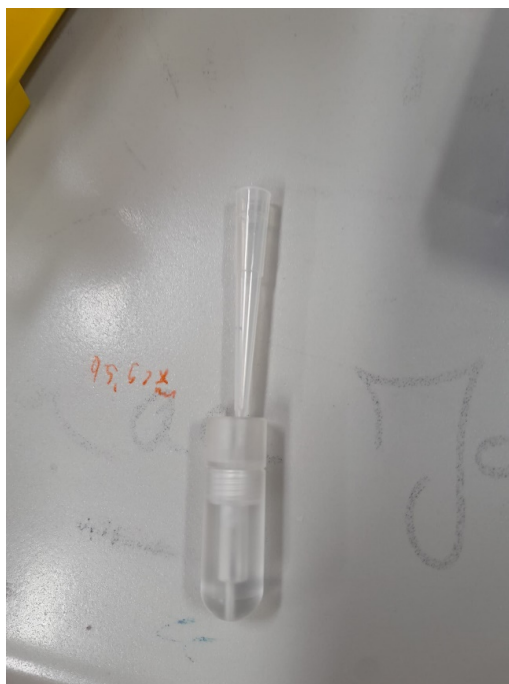
3. Fill the prepared tip with 30-50 μl water and spin at 5000 rpm in a swing-out rotor to create a visible line inside the tip. This will serve as volume reference. Invert the tip and centrifuge again to remove the water completely.
4. After ultracentrifugation, carefully remove the supernatant and use a portion of it ($\sim 60 \mu\text{l}$) to resuspend the pellet ($\sim 20 \mu\text{l}$ in size). Note: This step requires patience, as the pellet may take time to fully dissolve into the supernatant.
5. Transfer the resuspended fibril solution into the prepared closed pipette tip. Place the pipette tip in a cut Eppendorf tube and centrifuge in a swing out rotor at 5000 rpm for 30 minutes to sediment the fibrils in the pipette tip.



6. After centrifugation, a visible gradient should appear within the pipette tip: a dense, whitish or cloudy fibril layer at the bottom and a clearer, water-like supernatant above. Carefully remove the supernatant without touching the sedimented fibrils in the pipette tip. Repeat this steps several times to reach the volume mark with sedimented fibrils. With each round of centrifugation, the phase separation becomes easier to observe.



7. After the desired volume marked is reached, cut the pipette tip at the sealed edge and insert it into the rotor filling device containing the rotor. Centrifuge at 5000 rpm in a swing out rotor for one minute. Carefully remove the rotor using tweezers and place the cap onto the rotor.



Bruker Topspin Code for REDOR-SD pulse sequence

The following segment provides the pulse program code for the REDOR-SD 2D experiment described in Section 3.6, implemented in TopSpin version 4.1:

```

;hC_fsREDOR.cp
;version TS3.5 /2017/01/10
;written for TopSolids Bio: JOS 10.1.2017, 24.10.2023 changed to our convention LG, LL, NR,
; tested on 26.10.2023, HH LG. Works as 2D and 1D
;2D frequency selective REDOR experiment for exact NC heteronuclear distance measurments
;Tested 01/10/2016 on AV III console 700SB HCN 1.3mm probe
;Avance III version
;parameters:
;d1      : recycle delay
;p1      : C 90 at p11
;p2      : H 90 at p12
;p4      : C90 selective pulse at sp4
;p9      : z-filter delay p114 on H
;p15     : HC CP at sp41 (C) and sp40 (H)
;p19     : pulse length 180 deg gaussian shape N
;p20     : pulse length 180 deg gaussian shape C
;p13     : N 90 at p121
;p12     : N 180 at p121
;p11     : C pulse power
;p12     : H pulse power
;p15     : C (f1) power level for CP
;p16     : N NCO CP power
;p112    : H high dec power
;p113    : H high dec power
;p114    : H low power decoupling during z filter approx 1* rotation frequency (DARR)
;p116    : N Dec. during AQ (using 'waltz16', 2 kHz)
;p121    : N pulse power
;sp0     : H CP power (n=1 HH condition)
;sp4     : power level selective 90 deg pulse C (gaussian shape)
;sp20    : gaussian shape 180 deg pulse C
;sp21    : gaussian shape 180 deg pulse N
;d0      : incremented delay (t1)
;d1      : recycle delay; 1 to 5 times T1
;d8      : TEDOR total mixing time
;d30     : extra time for -DCDC2D/3D (t1)
;d31     : taur
;pcpd2   : H dec pulse cpdprg2 (~H180 + 0.3 us for swftppm)

```

Appendix

```
;cpdprg2: Decoupling program during REDOR element. use 'swftppm' (at pl12 or pl13) or sltppm for fast
MAS >40 kHz and low power decoupling
;cpdprg3 : Decoupling program during data acquisition in t1 and t2 use 'swftppm' (at pl12 or pl13) or
sltppm for fast MAS >40 kHz and low power decoupling
;spnam4 : selective 90 deg pulse C (sinc shape)
;spnam20 : selective 180 pulse on C Gauss or similar
;spnam21 : selective 180 pulse on N Gauss or similar
;cnst10 : Frequency reset to Carrier (o1, usually ~100 ppm)
;cnst21 : Frequency offset for CO (~175 ppm)
;cnst22 : Frequency offset for Ca (~55 ppm)
;cnst31 : MAS rotation rate in Hz
;in0 : increment of dephasing steps
;l1 : loopcounter for REDOR mixing period
;l2 : integer for C selective, rotor synchronized 180 pulse
;l4 : L2<=L4 integer for N selective, rotor synchronized 180 pulse
;l3 : loopcounter for REDOR mixing time increment
;ZGOPTNS : -DlargeSW for large SW (unlikely)
; -DXY16 : for xy16 phase cycle during REDOR block - hardly required and potentially detrimental for
signal
; or blank
;FnMODE : States-TPPI is recommended (for easy rotor sync.)
;but mc command allows for other FnModes as well
;ns : n*8
;mix : longest REDOR mixing time
;redmix : TEDOR block total mixing time duration determined by 0.5*td1

#####
;# #
;# Frequency Selective Heteronuclear Dipolar Recoupling #
;# in Rotating Solids: Accurate 13C-15N Distance #
;# Measurement in Uniformly 13C, 15N-Labeled Solids #
;# C.P.Jaroniec, B.A.Tounge, J.Herzfeld and R.G.Griffin #
;# JACS 2001, 124, 3507 - 3519 #
;# #
#####
;$COMMENT=heteronuclear correlation CN
;$CLASS=BioSolids
;$DIM=2D
;$TYPE=cross polarisation
;$SUBTYPE=REDOR
```

```

prosol relations=<biosolCHN>
#include <CHN_defs.incl>
    ; defines which channel corresponds to which nucleus
#include <trigg.incl>
    ; definition of external trigger output
#include <TauR_def.incl>
    ; definition of rotor period dependent delays
"p22=2*p3"
"d25=0.25s/cnst31"
"d26=0.25s/cnst31-(p22/2)"      ; one-quarter rotor cycle ...
"d28=0.75s/cnst31-1u"
"d29=1.25s/cnst31-1u"
"d31=1s/cnst31"
;"p2=2*p1"
"p20=2*12*1s/cnst31"
"p19=2*14*1s/cnst31"
"d0=0.2u"

;set t1 increment for States-TPPI
"in0=inf1" ;
;"acqt0=0"
define delay REDincr
define delay mix
"mix=4*11/cnst31"
"REDincr=4*11/cnst31"
if "l1=0" {
"mix=4*11/cnst31"
"REDincr=4*11/cnst31"
}
define delay ONTIME      ;#####
                        ;# Power Deposition Calcs #
                        ;#####
                        ;#####
;$EXTERN                ;# python insertion point #
                        ;#####

Prepare, ze
"ONTIME=aq+mix+p15"
ONTIME
;#####
;#      Protections: Pre-Check      #
;#####

```

Appendix

```
#include <TauR_prot.incl>
    ;min. spinning 2.5 kHz
#ifdef lacq
#include <acq_prot.incl>
    ;Max. 50 ms acquisition time
#include <ONTIME_prot.incl>
    ;total RF deposition restriction
#endif
#include <p15bio_prot.incl>
    ;p15 max. 10 ms
#include <t1_prot.incl>
    ;check d0 and d30
;REDincr
;mix
Start, d1 do:H
    10u pl1:C          ;set power level to drive HP amplifier
    10u pl2:H          ;set decoupler power
    10u pl3:N
    1m rpp17           ; reset phase list ph8 ...
    1m rpp18
    trigg              ;additional trigger available on HP router
    1u fq=0.0:H        ;set 1H on resonance
;#####
;#      Cross/Direct Polarization      #
;#####
0.5u fq=cnst9(bf ppm):C
(p2 pl2 ph1):H
(p15 pl5 ph2):C (p15:sp0 ph0):H
;t1 evolution
    1u pl12:H
    1u cpds2:f2
    d0
;#####
;#      Spin Diffusion      #
;#####
    1u cpds2:H ;f2
(p4:sp4 ph13):C
    1u do:H      ;f2
    d11          pl1:C; (p14 ph7):H
    0.5u fq=cnst10(bf ppm):C ; offset to CO
```



```

(p1 ph14):C
;#####
;#          REDOR Mixing S signal          #
;#####
TauR cpds2:H ; tppm decoupling at pl12 if synchronized with TPPM use special decoupling sequence
d25 pl1:C
5 d26          ; First 1/2 REDOR period several F3 pulses ...
(p22 ph17^):N ; ... at intervals of ...
d26          ; ... one-half rotor cycle
d26
(p22 ph17^):N ; ... at intervals of ...
d26          ; F1 pulse
d26          ; several F3 pulses ...
(p22 ph17^):N ; ... at intervals of ...
d26          ; ... at intervals of ...
d26          ; ... one-half rotor cycle
(p22 ph17^):N ; ... at intervals of ...
d26          ; F1 pulse
lo to 5 times l1
d28
1u do:H
(center (p19:sp21 ph4):N (p20:sp20 ph3):C (p19 pl12 ph10):f2 ) ; Hahn echo refocussing pulse on
F1
1u cpds2:f2
d29
6 d26          ; second 1/2 REDOR period several F3 pulses ...
(p22 ph18^):f3 ; ... at intervals of ...
d26          ; ... at intervals of ...
d26          ; ... one-half rotor cycle
(p22 ph18^):N ; ... at intervals of ...
d26          ;
d26          ; several F3 pulses ...
(p22 ph18^):N ; ... at intervals of ...
d26          ; ... at intervals of ...
d26          ; ... one-half rotor cycle
(p22 ph18^):N ; ... at intervals of ...
d26          ; F1 pulse
lo to 6 times l1 ;
d28          ; End of REDOR period
1u fq=0:C

```

Appendix

```
go=Start ph31
30m do:f2 mc #0 to Start F1PH(ip2, id0)
HaltAcqu, 1m
exit
/*
12 d1 do:H
  10u pl1:C          ;set power level to drive HP amplifier
  10u pl2:H          ;set decoupler power
  10u pl3:N

  1m rpp17           ; reset phase list ph8 ...
  1m rpp18

  trigg              ;additional trigger available on HP router
;#####
;#      Cross/Direct Polarization      #
;#####
  (p3 pl2 ph1):H
  (p15:sp41 ph2):C (p15:sp40 ph0):H
;#####
;#      REDOR Mixing S0 signal      #
;#####
  d31 cpds2:H
  d25 pl1:C          ; tppm decoupling at pl12 if synchronized with TPPM use special decoupling
sequence
15 d26              ; First 1/2 REDOR period several F3 pulses ...
  (p22 ph17^):N      ; ... at intervals of ...
  d26                ; ... one-half rotor cycle
  d26
  (p22 ph17^):N      ; ... at intervals of ...
  d26                ; F1 pulse
  d26                ; several F3 pulses ...
  (p22 ph17^):N      ; ... at intervals of ...
  d26                ; ... at intervals of ...
  d26                ; ... one-half rotor cycle
  (p22 ph17^):N      ; ... at intervals of ...
  d26                ; F1 pulse
lo to 15 times l1
  d28
  1u do:H
  (center (p20:sp20 ph3):C (p20 pl13 ph10):H ) ; Hahn echo refocussing pulse on F1
  1u cpds2:H
```

```

d29
16 d26 ; second 1/2 REDOR period several F3 pulses ...
(p22 ph18^):N ; ... at intervals of ...
d26 ; ... at intervals of ...
d26 ; ... one-half rotor cycle
(p22 ph18^):N ; ... at intervals of ...
d26 ;
d26 ; several F3 pulses ...
(p22 ph18^):N ; ... at intervals of ...
d26 ; ... at intervals of ...
d26 ; ... one-half rotor cycle
(p22 ph18^):N ; ... at intervals of ...
d26 ; F1 pulse
lo to 16 times l1 ;
d28 ; End of REDOR period
go=12 ph31
1m do:H
30m wr #0 if #0 zd ; Recording REDOR reference signal S0 */

ph0= 1 ; phHCP
ph1= 0 ; phH90
ph2= 1 1 1 1 3 3 3 3 ; phCCP
ph3= 0 1 2 3 ; phC180sel
ph4= 0 ; phN180sel
ph10= 0 ; pHdec
ph13= 0 ; ph 90 deg selective pulse spin diffusion
ph14= 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2
      3 3 3 3 3 3 3 3 0 0 0 0 0 0 0 0 ; 90 deg hard pulse spin diffusion (check!!!!)

ph17= 0 1 0 1 ; phNredor xy4
ph18= 0 1 0 1 ; phNredor xy4
ph31= 0 2 0 2 2 0 2 0 3 1 3 1 1 3 1 3
      2 0 2 0 0 2 0 2 1 3 1 3 3 1 3 1
; $Id:$

```

Full journal versions of Section 2.1

Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy

Luis Gardon⁺,^[a, b] Nina Becker⁺,^[a, b] Lothar Gremer,^{*[a, b]} and Henrike Heise^{*[a, b]}

Extracellular amyloid- β (A β) plaques, primarily formed by A β (1-40) and A β (1-42) fibrils, are a hallmark of Alzheimer's disease. The A β peptide can undergo a high variety of different post-translational modifications including formation of a pyroglutamate (pGlu, pE) at N-terminal Glu3 or Glu11 of truncated A β (3-x) or A β (11-x), respectively. Here we studied structural similarities and differences between pEA β (3-42) and LS-shaped A β (1-42) fibrils grown under identical conditions (pH 2) using solid-

state NMR spectroscopy. We show that the central region of pEA β (3-42) fibrils including the turn region around V24 is almost identical to A β (1-42) showing similar β -strands also at the N-terminus. The missing N-terminal residues D1-A2 along with pE3 formation in pEA β (3-42) preclude a salt bridge between K28-D1' as in A β (1-42) fibrils. G37 and G38 act as highly sensitive internal sensors for the modified N-terminus, which remains rigid over ~five pH units.

Introduction

Aggregation of amyloid- β (A β) is closely linked to Alzheimer's disease (AD).^[1] The dominant components of the senile A β plaques are A β (1-40) and A β (1-42), the latter has been shown to be more aggregation prone and more toxic.^[2] In addition to the full-length peptides, different N- and C-terminally truncated A β species (e.g. 3-x, 11-x), as well as various post-translational modifications are found in the plaques of AD patients and were shown to have altered biological and biophysical properties.^[3] N-terminal Glu residues in truncated A β (3-x) or A β (11-x) are usually converted to a cyclic pyroglutamate residue (pyroGlu, pE) (Figure 1A) catalyzed by glutaminyl cyclases, QC,^[4] leading to pE-modified A β species (pEA β).

Compared to A β (1-42), pEA β (3-42) shows higher toxicity in mouse models,^[5] a faster aggregation rate, and it cross seeds and accelerates the aggregation of A β (1-42).^[6] NMR studies on monomeric pEA β (3-40) and pEA β (3-42) revealed significant

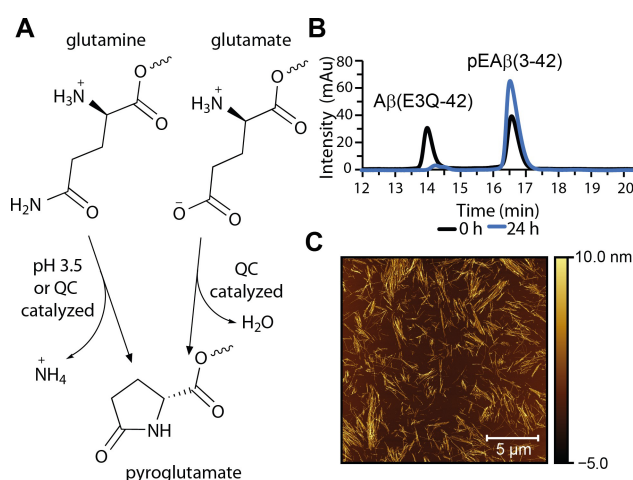


Figure 1. Production and detection of pEA β (3-42) fibrils. A) Scheme of pyroglutamylation of A β (3-42) or A β (E3Q-42) to pEA β (3-42), and B) analysis by RP-HPLC. HPLC runs are shown after 0 h (black) and 24 h (blue) incubation time at pH 3.5. pEA β (3-42) is already visible at time 0 h since its formation already starts during TEV cleavage of the fusion construct. C) AFM image of pEA β (3-42) fibrils.

chemical shift perturbations for the N-terminal residues up to G9, while the rest of the sequence remains relatively unperturbed compared to A β (1-40) and A β (1-42), respectively.^[7] In contrast to pEA β (3-42) fibrils, pEA β (3-40) fibrils and pEA β (11-40) fibrils have already been well characterized by solid-state NMR^[8] and revealed only small structural changes limited to the N-terminus when compared to A β (1-40) fibrils. The N-terminus is, however, flexible in the systems studied. Different to *in vitro* A β (1-42) fibril structures with flexible N-termini,^[9] an LS-shaped A β (1-42) fibril morph with the entire N-terminus being part of the rigid core can be produced at pH 2 in 30% acetonitrile, and its atomic structure was determined in a combined cryo-EM/solid-state NMR study.^[10]

These fibrils retained their structure including the fixed rigid N-terminus, apart from few salt bridge rearrangements, when

[a] L. Gardon,⁺ N. Becker,⁺ L. Gremer, H. Heise
Institute of Biological Information Processing (IBI-7: Structural Biochemistry)
and JuStruct: Jülich Center for Structural Biology
Forschungszentrum Jülich
52425 Jülich, Germany
E-mail: l.gremer@fz-juelich.de
h.heise@fz-juelich.de

[b] L. Gardon,⁺ N. Becker,⁺ L. Gremer, H. Heise
Physikalische Biologie
Heinrich-Heine-Universität Düsseldorf
40225 Düsseldorf, Germany

[*] sharing 1st authorship

Supporting information for this article is available on the WWW under
<https://doi.org/10.1002/chem.202303007>

© 2023 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

exposed to pH 7.^[11] Here, we used identical fibril preparation conditions^[10–11] for recombinant pEAβ(3–42), report on a complete NMR resonance assignment and secondary structure analysis of pEAβ(3–42) fibrils including the influence of a subsequent shift from pH 2 to pH 6.5 and compare it to the structure of LS-shaped Aβ(1–42) fibrils.

Results and Discussion

For recombinant production of uniformly [^{13}C , ^{15}N]-labeled pEAβ(3–42) we used an Aβ(E3Q–42) construct because an N-terminal Gln, in contrast to Glu, is able to convert chemically at acidic pH (3.5) into pE (Figure 1A) without the need of a QC catalyst.^[7a] pEAβ(3–42) formation was monitored by RP-HPLC (Figure 1B). pEAβ(3–42) has a longer retention time than Aβ(EQ3–42) due to the lack of the hydrophilic amino group at the N-terminus (Figure 1A). Fibril growth of purified pEAβ(3–42) was completed after several weeks under quiescent conditions at room temperature. Mostly nonbranched fibrils without any amorphous aggregates were obtained, as obvious from atomic force microscopy (AFM) (Figure 1C). Far-UV CD spectra of both

pEAβ(3–42) fibrils and LS-shaped Aβ(1–42) fibrils show same characteristics for β -sheet dominated structures (Figure S1).

We compared 2D Proton Driven Spin Diffusion (PDS) ^{13}C - ^{13}C fingerprint spectra of Aβ(1–42) and pEAβ(3–42) fibrils (Figure 2A, Table S1). Both spectra show clear similarities, particularly for the range Y10 to I32. Of note, chemical shifts of V24 located in the turn region of Aβ(1–42) fibrils are highly similar for pEAβ(3–42) fibrils (Figure 2A, C). These shifts are characteristic as they show a relatively low C_α chemical shift and two distinct C_γ resonances typical for steric restriction, which were not observed in the spectra of other Aβ(1–42) fibril polymorphs with flexible N-termini.^[9] Furthermore, both fibrils show identical resonances for S26 and K28 in the PDS spectra (Figure 2A).

As the two spectra show distinct differences for several amino acids located in other regions (e.g. the N-terminus, G37 and G38, detailed below), we performed a *de novo* resonance assignment for pEAβ(3–42) fibrils. Using a combination of different 2D and 3D ^{13}C - ^{13}C and NCACX/NCOCX spectra, we assigned all residues unambiguously, except H14, which was not visible (Figure 2E, S2–S4, Table S2). As for LS-shaped Aβ(1–42) fibrils,^[10–11] no protein signals are observed in Insensitive Nuclei Enhancement by Polarization Transfer (INEPT) spectra of

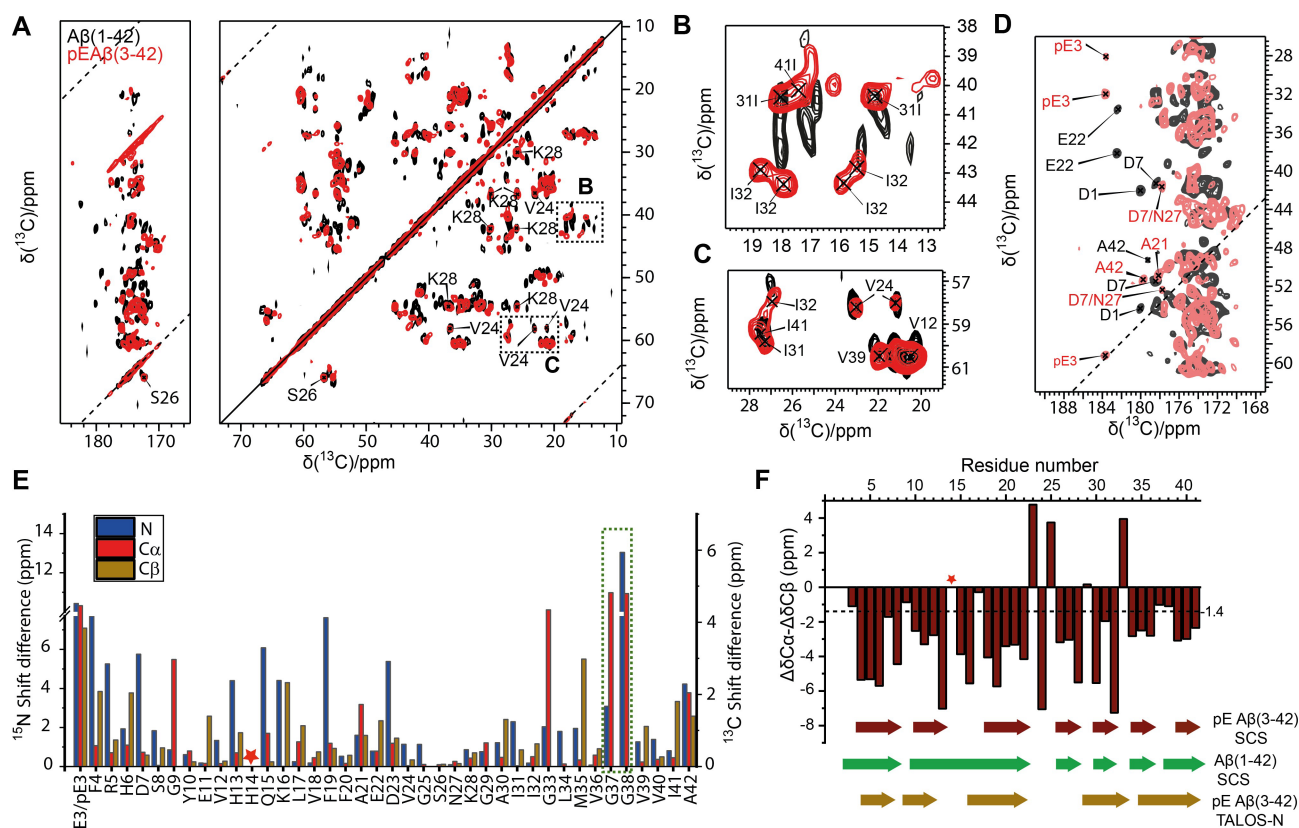


Figure 2. Comparison of pEAβ(3–42) and Aβ(1–42) fibrils produced under identical conditions. A) Overlay of 2D ^{13}C - ^{13}C PDS spectra with 20 ms mixing time at 11 kHz Magic Angle Spinning (MAS) frequency: pEAβ(3–42) fibrils (red) and LS-shaped Aβ(1–42) fibrils (black).^[10–11] The complete assignment of pEAβ(3–42) fibrils is shown in Figures S2–S4. B, C) Zoomed regions from A: C β and C γ /C δ cross peaks for Ile residues (B) and C α and C γ cross peaks for Val (in particular identical V24) and Ile residues (C). D) CO-region of 2D ^{13}C - ^{13}C PDS spectra with 200 ms mixing time and 12.5 kHz MAS frequency of Aβ(1–42) fibrils (black) and 2D ^{13}C - ^{13}C DARR with 100 ms mixing time and 20 kHz MAS frequency of pEAβ(3–42) fibrils (bright red). pE3 resonances are clearly visible for the pEAβ(3–42) fibrils, whereas resonances for D1–A2 are missing. E) Chemical shift difference of N, C α and C β chemical shifts for pEAβ(3–42) and Aβ(1–42) fibrils. F) Secondary chemical shifts (SCS) and TALOS-N analysis. A β -strand in SCS is defined when three consecutive residues exhibit (C α -C α)-(C β -C β) < -1.4.^[12] The asterisk marks missing resonances for H14.

pEAβ(3-42) fibrils, excluding any highly mobile and flexible parts (Figure S8).^[13] The carbonyl region of the ^{13}C - ^{13}C correlation spectra (PDSD/ Dipolar Assisted Rotational Resonance (DARR)) exhibits resonances for pE3 (Figure 2D) with chemical shifts typical for N-terminal pE,^[14] indicating a rigid N-terminus in pEAβ(3-42) fibrils. Some residues, e.g. S26 (as already reported for Aβ(1-42) fibrils^[10]), as well as some Ala and Ile residues (Figure 2B) show peak doubling, the latter due to conformational sidechain disorder.^[15] Peak doubling for G37 and G38 in NCOCX spectra indicates local structural variations for both residues (Figure S3). By comparing the chemical shifts of pEAβ(3-42) fibrils with those of Aβ(1-42) fibrils produced under same conditions,^[10] a conserved region (from V24 to K28) can be observed (Figure 2E). The missing D1-A2 residues and pE3 formation in pEAβ(3-42) fibrils led to changes in chemical shifts at the N-terminus up to G9, but also at the C-terminus starting from position G33 with largest changes for ^{13}C chemical shifts of G33, G37 and G38 (Figure 2E). Typically, Gly residues allow more flexibility for the peptide backbone and thus are expected to be more sensitive to structural changes induced by the adjacent modified N-terminus. Indeed, G37 and G38 are most affected by the D1-A2 truncation and pE3 modification. Likewise, the ^{15}N chemical shift of G38 changed by more than 10 ppm to a low value of 103 ppm (Table S2). In LS-shaped Aβ(1-42) fibrils G37 and G38 are in close contact with A2 and part of the hydrophobic cluster composed of A2, V36, F4, L34, G37 and G38.^[10]

Information about torsion angles (Figure S5), secondary structure and position of the β-strands in pEAβ(3-42) fibrils is obtained from secondary chemical shifts^[12] and TALOS-N.^[16] A comparison with results obtained for LS-shaped Aβ(1-42) fibrils^[10] shows similar positions of β-strands (Figure 2F). Correlation plots which relate residue-specific secondary chemical shifts reported *in vitro* structures^[9-10] with those obtained in this work report larger differences relative to S-shaped fibril structures^[9] than for LS-shaped fibrils^[10] (Figure S6).

In LS-shaped Aβ(1-42) fibrils, F19 and I31 are in close spatial proximity (Figure 3A), which can be probed by ^{13}C - ^{13}C spin diffusion at rotational resonance.^[17] Cross correlation peaks indicating a contact between I31 Cδ and the aromatic F19 residue were observed in PDSD spectra, where the spinning speed of 17.2 kHz was matching the chemical shift difference between aromatic Phe and Ile Cδ signals (at 600 MHz ^1H frequency) in both, pEAβ(3-42) and Aβ(1-42) fibrils (Figure 3B, Figure S7). This supports the conservation of the turn region comprising residues F19 to I31. Interestingly, chemical shifts of C-terminal residues in LS-shaped Aβ(1-42) fibrils (at pH 2) changed upon a pH shift to pH 7^[11] towards those observed in pEAβ(3-42) fibrils (at pH 2) (Figure 4 A, B). Especially for ^{15}N and $^{13}\text{C}\alpha$ of G38 chemical shift differences reduce from 13.0 and 4.8 ppm to 1.7 and 0.4 ppm, respectively.

For LS-Aβ(1-42) fibrils shifted to pH 7, the changes at the C-terminus are explained by a rearrangement of the salt bridge from K28-D1' at pH 2 to K28-A42 at pH 7.^[11] A salt bridge between K28 and a D1' residue is obviously absent in pEAβ(3-42) fibrils. We also studied possible alterations of the pEAβ(3-42) fibril structure after pH adjustment to pH 6.5 as performed as

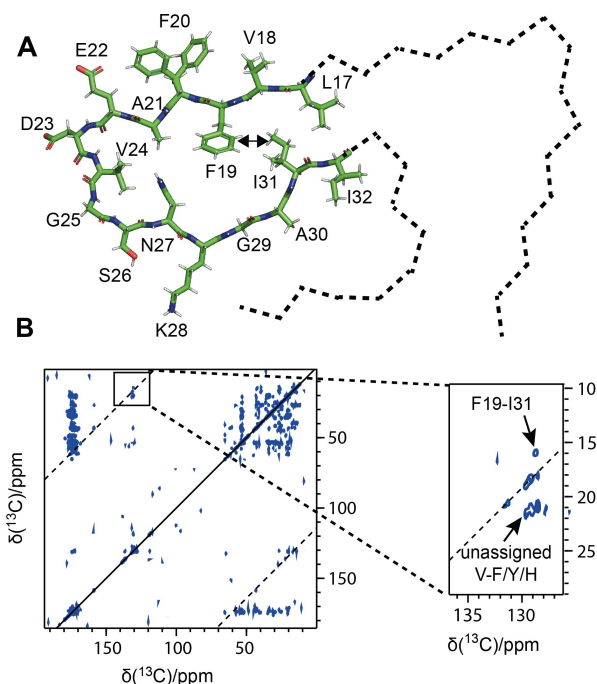


Figure 3. Rotational resonance dipolar recoupling of F19 and I31 sidechains. A) L17 to I32 region of LS-shaped Aβ(1-42) fibrils (PDB:5OQV).^[10] The turn region includes a close contact between F19 and I31 sidechains. B) PDSD spectra at 17.2 kHz MAS of pEAβ(3-42) fibrils show a cross peak indicating dipolar interaction of an aromatic F19 carbon and I31 Cδ. Cross correlation peaks for V-H/F/Y contacts could not be assigned unambiguously (e.g. V18-F19 or V12-H13).

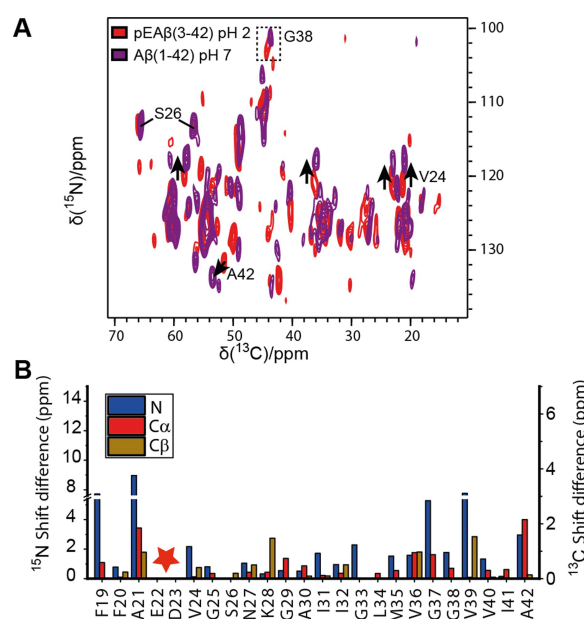


Figure 4. Comparison of pEAβ(3-42) at pH 2 and pH shifted Aβ(1-42) fibrils at pH 7 by addition of citrate-phosphate buffer. A) NCACX spectra at 11 kHz MAS frequency of pEAβ(3-42) at pH 2 and pH shifted Aβ(1-42) fibrils at pH 7.^[11] Resonances for S26 remain unchanged in ^{13}C and ^{15}N dimension, for G38 and V24 almost unchanged in ^{13}C , but shifted in ^{15}N dimension, for A42 shifted in both dimensions (due to change of its protonation state). Shifts of these resonances are indicated by arrows. B) Chemical shift difference of N, Cα and Cβ chemical shifts for pEAβ(3-42) at pH 2 and pH-shifted Aβ(1-42) fibrils at pH 7. The asterisk marks missing E22/D23 resonance assignments for Aβ(1-42) fibrils at pH 7.

recently described for A β (1–42).^[11] The added citrate/phosphate buffer was used as internal pH sensor (Figure S8).

Figure 5A shows the overlay of the 2D ^{13}C – ^{13}C PDSD spectra of pEA β (3–42) fibrils at pH 2 and pH 6.5. As most of the resonances overlap, we can conclude that the global fold of the pEA β (3–42) fibrils remains unchanged from pH 2 to pH 6.5. The C-terminal A42 of pEA β (3–42) fibrils changed from a protonated (at pH 2) to a deprotonated (at pH 6.5) state (Figure 5B), as already observed for LS-shaped A β (1–42) fibrils upon a pH shift^[11] with slow proton exchange on the NMR time scale. The fact that A42 is protonated excludes a salt bridge between K28 and A42 at low pH. Strong correlation signals typical for C α –C δ in deprotonated Glu, likely E22, appear at pH 6.5 (Figure 5B) which is presumably also seen as a shift in the C β –C γ region of E22 (Figure 5D). Likewise, the D7 C β resonances shifted by ~2 ppm to higher ppm values (from 41.5 ppm to 43.6 ppm) upon the pH-shift, typical for deprotonation (Figure 5C). For D7 of A β (1–42) fibrils a fast proton exchange between the protonated and deprotonated state was observed^[11] which we also assume for pEA β (3–42) fibrils. No changes are detected for K28, in contrast to pH 7 shifted A β (1–42). Compared to our previous work we do not observe any significant intensity losses for pEA β (3–42) fibrils at pH 6.5 as seen for A β (1–42) fibrils at pH 7.^[11]

At pH 6.5 the 1D Direct Excitation (DE) compared to the Cross Polarization (CP) spectra (Figure S8C–D) do not show significant differences, and no INEPT signals were observed. This confirms the absence of any highly mobile parts and further supports a rigid N-terminus also at pH 6.5.

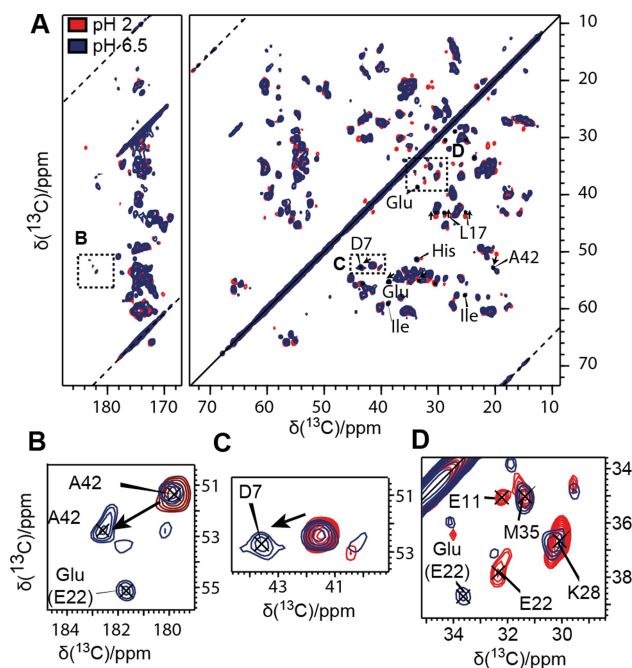


Figure 5. pEA β (3–42) fibrils at pH 2 and adjusted to pH 6.5. A) Overlay of 2D ^{13}C – ^{13}C PDSD spectra with 20 ms mixing time of pEA β (3–42) fibrils at pH 2 (red) and pH 6.5 (navy). B–D) Zoomed regions from A: carbonyl region of A42 for longer mixing time (200 ms) (B) and the shift of the C-terminal A42 indicate a transition from a protonated to a deprotonated state. C α –C β region of D7 (C). C β –C γ region of Glu residues (D).

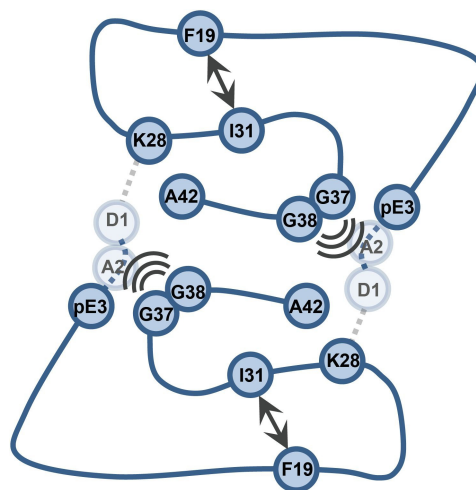


Figure 6. Characteristics of pEA β (3–42) fibrils. The missing N-terminal residues D1–A2 along with pE3 formation in pEA β (3–42) preclude the formation of a salt bridge between K28–D1' as in A β (1–42) fibrils. Residues G37 and G38 sense N-terminal modifications present in pEA β (3–42) fibrils compared to A β (1–42) fibrils. The F19–I31 contact is preserved in both structures.

Water-edited experiments (Figure S9) which probe the global water accessibility also revealed that the overall structure is retained over ~five pH units, as the build-up is only slightly enhanced for pH 6.5 compared to pH 2 due to faster chemical exchange.^[11,18]

Conclusions

To conclude, in this study we report an in-depth NMR characterization of a pEA β (3–42) fibril morph at pH 2 and pH 6.5. Comparison with A β (1–42) fibrils prepared under identical conditions reveals remarkable structural similarities and indicates a conserved central region spanning residues L17 to I32, around the turn at V24 in both fibrils (Figure 6). The missing N-terminal residues D1–A2 along with pE3 formation in pEA β (3–42) preclude the formation of a salt bridge between K28–D1' present in A β (1–42) fibrils. Obviously, this salt bridge is not needed for a rigid N-terminus in this fibril morph. G37 and G38 act as highly sensitive sensors for the modified N-terminus as seen by their shift perturbations. Notably, pEA β (3–42) fibrils harbor β -strand positions highly similar to those in LS-shaped A β (1–42) fibrils including the rigid N-terminal region.

Experimental Section

Sample preparation

Expression of the His-tagged A β (E3Q–42) fusion construct was essentially performed as described^[7a] in 2.5 l cultures of [^{13}C , ^{15}N]-labeled M9 minimal medium with [^{13}C]-glucose and [^{15}N]- NH_4Cl as sole carbon and nitrogen sources. The medium was inoculated with starter cultures (1:100) and incubated at 37 °C and 120 rpm until an OD_{600} of 0.3 was reached. Protein expression was induced

by adding 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The culture was then incubated at 25 °C at 120 rpm overnight. Cells were harvested by centrifugation (5.000 xg, 15 min) and cell pellets were resuspended with ~5 to 8 ml per g cell mass of lysis buffer (50 mM Na phosphate buffer, pH 7.4, 200 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml lysozyme). Cell disruption was done by sonification under ice cooling (15x10 s on, 60 s off, at 80% amplitude, Bandelin Sonopuls, VS70T sonotrode), and subsequent ultracentrifugation (55.000 xg, 1 h) was used to separate the soluble fraction containing the [U- ^{13}C , ^{15}N]-A β (E3Q-42) fusion construct from cell debris. Further purification was done by immobilized metal ion affinity chromatography (IMAC). For that the supernatant was loaded on Ni $^{2+}$ -NTA material (two in series connected 5 ml Ni-NTA Protino columns, Macherey-Nagel, Düren, Germany), equilibrated with 50 mM Na phosphate buffer, pH 7.4, 200 mM NaCl, 20 mM imidazole, pH 7.4, and connected to an Äkta prime plus system (Cytiva, Germany). Elution of the fusion construct was achieved by using an imidazole gradient of 20 to 500 mM imidazole in 50 mM Na phosphate buffer, pH 7.4, 200 mM NaCl, using a gradient volume of 87.5 ml at a flow rate of 2.5 ml/min. Subsequently, tobacco etch virus (TEV) protease digestion was performed using 1 mg TEV per 8.5 mg A β (E3Q-42) fusion construct in 50 mM Na phosphate buffer, pH 7.4, 200 mM NaCl for 7 days at 4 °C. After 3 days incubation 15 mM Tris(2-carboxyethyl)phosphine (TCEP) was added as reducing agent. [U- ^{13}C , ^{15}N]-A β (E3Q-42) was then further purified by semipreparative RP-HPLC on Zorbax SB-300 C8 (9.4 mm x 250 mm column, Agilent, Böblingen, Germany) connected to an Agilent 1260 Infinity system with UV detection at 214 nm and a column temperature of 80 °C. The mobile phase used was isocratic aqueous 30% (v/v) acetonitrile (ACN), 0.1% (v/v) trifluoroacetic acid (TFA). Purified [U- ^{13}C , ^{15}N]-A β (E3Q-42) was freeze dried, and pyroGlu formation of the N-terminal was performed as described^[7a] by dissolving lyophilized [U- ^{13}C , ^{15}N]-A β (E3Q-42) in 30 mM sodium acetate, pH 3.5 (pH adjusted with acetic acid) at a concentration of 0.25 mg/ml and incubation at 4 °C for 3 days. A further semipreparative HPLC purification step under the same isocratic conditions (aqueous 30% (v/v) ACN, 0.1% (v/v) TFA, pH 2) as described above yields pure [U- ^{13}C , ^{15}N]-pEA β (3-42) with typical purities of above 97%. Fibril growth of pEA β (3-42) was achieved by quiescent incubation of ~6 μM pEA β (3-42) directly in the mobile HPLC phase (aqueous 30% (v/v) ACN, 0.1% (v/v) TFA, pH 2) for one month at RT as already described for A β (1-42).^[10]

Atomic force microscopy (AFM) and far-UV circular dichroism (CD) spectroscopy

For AFM, typically 4 μl samples of pEA β (3-42) fibrils in aqueous 30% (v/v) ACN, 0.1% (v/v) TFA, pH 2 at concentrations of 10 to 50 μM (monomer equivalents) were pipetted on a freshly cleaved mica support and incubated for 10 min. Then the mica was dried with a gas stream of nitrogen. AFM micrographs were recorded in peak-force tapping mode on a Bruker Multimode 8 (Billerica, Massachusetts, USA) using OMCL-AC160TS cantilevers (Shinjuku, Tokyo, Japan) using ScanAsyst software at 1024x1024 pixel resolution. The images were processed with Gwyddion 2.61.^[19]

For far-UV CD spectroscopy, samples of pEA β (3-42) fibrils or A β (1-42) fibrils in aqueous 30% (v/v) ACN, 0.1% (v/v) TFA, pH 2 at typical concentrations in the range of 12 to 32 μM (monomer equivalents) were placed into 1 mm quartz cuvettes (Helma, Germany) and measured with following instrument settings on a Jasco J-1100 CD spectropolarimeter: 0.1 nm step size, 50 nm/min scan speed, 1 nm bandwidth, 10 accumulations, 20 °C.

Rotor filling and pH shift

[U- ^{13}C , ^{15}N]-pEA β (3-42) fibrils in aqueous 30% (v/v) ACN, 0.1% (v/v) TFA, pH 2 were harvested by centrifugation (15.000 xg, 60 min) and applied by centrifugation (500 xg, 60 sec), into a 3.2 mm thick wall rotor (Bruker, Germany) by homemade rotor filling tools. For the pH 6.5 shifted fibril sample harvested [U- ^{13}C , ^{15}N]-pEA β (3-42) fibrils (grown at pH 2 in 30% (v/v) ACN, 0.1% (v/v) TFA) were adjusted to pH 6.5 by addition and mixing of 10 μl citrate-phosphate buffer stock, pH 7.0^[20] to 40 μl harvested fibril pellet as described previously.^[11] The citrate-phosphate buffer stock was obtained by mixing aqueous solutions of 17.65 vol% of 0.25 M citric acid and 82.35 vol% of 0.5 M Na $_2$ HPO $_4$. The pH 6.5 adjusted [U- ^{13}C , ^{15}N]-pEA β (3-42) fibril sample thus contained aqueous 24% (v/v) ACN, 0.08% (v/v) TFA, 82.35 mM Na $_2$ HPO $_4$, 8.825 mM citric acid, and placed in a 3.2 mm thin wall rotor (Bruker, Germany) as described above. As previously described for [U- ^{13}C , ^{15}N]-A β (1-42) fibrils^[11] each 50 μl rotor sample contains ~1.7 to 2.1 mg [U- ^{13}C , ^{15}N]-pEA β (3-42) fibrils, equivalent to 380 to 460 nmol monomer equivalents of [U- ^{13}C , ^{15}N]-pEA β (3-42), which corresponds to final [U- ^{13}C , ^{15}N]-pEA β (3-42) concentrations of 7.5 to 9.3 mM. Thus, the fibril preparations contain ~96% solvent and are highly hydrated. In our previous study on pH 2 to pH 7 adjusted A β (1-42) fibrils, we could exclude an effect of the citrate-phosphate buffer on the A β (1-42) fibril structure.^[11]

Solid-state NMR experiments

Solid-state NMR experiments were conducted on an 18.8 T (800 MHz ^1H frequency) Avance III and 14.1 T (600 MHz ^1H frequency) Bruker AVANCE NEO spectrometer equipped with triple resonance HCN 3.2 mm MAS Efree probes. Typical radiofrequency field strengths were 91–100 kHz for ^1H , 55.6 kHz for ^{13}C and 45.5 kHz for ^{15}N . The VT gas temperature was set to 263 K (thermocouple reported temperature); the sample temperature was estimated to be around 5–10 K higher due to frictional heating under MAS. Spinal64^[21] ^1H decoupling (rf field of 85 kHz) was applied during ^{13}C acquisition. The MAS frequency was set to 11 kHz for most of the Proton Driven Spin Diffusion (PDS) and NCACX/NCOCX experiments, 12.5 kHz for other PDS, 17.2 kHz for Rotational Resonance experiments and 20 kHz for Dipolar Assisted Rotational Resonance (DARR). Detailed experimental parameters can be found in Table S1. ^{13}C chemical shifts were externally referenced using adamantane by setting its CH signal to 31.4 ppm (corresponding to the DSS reference scale). The ^{15}N chemical shifts were indirectly referenced to liquid NH $_3$. All spectra were processed using TopSpin 3.5 and 4.0.9 (Bruker). The analysis was performed using CcpNMR Analysis 2.4.^[22]

Water-edited experiments: To probe water-accessibility of the fibrils, we performed water-edited 1D experiments, where ^1H polarization of the protein is destroyed using a spin-echo T_2 -filter with a τ of 2.5 ms duration, followed by a transfer of ^1H polarization from water back onto the protein by longitudinal ^1H - ^1H mixing^[23] (Figure S8A). Chemical exchange of protons as well as spin diffusion at the water-protein interface lead to higher intensities for water-exposed residues compared to residues in the dry interior of the protein.^[24] For the global water accessibility, we integrated the aliphatic regions of the water-edited 1D ^{13}C CP spectra for the pEA β (3-42) and A β (1-42) fibrils at pH 2 and pH 6.5 (Figure S8B, C). Comparing water build-up times, we observe a slower build-up for pEA β (3-42) than for A β (1-42) fibrils at both pH values of pH 2 and pH 6.5 (Figure S8D), which might be caused by the higher hydrophobicity, as three charges are missing for the shorter peptide. Additionally, the build-up is only slightly enhanced for pH 6.5 compared to pH 2 due to faster chemical exchange,^[18] also indicating that the global structure is retained over ~five pH units.

Acknowledgements

The authors gratefully acknowledge experimental assistance by Robin Backer and Celina Schulz, Heinrich-Heine-Universität Düsseldorf (HHU). Access to the Jülich-Düsseldorf Biomolecular NMR Center jointly run by Forschungszentrum Jülich and HHU is acknowledged. HH was supported by the Entrepreneur Foundation at HHU and the DFG (HE 3243/4-1 and INST 208/771-1 FUGG). NMR data are deposited in the Biological Magnetic Resonance Data Bank (BMRB) under accession number 51993. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in BMRB at <https://bmr.io>, reference number 51993.

Keywords: Amyloid beta-peptides • NMR spectroscopy • Neurodegenerative diseases • Alzheimer's disease • Pyroglutamate formation

- [1] M. Hashimoto, E. Rockenstein, L. Crews, E. Masliah, *NeuroMol. Med.* **2003**, *4*, 21–36.
- [2] a) G. Bitan, M. D. Kirkitadze, A. Lomakin, S. S. Vollers, G. B. Benedek, D. B. Teplow, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 330–335; b) M. Folin, S. Baiguera, M. Tommasini, D. Guidolin, M. T. Conconi, E. De Carlo, G. G. Nussdorfer, P. P. Parnigotto, *Int. J. Mol. Med.* **2005**, *15*, 929–935; c) T. Iwatsubo, A. Odaka, N. Suzuki, H. Mizusawa, N. Nukina, Y. Ihara, *Neuron* **1994**, *13*, 45–53.
- [3] a) O. Wirths, S. Zampar, *Expert Opin. Ther. Targets* **2019**, *23*, 991–1004; b) M. Wulff, M. Baumann, A. Thummler, J. K. Yadav, L. Heinrich, U. Knupfer, D. Schlénzig, A. Schierhorn, J. U. Rahfeld, U. Horn, J. Balbach, H. U. Demuth, M. Fandrich, *Angew. Chem. Int. Ed. Engl.* **2016**, *55*, 5081–5084.
- [4] a) M. P. Kummer, M. T. Heneka, *Alzheimer's Res. Ther.* **2014**, *6*, 28; b) S. Schilling, T. Hoffmann, S. Manhart, M. Hoffmann, H. U. Demuth, *FEBS Lett.* **2004**, *563*, 191–196.
- [5] a) O. Wirths, H. Breyhan, H. Cynis, S. Schilling, H. U. Demuth, T. A. Bayer, *Acta Neuropathol.* **2009**, *118*, 487–496; b) L. C. Camargo, M. Schoneck, N. Sangarapillai, D. Honold, N. J. Shah, K. J. Langen, D. Willbold, J. Kutzsche, S. Schemmert, A. Willuweit, *Int. J. Mol. Sci.* **2021**, *22*; c) T. Dunkelmann, S. Schemmert, D. Honold, K. Teichmann, E. Butzkuven, H. U. Demuth, N. J. Shah, K. J. Langen, J. Kutzsche, D. Willbold, A. Willuweit, *J. Alzheimer's Dis.* **2018**, *63*, 115–130.
- [6] C. Dammers, M. Schwarten, A. K. Buell, D. Willbold, *Chem. Sci.* **2017**, *8*, 4996–5004.
- [7] a) C. Dammers, L. Gremer, P. Neudecker, H. U. Demuth, M. Schwarten, D. Willbold, *PLoS One* **2015**, *10*, e0139710; b) C. Dammers, L. Gremer, K. Reiss, A. N. Klein, P. Neudecker, R. Hartmann, N. Sun, H. U. Demuth, M. Schwarten, D. Willbold, *PLoS One* **2015**, *10*, e0143647; c) C. Dammers, K. Reiss, L. Gremer, J. Lecher, T. Ziehm, M. Stoldt, M. Schwarten, D. Willbold, *Biophys. J.* **2017**, *112*, 1621–1633.
- [8] a) H. A. Scheidt, J. Adler, M. Krueger, D. Huster, *Sci. Rep.* **2016**, *6*, 33531; b) H. A. Scheidt, J. Adler, U. Zeitschel, C. Höfling, A. Korn, M. Krueger, S. Roßner, D. Huster, *Chem. Eur. J.* **2017**, *23*, 15834–15838.
- [9] a) Y. Xiao, B. Ma, D. McElheny, S. Parthasarathy, F. Long, M. Hoshi, R. Nussinov, Y. Ishii, *Nat. Struct. Mol. Biol.* **2015**, *22*, 499; b) M. T. Colvin, R. Silvers, Q. Z. Ni, T. V. Can, I. Sergeev, M. Rosay, K. J. Donovan, B. Michael, J. Wall, S. Linse, R. G. Griffin, *J. Am. Chem. Soc.* **2016**, *138*, 9663–9674; c) M. A. Wälti, F. Ravotti, H. Arai, C. G. Glabe, J. S. Wall, A. Böckmann, P. Güntert, B. H. Meier, R. Riek, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E4976–E4984; d) A. Wickramasinghe, Y. Xiao, N. Kobayashi, S. Wang, K. P. Scherpelz, T. Yamazaki, S. C. Meredith, Y. Ishii, *J. Am. Chem. Soc.* **2021**, *143*, 11462–11472.
- [10] L. Gremer, D. Schölzel, C. Schenk, E. Reinartz, J. Labahn, R. B. G. Ravelli, M. Tusche, C. Lopez-Iglesias, W. Hoyer, H. Heise, D. Willbold, G. F. Schröder, *Science* **2017**, *358*, 116–119.
- [11] N. Becker, B. Friege, L. Gremer, T. Kupreichyk, L. Gardon, P. Freiburg, P. Neudecker, D. Willbold, H. Gohlke, H. Heise, *J. Am. Chem. Soc.* **2023**, *145*, 2161–2169.
- [12] D. S. Wishart, B. D. Sykes, *J. Biomol. NMR* **1994**, *4*, 171–180.
- [13] O. C. Andronesi, S. Becker, K. Seidel, H. Heise, H. S. Young, M. Baldus, *J. Am. Chem. Soc.* **2005**, *127*, 12965–12974.
- [14] A. Hinterholzer, V. Stanojlovic, C. Cabrele, M. Schubert, *Anal. Chem.* **2019**, *91*, 14299–14305.
- [15] L. Siemons, B. Ulucay-Yazgi, R. B. Pritchard, S. McCarthy, H. Heise, D. F. Hansen, *Chem. Commun. (Camb.)* **2019**, *55*, 14107–14110.
- [16] Y. Shen, A. Bax, *J. Biomol. NMR* **2013**, *56*, 227–241.
- [17] a) F. Creuzet, A. McDermott, R. Gebhard, K. van der Hoef, M. B. Spijker-Assink, J. Herzfeld, J. Lugtenburg, M. H. Levitt, R. G. Griffin, *Science* **1991**, *251*, 783–786; b) T. G. Oas, R. G. Griffin, M. H. Levitt, *J. Chem. Phys.* **1988**, *89*, 692–695.
- [18] V. S. Mandala, A. R. Loftis, A. A. Shcherbakov, B. L. Pentelute, M. Hong, *Nat. Struct. Mol. Biol.* **2020**, *27*, 160–167.
- [19] D. Necas, P. Klapetek, *Cent. Eur. J. Phys.* **2012**, *10*, 181–188.
- [20] T. C. McIlvaine, *J. Biol. Chem.* **1921**, *49*, 183–186.
- [21] B. M. Fung, A. K. Khitrin, K. Ermolaev, *J. Magn. Reson.* **2000**, *142*, 97–101.
- [22] W. F. Vranken, W. Boucher, T. J. Stevens, R. H. Fogh, A. Pajon, M. Llinas, E. L. Ulrich, J. L. Markley, J. Ionides, E. D. Laue, *Proteins* **2005**, *59*, 687–696.
- [23] a) A. Lesage, A. Bockmann, *J. Am. Chem. Soc.* **2003**, *125*, 13336–13337; b) C. Ader, R. Schneider, K. Seidel, M. Etzkorn, S. Becker, M. Baldus, *J. Am. Chem. Soc.* **2009**, *131*, 170–176; c) J. K. Williams, M. Hong, *J. Magn. Reson.* **2014**, *247*, 118–127.
- [24] M. D. Gelenter, K. J. Smith, S.-Y. Liao, V. S. Mandala, A. J. Dregni, M. S. Lamm, Y. Tian, W. Xu, D. J. Pochan, T. J. Tucker, Y. Su, M. Hong, *Nat. Struct. Mol. Biol.* **2019**, *26*, 592–598.

Manuscript received: November 20, 2023

Accepted manuscript online: December 15, 2023

Version of record online: December 29, 2023

Chemistry–A European Journal

Supporting Information

Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy

Luis Gardon, Nina Becker, Lothar Gremer,* and Henrike Heise*

Structural Impact of N-terminal Pyroglutamate in an Amyloid- β (3-42) Fibril Probed by Solid-State NMR Spectroscopy

Luis Gardon, ‡^{[a][b]} Nina Becker, ‡^{[a][b]} Lothar Gremer, *^{[a][b]} and Henrike Heise *^{[a][b]}

[a] L. Gardon, N. Becker, L. Gremer, H. Heise

Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology

Forschungszentrum Jülich

52425 Jülich, Germany

E-mail: l.gremer@fz-juelich.de, h.heise@fz-juelich.de

[b] L. Gardon, N. Becker, L. Gremer, H. Heise

Physikalische Biologie

Heinrich-Heine-Universität Düsseldorf

40225 Düsseldorf, Germany

‡ sharing 1st authorship

* corresponding authors

Contents

Far-UV CD spectra of LS-shaped A β (1-42) fibrils and pEA β (3-42) fibrils.....	3
PDSD spectrum and assignment graph of pEA β (3-42) fibrils	4
2D NCOCX and 2D NCACX spectra of pEA β (3-42) fibrils	5
Sequential walk of pEA β (3-42) fibrils using 3D NCACX and 3D NCOCX spectra.....	6
Predicted torsion angles.....	7
Correlation plots.....	8
Contact between F19 and I31 sidechains in LS-shaped A β (1-42) fibrils and pEA β (3-42) fibrils	9
No highly mobile protein signals and internal pH sensor	10
Water build up curves	11
Experimental parameters for NMR	12
Chemical shift table for pEA β (3-42) fibrils	13
References.....	15

Far-UV CD spectra of LS-shaped A β (1-42) fibrils and pEA β (3-42) fibrils

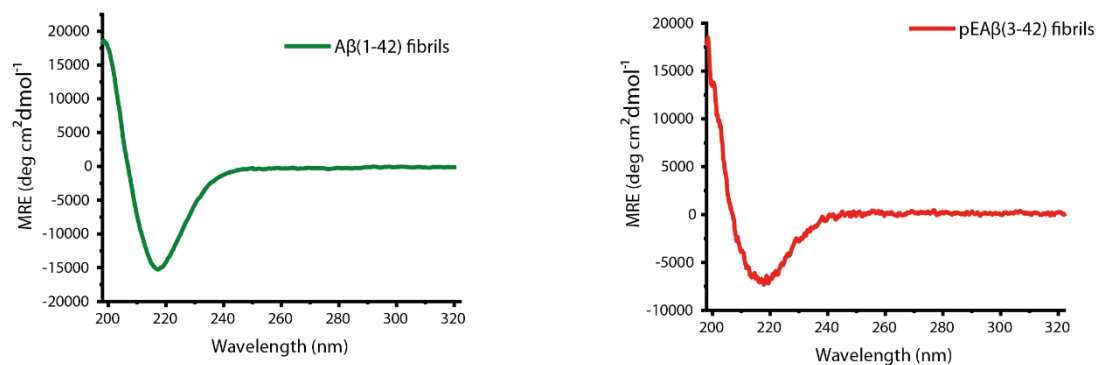
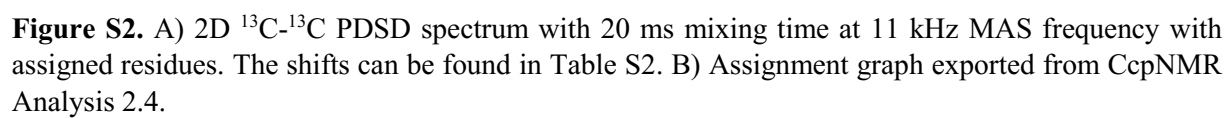
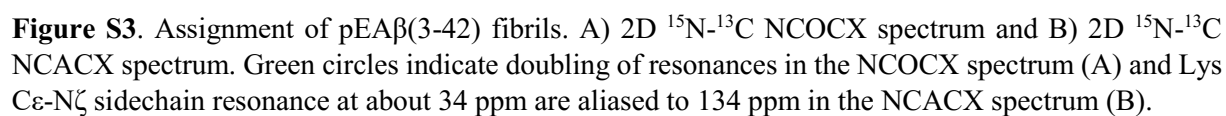


Figure S1. Far-UV CD spectra of A β (1-42) fibrils (left) and pEA β (3-42) (right) exhibit minima at 218 nm and x-axis intercepts at 207 nm (pH 2.0) characteristic for β -sheet dominated structures. As pEA β (3-42) fibrils tend to fibril bundling a reduced mean residue ellipticity (MRE) and an increased S/N ratio compared to A β (1-42) fibrils is observed.

A



A



Sequential walk of pEA β (3-42) fibrils using 3D NCACX and 3D NCOCX spectra

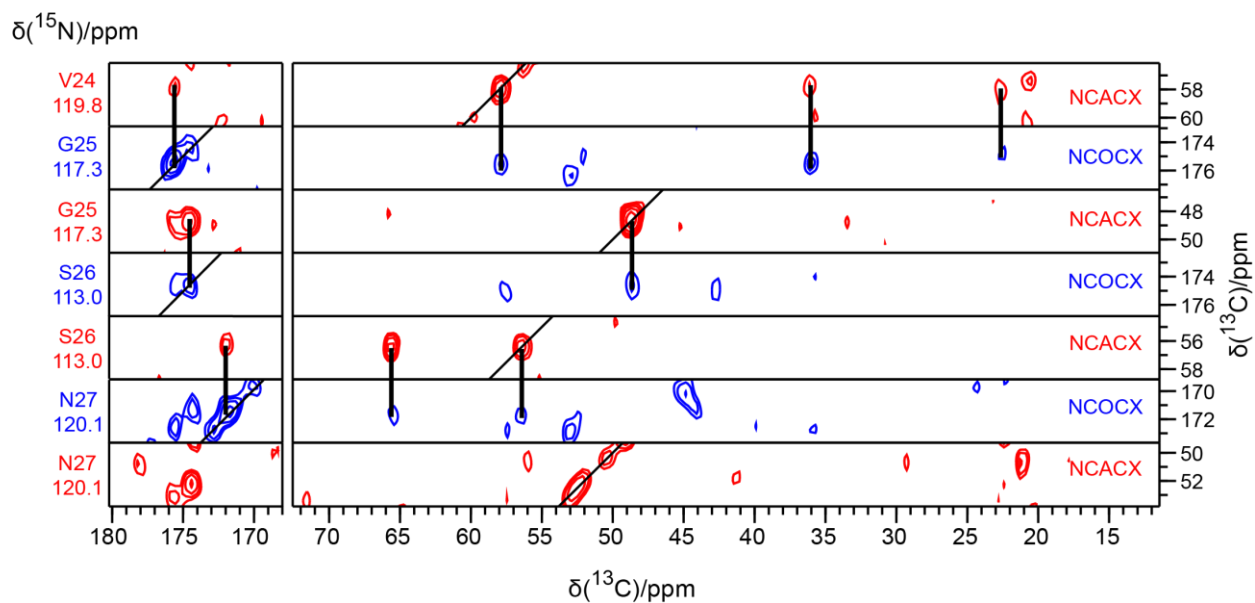


Figure S4. Extract from the sequential walk of amino acid residues V24 to N27 with 3D-NCACX (red) and 3D-NCOCX (blue) spectra of pEA β (3-42) represented by horizontal strips.

Predicted torsion angles

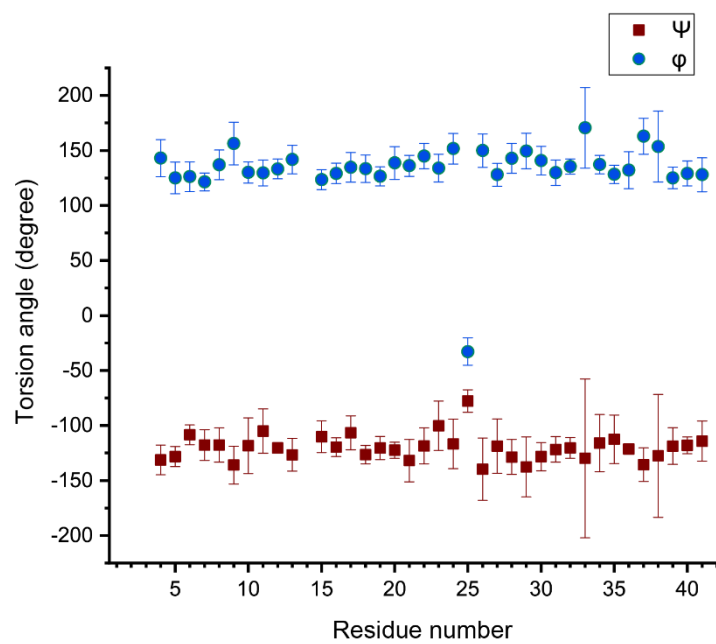


Figure S5. Torsion angles predicted by TALOS-N^[1] for pEAβ(3-42) fibrils.

Correlation plots

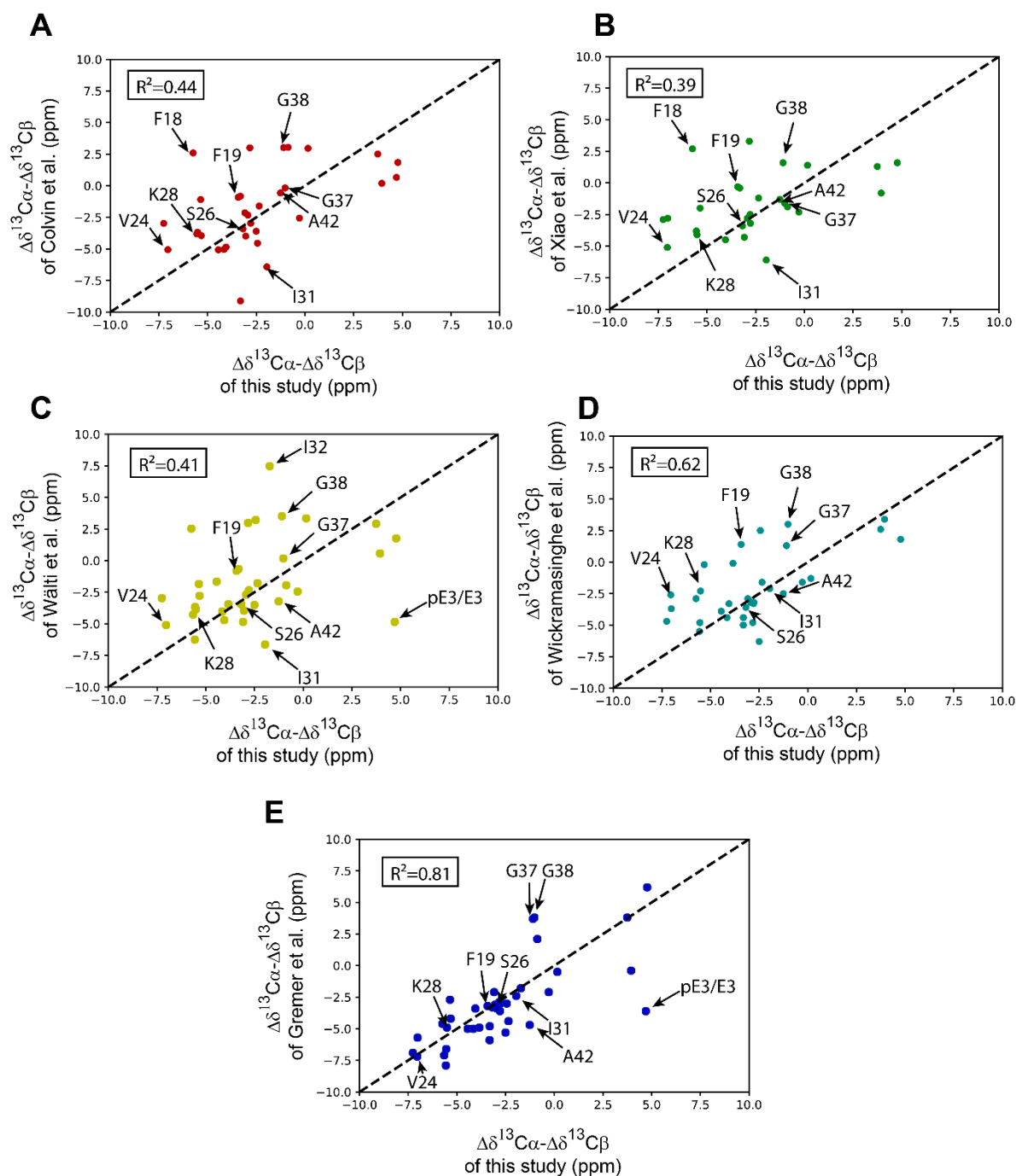


Figure S6. Correlation plots of pEA β (3-42) fibrils compared to different A β (1-42) fibril polymorphs. Comparison is represented by the difference of the secondary chemical shifts for pEA β (3-42) fibril obtained in this work and the shifts reported by Colvin et al.^[2], BMRB: 30121 (A), Xiao et al.^[3], BMRB: 25429 (B), Wälti et al.^[4], BMRB: 26692, (C), Wickramasinghe et al.^[5], BMRB: 26307, (D), and Gremer et al.^[6] BMRB: 27212 (E). Each residue is compared by plotting the secondary chemical shift value of this study on the x-axis and the secondary chemical shift value of the respective work on the y-axis. Selected residues from different regions of the peptide are labeled. The comparison pE3/E3 was not included in the calculation of the R^2 values.

Contact between F19 and I31 sidechains in LS-shaped A β (1-42) fibrils and pEA β (3-42) fibrils

A

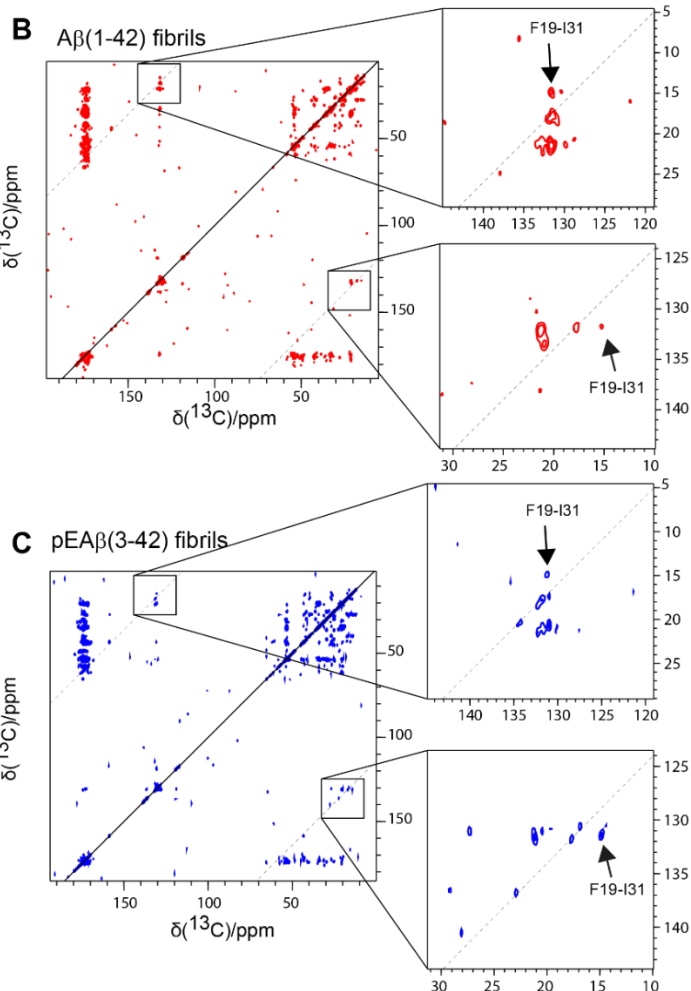
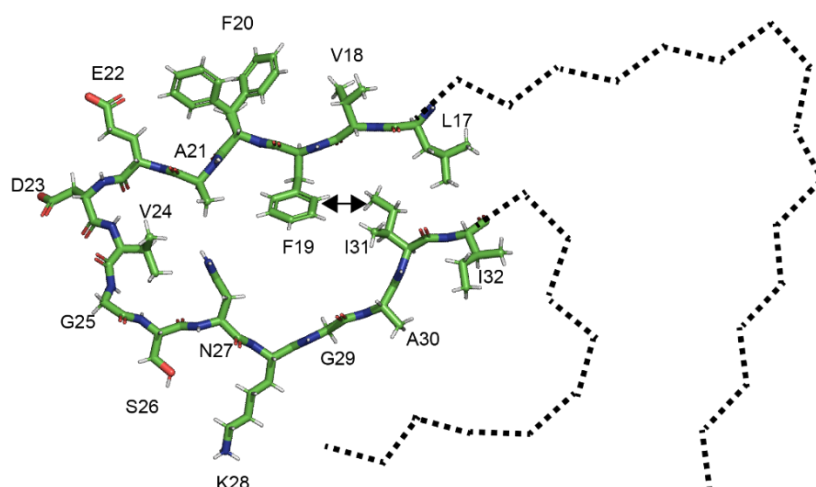
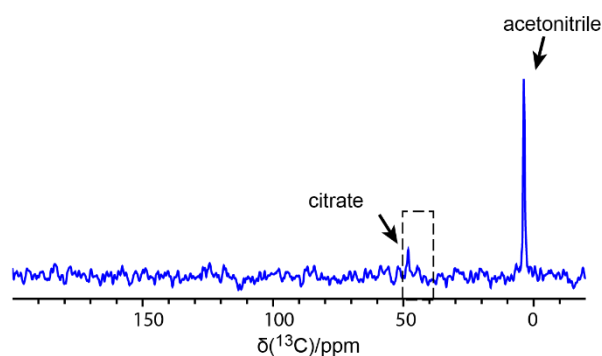


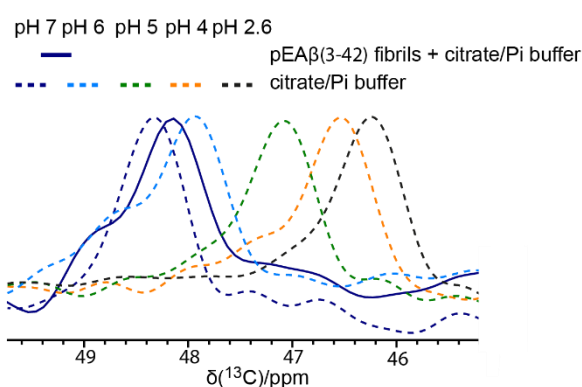
Figure S7. Rotational resonance dipolar recoupling of F19 and I31 sidechains. A) L17 to I32 region of LS-shaped A β (1-42) fibrils (PDB:5OQV)^[6]. The turn region includes a close contact between F19 and I31 sidechains. B, C) PDSD spectra at 17.2 kHz MAS frequency and 200 ms mixing time of A β (1-42) fibrils (B) and pEA β (3-42) fibrils (C) show a cross peak indicating dipolar interaction of an aromatic F19 carbon and I31 C δ and, therefore, identify a close contact between both residues.

No highly mobile protein signals and internal pH sensor

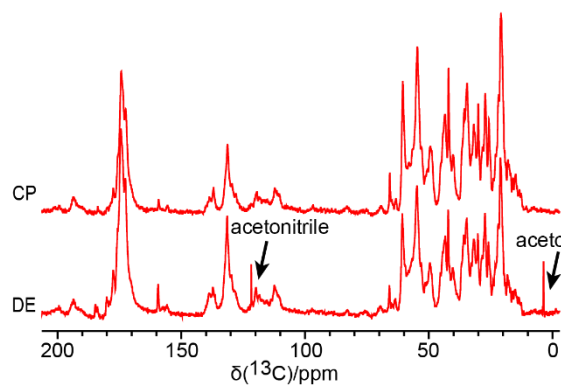
A



B



C



D

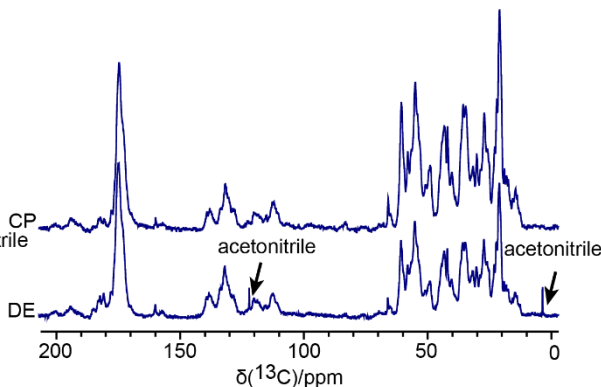


Figure S8. No highly mobile protein signals are observed exemplified for pH 6.5 shifted pEA β (3-42) fibrils and pH determination by NMR in the rotor-filled fibril sample. A) The ^{13}C detected refocused INEPT spectrum of pEA β (3-42) fibrils at pH 6.5 is devoid of protein signals, only solvent and buffer signals are visible. B) The chemical shift of the signal of the CH_2 groups of citrate is pH sensitive and can be used as an internal pH sensor for the pH in the fibril samples. Chemical shift differences between the pH shifted fibril sample (continuous line) and the buffer solutions (24% (v/v) ACN, 0.08% (v/v) TFA, dashed lines) at given pH values is around 27 Hz to pH 7 and 30 Hz to pH 6 indicating a pH of ~ 6.5 for the pH shifted pEA β (3-42) fibril sample. C, D) Comparison of the 1D ^{13}C cross-polarization (CP) and the direct excitation (DE) spectra at pH 2 (C) and pH 6.5 (D). The acetonitrile peaks were cut off. No additional protein signals were detected for direct excitation compared to the CP spectrum and exclude any highly mobile or flexible parts in pEA β (3-42) fibrils at pH 2 and 6.5.

Water build up curves

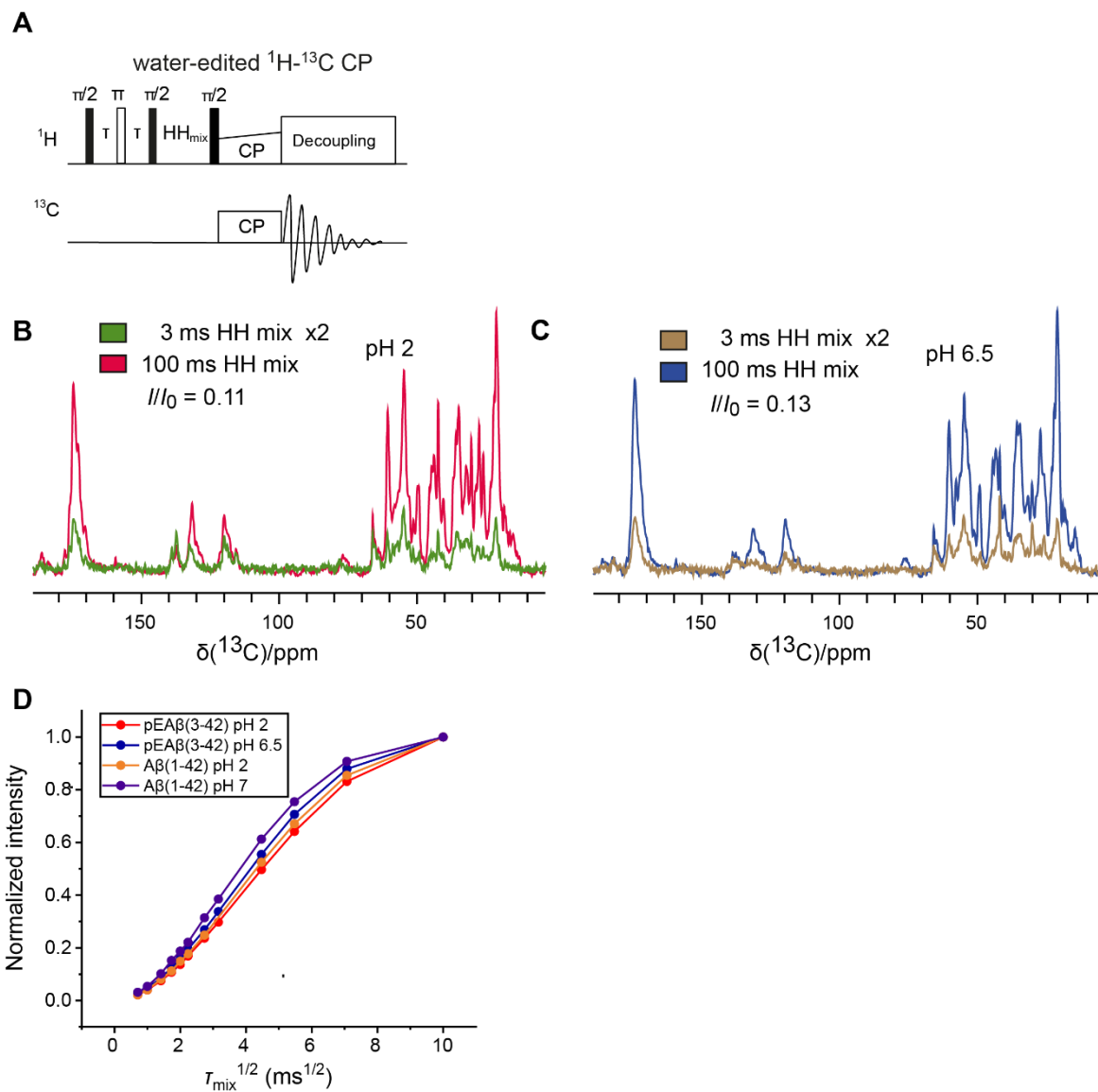


Figure S9. Water-edited experiments of pEAβ(3-42) and Aβ(1-42) fibrils at pH 2 or adjusted to pH 6.5 and pH 7, respectively. A) Pulse sequence of 1D water-edited CP spectra. B, C) 1D water-edited CP spectra of pEAβ(3-42) at different ^1H - ^1H mixing times (3 ms, 2-fold magnified, and 100 ms) at pH 2 (B) and pH 6.5 (C). D) Water-build up curves of pEAβ(3-42) and Aβ(1-42) fibrils at different pH values. A faster buildup with neutral pH values compared to pH 2 can be observed due to faster chemical exchange at increased pH values.

Experimental parameters for NMR

Table S1. Detailed experimental parameters for solid-state NMR measurements.

	PDSD/DARR 2D	NCACX 2D&3D	NCOCX 2D&3D
¹ H frequency (MHz)	800	600	600
MAS frequency (kHz)	1)&2) 11 3) 12.5 4&5)17.2 6) 20	11	11
Mixing time (ms)	1) 20 2) 200 3) 20 4&5) 200 6) 100	2D+3D: 50	2D+3D: 40
Transfer 1	HC CP	HN CP	HN CP
Carrier (ppm)	100	122	122
Duration of 1 st transfer (μs)	1100	1000	1000
Transfer 2		N-Ca CP	N-CO CP
Carrier (ppm)		55	173
Duration of 2 nd transfer (μs)		3200	2600
¹³ C rf field (kHz)		26	26
¹⁵ N rf field (kHz)		15	15
t1 increments	480	2D: 32 3D: 24	2D: 32 3D: 24
t1 spectral width (ppm)	200	2D: 50 3D: 40	2D: 50 3D: 40
t1 acquisition time (ms)	6	2D: 5.3 3D: 4.9	2D: 5.3 3D: 4.9
t2 increments		32	20
t2 spectral width (ppm)		30	20
t2 acquisition time (ms)		3.5	3.3
Number of scans	1)&3) 160 2) 120 4&5) 192 6) 224	2D: 2048 3D: 7	2D: 2048 3D: 8
Decoupling power during acquisition (kHz)	85	85	85
Decoupling power during spin diffusion (kHz)	6) 19		
Duration (h)	1) 43.5 h 2) 35.5 h 3) 54 h 4&5) 46 h 6) 45 h	2D: 38 h 3D: 311 h	2D: 38 h 3D: 213 h
Window function for processing	1,2,3&6) qsin(2,4) 4&5) qsin(4,4)	2D: qsin(4,4) 3D: qsin(3.5,3.5,3.5)	2D: qsin(4,4) 3D: qsin(3.5,3.5,3.5)
Contour level spacing	1.2	2D: 1.2 3D: 1.5	2D: 1.2 3D: 1.5

Chemical shift table for pEAβ(3-42) fibrils

Table S2. Obtained chemical shifts of the pEAβ(3-42) fibril polymorph at pH 2 (in ppm).

Residue	AS	N	C'	CA	CB	CG	CD	CE	CZ	NZ
3	pE	126.52	-	58.86	28.16	31.92	184.14			
4	F	129.83	-	56.15	44.45					
5	R	126.45	-	54.22	34.64	27.38	43.79		159.48	
6	H	129.31	-	50.78	31.34					
7	D	127.25	174.81	52.39	41.52	177.75				
8	S	118.82	172.71	55.33	65.78					
9	G	110.94	171.72	44.33	-					
10	Y	124.19	173.91	57.23	41.47					
11	E	125.31	174.07	54.69	32.00	34.80	-			
12	V	125.84	174.22	60.48	34.65	20.67				
13	H	122.30	-	51.02	32.84					
14	H	-	-	-	-					
15	Q	127.68	172.58	54.02	31.77	33.88	-			
16	K	128.20	173.60	54.51	36.67	25.38	30.02			
17	L	128.35	175.29	55.58	43.87	30.47	28.83			
							25.88			
18	V	121.32	172.65	60.35	35.86	20.96				
						22.18				
19	F	129.82	173.10	55.96	44.70					
20	F	130.73	-	56.39	42.81					
21	A	120.90	174.46	50.92	21.44					
22	E	122.09	173.31	54.07	32.23	37.83				
23	D	121.32	176.30	53.26	35.89					
24	V	119.94	175.81	58.07	36.52	21.14				
						23.07				
25	G	117.43	174.82	48.94	-					
26	S	113.10	172.64	56.65	65.83					
27	N	119.79	174.91	52.54	41.48					
28	K	127.74	173.72	54.67	36.78	25.87	30.14	42.18		34.09
29	G	110.84	170.48	45.36	-					
30	A	121.82	177.15	49.55	22.29					
30'	A'	128.13	174.40	48.85	22.72					
31	I	123.79	174.98	60.08	40.34	27.30	14.79			
						17.76				
32	I	125.18	176.34	57.77	43.33	26.93	15.65			
						17.99				
33	G	115.74	172.94	49.14	-					
34	L	124.21	173.53	54.57	45.40	28.75	23.81			
							-			
35	M	128.45	173.54	54.12	34.92	31.41	-	-		
36	V	126.23	174.64	60.52	34.71	20.68				
						21.82				

37	G	111.51	171.53	44.18	-		
38	G	102.96	169.51	44.10	-		
39	V	126.66	174.48	60.52	35.01	20.14	
						21.82	
40	V	128.19	174.38	60.60	34.93	22.00	
						20.58	
41	I	127.11	174.30	59.35	40.00	27.33	12.84
						16.84	
42	A	131.02	179.90	51.34	19.80		

References

- [1] Y. Shen, A. Bax, *Journal of Biomolecular NMR* **2013**, *56*, 227-241.
- [2] M. T. Colvin, R. Silvers, Q. Z. Ni, T. V. Can, I. Sergeyev, M. Rosay, K. J. Donovan, B. Michael, J. Wall, S. Linse, R. G. Griffin, *Journal of the American Chemical Society* **2016**, *138*, 9663-9674.
- [3] Y. Xiao, B. Ma, D. McElheny, S. Parthasarathy, F. Long, M. Hoshi, R. Nussinov, Y. Ishii, *Nature Structural & Molecular Biology* **2015**, *22*, 499.
- [4] M. A. Wälti, F. Ravotti, H. Arai, C. G. Glabe, J. S. Wall, A. Böckmann, P. Güntert, B. H. Meier, R. Riek, *Proceedings of the National Academy of Sciences of the United States of America* **2016**, *113*, E4976-E4984.
- [5] A. Wickramasinghe, Y. Xiao, N. Kobayashi, S. Wang, K. P. Scherpelz, T. Yamazaki, S. C. Meredith, Y. Ishii, *J Am Chem Soc* **2021**, *143*, 11462-11472.
- [6] L. Gremer, D. Schölzel, C. Schenk, E. Reinartz, J. Labahn, R. B. G. Ravelli, M. Tusche, C. Lopez-Iglesias, W. Hoyer, H. Heise, D. Willbold, G. F. Schröder, *Science* **2017**, *358*, 116-119.



OPEN ACCESS

EDITED BY

Javier Oroz,
Spanish National Research Council
(CSIC), Spain

REVIEWED BY

Thibault Viennet,
Aarhus University, Denmark
Filippo Favretto,
University of Verona, Italy

*CORRESPONDENCE

Lothar Gremer,
✉ l.gremer@fz-juelich.de
Henrike Heise,
✉ h.heise@fz-juelich.de
Nils-Alexander Lakomek,
✉ n.lakomek@fz-juelich.de

[†]These authors have contributed equally
to this work and share first authorship

RECEIVED 07 July 2023

ACCEPTED 06 November 2023

PUBLISHED 17 November 2023

CITATION

Gardon L, Becker N, Rähse N, Hölbling C,
Apostolidis A, Schulz CM, Bochinsky K,
Gremer L, Heise H and Lakomek N-A
(2023), Amyloid fibril formation kinetics of
low-pH denatured bovine PI3K-SH3
monitored by three different
NMR techniques.
Front. Mol. Biosci. 10:1254721.
doi: 10.3389/fmolb.2023.1254721

COPYRIGHT

© 2023 Gardon, Becker, Rähse, Hölbling,
Apostolidis, Schulz, Bochinsky, Gremer,
Heise and Lakomek. This is an open-
access article distributed under the terms
of the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution or
reproduction in other forums is
permitted, provided the original author(s)
and the copyright owner(s) are credited
and that the original publication in this
journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques

Luis Gardon^{1,2†}, Nina Becker^{1,2†}, Nick Rähse², Christoph Hölbling²,
Athina Apostolidis², Celina M. Schulz², Kevin Bochinsky¹,
Lothar Gremer^{1,2*}, Henrike Heise^{1,2*} and
Nils-Alexander Lakomek^{1,2*}

¹Institute of Biological Information Processing (IBI-7: Structural Biochemistry), JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, Jülich, Germany, ²Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

Introduction: Misfolding of amyloidogenic proteins is a molecular hallmark of neurodegenerative diseases in humans. A detailed understanding of the underlying molecular mechanisms is mandatory for developing innovative therapeutic approaches. The bovine PI3K-SH3 domain has been a model system for aggregation and fibril formation.

Methods: We monitored the fibril formation kinetics of low pH-denatured recombinantly expressed [¹³C, ¹⁵N] labeled bovine PI3K-SH3 by a combination of solution NMR, high-resolution magic angle spinning (HR-MAS) NMR and solid-state NMR spectra. Solution NMR offers the highest sensitivity and, therefore, allows for the recording of two-dimensional NMR spectra with residue-specific resolution for individual time points of the time series. However, it can only follow the decay of the aggregating monomeric species. In solution NMR, aggregation occurs under quiescent experimental conditions. Solid-state NMR has lower sensitivity and allows only for the recording of one-dimensional spectra during the time series. Conversely, solid-state NMR is the only technique to detect disappearing monomers and aggregated species in the same sample by alternately recoding scalar coupling and dipolar coupling (CP)-based spectra. HR-MAS NMR is used here as a hybrid method bridging solution and solid-state NMR. In solid-state NMR and HR-MAS NMR the sample is agitated due to magic angle spinning.

Results: Good agreement of the decay rate constants of monomeric SH3, measured by the three different NMR methods, is observed. Moderate MAS up to 8 kHz seems to influence the aggregation kinetics of seeded fibril formation only slightly. Therefore, under sufficient seeding (1% seeds used here), quiescent conditions (solution NMR), and agitated conditions deliver similar results, arguing against primary nucleation induced by MAS as a major contributor. Using solid-state NMR, we find that the amount of disappeared monomer corresponds approximately to the amount of aggregated species under the applied experimental conditions (250 μ M PI3K-SH3, pH 2.5, 298 K, 1% seeds) and within the experimental error range. Data can be fitted by simple mono-exponential conversion kinetics, with lifetimes τ in the 14–38 h range. Atomic

force microscopy confirms that fibrils substantially grew in length during the aggregation experiment. This argues for fibril elongation as the dominant growth mechanism in fibril mass (followed by the CP-based solid-state NMR signal).

Conclusion: We suggest a combined approach employing both solution NMR and solid-state NMR, back-to-back, on two aliquots of the same sample under seeding conditions as an additional approach to follow monomer depletion and growth of fibril mass simultaneously. Atomic force microscopy images confirm fibril elongation as a major contributor to the increase in fibril mass.

KEYWORDS

amyloid fibrils, fibrillation, aggregation kinetics, NMR spectroscopy, PI3K-SH3, neurodegeneration, atomic force microscopy

Introduction

Protein misfolding, aggregation, and formation of amyloid fibrils are a hallmark of neurodegenerative diseases (Bucciantini et al., 2002; Dobson, 2003; Polverino de Laureto et al., 2003; Ross and Poirier, 2004; Chiti and Dobson, 2006; Chiti and Dobson, 2017; Iadanza et al., 2018; Willbold et al., 2021). A detailed understanding of the aggregation mechanism is mandatory to counteract the progression of neurodegenerative diseases and to develop appropriate medication. The Src-homology 3 domain from the p85 α subunit of phosphatidylinositol-3-kinase (PI3K-SH3) is one of the best-investigated systems in terms of protein folding, aggregation, and fibrillation (Guijarro et al., 1998; Zurdo et al., 2001; Polverino de Laureto et al., 2003; Bader et al., 2006; Vettore and Buell, 2019). SH3 domains play a role in multiple signaling pathways by recognizing proline-rich PxxP motifs and mediating protein-protein interactions (Feng et al., 1994; Musacchio et al., 1994; Batra-Safferling et al., 2010; Saksela and Permi, 2012; Kurochkina and Guha, 2013; Dionne et al., 2022). Below pH 4, the PI3K-SH3 domain denatures, however, and forms fibrils (Guijarro et al., 1998; Zurdo et al., 2001). Solid-state NMR and cryo-electron microscopy have obtained detailed, high-resolution 3D-structural insights into bovine PI3K-SH3 fibrils (Bayro et al., 2010; Röder et al., 2019).

Several methods exist to measure the aggregation kinetics of amyloid formation. Amyloid dyes like Thioflavin T (ThT) or Congo red are frequently used as extrinsic probes to study the formation or presence of fibrils (Levine, 1993; Chiti et al., 1999; Biancalana and Koide, 2010; Schutz et al., 2011; Frieg et al., 2020). Also, methods employing intrinsic probes, such as tryptophan fluorescence or dynamic light scattering, can be used to follow the kinetics of amyloid formation or amyloid decomposition (Röder et al., 2019; Vettore and Buell, 2019; Housmans et al., 2021). NMR spectroscopy has been very powerful in elucidating the structures of amyloid fibrils (Balbach et al., 2000; Petkova et al., 2002; Jaroniec et al., 2004; Heise et al., 2005; Luhrs et al., 2005; Paravastu et al., 2008; Wasmer et al., 2008; Bayro et al., 2010; Schutz et al., 2011; Colvin et al., 2016; Tuttle et al., 2016; Wälti et al., 2016; Weirich et al., 2016; Gremer et al., 2017; Iadanza et al., 2018; Becker et al., 2023). But NMR can also follow protein fibrillation and aggregation kinetics using nuclear spins as an intrinsic probe. For example, Zurdo et al. studied the aggregation behavior of denatured PI3K-SH3 by employing one-dimensional solution NMR (Zurdo et al., 2001), combined with far-UV circular dichroism (CD) spectroscopy, Fourier-transform

infrared (FTIR) spectroscopy and electron microscopy (EM). More recently, several studies investigated the fibril formation kinetics of Amyloid- β A β (1–40) and A β (1–42) and α -synuclein. Roche et al. and Bellomo et al. followed the fibril formation kinetics of A β (1–40) and A β (1–42) by a combination of solution NMR spectroscopy and ThT fluorescence (Roche et al., 2016; Bellomo et al., 2018), and Horvath et al. fibril elongation of α -synuclein (Horvath et al., 2023). In these studies, the disappearance of the monomer signal in solution was monitored. Fibrils or larger aggregates are difficult to detect by solution NMR due to the overall tumbling limitation, restricting the maximum weight of the molecular complex that is still visible to solution NMR. ThT fluorescence was chosen as a complementary method to detect the fibrils or oligomers originating from the aggregation process.

In contrast to solution NMR, solid-state NMR does not face any overall tumbling limitation, and high molecular weight systems can be studied. Fibrils or large aggregates become accessible via dipolar CP (cross-polarization) transfer. Also, mobile/flexible monomers can be detected via scalar-based transfers, such as, e.g., INEPT (Insensitive Nuclei Enhanced by Polarization Transfer). However, the absence of weight limitation in solid-state NMR comes at the cost of line broadening due to anisotropic interactions. To reduce the line broadening, magic angle spinning (MAS) is applied in solid-state NMR, which, to first order, averages the anisotropic interactions (anisotropic chemical shift and dipolar couplings). Ravera and coworkers investigated the fibril formation kinetics of A β (1–40) and A β (1–42) by solid-state NMR under MAS conditions (*in situ* SedNMR) (Bertini et al., 2013). The disappearance of the monomeric signal was monitored by one-dimensional ^1H - ^{13}C INEPT spectra and the appearance of the aggregated species by one-dimensional ^1H - ^{13}C CP spectra. Debelouchina and coworkers observed the maturation of liquid droplets of a low-complexity domain of FUS by MAS NMR-spectroscopy (Berkeley et al., 2021). One concern with studying fibril formation and aggregation kinetics by solid-state NMR spectroscopy has been magic angle spinning. MAS with frequencies of several kHz leads to centrifugal forces inside the rotor comparable to those experienced in an ultracentrifuge (Ravera, 2014; Wang et al., 2018). The aggregated protein in the sample is compacted at the rotor walls during spinning. The influence of agitated conditions induced by magic angle spinning on fibril formation and aggregation kinetics has not been thoroughly investigated.

High-resolution magic angle spinning (HR-MAS) solid-state NMR is a hybrid between solution NMR and solid-state NMR. In a simplified view, it can be considered as applying mainly solution NMR spectroscopy under slow magic angle spinning conditions to average sample inhomogeneities. HR-MAS has been used in the context of metabolomics or pharmaceutical research when the investigated samples are too dense or inhomogeneous to be studied by standard solution NMR (Lindon et al., 2009; Sitter et al., 2009; Holzgrabe, 2010). Only a few studies have applied HR-MAS in the context of aggregation processes (Warrass et al., 2000; Wang et al., 2018; Sahoo et al., 2020).

In the current study, we report the seeded fibril formation kinetics for the bovine PI3K-SH3 domain as an amyloid model protein using three different NMR methods: solution NMR, solid-state MAS NMR, and HR-MAS NMR spectroscopy. We used identical protein concentration, buffer conditions, and temperature for the aggregation measurements to allow maximal comparability between the different methods. We find that under the chosen conditions, notably including fibril seeds as nucleation points, magic angle spinning has only a weak influence on the aggregation kinetics, and we observe similar aggregation kinetics for solution NMR under quiescent conditions and HR-MAS and solid-state MAS experiments representing agitated conditions. Hence, solid-state MAS NMR seems suitable for following amyloid fibril formation and aggregation kinetics of proteins in general. In combination with solution NMR, residue-specific resolution of the decaying monomeric species can be obtained in a time series of two-dimensional solution NMR spectra.

Materials and methods

Protein expression and purification of bovine [U-¹³C, ¹⁵N] PI3K-SH3

Bovine PI3K-SH3 domain was expressed in *Escherichia coli* BL21 (DE3) as an N-terminal glutathione S-transferase (GST)-fusion followed by a thrombin protease cleavage site using pGEX-4T (GenScript Biotech, Netherlands) as vector plasmid. The 86 amino acid sequence of the purified bovine PI3K-SH3 domain used in this study is GS MSAEGYQYRA LYDYKKEREE DIDLHLGDIL TVNKGSLVAL GFSDGQEAKP EEIGWLNLYN ETTGERGDFP GTYVEYIGRK KISP with the N-terminal Gly-Ser residues remaining as overhang from the thrombin cleavage.

BL21 cells were grown in M9 medium (for [U-¹³C, ¹⁵N] labeling with 3.2 g U-¹³C-glucose and 2 g ¹⁵N-ammonium chloride per 1 culture medium as the sole carbon and nitrogen sources) at 37°C for ~4.5 h until an OD₆₀₀ of 0.6 was reached. Protein expression was induced by the addition of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were then grown overnight at 28°C, harvested by centrifugation (5,000xg, 15 min) and resuspended in 5–8 mL per g cell mass of lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 100 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.6 containing 5 mg/mL lysozyme). After sonication with a Bandelin Sonopuls sonicator (Bandelin) using a VS 70T probe (60% amplitude, 3 × 5 min, 3 s “on,” 5 s “off,” ice cooling) and ultracentrifugation in an Optima XPN-80 ultracentrifuge

(Beckman) equipped with a 70Ti rotor at 4°C and 42,000 rpm for 1 h, the supernatant was loaded on two serially connected 5 mL Protino GST/4B columns (Macherey-Nagel, Düren, Germany) equilibrated with 50 mM HEPES-NaOH buffer, 100 mM NaCl, pH 7.6, and 3 mM Dithiothreitol (DTT). The protein was eluted with 20 mM reduced glutathione in 50 mM HEPES, 100 mM NaCl, 3 mM DTT, and pH 7.6. The fractions containing the eluted GST-PI3K-SH3 fusion protein were cleaved using 5–7.5 NIH units of thrombin (SERVA Electrophoresis GmbH, Heidelberg, Germany) per mg protein for 2 days at 4°C under mild shaking conditions. Then, the solution was loaded on a Superdex 75 HiLoad 16/600 size exclusion column (GE Healthcare Europe GmbH, Freiburg, Germany), equilibrated, and run with 5 mM ammonium acetate (pH 7.7) as a volatile buffer system, leading to the separation of the cleaved GST from PI3K-SH3. To ensure complete removal of GST traces, fractions containing PI3K-SH3 were pooled and loaded again on two serially connected 5 mL Protino GST/4B columns (Macherey-Nagel, Düren, Germany) equilibrated with 5 mM ammonium acetate. The flow-through fractions containing purified bovine [U-¹³C, ¹⁵N] PI3K-SH3 were pooled, aliquoted, and freeze-dried for further usage. Analysis by SDS-PAGE and NMR suggest bovine PI3K-SH3 purities above 97%, as neither method detected impurities.

Production of unlabeled PI3K-SH3 fibrils seeds

The production of bovine PI3K-SH3 fibril seeds was essentially carried out according to Röder et al. (2019). In brief, 100 nmol lyophilized non-isotopically labeled PI3K-SH3 was resuspended in 500 μL 10 mM glycine-hydrochloride pH 2.5 buffer, leading to a final concentration of 200 μM PI3K-SH3. The glycine-hydrochloride buffer comprised 10 mM glycine and 5.66 mM hydrochloric acid, resulting in a final pH 2.5. The solution was shaken in an Eppendorf tube at 1400 rpm at 42°C for 24 h. A ~5 μL sample was withdrawn for Atomic Force Microscopy (AFM) measurements (see below). The remaining 495 μL were transferred into a 15 mL Falcon tube and then diluted to approximately 100 μM with another 500 μL 10 mM glycine-hydrochloride pH 2.5 buffer. The solution was then sonicated under ice-cooling for 15 s (1 s “on,” 2 s “off,” 10% amplitude) with a Bandelin Sonopuls sonifier using an M72 probe to disrupt the fibrils into smaller species called seeds.

NMR sample preparation

100 nmol lyophilized bovine PI3K-SH3 was resuspended in 390 μL 10 mM glycine-hydrochloride buffer (pH 2.5, 8% D₂O). The sample was divided into three parts (one for solution NMR, one for HR-MAS, and one for MAS solid-state NMR experiments). A total volume of 10 μL unlabeled PI3K-SH3 fibril seeds (100 μM stock) was added proportionally to each PI3K-SH3 sample, corresponding to a 1% (mol/mol monomer equivalents) seed mass. The final PI3K-SH3 protein concentration was 250 μM. The “dead time” (the time between the addition of seeds but

before NMR spectra were recorded) was measured after adding the PI3K-SH3 fibril seeds.

NMR spectroscopy

All NMR experiments described in the following were conducted at 298 K (25°C). The temperature at every spectrometer was calibrated using either Methanol (solution NMR probes, HR-MAS probe) or DSS (solid-state NMR probe). Details on all NMR experiments are listed in [Supplementary Tables S1, S2](#).

Solution NMR measurements

A series of two-dimensional solution NMR ^1H - ^{15}N HSQC spectra was recorded, with approximately 1 h duration each, to follow the disappearance of the monomeric species. Solution NMR measurements were conducted on a Bruker 900 MHz Avance Neo spectrometer equipped with a triple resonance ^1H , ^{13}C , ^{15}N TCI cryoprobe, and for a second series of NMR experiments using a Bruker 600 MHz Avance III HD spectrometer equipped with a ^1H , ^{13}C , ^{15}N triple resonance TCI cryoprobe.

HR-MAS experiments

A series of two-dimensional HR-MAS ^1H - ^{15}N HSQC (^1H detection) and one-dimensional ^1H - ^{13}C INEPT (^{13}C detection) spectra were recorded alternately; each experiment had a duration of approximately 1 h. HR-MAS NMR experiments were conducted on a Bruker Avance III HD 800 MHz spectrometer equipped with a 4 mm triple resonance ^1H , ^{13}C , ^{15}N HR-MAS probe. The MAS frequency was set to 6 kHz.

Solid-state MAS experiments

A series of one-dimensional ^{13}C -detected solid-state NMR ^1H - ^{13}C CP experiments (duration: 1 h) and ^1H - ^{13}C INEPT experiments (duration 1 h) were recorded alternately. The solid-state MAS experiments were conducted on a Bruker 600 MHz Avance NMR spectrometer equipped with a double resonance ^1H , ^{13}C 3.2 mm probe; the MAS frequency was set to 8 kHz. Field strengths for hard 90° pulses were 83 kHz for ^1H and 45 kHz for ^{13}C . For CP transfer, a field strength of 37 kHz for ^1H and 45 kHz for ^{13}C were used (Hartmann-Hahn zero quantum condition: $\omega_{13\text{C}} - \omega_{1\text{H}} = \omega_{\text{MAS}}$). The field strength for proton decoupling during acquisition was 82 kHz. The ^{13}C -detected spectra were referenced indirectly using adamantane by setting its CH peak to 31.4 ppm (corresponding to the DSS reference scale).

Spectra analysis

All spectra were processed and integrated using the Bruker Topspin 4 software (Bruker, Billerica, MA, United States). Two-dimensional spectra were plotted using the CCPN Analysis 2.3 software ([Vranken et al., 2005](#)).

The first increment/FID for two-dimensional spectra was Fourier-transformed and integrated as a one-dimensional spectrum. The integration regions were 6.4–10.2 ppm for ^1H -detected spectra and 5–75 ppm for the ^{13}C -detected spectra. Error estimates were obtained by integrating a noise region in the 1D spectra. For CP spectra: 225 to 200 ppm (^{13}C), for HSQC-based: 1.8 to −2 ppm (^1H), for INEPT HR-MAS and solid state: −5 to −45 ppm (^{13}C). For solid-state NMR spectra with ^{13}C

detection, the chemical shift dispersion is larger than for protons, limiting the (signal-free) noise regions. Therefore, for the ^{13}C -detected CP and INEPT based spectra the integrated noise region differed, but was scaled to be proportional to the integrated signal region by multiplying it with the square root of the ratio (signal region width/noise region width).

The integrated intensities were fitted using the following equation:

$$I(t) = I_0 * e^{-\frac{t}{\tau}} + \text{const} \text{ (mono - exponential fit)} \quad (1)$$

For the INEPT experiments, $I(t)$ corresponds to the monomer concentration in the solution. Besides the half-life time, τ , the initial signal intensity, I_0 , is a fitting parameter. Further, we have introduced a constant, *const*, considering that the monomer signal does not fully decay to zero (within the measurement time). This suggests a monomer/fibril equilibrium is reached, with a residual amount of monomer in the solution. For the CP-based experiments, $I(t)$ corresponds to the monomer concentration that has become part of the fibril. The software Origin 19 (OriginLab Corporation, Northampton, MA, United States) was used for fitting the data.

Integration of the peaks in two-dimensional solution NMR spectra ([Supplementary Figure S3](#)) was performed using Topspin 4, integrating over a square of 0.1 ppm lateral length in the ^1H and 1 ppm in the ^{15}N dimension. The noise was integrated over a square of the same dimensions in an empty spectrum region.

Atomic force microscopy (AFM)

After the NMR experimental series, all PI3K-SH3 samples were investigated using Atomic Force Microscopy (AFM). The aggregated solutions of HR-MAS and solid-state NMR rotors and the NMR tube were transferred to 2 mL Eppendorf tubes. For AFM samples of the fibril seeds, the solution was diluted in 10 mM glycine-hydrochloride, pH 2.5 buffer to a protein concentration of 0.5 μM ; for HR-MAS and solid-state NMR AFM samples to 50 μM , and the liquid NMR sample to 5 μM . A 5–10 μL volume of the solutions was pipetted on a mica surface. After 10 min of incubation, the mica was washed extensively with Milli-Q water and dried under a nitrogen gas flush. The AFM micrographs were recorded in ScanAsyst mode using the peak-force tapping mechanism on a Bruker Multimode 8 (Billerica, Massachusetts, United States) using OMCL-AC160TS cantilevers (Shinjuku, Tokyo, Japan) at 1024 × 1024 pixel resolution. The images were processed with Gwyddion 2.61 ([Nečas and Klapetek, 2012](#)).

Circular dichroism (CD) spectrometry

Far-UV-CD spectroscopy of bovine PI3K-SH3 samples was performed on a JASCO J-1500 CD spectropolarimeter (Jasco, Gross-Umstadt, Germany) using 1 mm path length quartz cuvettes (Hellma, Müllheim, Germany). Spectra were recorded at 20°C after dissolving lyophilized PI3K-SH3 to a protein concentration of 20 μM in 10 mM glycine-hydrochloride buffer, pH 2.5, or in 20 mM Na phosphate buffer, pH 6.8, with instrument settings as follows: step size 1 nm, scan speed 100 nm min^{−1}, bandwidth 2 nm, DIT 4. Far-UV-CD spectroscopy data

of bovine PI3K-SH3 fibrils were recorded at a protein concentration of 20 μM (monomer equivalents) in 10 mM glycine-hydrochloride buffer, pH 2.5, as described above. The signal-to-noise ratio was improved by accumulation of 6 scans per sample. The mean residue ellipticity, MRE or $[\theta]_{\text{mrw}}$ in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ was calculated from the equation $[\theta]_{\text{mrw}} = (\theta_{\text{obs}} \times \text{MRW}) / (c \times d \times 10)$, with θ_{obs} , observed ellipticity (in millidegrees); c , concentration (in g/l); d , cell path length (in cm); MRW (mean residue weight), molecular weight divided by number of peptide bonds.

Thioflavin T (ThT) assay

For the fibril elongation assay, which was monitored through the amyloid-specific dye Thioflavin-T (ThT), fibril growth experiments were conducted in triplicates (using three separate batches). Each batch/sample contained 250 μM PI3K-SH3, 0.04% NaN_3 , and 1% seed material in 10 mM glycine-hydrochloride buffer, pH 2.5, using the same experimental conditions as the NMR measurements. The sample was incubated under quiescent conditions at 25°C for 80 h. Due to the limitations of ThT fluorescence measurements at low pH (Becker et al., 2023), every 8 h a 10 μL aliquot of the reaction mixture was withdrawn and diluted 1:10 in 20 mM sodium acetate buffer (pH 5.0), 50 mM NaCl, containing 20 μM ThT. Subsequently, the diluted samples were transferred to a 96-well half-area, non-binding surface, polystyrene plate (Corning, No. 3881, black with clear bottom), and the plate was sealed with clear sealing tape (232701, Thermo Scientific). The plate was then loaded into a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Fluorescence measurements were performed with an excitation wavelength of 448 nm and emission measurements at 482 nm at 25°C. Measurements were repeated every 20 s in cycles 1 to 500 to measure the sample for a total of 2.7 h for each data point of the original aggregation assay. For analysis, the plateau values of the ThT fluorescence curve between 2.2 and 2.7 h were averaged and plotted against the time.

Results and discussion

Solution NMR detects the decay of the monomeric species

Bovine PI3K-SH3 loses its stable globular fold below pH 4 and aggregates into fibrils, as found previously by Dobson and coworkers (Zurdo et al., 2001). Here, we studied the PI3K-SH3 aggregation at low pH and under seeding conditions using three different NMR techniques (solution, HR-MAS, solid-state) and, for comparison, using a Thioflavin T assay. As a starting point, we dissolved lyophilized bovine PI3K-SH3 at pH 2.5 in 10 mM glycine-hydrochloride pH 2.5 buffer. We divided the sample into three aliquots of 250 μM final concentration (PI3K-SH3 monomer) for the three NMR experiments (solution, HR-MAS, solid-state) at a sample temperature of 25°C. To initiate immediate fibrillation, fibril seeds were added to each sample at a concentration of 1% molarity relative to the PI3K-SH3 monomer concentration in the solution. Initially, before fibrillation started, all PI3K-SH3 monomers were in solution and fully visible in the solution-state NMR spectra. To

monitor the presence of the monomeric PI3K-SH3 species, we recorded a time series of consecutive two-dimensional (2D) solution NMR ^1H - ^{15}N HSQC spectra of approximately one-hour duration each. Figure 1A shows 2D solution NMR ^1H - ^{15}N HSQC spectra of unfolded monomeric bovine PI3K-SH3 at three exemplary time points after adding fibril seeds (1 h, 51 h, and 101 h). The narrow dispersion in the ^1H dimension of the most intense peaks indicates that the major population of monomeric PI3K-SH3 is disordered under these conditions. However, a minor population of PI3K-SH3 with β -sheet conformation, similar to the globular conformation, is still present, visible as the less intense peaks with ^1H chemical shifts ranging between 6 and 10 ppm. A direct comparison of ^1H - ^{15}N HSQC spectra of PI3K-SH3 at pH 2.5 and PI3K-SH3 at pH 6.8 (globular conformation), see Supplementary Figure S1, underlines the presence of a minor folded species, similar to the globular protein at pH 6.8 and a major unfolded population. From the first increment of the solution NMR 2D spectra, shown in Figure 1C, we estimated the population of the minor folded species to approx. 15%–20%, based on intensities associated with the folded β -sheet region (9–10 ppm) compared to the central region between 7.5 and 8.5 ppm, which is dominated by intensities of the disordered conformation. Supplementary Figure S1 shows a direct comparison (overlay) of the 2D solution NMR spectra of SH3 at low and neutral pH. Supplementary Figure S2 shows the circular dichroism (CD) spectrum of PI3K-SH3 at pH 2.5, underlining that at pH 2.5 and 25°C, SH3 is mainly disordered. A CD spectrum of SH3 fibrils at the same pH of globular folded SH3 at neutral pH is compared. CD spectra agree with data shown in (Zurdo et al., 2001).

During the time course of aggregation, a series of solution NMR ^1H - ^{15}N HSQC spectra were recorded. Spectra recorded at later time points show lower signal intensities of monomeric PI3K-SH3. Spectral intensities declined exponentially. The decay rate for the disordered and the minor folded conformation appears similar, as the inspection and comparison of the one-dimensional (1D) spectra of the first increments of the HSQC spectra indicates, see Figure 1C. Also, the intensities of representative peaks in the 2D solution NMR spectra decay with similar rate constants (Supplementary Figure S3). This suggests a dynamic equilibrium between the major unfolded population and the minor folded population, which must be slow on the NMR timescale (slower than about 10 ms, as two separate conformations are visible) but fast on the aggregation time scale (as all resonances decay with similar rate constants, in the order of hours by inspection of spectral intensities, see Figure 1 and Supplementary Figure S3). Intensities are decreasing because of increasing monomer aggregates during the experiment. The aggregated PI3K-SH3 species is not visible to solution NMR because of the high molecular weight of the aggregated species and the associated slow overall tumbling correlation time.

Solid-state NMR detects the emerging aggregated species by CP-based methods and the decay of monomeric species by INEPT-based methods

The emergence of the aggregated species can be followed by solid-state NMR, which does not face any overall weight limitation,

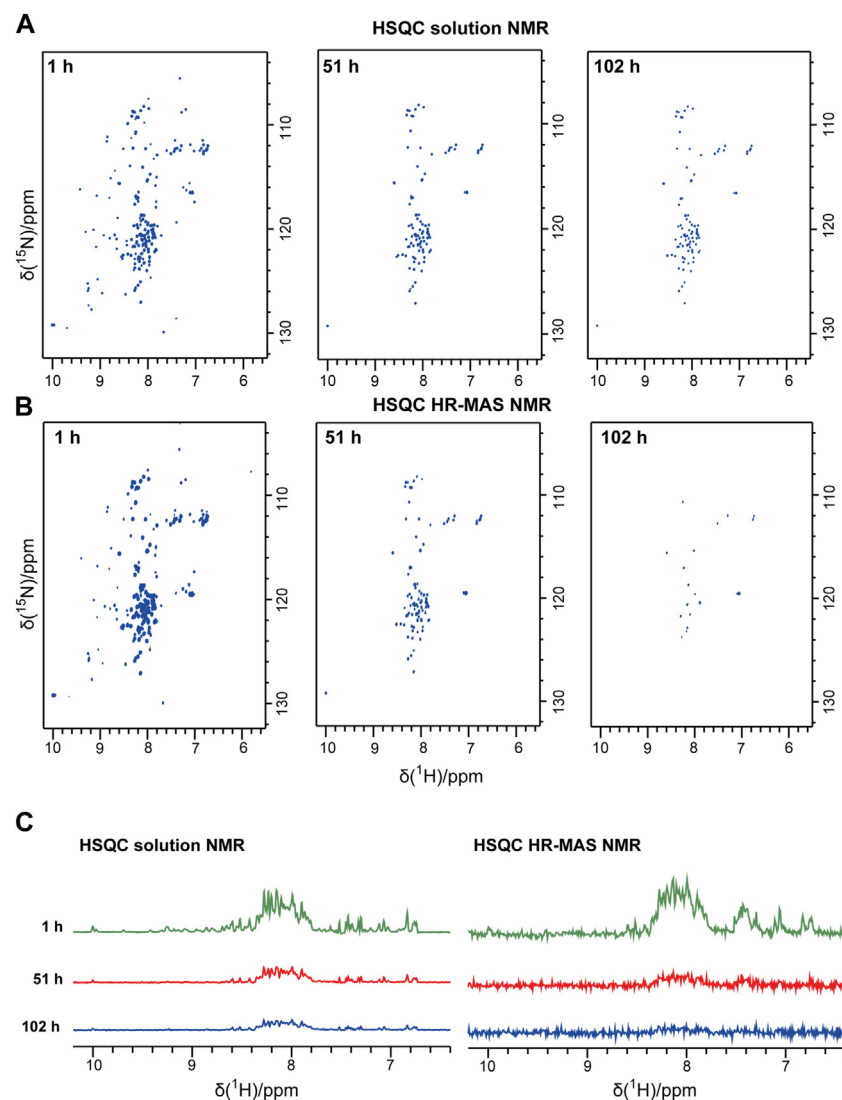


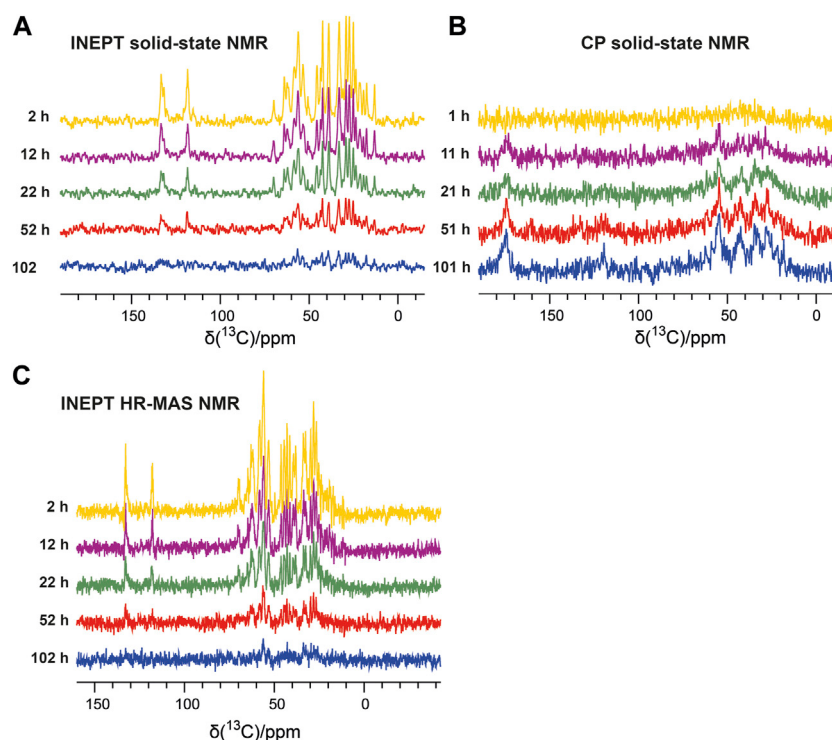
FIGURE 1

Solution NMR and HR-MAS NMR time series following the aggregation of the PI3K-SH3 monomers. Representative spectra recorded during the time series are shown: **(A)** Solution NMR ^1H - ^{15}N HSQC spectra recorded under quiescent conditions. **(B)** HR-MAS ^1H - ^{15}N NMR HSQC spectra were recorded at a MAS frequency of 6 kHz. In both ^1H - ^{15}N spectra, the ^1H resonance is correlated to the ^{15}N resonance of the same amide group. Thus, one peak corresponds typically to one amino acid residue. **(C)** The first 1D increment of the 2D spectra is shown in **(A)** and **(B)**. All spectra were recorded at 25°C.

as overall tumbling is absent in the solid state. Therefore, in parallel to the solution NMR time series, we recorded one-dimensional ^{13}C -detected solid-state NMR spectra on the second aliquot of the PI3K-SH3 sample (250 μM with 1% seeds added). Two types of magnetization transfer were used for the solid-state NMR spectra: In the first spectrum, a dipolar cross-polarization (CP) based transfer was employed, which is sensitive to the more rigid part of the sample, namely the aggregated species. Second, a scalar coupling-based INEPT (insensitive nuclei enhanced polarization transfer) was used, which is sensitive to the more flexible species, which is the unbound monomer in solution. Therefore, the INEPT-based 1D spectra connect to the solution-state NMR spectra on the monomeric species. **Figures 2A, B** show representative spectra of both time series, which were recorded inter-leaved. The CP-spectra (**Figure 2B**) show increasing intensities indicating the emergence of

the aggregated species. This corresponds to a decay of the monomeric species, as visible in the INEPT-based spectra (**Figure 2A**).

To connect the observations by solution-state NMR and solid-state NMR, we also recorded a time series of interleaved ^1H - ^{15}N HSQC spectra and ^{13}C -detected INEPT spectra on the third aliquot of the sample, using an HR-MAS probe. This probe can record solution NMR-like spectra using proton-detection under MAS conditions, as in solid-state NMR. The influence of the magic angle spinning on the aggregation kinetics and the resulting product has not been investigated in detail yet. **Figure 1B** shows the time series of ^1H - ^{15}N HSQC spectra recorded on bovine PI3K-SH3 under MAS conditions, with MAS frequencies of 6 kHz, recorded using an HR-MAS probe. Comparison of **Figures 1A, B** reveals that the initial PI3K-SH3 monomer spectra appear similar

**FIGURE 2**

Solid-state NMR time series following the aggregation of the PI3K-SH3 monomers via the aliphatic (15–70 ppm) and aromatic carbon resonances (110–140 ppm). **(A)** ^{13}C -detected ^1H - ^{13}C INEPT solid-state spectra recorded at a MAS frequency of 8 kHz show the disappearance of the monomer. **(B)** One-dimensional ^{13}C -detected ^1H - ^{13}C cross-polarization (CP) MAS solid-state spectra recorded at a MAS frequency of 8 kHz detect the emergence of the aggregated species. **(C)** ^{13}C -detected ^1H - ^{13}C INEPT HR-MAS spectra were recorded at a MAS frequency of 6 kHz (without decoupling).

under both conditions, the quiescent condition during solution NMR experiments and under agitated conditions, namely centrifugal forces experienced during MAS conditions. However, the intensity decay rate appears slightly faster for the HR-MAS experiments than for the solution NMR experiments under quiescent conditions. This is also visible in the 1D spectra corresponding to the first increments of the respective HSQC spectra (Figure 1C) and will be discussed later. ^1H - ^{15}N HSQC spectra, or their first increments, respectively, report on the protein backbone. A similar trend is observed for the HR-MAS ^{13}C -detected INEPT spectra (Figure 2C), which are sensitive to protein sidechains. Consistent with the solid-state NMR spectra (Figure 2A), we observe that the concentration of the monomeric species decreases exponentially (see below) with time (Figure 2C).

To analyze the aggregation kinetics extracted from the various NMR spectra more quantitatively, we integrated and normalized spectral intensities of the 1D spectra (or first increments of the 2D spectra), as described in the Materials and Methods section. The normalized integrated intensities as a function of time (after the start of the aggregation experiment) are shown in Figure 3A. Interestingly, in the case of the solid-state NMR CP- vs. INEPT-based 1D spectra, the sum of the two integrated intensities gives an almost constant value (Figure 3B). (We interpret the observed slight decrease of the sum of integrated intensities over several days as a slight detuning of the probe over time.) Thus, the aggregation appears as a two-state process, with a relatively direct transition from the monomeric to the fibrillar state, without a high population

of an invisible oligomeric on-pathway intermediate. (Loss to an invisible off-pathway (“dead end”) oligomeric species that does not give rise to any CP-detected solid-state NMR signal, however, cannot entirely be excluded.) Mono-exponential decay curves (or the asymptotic growth curves, respectively) were fitted to the integrated intensities using Eq. 1 in the Materials and Methods section. The obtained half-life times, τ , are shown in Table 1. Supplementary Figure S4 gives details on the fits. Overall, the lifetimes measured using the different NMR techniques (solution-state, HR-MAS, solid-state) show good qualitative agreement. Under quiescent solution NMR conditions, the monomeric species seems to have a slightly longer lifetime than under MAS solid-state NMR conditions (37.4 ± 0.6 h vs. 30.2 ± 1.1 h), representing agitated conditions.

For comparison, a ThT assay was recorded (Figure 3C), monitoring the increase of the fibrillar state. The observed increase in fibrillar structure is overall in good qualitative agreement with the solid-state NMR CP-based experiment, sensitive to the emergence of aggregated species (squared brown data points shown in Figure 3A). A comparison of the timeline of the CP-based solid-state NMR experiment and the ThT assay suggests that most CP signals originate from fibrillar SH3.

Integrated intensities of the first increment HR-MAS ^1H - ^{15}N HSQC spectra, sensitive to the amide proton backbone signals of the monomer, decrease simultaneously to ^1H - ^{13}C INEPT spectra, sensitive to the side chain signals, with almost identical lifetimes (25.0 ± 1.2 h vs. 24.0 ± 0.6 h). This suggests that both backbone or

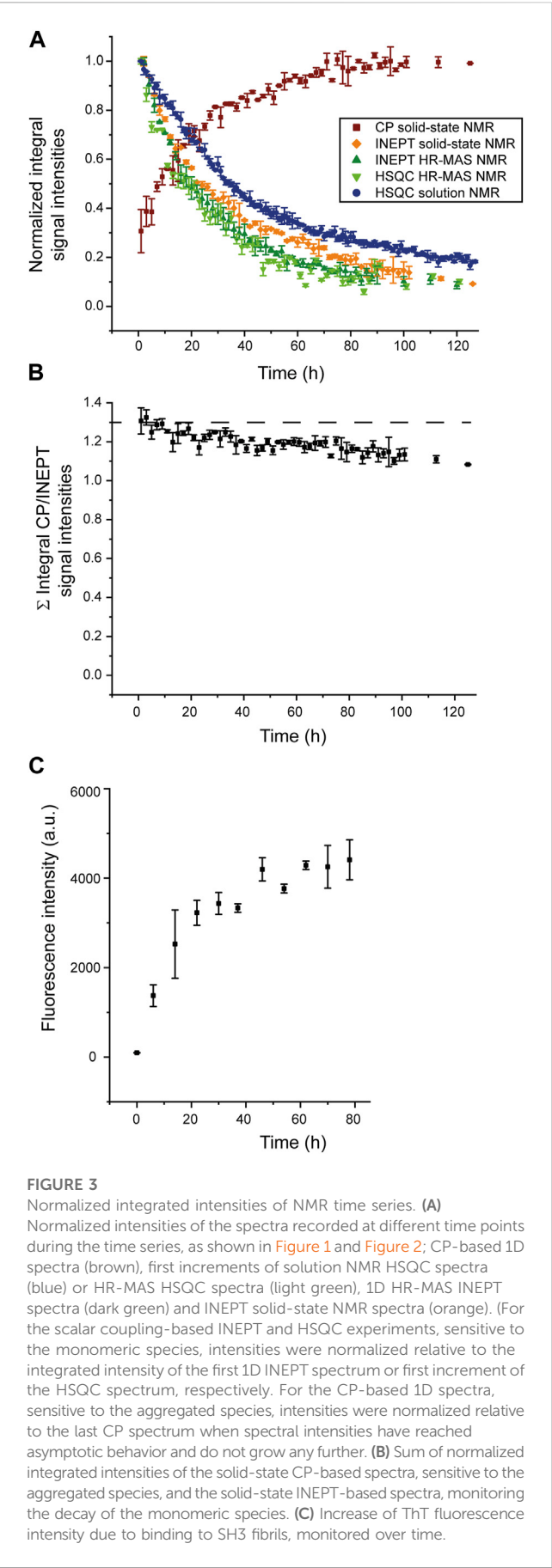
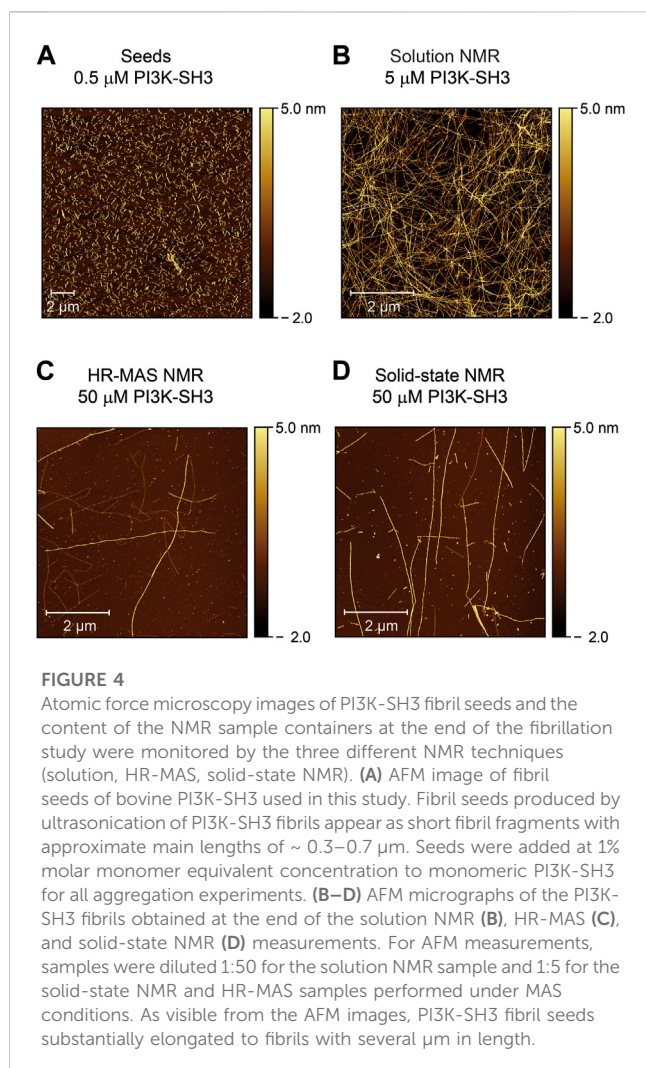


TABLE 1 Lifetime, τ , of the monomeric species (INEPT and HSQC) and build-up time, τ , of aggregated species (CP), as extracted from a mono-exponential fit of the normalized integrated intensities reported in Figure 3, see Supplementary Figure S4 for details on the fits.

Spectrum	τ/h	$\pm\Delta\tau/h$
CP solid-state	26.3	1.2
INEPT solid-state	30.2	1.1
INEPT HR-MAS (^{13}C)	24.0	0.6
HSQC HR-MAS (^1H)	25.0	1.2
HSQC solution NMR	37.4	0.6

side-chain signals report unanimously on the decrease of the monomeric species. Under HR-MAS conditions, the decay of the monomeric species appeared slightly faster than under solid-state NMR conditions ($\tau = 24.0 \pm 0.6$ h vs. 30.2 ± 1.1 h). Thus, under both MAS conditions, the lifetime of the monomeric species appears slightly shorter than under quiescent solution NMR conditions (37.4 ± 0.6 h). We rationalize the slightly faster decrease of the monomeric species and, consequently, somewhat more rapid aggregation kinetics observed under agitated conditions in the MAS rotor by the influence of centrifugal forces on the aggregation process. Centrifugal forces will increase the local mass density close to the rotor wall. They may, therefore, slightly increase the likelihood of primary nucleation events, thus slightly speeding up the aggregation process.

To assess the reproducibility of the experimental setup, we repeated all experiments under identical conditions (Supplementary Figures S5, S6). While the results are in qualitative agreement, we observe differences when fitting the lifetimes of the monomeric species (from the INEPT spectra) and, in particular, for the build-up time of aggregated species (from the CP-based spectra), as described in Supplementary Table S3. Here, the observed differences in the lifetime of the monomeric species appear larger than the variation between the different NMR techniques (solution, solid-state, HR-MAS). Notably, in the second set of experiments, build-up times of aggregation as well as lifetimes of monomers in all experiments appear to be shortened to 50%–60% of the values in the original experiment, suggesting that this deviation is caused by an (unintended) variation of the starting conditions (e.g., slightly local differences in seed concentrations within the rotor or the NMR tube, respectively). This variation seems to have a larger influence than the NMR technique, making it difficult to judge or rationalize differences between the different NMR techniques fully. The way of seeding, or the seed itself, seems to have a major influence on the obtained aggregation kinetics. Also, we observe differences in the “appearance” time of the aggregated species extracted from the CP-based spectra. There will be a size distribution of aggregates during the aggregation process (see below). More stable and rigid aggregates like fibrils will contribute more strongly to the CP signal than smaller, more flexible species. Therefore, a slight variation of kinetics in the emergence of fibrils of different sizes will impact the growth of the CP signal over time.



Atomic force microscopy images confirm fibril growths

To check the morphology of the bovine PI3K-SH3 fibril seeds before and after the NMR time series, we recorded atomic force microscopy (AFM) images: First of the fibril seeds (Figure 4A) and second of all NMR samples, once the recording of the fibrillation time series was finished. Samples were taken from all sample containers of the three different NMR methods employed: solution NMR (Figure 4B), HR-MAS (Figure 4C), and solid-state NMR (Figure 4D). The consistency of all samples was solution-like and not gel-like. The AFM images show that elongated amyloid fibrils with several μm length were formed during all measurements, substantially longer than the sub-μm short fibrils used for seeding (Figure 4A). For the sample obtained from the quiescent solution NMR conditions (Figure 4B), a wealth of fibrils is visible in the AFM images. The observed fibril density is less for the HR-MAS (Figure 4C) and the solid-state NMR sample (Figure 4D), both recorded under MAS conditions. At the same time, the fibrils in the solution NMR sample show a homogenous distribution; fibrils in the samples obtained after the MAS experiments are distributed more inhomogeneous. However, the sample was obtained from the center

of the rotor. At the same time, the highest local density of fibrils will be found close to the rotor wall due to the centrifugal forces present during the MAS sample rotation. In addition to separated PI3K-SH3 fibrils, a few large aggregates with unresolvable structures can be observed in the AFM images of the experiments involving a MAS rotor and sample rotation (solid-state NMR and HR-MAS). The faster aggregation kinetics observed in the second NMR time series results in shorter but also more fibrils (Supplementary Figure S7).

The observance of substantially elongated fibrils suggests that fibril elongation is the dominant mechanism for fibril formation. This is also in line with the observed exponential decay of the monomer concentration, considering that the monomer concentration decreases upon fibril formation and the monomer supply is not unlimited. Following Occam's razor, we fitted the observed decay on the monomer concentration using a mono-exponential decay function (Supplementary Figure S4). If the decay rate of the monomer concentration, $\frac{d}{dt}c(\text{monomer})$, solely depends on the available monomer concentration (and the decay rate constant α), a simple differential equation can describe this $\frac{d}{dt}c(\text{monomer}) = -\alpha c(\text{monomer})$. The solution of that differential equation is a mono-exponential decay function $c(\text{monomer}) = c(0) * \exp(-\alpha * t)$, with the initial monomer concentration $c(0)$ and the decay constant $\alpha = \frac{1}{\tau}$ where τ is the fitted half-life time. This is consistent with the fits described in Supplementary Figure S4, where the fitted intensity corresponds to the monomer concentration (see also Materials and Methods).

Conclusion

We have followed the seeded aggregation kinetics of denatured bovine PI3K-SH3, a model system for studying aggregation and amyloid fibril formation (Guijarro et al., 1998; Zurdo et al., 2001; Polverino de Laureto et al., 2003; Chiti and Dobson, 2006; Bayro et al., 2010; Chiti and Dobson, 2017; Röder et al., 2019), by a combination of three different NMR techniques: solution NMR, solid-state NMR, and HR-MAS NMR, providing the link between solution NMR and solid-state NMR. Scalar coupling-based INEPT experiments, which have been applied in all three NMR techniques, are sensitive to the disappearance of the monomeric species. In contrast, cross-polarization (CP) based solid-state NMR experiments detect the emergence of the aggregated species. Overall, we find good agreement between all three NMR techniques. The solid-state NMR experiments have the advantage of monitoring monomeric species' disappearance and aggregated species' emergence on the same sample (inside the solid-state NMR rotor). Here, the MAS spinning (representing agitated conditions) seems to accelerate the fibril formation kinetics only slightly compared to the quiescent solution NMR conditions. However, AFM data from the reaction under quiescent solution NMR conditions show the most homogenous fibril distribution. By solid-state NMR, under the investigated conditions, we find that the amount of disappeared monomer (visible by INEPT) corresponds to the increasing amount of the aggregated species (visible by CP), suggesting a two-state model for PI3K-SH3 aggregation and the absence of an oligomeric on-pathway intermediate, under

the used conditions. Following Occam's razor, the observed monomer-to-fibril kinetics, under seeding conditions, can be fitted using simple mono-exponential functions, both for the decay of PI3K-SH3 monomers as well as for the PI3K-SH3 fibrils' build-up. AFM images, taken before and after the measurements, show that the initial fibril seeds substantially elongated during the NMR time series, suggesting fibril elongation as the dominant fibril growth mechanism.

For α -synuclein at neutral pH, fibril elongation has also been shown to be the dominant fibrillation mechanism (Buell et al., 2014b). However, below pH 6, secondary nucleation becomes the dominant mechanism for fibril generation. Here, one must distinguish between fibril growth and nucleation (Michaels et al., 2018). Fibril elongation is frequently the primary mechanism of growth in fibril mass (Meisl et al., 2017), while primary and secondary nucleation lead to the emergence of new fibrils (Meisl et al., 2017; Michaels et al., 2018). In the absence of seeds, primary nucleation is the only option for nucleation, whereas with increasing seed concentration secondary nucleation will become more important, as in the case of A β (Meisl et al., 2014). Indeed, a comprehensive set of parameters (monomer concentration, seed concentration, pH, temperature, quiescent vs agitated, etc.) will determine the balance between different fibrillation mechanisms (Buell et al., 2014a; Buell et al., 2014b; Michaels et al., 2018). Knowles and co-workers have derived a sophisticated Master equation approach to delineate the contribution of the various mechanisms (Knowles et al., 2009; Cohen et al., 2011b; Cohen et al., 2011c). Application of this approach requires a large set of experimental data, including variations in monomer concentration and seed concentration (Buell et al., 2014b; Meisl et al., 2017). The contributions of different nucleation and elongation mechanisms to the total fibrillation process can be analyzed by measuring the reaction half-time as a function of monomer concentration (Meisl et al., 2017). Nevertheless, fibril elongation is frequently the primary process of generating new fibril mass (Meisl et al., 2017).

As the intensity of the CP-based solid-state NMR spectra is proportional to the amount of fibril mass, and AFM data support the fibril growth by fibril elongation in the case of PI3K-SH3 (Figure 4), we argue for fibril elongation as the dominant mechanism of the observed increase of the CP-based solid-state NMR signal (Figure 3A). Indeed, in the presence of a large number of seeds and when fibril elongation is the dominant growth mechanism for generating new fibril mass, the aggregation profile is expected to be a single exponential function (Cohen et al., 2011a; Buell et al., 2014b). Secondary nucleation as an alternative mechanism, however, cannot be excluded. It may have occurred as a secondary mechanism responsible for the faster aggregation kinetics observed in the "reproduced" experiment (see above), where shorter and more fibrils were observed after fibrillation. However, faster monomer decay rates at higher temperatures (data not shown) argue against secondary nucleation as the dominant fibrillation mechanism, see, e.g. (Törnquist et al., 2018).

Our data support that solid-state NMR, in combination with AFM, presents a valuable addition to measure the kinetics of fibril

mass increase. By comparing scalar coupling-based and CP-based solid-state NMR intensities (agitated conditions, by MAS rotation) with quiescent condition (solution NMR), we find that under sufficiently high seed concentrations (1% seeds in our case), primary nucleation events induced by MAS rotation, have only a small influence. By comparing solution NMR measurements, sensitive to the depletion of monomer, and solid-state NMR sensitive to fibril mass increase by CP-based measurements (but also to monomer depletion by conducting scalar coupling-based experiments as an internal reference), we followed fibril mass increase and monomer depletion simultaneously.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

LuG: Methodology, Writing-original draft, Formal Analysis, Investigation, Resources. NB: Methodology, Writing-original draft, Formal Analysis, Investigation. NR: Formal Analysis, Investigation, Resources, Writing-review and editing. CH: Resources, Writing-review and editing. AA: Resources, Writing-review and editing. CS: Investigation, Methodology, Writing-review and editing. KB: Investigation, Methodology, Writing-review and editing. LoG: Investigation, Methodology, Conceptualization, Resources, Supervision, Writing-review and editing. HH: Conceptualization, Methodology, Supervision, Writing-review and editing, Funding acquisition. N-AL: Conceptualization, Funding acquisition, Methodology, Supervision, Writing-review and editing, Writing-original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. N-AL acknowledges the German Science Foundation for funding through the Heisenberg Program (DFG grant number 433700474). HH was supported by the Entrepreneur Foundation at the Heinrich-Heine-University (HHU) of Düsseldorf and by the German Science Foundation (DFG grant numbers HE 3243/4-1 and INST 208/771-1 FUGG).

Acknowledgments

We thank Tsering Sherpa (HHU) for technical help with NMR sample preparation and data analysis and acknowledge access to the Jülich-Düsseldorf Biomolecular NMR Center, jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2023.1254721/full#supplementary-material>

References

- Bader, R., Bamford, R., Zurdo, J., Luisi, B. F., and Dobson, C. M. (2006). Probing the mechanism of amyloidogenesis through a tandem repeat of the PI3-SH3 domain suggests a generic model for protein aggregation and fibril formation. *J. Mol. Biol.* 356, 189–208. doi:10.1016/j.jmb.2005.11.034
- Balbach, J. J., Ishii, Y., Antzutkin, O. N., Leapman, R. D., Rizzo, N. W., Dyda, F., et al. (2000). Amyloid fibril formation by A beta 16–22, a seven-residue fragment of the Alzheimer's beta-amyloid peptide, and structural characterization by solid state NMR. *Biochemistry* 39, 13748–13759. doi:10.1021/bi0011330
- Batra-Safferling, R., Granzin, J., Modder, S., Hoffmann, S., and Willbold, D. (2010). Structural studies of the phosphatidylinositol 3-kinase (PI3K) SH3 domain in complex with a peptide ligand: role of the anchor residue in ligand binding. *Biol. Chem.* 391, 33–42. doi:10.1515/BC.2010.003
- Bayro, M. J., Maly, T., Birkett, N. R., Macphree, C. E., Dobson, C. M., and Griffin, R. G. (2010). High-resolution MAS NMR analysis of PI3-SH3 amyloid fibrils: backbone conformation and implications for protofilament assembly and structure. *Biochemistry* 49, 7474–7484. doi:10.1021/bi100864t
- Becker, N., Frieg, B., Gremer, L., Kupreichyk, T., Gardon, L., Freiburg, P., et al. (2023). Atomic resolution insights into pH shift induced deprotonation events in LS-shaped Aβ(1–42) amyloid fibrils. *J. Am. Chem. Soc.* 145, 2161–2169. doi:10.1021/jacs.2c09231
- Bellomo, G., Bologna, S., Gonnelli, L., Ravera, E., Fragai, M., Lelli, M., et al. (2018). Aggregation kinetics of the Aβ1–40 peptide monitored by NMR. *Chem. Commun.* 54, 7601–7604. doi:10.1039/c8cc01710g
- Berkeley, R. F., Kashefi, M., and Debelouchina, G. T. (2021). Real-time observation of structure and dynamics during the liquid-to-solid transition of FUS L.C. *Biophys. J.* 120, 1276–1287. doi:10.1016/j.bpj.2021.02.008
- Bertini, I., Gallo, G., Korsak, M., Luchinat, C., Mao, J., and Ravera, E. (2013). Formation kinetics and structural features of Beta-amyloid aggregates by sedimented solute NMR. *ChemBiochem* 14, 1891–1897. doi:10.1002/cbic.201300141
- Biancalana, M., and Koide, S. (2010). Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochimica Biophysica Acta-Proteins Proteomics* 1804, 1405–1412. doi:10.1016/j.bbapap.2010.04.001
- Bucciattini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J. S., et al. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416, 507–511. doi:10.1038/416507a
- Buell, A. K., Dobson, C. M., and Knowles, T. P. J. (2014a). The physical chemistry of the amyloid phenomenon: thermodynamics and kinetics of filamentous protein aggregation. *Amyloids Health Dis.* 56, 11–39. doi:10.1042/bse0560011
- Buell, A. K., Galvagnion, C., Gaspar, R., Sparr, E., Vendruscolo, M., Knowles, T. P. J., et al. (2014b). Solution conditions determine the relative importance of nucleation and growth processes in α-synuclein aggregation. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7671–7676. doi:10.1073/pnas.1315346111
- Chiti, F., and Dobson, C. M. (2006). Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* 75, 333–366. doi:10.1146/annurev.biochem.75.101304.123901
- Chiti, F., and Dobson, C. M. (2017). "Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade," in *Annu. Rev. Biochem.* doi:10.1146/annurev-biochem-061516-045115
- Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., et al. (1999). Designing conditions for *in vitro* formation of amyloid protofilaments and fibrils. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3590–3594. doi:10.1073/pnas.96.7.3590
- Cohen, S. I. A., Vendruscolo, M., Dobson, C. M., and Knowles, T. P. J. (2011a). Nucleated polymerisation in the presence of pre-formed seed filaments. *Int. J. Mol. Sci.* 12, 5844–5852. doi:10.3390/ijms12095844
- Cohen, S. I. A., Vendruscolo, M., Dobson, C. M., and Knowles, T. P. J. (2011b). Nucleated polymerization with secondary pathways. II. Determination of self-consistent solutions to growth processes described by non-linear master equations. *J. Chem. Phys.* 135, 065106. doi:10.1063/1.3608917
- Cohen, S. I. A., Vendruscolo, M., Welland, M. E., Dobson, C. M., Terentjev, E. M., and Knowles, T. P. J. (2011c). Nucleated polymerization with secondary pathways. I. Time evolution of the principal moments. *J. Chem. Phys.* 135, 065105. doi:10.1063/1.3608916
- Colvin, M. T., Silvers, R., Ni, Q. Z., Can, T. V., Sergeyev, I., Rosay, M., et al. (2016). Atomic resolution structure of monomeric Aβ42 amyloid fibrils. *J. Am. Chem. Soc.* 138, 9663–9674. doi:10.1021/jacs.6b05129
- Dionne, U., Percival, L. J., Chartier, F. J. M., Landry, C. R., and Bisson, N. (2022). SRC homology 3 domains: multifaceted binding modules. *Trends Biochem. Sci.* 47, 772–784. doi:10.1016/j.tibs.2022.04.005
- Dobson, C. M. (2003). Protein folding and misfolding. *Nature* 426, 884–890. doi:10.1038/nature02261
- Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994). Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science* 266, 1241–1247. doi:10.1126/science.7526465
- Frieg, B., Gremer, L., Heise, H., Willbold, D., and Gohlke, H. (2020). Binding modes of thioflavin T and Congo red to the fibril structure of amyloid-β(1–42). *Chem. Commun.* 56, 7589–7592. doi:10.1039/d0cc01161d
- Gremer, L., Scholzel, D., Schenk, C., Reinartz, E., Labahn, J., Ravelli, R. B. G., et al. (2017). Fibril structure of amyloid-β(1–42) by cryo-electron microscopy. *Science* 358, 116–119. doi:10.1126/science.aao2825
- Guijarro, J. I., Sunde, M., Jones, J. A., Campbell, I. D., and Dobson, C. M. (1998). Amyloid fibril formation by an SH3 domain. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4224–4228. doi:10.1073/pnas.95.8.4224
- Heise, H., Hoyer, W., Becker, S., Andronesi, O. C., Riedel, D., and Baldus, M. (2005). Molecular-level secondary structure, polymorphism, and dynamics of full-length alpha-synuclein fibrils studied by solid-state NMR. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15871–15876. doi:10.1073/pnas.0506109102
- Holzgrabe, U. (2010). Quantitative NMR spectroscopy in pharmaceutical applications. *Prog. Nucl. Magnetic Reson. Spectrosc.* 57, 229–240. doi:10.1016/j.pnmrs.2010.05.001
- Horvath, I., Welte, H., Schmit, J. D., Kovermann, M., and Wittung-Stafshede, P. (2023). Distinct growth regimes of α-synuclein amyloid elongation. *Biophysical J.* 122, 2556–2563. doi:10.1016/j.bpj.2023.05.009
- Housmans, J. A. J., Wu, G., Schymkowitz, J., and Rousseau, F. (2021). A guide to studying protein aggregation. *FEBS J.* 290, 554–583. doi:10.1111/febs.16312
- Iadanza, M. G., Jackson, M. P., Hewitt, E. W., Ranson, N. A., and Radford, S. E. (2018). A new era for understanding amyloid structures and disease. *Nat. Rev. Mol. Cell Biol.* 19, 755–773. doi:10.1038/s41580-018-0060-8
- Jaroniec, C. P., Macphree, C. E., Bajaj, V. S., McMahon, M. T., Dobson, C. M., and Griffin, R. G. (2004). High-resolution molecular structure of a peptide in an amyloid fibril determined by magic angle spinning NMR spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 101, 711–716. doi:10.1073/pnas.0304849101
- Knowles, T. P. J., Waudby, C. A., Devlin, G. L., Cohen, S. I. A., Aguzzi, A., Vendruscolo, M., et al. (2009). An analytical solution to the kinetics of breakable filament assembly. *Science* 326, 1533–1537. doi:10.1126/science.1178250
- Kurochkina, N., and Guha, U. (2013). SH3 domains: modules of protein-protein interactions. *Biophys. Rev.* 5, 29–39. doi:10.1007/s12551-012-0081-z
- Levine, H. (1993). Thioflavine-T Interaction With Synthetic Alzheimer's-Disease Beta-Amyloid Peptides - Detection of Amyloid Aggregation in Solution. *Prot. Sci.* 2, 404–410. doi:10.1002/pro.5560020312
- Lindon, J. C., Beckonert, O. P., Holmes, E., and Nicholson, J. K. (2009). High-resolution magic angle spinning NMR spectroscopy: application to biomedical studies. *Prog. Nucl. Magnetic Reson. Spectrosc.* 55, 79–100. doi:10.1016/j.pnmrs.2008.11.004

- Luhers, T., Ritter, C., Adrian, M., Riek-Loher, D., Bohrmann, B., Doeli, H., et al. (2005). 3D structure of Alzheimer's amyloid-beta(1-42) fibrils. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17342–17347. doi:10.1073/pnas.0506723102
- Meisl, G., Rajah, L., Cohen, S. A. I., Pfammatter, M., Saric, A., Hellstrand, E., et al. (2017). Scaling behaviour and rate-determining steps in filamentous self-assembly. *Chem. Sci.* 8, 7087–7097. doi:10.1039/c7sc01965c
- Meisl, G., Yang, X. T., Hellstrand, E., Frohm, B., Kirkegaard, J. B., Cohen, S. I. A., et al. (2014). Differences in nucleation behavior underlie the contrasting aggregation kinetics of the A β 40 and A β 42 peptides. *Proc. Natl. Acad. Sci. U. S. A.* 111, 9384–9389. doi:10.1073/pnas.1401564111
- Michaels, T. C. T., Saric, A., Habchi, J., Chia, S., Meisl, G., Vendruscolo, M., et al. (2018). Chemical kinetics for bridging molecular mechanisms and macroscopic measurements of amyloid fibril formation. *Annu. Rev. Phys. Chem.* 69 (69), 273–298. doi:10.1146/annurev-physchem-050317-021322
- Musacchio, A., Wilmanns, M., and Saraste, M. (1994). Structure and function of the SH3 domain. *Prog. Biophys. Mol. Biol.* 61, 283–297. doi:10.1016/0079-6107(94)90003-5
- Nečas, D., and Klapetek, P. (2012). Gwyddion: an open-source software for SPM data analysis. *Open Phys.* 10, 181–188. doi:10.2478/s11534-011-0096-2
- Paravastu, A. K., Leapman, R. D., Yau, W. M., and Tycko, R. (2008). Molecular structural basis for polymorphism in Alzheimer's beta-amyloid fibrils. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18349–18354. doi:10.1073/pnas.0806270105
- Petkova, A. T., Ishii, Y., Balbach, J. J., Antzutkin, O. N., Leapman, R. D., Delaglio, F., et al. (2002). A structural model for Alzheimer's beta-amyloid fibrils based on experimental constraints from solid state NMR. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16742–16747. doi:10.1073/pnas.262663499
- Polverino De Laureto, P., Taddei, N., Frare, E., Capanni, C., Costantini, S., Zurdo, J., et al. (2003). Protein aggregation and amyloid fibril formation by an SH3 domain probed by limited proteolysis. *J. Mol. Biol.* 334, 129–141. doi:10.1016/j.jmb.2003.09.024
- Ravera, E. (2014). The bigger they are, the harder they fall: a topical review on sedimented solutes for solid-state NMR. *Concepts Magn. Reson. Part A* 43, 209–227. doi:10.1002/cmr.a.21318
- Roche, J., Shen, Y., Lee, J. H., Ying, J., and Bax, A. (2016). Monomeric A β 1–40 and A β 1–42 peptides in solution adopt very similar ramachandran map distributions that closely resemble random coil. *Biochemistry* 55, 762–775. doi:10.1021/acs.biochem.5b01259
- Röder, C., Vettore, N., Mangels, L. N., Gremer, L., Ravelli, R. B. G., Willbold, D., et al. (2019). Atomic structure of PI3-kinase SH3 amyloid fibrils by cryo-electron microscopy. *Nat. Comm.* 10, 3754. doi:10.1038/s41467-019-11320-8
- Ross, C. A., and Poirier, M. A. (2004). Protein aggregation and neurodegenerative disease. *Nat. Med.* 10, S10–S17. doi:10.1038/nm1066
- Sahoo, B. R., Cox, S. J., and Ramamoorthy, A. (2020). High-resolution probing of early events in amyloid- β aggregation related to Alzheimer's disease. *Chem. Commun.* 56, 4627–4639. doi:10.1039/d0cc01551b
- Saksela, K., and Permi, P. (2012). SH3 domain ligand binding: what's the consensus and where's the specificity? *FEBS Lett.* 586, 2609–2614. doi:10.1016/j.febslet.2012.04.042
- Schutz, A. K., Soragni, A., Hornemann, S., Aguzzi, A., Ernst, M., Bockmann, A., et al. (2011). The amyloid-Congo red interface at atomic resolution. *Angew. Chem. Int. Ed.* 50, 5956–5960. doi:10.1002/anie.201008276
- Sitter, B., Bathen, T. F., Tessem, M. B., and Gribbestad, I. S. (2009). High-resolution magic angle spinning (HR-MAS) MR spectroscopy in metabolic characterization of human cancer. *Prog. Nucl. Magnetic Reson. Spectrosc.* 54, 239–254. doi:10.1016/j.pnmrs.2008.10.001
- Törnquist, M., Michaels, T. C. T., Sanagavarapu, K., Yang, X. T., Meisl, G., Cohen, S. I. A., et al. (2018). Secondary nucleation in amyloid formation. *Chem. Commun.* 54, 8667–8684. doi:10.1039/c8cc02204f
- Tuttle, M. D., Comellas, G., Nieuwkoop, A. J., Covell, D. J., Berthold, D. A., Kloepper, K. D., et al. (2016). Solid-state NMR structure of a pathogenic fibril of full-length human alpha-synuclein. *Nat. Struct. Mol. Biol.* 23, 409–415. doi:10.1038/nsmb.3194
- Vettore, N., and Buell, A. K. (2019). Thermodynamics of amyloid fibril formation from chemical depolymerization. *Phys. Chem. Chem. Phys.* 21, 26184–26194. doi:10.1039/c9cp04524d
- Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, M., et al. (2005). The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* 59, 687–696. doi:10.1002/prot.20449
- Wälti, M. A., Ravotti, F., Arai, H., Glabe, C. G., Wall, J. S., Bockmann, A., et al. (2016). Atomic-resolution structure of a disease-relevant A β (1–42) amyloid fibril. *Proc. Natl. Acad. Sci. U.S.A.* 113, E4976–E4984. doi:10.1073/pnas.1600749113
- Wang, J., Yamamoto, T., Bai, J., Cox, S. J., Korshavn, K. J., Monette, M., et al. (2018). Real-time monitoring of the aggregation of Alzheimer's amyloid-beta via (1)H magic angle spinning NMR spectroscopy. *Chem. Commun. (Camb)* 54, 2000–2003. doi:10.1039/c8cc00167g
- Warrass, R., Wieruszkeski, J. M., Boutillon, C., and Lippens, G. (2000). High-resolution magic angle spinning NMR study of resin-bound polyalanine peptides. *J. Am. Chem. Soc.* 122, 1789–1795. doi:10.1021/ja993718r
- Wasmer, C., Lange, A., Van Melckebeke, H., Siemer, A. B., Riek, R., and Meier, B. H. (2008). Amyloid fibrils of the HET-s(218–289) prion form a beta solenoid with a triangular hydrophobic core. *Science* 319, 1523–1526. doi:10.1126/science.1151839
- Weirich, F., Gremer, L., Mirecka, E. A., Schiefer, S., Hoyer, W., and Heise, H. (2016). Structural characterization of fibrils from recombinant human islet amyloid polypeptide by solid-state NMR: the central FGAILS segment is part of the beta-sheet core. *PLoS One* 11, e0161243. doi:10.1371/journal.pone.0161243
- Willbold, D., Strodel, B., Schroder, G. F., Hoyer, W., and Heise, H. (2021). Amyloid-type protein aggregation and prion-like properties of amyloids. *Chem. Rev.* 121, 8285–8307. doi:10.1021/acs.chemrev.1c00196
- Zurdo, J., Guijarro, J. I., Jiménez, J. L., Saibil, H. R., and Dobson, C. M. (2001). Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain. *J. Mol. Biol.* 311, 325–340. doi:10.1006/jmbi.2001.4858

Supplementary Material

Amyloid fibril formation kinetics of low-pH denatured bovine

PI3K-SH3 monitored by three different NMR techniques

Luis Gardon^{1,2#}, Nina Becker^{1,2#}, Nick Rähse², Christoph Hölbling², Athina Apostolidis²,
Celina M. Schulz², Kevin Bochinsky¹, Lothar Gremer^{1,2*}, Henrike Heise^{1,2*}, Nils-Alexander
Lakomek^{1,2*}

¹ Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct:
Jülich Center for Structural Biology, Forschungszentrum Jülich, Jülich, Germany;

² Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, Düsseldorf,
Germany.

authors contributed equally and share the first authorship

* correspondence: l.gremer@fz-juelich.de, h.heise@fz-juelich.de, n.lakomek@fz-juelich.de

Table of contents:

1. Supplementary Materials and Methods:

Supplementary Table S1: Experimental parameters of 2D ^1H - ^{15}N HSQC experiments (solution NMR and HR-MAS)

Supplementary Table S2: Experimental parameters of 1D ^{13}C -detected experiments

2. Supplementary Results:

Supplementary Figure S1: Comparison of solution NMR ^1H - ^{15}N HSQC spectra of bovine PI3K-SH3 at neutral and acidic pH conditions.

Supplementary Figure S2: Far-UV-CD spectra of bovine PI3K-SH3 at pH 6.8 and 2.5

Supplementary Figure S3: Decay of representative intensities in two-dimensional solution NMR spectra.

Supplementary Figure S4: Fit of integrated intensities shown in Figure 3 and Figure S6

Supplementary Figure S5: Comparison of the integrated intensities of our first NMR measurements and our repeated (reproduced) NMR measurements.

Supplementary Figure S6: Fit of integrated intensities shown in Figure 3 and Figure S4.

Supplementary Table S3: Fitting parameters of decay and build-up curves (mono-exponential fit).

Supplementary Figure S7: AFM images of the repeated (reproduced) measurement.

1. Supplementary Materials and Methods

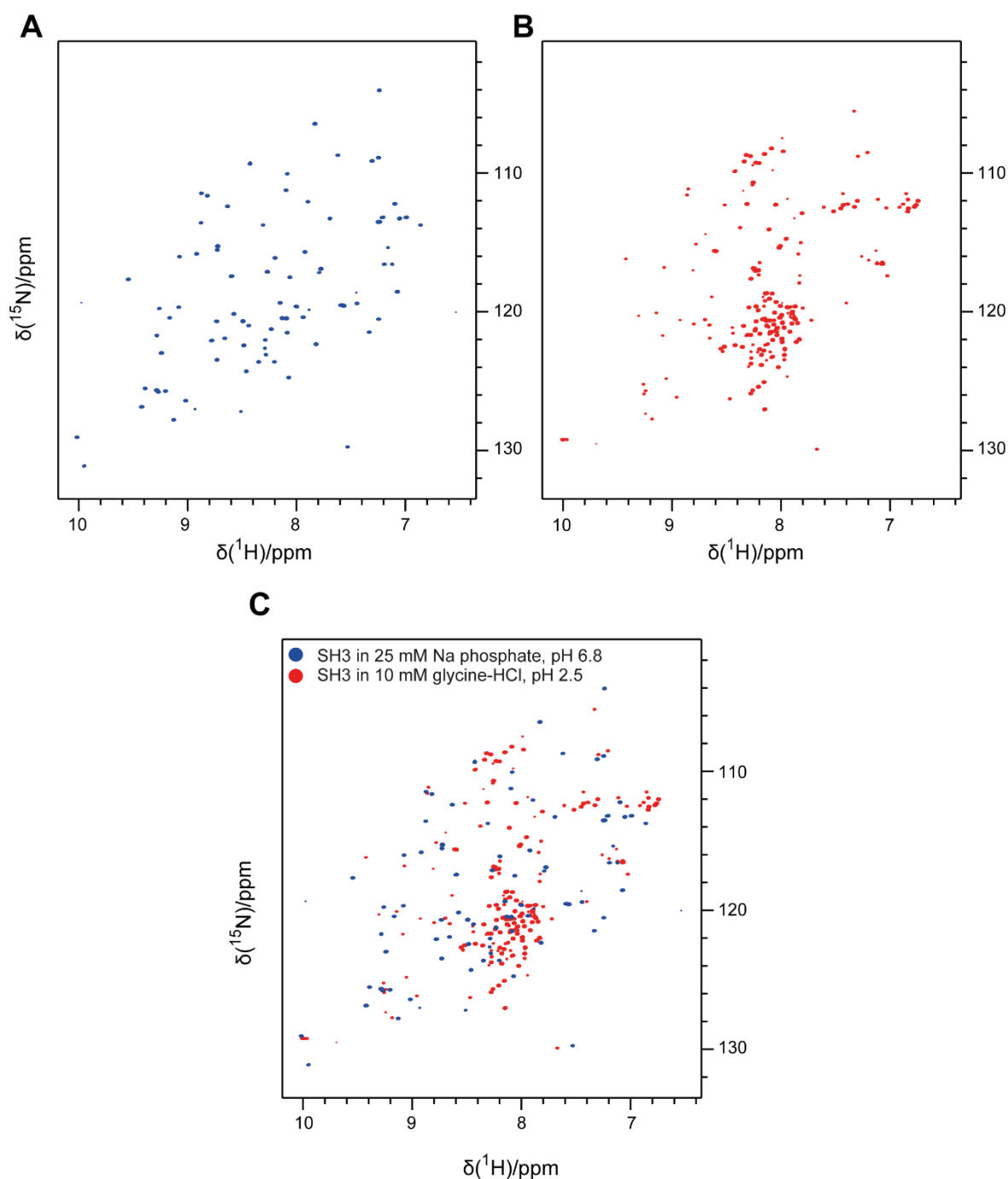
Supplementary Table S1: Experimental parameters of 2D ^1H - ^{15}N HSQC experiments (solution NMR and HR-MAS).

	HSQC (solution NMR)	HSQC (solution NMR)	HSQC (HR-MAS)
Field (T)	21.15	14.1	18.8
^1H Larmor frequency	900 MHz	600 MHz	800 MHz
Sample volume	200 μl (3 mm tube)	200 μl (3 mm tube)	~110 μl (4 mm rotor)
MAS frequency (kHz)	-	-	6
Bruker pulse program	hsqcfpf3gp phwg	fhsqcf3gp hh	hsqcfpf3gp phwg
Interscan recovery delay (s)	1.3	1.2	1
Number of scans	8	8	16
Dummy scans	4	16	4
Total experimental time	56 min 36 sec	56 min 39 sec	59 min 42 sec
Direct dimension (^1H)			
^1H carrier (ppm)	4.7	4.698	4.699
Spectral width (ppm)	16.3427	16.0205	16.0294
Data points	2048	1024	2048
Acquisition time (ms)	69.6	53.2	79.9
Indirect dimension (^{15}N)			
^{15}N carrier (ppm)	117	99.877	117
Spectral width (ppm)	32	79.9	35
Data points	300	328	200
Acquisition time (ms)	51.4	33.7	35.2

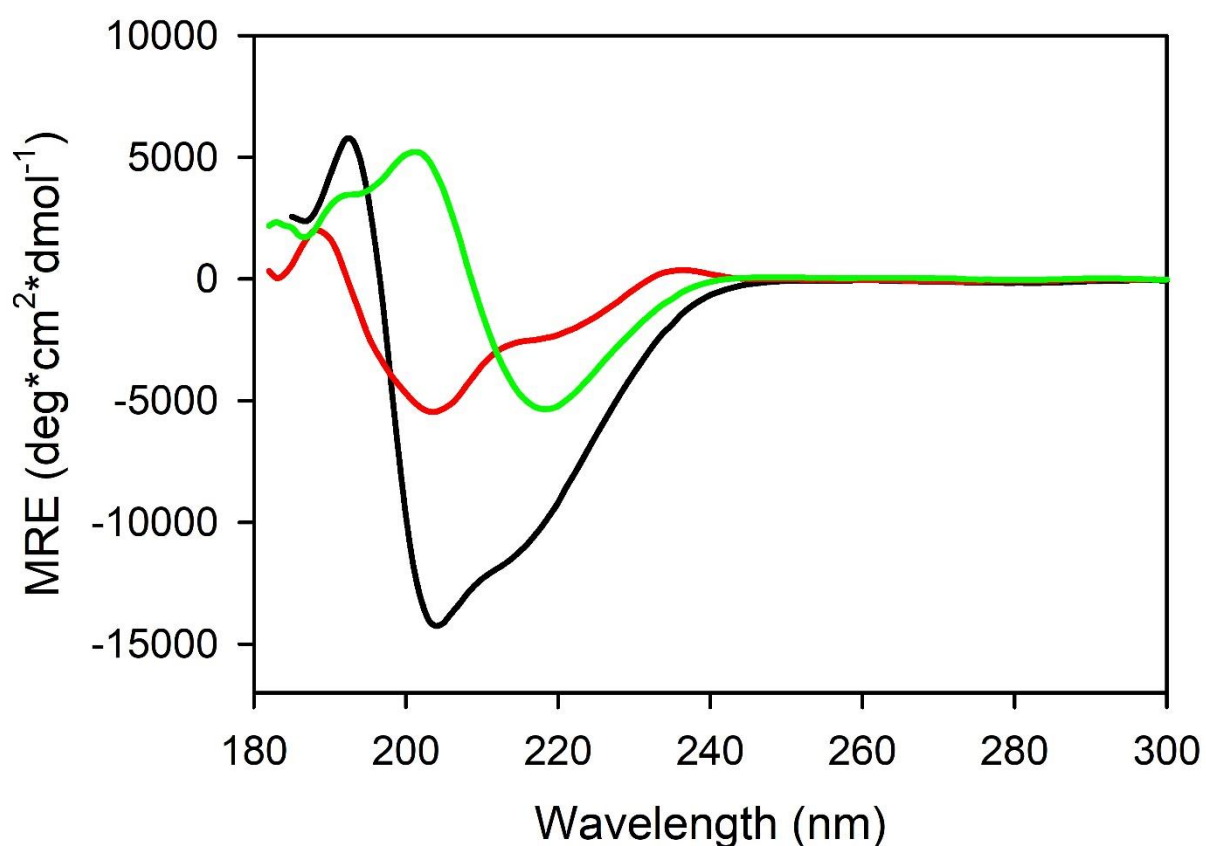
Supplementary Table S2: Experimental parameters of 1D ¹³C-detected experiments.

	INEPT (HR-MAS)	INEPT (solid-state)	CP (solid-state)
Field (T)	18.8	14.1	14.1
¹ H Larmor frequency	800	600	600
Sample volume	~110 µl (4 mm rotor)	~32 µl (3.2 mm rotor)	~32 µl (3.2 mm rotor)
MAS frequency (kHz)	6	8	8
Interscan recovery delay (s)	1.5	2	2
Number of scans	2368	1780	1780
Dummy scans	0	2	2
Total experimental time	1h 0 min 34 sec	1h 0 min 5sec	1h 0 min 1 sec
Transfer	¹ H- ¹³ C INEPT	¹ H- ¹³ C INEPT	¹ H- ¹³ C CP
¹³ C Carrier (ppm)	56	70	100
Duration of transfer (ms)	3.4	3.4	0.7
Spectral width (ppm)	202.7	251	251
Data points	2048	1024	1024
Acquisition time (ms)	25.1	13.5	13.5
Decoupling power during acquisition (kHz)	no decoupling	82	82

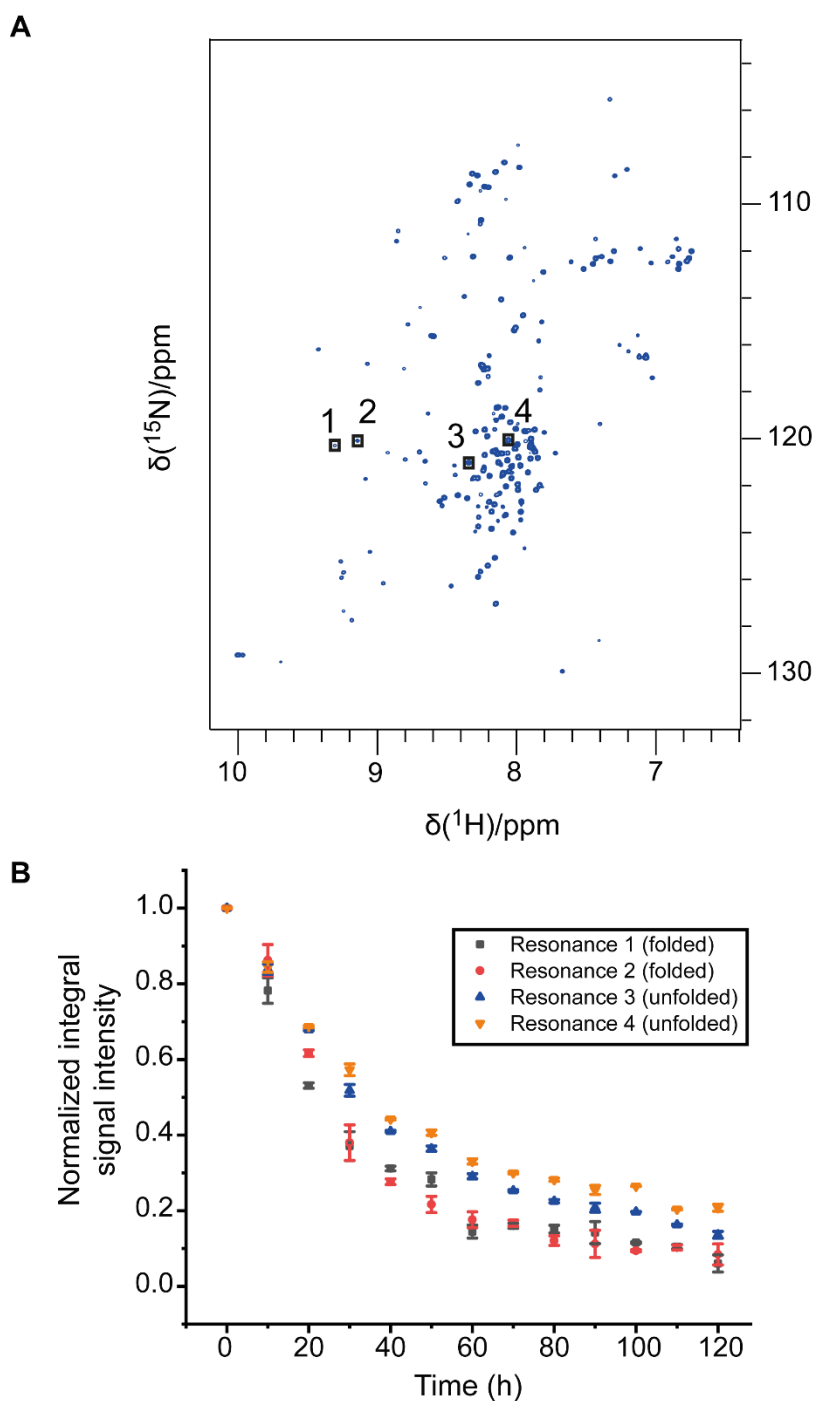
2. Supplementary Results



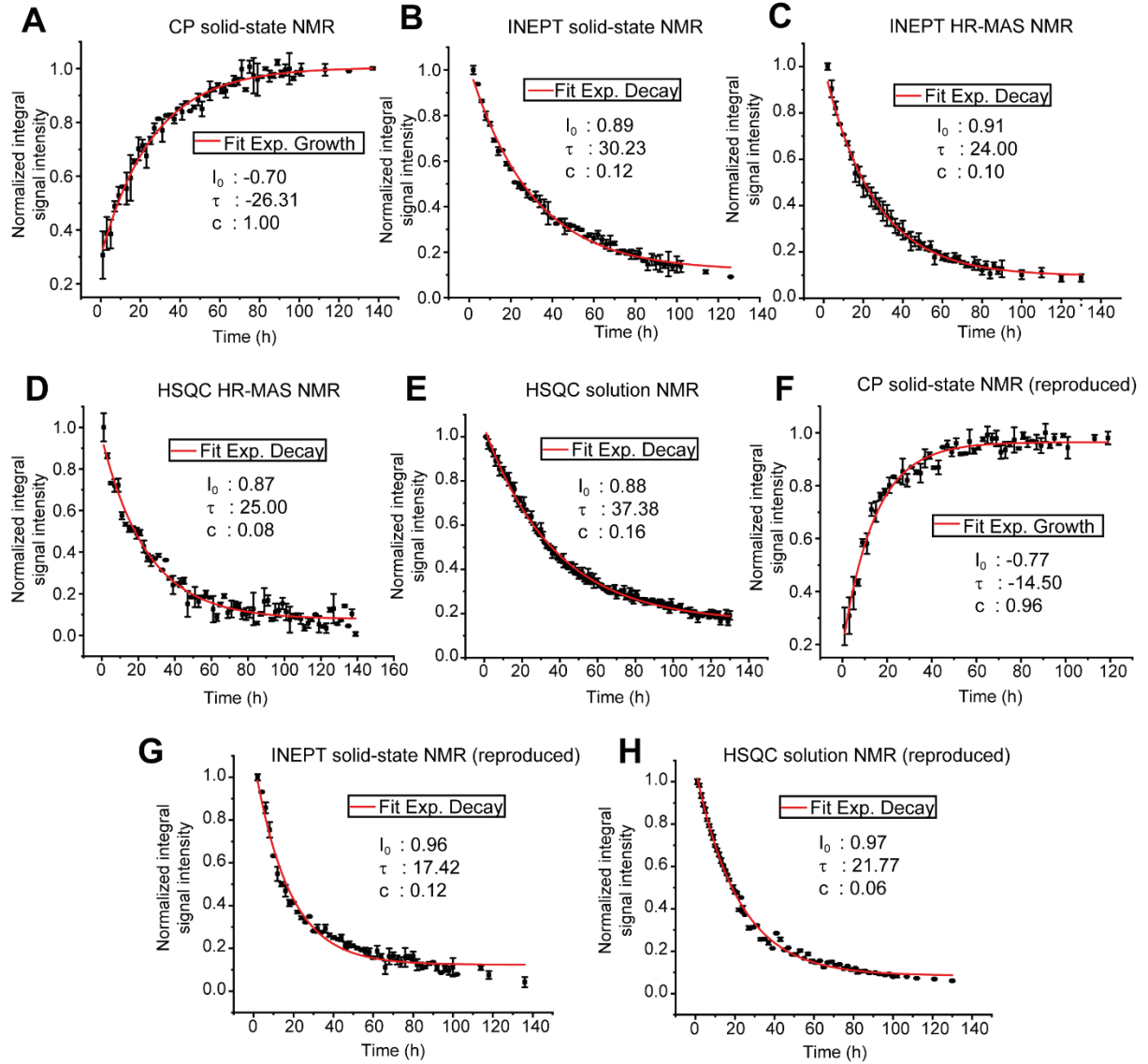
Supplementary Figure S1: Comparison of solution NMR ^1H - ^{15}N HSQC spectra of bovine PI3K-SH3 at neutral (A, blue) and acidic (B, red) pH conditions. A) The blue spectrum was recorded on 250 μM $^{15}\text{N}^{13}\text{C}$ PI3K-SH3 sample dissolved in 25 mM sodium phosphate buffer at pH 6.8 and 900 MHz and 25 $^\circ\text{C}$. It shows a wide signal dispersion expected for a β -strand-native structure of the PI3K-SH3 domain. B) The red spectrum was recorded on 250 μM $^{15}\text{N}^{13}\text{C}$ PI3K-SH3 sample dissolved in 10 mM glycine-HCl buffer at pH 2.5 and 900 MHz and 25 $^\circ\text{C}$. A collapse of peaks into the region of 7.5 and 8.5 ppm, indicating disordered conformation, is observed in the red spectrum. However, peaks still appear in the region above 8.5 ppm, indicating β -strand content. C) Overlay of the solution NMR ^1H - ^{15}N HSQC spectra of PI3K-SH3 at acidic (red) and neutral (blue) pH conditions shown in panels A and B).



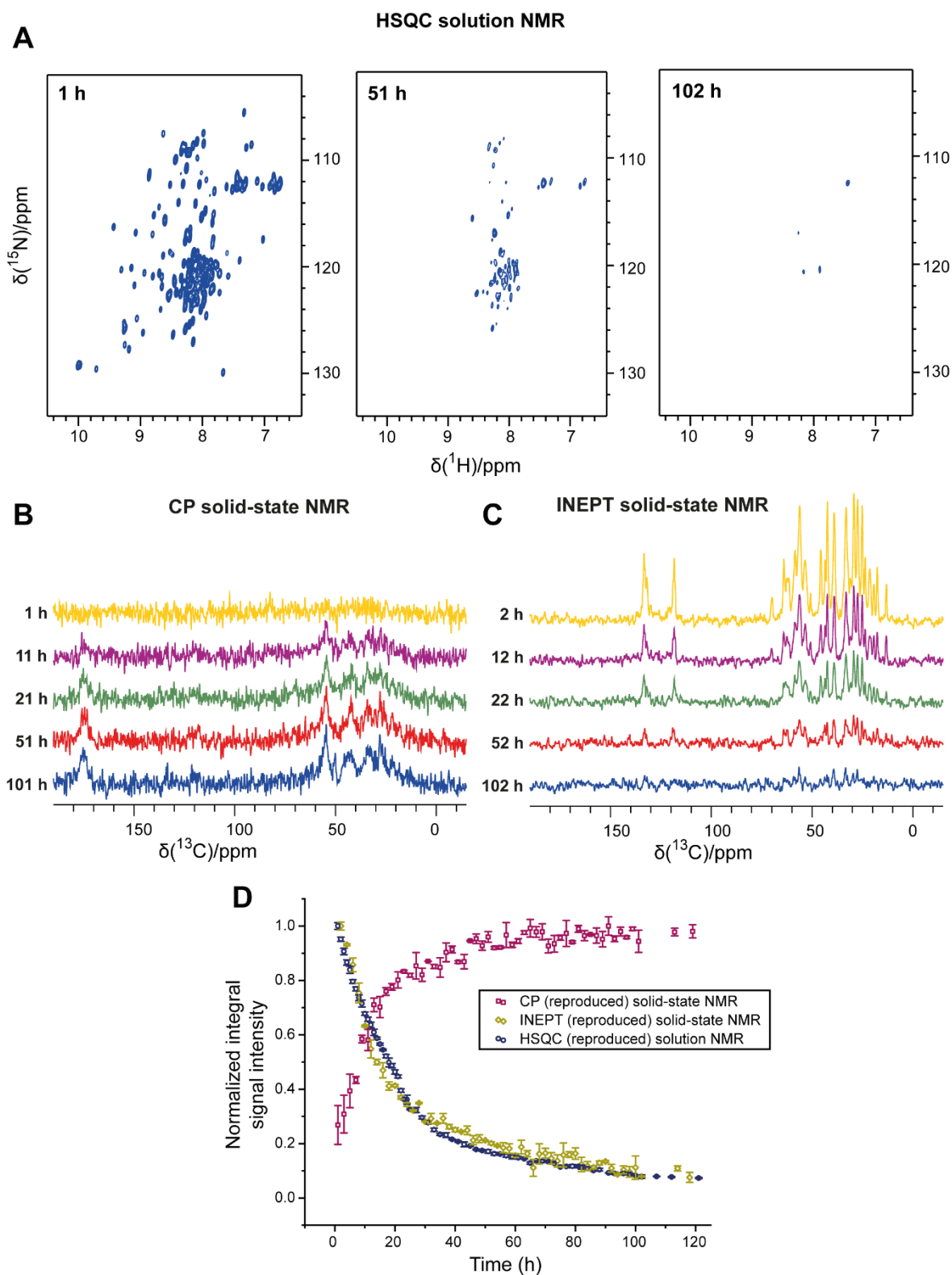
Supplementary Figure S2. Far-UV-CD spectra of bovine PI3K-SH3 in its monomeric state at pH 6.8 (red) or 2.5 (black) or in its fibrillar state at pH 2.5 (green), respectively. Far-UV-CD spectra were recorded at 20 μ M protein concentration after dissolving lyophilized bovine PI3K-SH3 in 10 mM glycine-hydrochloride buffer, pH 2.5, or in 20 mM Na phosphate buffer, pH 6.8, respectively. Fibrils of bovine PI3K-SH3 were grown at 200 μ M protein concentration overnight at 42°C in 10 mM glycine-hydrochloride buffer, pH 2.5, and diluted to 20 μ M concentration (monomer equivalents) for CD measurements. MRE, mean residue ellipticity.



Supplementary Figure S3. Decay of representative intensities in two-dimensional solution NMR spectra. (A) Four representative peaks (NMR resonances) were selected from the two-dimensional solution NMR spectrum of SH3 at pH 2.5 and 25°C. Peaks (1) and (2) show ^1H chemical shifts that are characteristic of folded secondary structure elements, most likely β -sheet conformation. Peaks belonging to the unfolded region have a ^1H chemical shift range between 7.5 and 8.5 ppm. Most resonances in this region belong to the unfolded SH3 population. However, the resonances of the folded SH3 (minor) population overlap with this region. Peaks (3) and (4) presumably belong to the (minor) folded population. (B) Normalized intensity decay of the representative peaks (1-4) shown in (A).

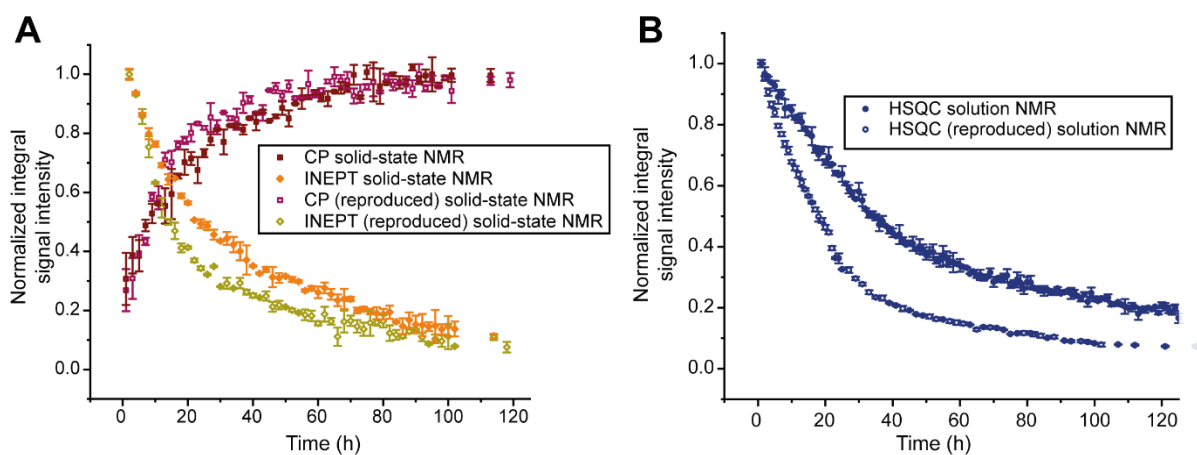


Supplementary Figure S4: Fit of integrated intensities shown in Figure 3 and Figure S6. Integrated intensities were fitted using the mono-exponential equation $I(t) = I_0 * e^{-\left(\frac{t}{\tau}\right)} + c$, with fitted parameters τ and c . A) CP-based solid-state NMR spectrum, B) INEPT-based solid-NMR spectrum, C) INEPT-based HR-MAS NMR spectrum, D) HSQC-based HR-MAS spectrum, E) HSQC-based solution NMR spectrum, F) CP-based solid-state NMR spectrum (reproduced), G) INEPT-based solid-NMR spectrum (reproduced), H) HSQC-based solution spectrum (reproduced).



Supplementary Figure S5: NMR spectra of the second (repeated) experiment. The NMR experiments were repeated a second time under identical conditions to assess the reproducibility of the data. NMR spectra and integrated intensities of the repeated (reproduced) aggregation experiments of bovine PI3K-SH3 at 25°C indicated duration after the start of the experiment. A) Representative solution NMR 2D HSQC spectra of the NMR time series, recorded at a magnetic field strength of 14.1 T (600 MHz ^1H Larmor frequency) and 25°C. B and C) Representative spectra of ^{13}C -detected 1D solid-state NMR

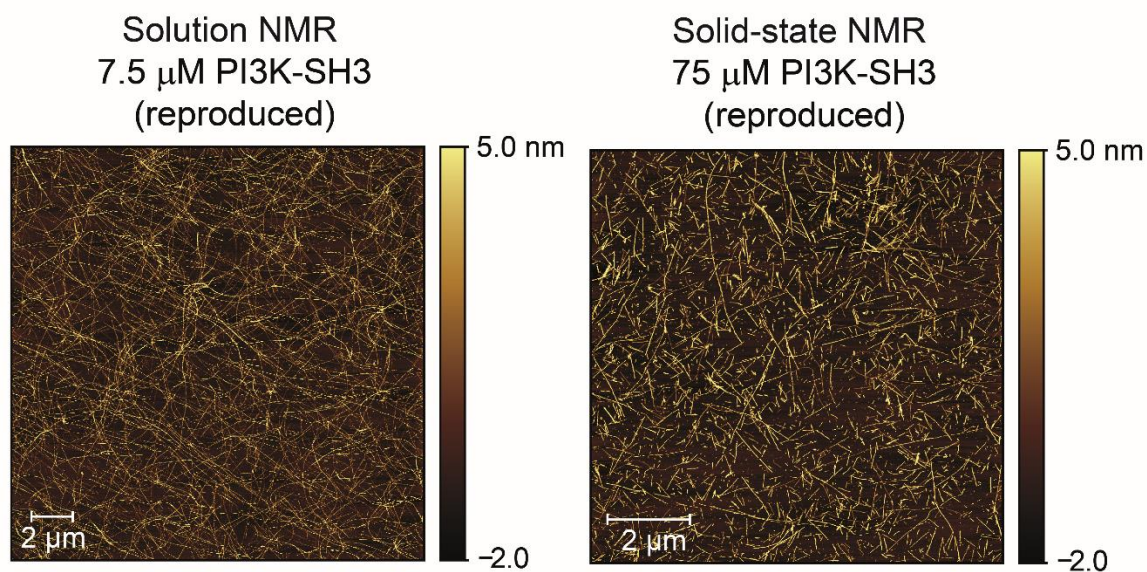
measurements: CP spectra (B) and INEPT spectra (C). D) Integrated CP, INEPT, and HSQC spectra intensities during the NMR time series.



Supplementary Figure S6: Comparison of the integrated intensities of our first NMR measurements and our repeated (reproduced) NMR measurements. A) ^{13}C -detected 1D solid-state NMR experiments, B) First increment of 2D solution NMR ^1H - ^{15}N HSQC experiments.

Supplementary Table S3: Fitting parameters of decay and build-up curves (mono-exponential fit).

Spectrum	τ	$\pm\Delta\tau$
CP solid-state	26.3	1.2
INEPT solid-state	30.2	1.1
INEPT HR-MAS	24.0	0.6
HSQC HR-MAS	25.0	1.2
HSQC solution NMR	37.4	0.6
CP solid-state (reproduced)	14.5	0.7
INEPT solid-state (reproduced)	17.4	0.8
HSQC solution NMR (reproduced)	21.8	0.5



Supplementary Figure S7: AFM images of the repeated (reproduced) measurement. The PI3K-SH3 fibril morphology is highly similar and reproducible to what has been observed before. In the sample obtained after solid-state NMR, in addition to several μ m long fibrils, shorter fibrils, likely due to fragmentation or secondary nucleation as an alternative mechanism, are also visible (see Figure 4 for comparison).

Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-Shaped A β (1–42) Amyloid Fibrils

Nina Becker, Benedikt Frieg, Lothar Gremer, Tatsiana Kupreichyk, Luis Gardon, Patrick Freiburg, Philipp Neudecker, Dieter Willbold, Holger Gohlke,* and Henrike Heise*

Cite This: *J. Am. Chem. Soc.* 2023, 145, 2161–2169

Read Online

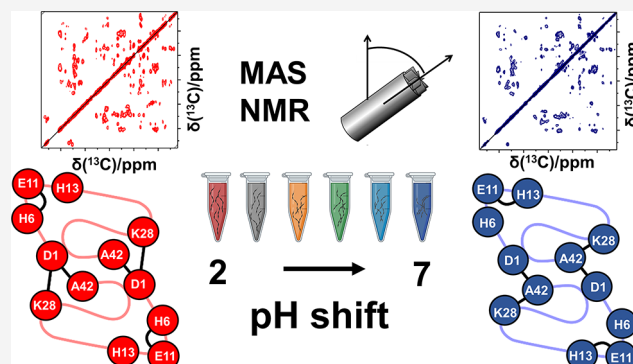
ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Alzheimer's disease is a neurodegenerative disorder associated with the deposition of misfolded aggregates of the amyloid- β protein (A β). A β (1–42) is one of the most aggregation-prone components in senile plaques of AD patients. We demonstrated that relatively homogeneous A β (1–42) fibrils with one predominant fold visible in solid-state NMR spectra can be obtained at acidic pH. The structure of these fibrils differs remarkably from some other polymorphs obtained at neutral pH. In particular, the entire N-terminal region is part of the rigid fibril core. Here, we investigate the effects of a pH shift on the stability and the fold of these fibrils at higher pH values. Fibril bundling at neutral pH values renders cryo-EM studies impractical, but solid-state NMR spectroscopy, molecular dynamics simulations, and biophysical methods provide residue-specific structural information under these conditions. The LS-fold of the A β (1–42) fibrils does not change over the complete pH range from pH 2 to pH 7; in particular, the N-terminus remains part of the fibril core. We observe changes in the protonation state of charged residues starting from pH 5 on a residue-specific level. The deprotonation of the C-terminal carboxyl group of A42 in the intermolecular salt bridge with D1 and K28 is slow on the NMR time scale, with a local pK_a of 5.4, and local conformations of the involved residues are affected by deprotonation of A42. Thus, we demonstrate that this fibril form is stable at physiological pH values.



INTRODUCTION

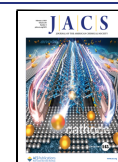
The deposition of amyloid plaques consisting of amyloid β peptide (A β) is one of the pathological hallmarks of Alzheimer's disease (AD).¹ The predominant species of A β in the brain are peptides containing 39 to 43 amino acid residues generated from the cleavage of amyloid precursor protein (APP) by β - and γ -secretases.² The two prevalent forms are A β (1–40) and A β (1–42). The latter is more toxic and shows a higher aggregation propensity.^{3,4}

In recent years, several 3D structures of A β (1–42) fibrils were solved using solid-state nuclear magnetic resonance (NMR) spectroscopy and cryogenic electron microscopy (cryo-EM).^{5–8} In a recent study on brain-derived A β (1–42) fibrils from brains of 10 individuals with different A β -associated neurodegenerative diseases (among them sporadic and familial Alzheimer's disease), two different structures were solved using cryo-EM. Interestingly, both fibril types seem to coexist in most AD-related brain tissues, although with different propensities.⁹ Likewise, recent solid-state NMR investigations of A β (1–42) fibrils showed that A β (1–42) fibrils seeded from brain material were polymorphic¹⁰ with distributions of polymorphs differing between nondemented older people and Alzheimer's disease patients.¹¹ Polymorphism

for brain-seeded A β (1–42) fibrils was also observed by Ishii and co-workers by ¹H detected solid-state NMR spectroscopy, together with a novel fibril type for recombinantly expressed *in vitro* A β (1–42).¹² Although the 3D fibril structures^{5–9} could show that the fibrils consist of two intertwined protofilaments with the C-terminus describing an S-shaped conformation, substantial structural differences exist. Some structural differences may result from the preparation conditions, differing especially in pH values. For most A β fibrils grown at neutral pH values (pH 7.4–8), the N-terminus is disordered and not part of the fibril core, an exception being fibrils of the A β (1–40) peptide with the Osaka mutation (E22 Δ).¹³ In most cases, for the generation of homogeneous A β (1–42) fibril preparations repeated seeding was necessary.^{5–7} *In vitro* fibrillization of A β (1–42) at an acidic pH value of 2 and in the presence of 30% acetonitrile, however, resulted in extremely

Received: August 30, 2022

Published: January 18, 2023



slow fibril growth over several weeks. In the resulting fibril preparations, fibrils are well separated from each other, as the acetonitrile prevents formation of large fibril bundles, perfect for characterization by cryo-EM. Solid-state NMR spectra showed only one set of resonances above the noise level at a signal-to-noise ratio of 10, thus suggesting that at least 90% of the A β monomers in the fibrils have the same fold^{8,14} without the need for further seeding. This suggests that the resulting fibril fold is the thermodynamically most stable structure at this pH value.⁸ In these “low-pH fibrils”, the full N-terminus is part of the β -sheet core, and the fibrils’ structure has been determined to high resolution by cryo-EM and solid-state NMR spectroscopy and has been described as an “LS-shape”.⁸

The effect of a pH shift on amyloid fibrils was studied previously by Shammas and co-workers.¹⁵ For insulin fibrils prepared at pH 2 and suspended in buffers with different pH values, they could show that the fibrils are highly stable at low pH values (pH 2.0–4.0). For pH values from 4.0 to 8.0, the disruption of specific electrostatic interactions leads to structural reorganization and a reduced β -strand network.¹⁵ For the model system β_2 -microglobulin (β_2 m), Tipping and co-workers reported that the stability of amyloid fibrils is highly pH-dependent and that even mild acidification enhances the formation of toxic fibril-derived oligomeric species.¹⁶ The observation that amyloid fibrils, once formed, are not stable at every pH value could be shown in several other studies: amyloid fibrils formed by hormones are able to release monomers at pH 6 and pH 7.4.¹⁷ Additionally, the pH-dependence of fibril disassembly of β -endorphin fibrils was investigated at pH 5.5 and pH 7.4 and found to be significantly faster at pH 5.5 compared to pH 7.4.¹⁸ For PI3K-SH3 fibrils, it was observed that a pH change from 2 to 7.4 leads to almost complete decomposition of the fibrils after 1 h.¹⁹

For biophysical investigations such as interaction studies with physiological and diagnostic binding partners, reliable and reproducible (see Figure S1) *in vitro* generation of A β (1–42) fibrils with a high degree of homogeneity and known structure is desirable. However, such studies should be conducted under physiological conditions, i.e., at pH values above 4. Thus, knowledge about the stability and structure of this fibril type at high pH values is essential for exploiting the high homogeneity of these well-characterized monomorphic LS fibrils, which are not obtained by fibrillization at higher pH values.

Here, we probe the stability and morphology of those low-pH A β (1–42) fibrils over the investigated pH range from 2 to 7. A change to higher pH values results in lateral association and bundling of amyloid filaments (*vide infra*, Figure 1A), thus preventing high-resolution structural analysis by cryo-EM. Solid-state NMR spectroscopy, on the other hand, probes the local environment of the nuclei and is, therefore, not affected by macroscopic rearrangements of filaments. Since solid-state NMR spectroscopy generally offers site-specific resolution, it can be used to probe the fibril structure as well as the protonation state of individual titratable groups. Further details were obtained from molecular dynamics (MD) simulations and complementary biophysical techniques, including AFM and CD spectroscopy and solution NMR spectroscopy.

RESULTS AND DISCUSSION

LS-Shaped A β (1–42) Fibrils Are Stable over the pH Range 2 to 7. A β (1–42) fibrils grown at acidic pH were adjusted to pH values ranging from 2.6 to 7.0 by adding a citrate-phosphate buffer to the fibril preparation. To test

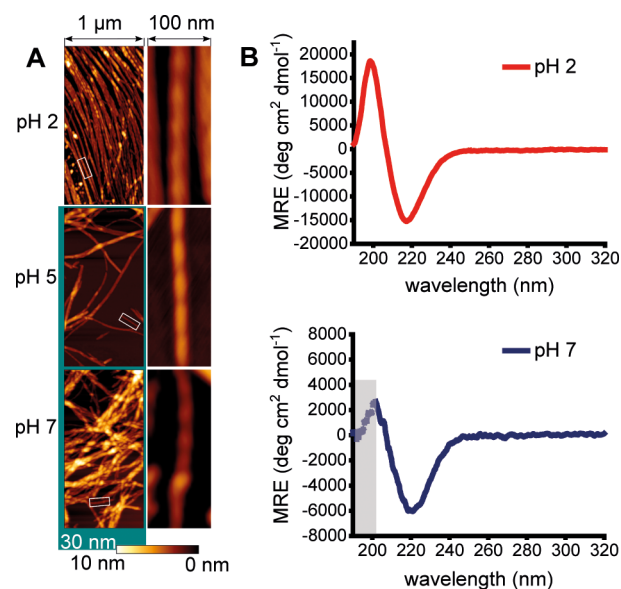


Figure 1. AFM images and CD spectra of A β (1–42) fibrils at different pH values. (A) AFM images of A β (1–42) fibrils under various pH conditions. Height profiles of the fibrils at original pH 2.0 and of fibrils shifted to pH 5.0 and 7.0 are shown: 1 μ m $\times$ 2 μ m overview images (left column) and 100 nm $\times$ 250 nm single fibril cut-outs (right column). The color scale from dark brown to white represents heights from 0 to 10 nm, or to 30 nm (highlighted in teal). Upon changing the pH from acidic toward neutral, the fibrils tend to form clusters instead of being present individually. Nevertheless, single fibrils are detectable also at neutral pH, exhibiting the characteristic periodicity. Its interval is in accordance with the 1160 $\pm$ 199 Å helical pitch of A β (1–42) fibrils grown under equivalent acidic conditions.⁸ (B) CD (mean residue ellipticity (MRE)) spectra of A β (1–42) fibrils exhibit minima at 218 nm (pH 2.0) or 218–222 nm (pH 7.0) and *x*-axis intercepts at 207 nm (pH 2.0) or 208 nm (pH 7.0), characteristic for β -sheet dominated structures. At pH 7.0 extended fibril bundling and precipitation leads to a reduced S/N ratio, and below wavelengths of 200 nm (gray box) the number of photons reaching the detector is insufficient for reliable quantification. Likewise, the exact position of the minimum in the pH 7.0 spectrum may be affected by the rather high noise level.

whether the fibrils are preserved up to pH 7.0, we recorded AFM images of the samples at all pH values. The AFM images of these pH-shifted fibrils are shown in Figure 1A and Figure S2A. The addition of citrate-phosphate buffer resulted in increased clustering of the fibrils, in particular at pH 5.0, which is close to the isoelectric point of the peptide (5.3). This clustering also leads to signal reduction in the CD spectra (due to absorption flattening^{20–22}), which otherwise are highly similar and typical for a β -sheet-dominated structure (Figure 1B). However, the overall appearance, diameter, and periodicity of the fibrils, which are the signatures of a fibril polymorph, did not change, showing that the fibrils did not dissolve upon pH shift and that their morphology was generally preserved (Figure S2). A commonly used probe to detect amyloid fibril formation *in vitro* is the fluorescent dye thioflavin T (ThT). However, the ThT absorption and fluorescence emission intensity is highly pH-dependent,²³ and there are reports of a decrease in ThT fluorescence intensity at low pH values.^{24,25} Indeed, A β fibrils grown in 30% acetonitrile (ACN), 0.1% TFA at pH 2.0 show no detectable ThT fluorescence increase (Figure S2B), but they are readily observable by AFM (Figure S2A). A pH variation between

pH 2.0 and 7.5 (at a constant ACN concentration of 24%) shows a strong pH dependence of ThT fluorescence intensity in the presence of A β fibrils with almost no fluorescence below pH 2.6 but increasing fluorescence with increasing pH (Figure S2B). Variation of the organic solvent content (between 1.5% and 24% ACN) at constant pH 2.0 or pH 7.0 in the presence of fibrils indicates that the ThT fluorescence is also highly ACN concentration-dependent (Figure S3B), with decreasing fluorescence at increasing ACN concentrations. Thus, our data indicate that ThT fluorescence alone is not suitable for detecting A β fibrils under the growth conditions applied (30% ACN, 0.1% TFA, pH 2).

Free ThT has been reported to undergo protonation of the dimethylamino group at low pH with a concomitant decrease in fluorescence intensity.²³ A pH titration of free ThT in citrate-phosphate buffer with 24% ACN monitored by NMR spectroscopy reveals that protonation is only detectable below pH 3.5 and remains negligibly small at pH 3.0 and pH 2.6 (Figure S4). Accordingly, the fluorescence intensity of ThT alone does not show any significant variation between pH 2.0 and 7.5 if the experimental uncertainties are taken into account (Figure S2B). We conclude that the lower sensitivity of the ThT assay at acidic pH cannot be explained by the properties of ThT alone without taking into account the interaction between ThT and A β fibrils. Consistent with the general notion that ThT primarily binds to grooves oriented parallel to the fibril axis,²⁶ MD simulations of ThT binding to LS-shaped A β fibrils at pH 2.0 revealed that protonated ThT binds along the fibril axis at a surface-exposed groove formed by the side chains of V18, F20, and E22 on either protofilament with calculated affinities in the low micromolar range.²⁷ It is conceivable that the pH-induced electrostatic changes of the A β fibrils, such as those described below, modulate ThT binding modes, affinities, and fluorescence properties in the bound state and that high ACN concentrations compete with ThT binding at this hydrophobic groove.

To test the effect of a pH shift on the fibril structure at the atomic level, we recorded $^{13}\text{C}/^{13}\text{C}$ and $^{15}\text{N}/^{13}\text{C}$ correlation solid-state NMR spectra on fibrils at different pH values. ^{13}C chemical shifts of backbone and uncharged side chain atoms are mainly influenced by backbone and side chain torsion angles,^{28–31} whereas shifts of carboxyl groups are sensitive reporters on the protonation state. In fact, NMR spectroscopy is the only method that can determine protonation on a residue-specific level.³² The addition of citrate-phosphate buffer, which was used for adjusting the pH value, does not affect the overall A β (1–42) fibril structure, as evident from the comparison of 2D PDSD (proton-driven spin diffusion) $^{13}\text{C}/^{13}\text{C}$ correlation spectra of fibrils near pH 2 in the presence and absence of buffer (Figure S5).

The effect of a pH shift from 2 to 7 on the fibril structure can be estimated from an overlay of two PDSD spectra of fibrils at pH 2 (red) and pH 7 (blue) (Figure 2A, more details can be found in Figure S6). Most of the resonances observed at pH 2 and pH 7 coincide, indicating that most of the fold of the fibrils does not change when the pH is changed. Decreased intensities were observed for the N-terminus (residues D1 to G9), the His residues (H6, H13, H14), and K16. Figure 2D gives an estimate of relative intensities between selected cross peaks in two pairs of PDSD spectra (with short and long initial CP contact times of 100 and 1000 μs) recorded on samples at pH 2 and pH 7, where the bulk Ca/C β cross peak of the overlapping resonances of five Val residues was used for

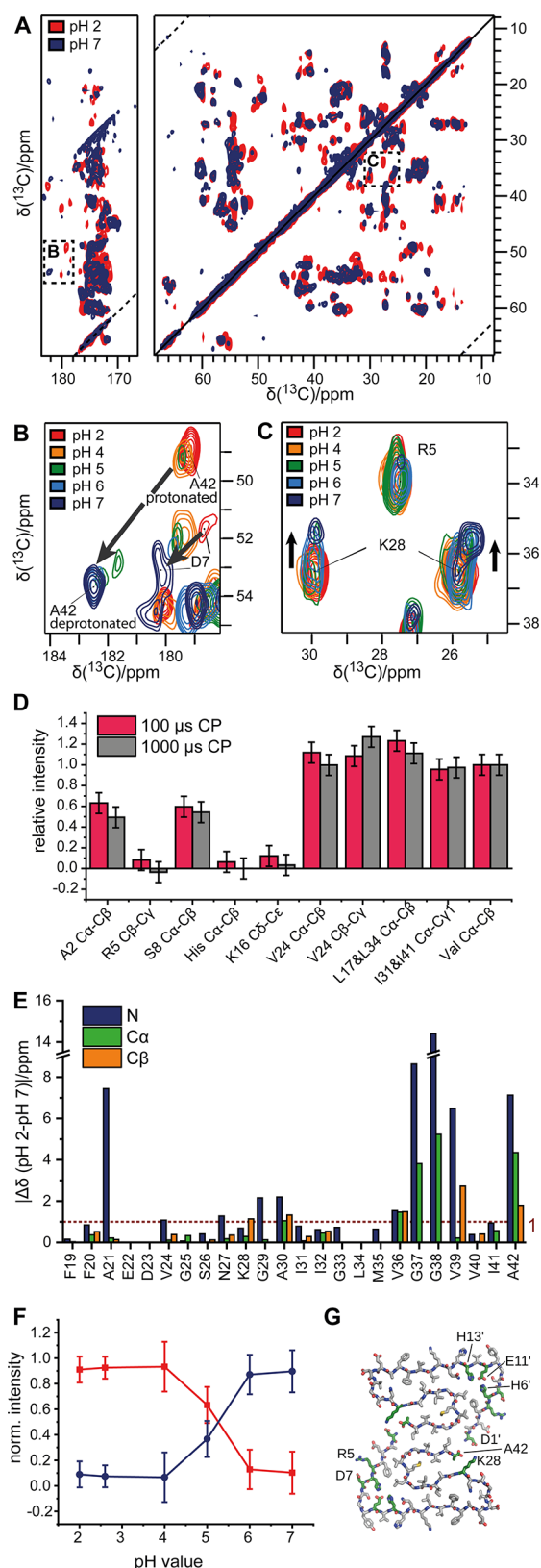


Figure 2. pH-induced local changes of A β (1–42) fibrils as seen by solid-state NMR spectroscopy. (A) Overlay of 2D ^{13}C – ^{13}C PDSD spectra of A β (1–42) fibrils at pH 2 (red) and pH 7 (navy). For a more detailed view, see Figure S5. The chemical shift assignment for pH 2 was published in ref 8. As most of the signals are superimposing, the fold of the A β (1–42) fibrils remains unchanged. (B, C) Zoomed-in region from the overlay of the carboxyl (B) and the aliphatic (C)

Figure 2. continued

regions of the PDS spectra at pH 2 (red), pH 4 (orange), pH 5 (green), pH 6 (blue), and pH 7 (navy). The pH shift influences especially the residues in the salt bridges (D1-K28 and adjacent A42, R5-D7). (D) Relative cross peak intensities of selected N- and C-terminal amino acid residues in PDS spectra of the pH 7 fibril sample, normalized to the respective cross peaks in PDS spectra recorded under identical conditions at pH 2. For each sample, two sets of PDS spectra were recorded with initial ^1H – ^{13}C contact times of 100 μs (red) and 1000 μs (gray), respectively, and a mixing time of 20 ms. Spectra of the two samples were referenced to the bulk $\text{Ca}/\text{C}\beta$ cross peak of five Val residues (without V24), and the relative peak intensities for cross peak (i), $I_{\text{rel}}^{(i)}$, were calculated as follows: $I_{\text{rel}}^{(i)} = I(\text{pH7})^{(i)} \cdot I(\text{pH2})^{(\text{Val bulk})} / I(\text{pH2})^{(i)} \cdot I(\text{pH7})^{(\text{Val bulk})}$. (E) Chemical shift perturbation plot for pH 2 vs pH 7. (F) Normalized intensities for the A42 CO– Ca correlation signals (in the protonated and deprotonated state) from the 2D CC are plotted against the pH value. The intensities were normalized using the following equations: $I_{\text{norm}}(\text{prot}) = (I(\text{prot}) / (I(\text{prot}) + I(\text{deprot})))$ and $I_{\text{norm}}(\text{deprot}) = 1 - I_{\text{norm}}(\text{prot})$. The C-terminal A42 carboxyl group is completely protonated up to pH 4. For pH 5 and 6, both states can be observed, while the carboxyl group is to a high degree deprotonated at pH 7. Further details with chemical shifts from acidic amino acids and A42 can be found in Table S1. (G) The atomic model of the $\text{A}\beta(1-42)$ fibril (PDB: 5OQV Gremer et al., 2017⁸) shown as a stick model. Amino acids assumed essential for stabilizing the protofilament are colored in green. The structure with all labels is also shown in Figure S8). Spacing of contour levels in 2D spectra is 1.4 for (A) and 1.2 for (B) and (C).

normalization. $\text{Ca}/\text{C}\beta$ cross peak intensities of the well-resolved N-terminal residues A2 and S8 were reduced to $\sim 50\%$ of the intensity of corresponding cross peaks in spectra of pH 2 samples; $\text{Ca}/\text{C}\beta$ cross peaks of His residues, which may be affected by changes in side chain protonation, and side chain cross peaks of R5 and K16 are reduced to below 10% of the intensities of respective signals at pH 2. $\text{Ca}/\text{C}\beta$ correlations as well as side chain cross peaks of C-terminal residues are not reduced after the pH change. These findings are indicative of slightly increased dynamics for the backbone of the N-terminus and strongly enhanced mobility for the side chains of N-terminal residues at pH 7.

Despite intensity losses, the correlation signals of all N-terminal residues remain visible in the cross-polarization (CP)-based ^{13}C -spectra (Figure 2A, Figure S6), in contrast to $\text{A}\beta(1-42)$ fibril structures having a flexible N-terminus.^{5–7} Intensity losses in CP spectra are an indication of enhanced mobility in the μs –ms regime. No protein resonance signals have been detected in Insensitive Nuclei Enhanced by Polarization Transfer (INEPT)³³ spectra at all pH values (Figure S7A,B), excluding isotropic motions in the sub- μs regime, which would be indicative of highly dynamic or flexible parts or the presence of monomers in the sample. We also could not observe any additional protein signal in the direct excitation (DE) spectra compared to the CP spectra (Figure S7C). These findings suggest that the full $\text{A}\beta(1-42)$ peptide stays within the cross- β core of the fibril even at neutral pH. The L-shape of the N-terminal residues is destabilized with increased dynamics at

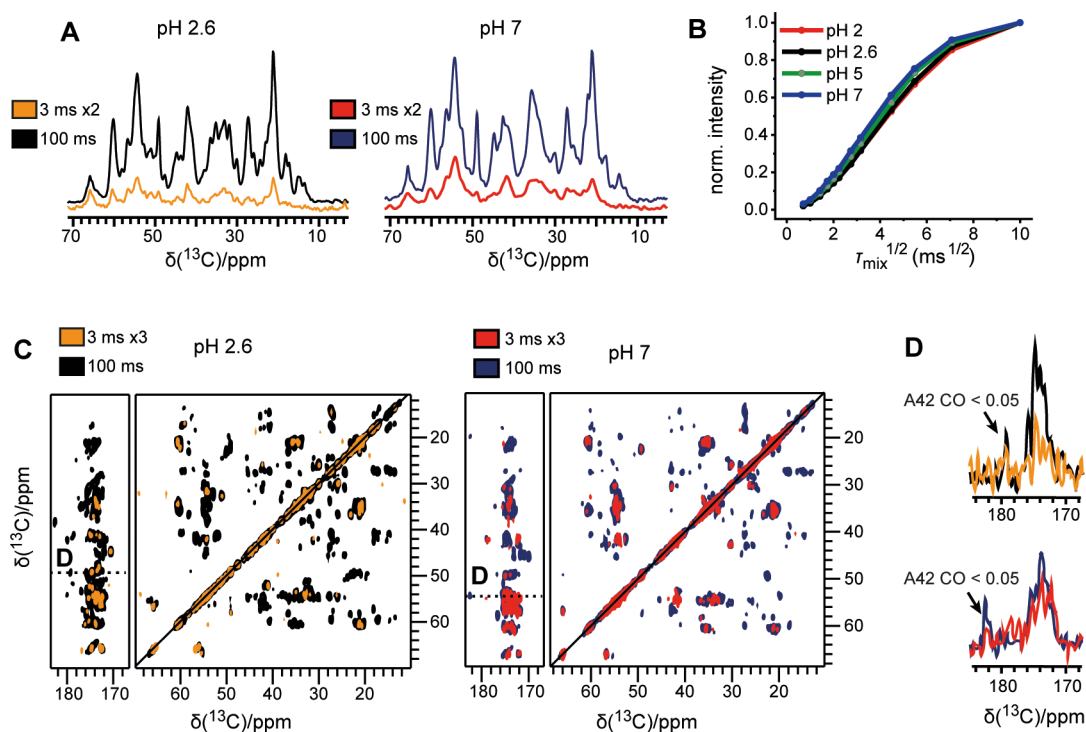


Figure 3. Water accessibility of $\text{A}\beta(1-42)$ fibrils at different pH conditions. (A) Aliphatic region of water-edited 1D ^{13}C CP spectra at pH 2.6 and pH 7, measured with ^1H – ^1H mixing times of 3 and 100 ms. Spectra recorded with short (3 ms) mixing time were recorded with twice the number of scans as those with long mixing time (100 ms). (B) pH-dependent water buildup curves for different pH values. A faster buildup with increasing pH values can be observed due to faster chemical exchange at increased pH values. (C) Water-edited 2D ^{13}C – ^{13}C PDS spectra of $\text{A}\beta(1-42)$ fibrils at pH 2.6 and pH 7 with a ^1H – ^1H mixing time of 3 and 100 ms. In spectra recorded with a 3 ms mixing time, the number of scans was three times higher than for those recorded with long mixing time (100 ms). (D) 1D ^{13}C cross sections taken from the A42 Ca chemical shifts at 49.3 ppm (pH 2.6) and 53.6 ppm (pH 7). A42 CO signals show low relative water-transferred intensities of less than 0.05. For the complete cross section of A42 and the cross section of the hydrophobic Ile side chains, see Figure S12. Spacing of contour levels in 2D spectra is 1.2.

pH 7, but some residual structure is retained, as indicated by weaker but unshifted CP signals.

For a more detailed insight, we also recorded 2D ^{15}N – ^{13}C (NCaCX and NCOCX, Figure S8) spectra for pH 2 and pH 7. As the $\text{A}\beta(1\text{--}42)$ fibril preparation is reproducible, most resonances are the same for the pH 2 sample compared to previously published results.⁸ Most backbone residues could be *de novo* sequentially assigned for pH 7 starting from F19 (chemical shifts are deposited in the BMRB under the accession code 51584). As the NC transfer is a stronger mobility filter than a $^{13}\text{C}/^{13}\text{C}$ spin diffusion transfer, only the most rigid parts of the fibrils contribute to the $^{15}\text{N}/^{13}\text{C}$ correlation spectra. Thus, N-terminal residues up to V18 and the side chains of some residues are not visible in the NC spectra at pH 7 (Figure S8), whereas most signals can still be found in the PDSD spectrum.

In Figure 2E a chemical shift perturbation plot displaying chemical shift differences for $\text{C}\alpha$, $\text{C}\beta$, and N between pH 2 and pH 7 is shown. While chemical shifts for most ^{13}C atoms N-terminal of residue V36 do not change substantially (i.e., not more than 1.5 ppm for ^{13}C and 2.5 ppm for ^{15}N) with the pH value, we observe relatively large differences for the very C-terminal residues starting from G37 and especially for the two Gly residues G37 and G38. Furthermore, the ^{15}N shift of A21 is strongly affected by the pH shift, an effect that may be due to electrostatic interaction with the side chain of E22 that is deprotonated at pH 7. These changes are most likely caused by small conformational alterations in the C-terminus due to an alternative salt bridge formation (see next paragraph).

Deprotonation of Acidic Side Chains and Its Impact on the D1-K28 Salt Bridge. As expected from their pK_a values (intrinsic pK_a values for Asp, Glu, and His as determined in blocked tri- or pentapeptides acetyl-Gly-X-Gly-amide or Ala-Ala-X-Ala-Ala are 3.9, 4.3, and 6.5, respectively^{32,34}), the protonation state of acidic amino acid residues is affected by a pH shift. This affects the stability of interresidual salt bridges, such as in the triad D1-K28-A42. Indeed, we observed shifts of several resonances for the carboxyl groups of D1, A42, D7, and the side chain resonances of K28 (Figure 2A and Figure S6). To observe at which pH value the changes start to occur, we performed a pH titration. In Figure 2B and Figure 2C, overlays of five spectra for fibrils between pH 2 and 7 are shown for two characteristic regions of the full spectrum. Up to pH 4, no changes were observed. Thus, we conclude that the overall protonation state of residues does not change up to this pH value. The effect of proton exchange at carboxyl and amino groups on the related NMR signals depends on the proton exchange rate relative to the frequency difference between the NMR resonances for the respective functional group in the protonated and the deprotonated state.^{35,36} For fast proton exchange, we observe a progressive change in chemical shift, which reflects the population-weighted average between the protonated and deprotonated state (as observed for deprotonation of D7 (Figure 2B), a water-exposed residue at the fibril surface). For slow exchange by contrast, two resonances with population-dependent intensities are observed for the protonated as well as the deprotonated state (as seen for the C-terminal carboxyl group of A42 and for the side chain resonances of K28 (Figure 2B,C)).³⁷

In the $\text{A}\beta(1\text{--}42)$ fibril structure (Figure 2G, Figure S9), the interaction between protofilaments is stabilized by intermolecular salt bridges between D1 and K28 (Figure 4B).

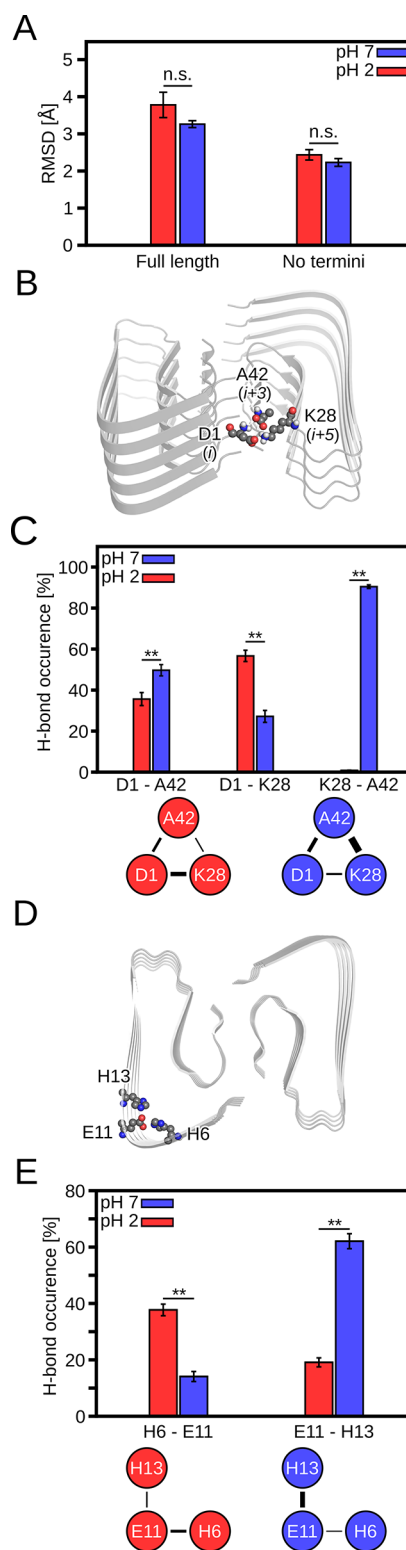


Figure 4. Interactions essential for fibril stability at acidic and neutral pH. (A) Mean backbone RMSD ($n = 10$) for the full-length fibril and the fibril core in which the four terminal $\text{A}\beta$ peptides were not considered during RMS fitting. (B) Fibril cryo-EM structure⁸ with amino acids crucial for inter-subunit stability depicted as spheres. (C) Average occurrence frequency of hydrogen bond interactions (from left to right $n = 17$, $n = 15$, and $n = 18$) between amino acids shown in (B). The lower panel shows a schematic of the interactions, with black lines depicting the strength of interactions (scaled according to average occurrence). (D) Fibril cryo-EM structure⁸ with amino acids

Figure 4. continued

crucial for the stability of the N-terminal region depicted as spheres. (E) Average occurrence frequency of hydrogen bond interactions ($n = 20$) between residues shown in (D). The lower panel shows a schematic of the interactions, with black lines depicting the strength of interactions (scaled according to average occurrence). In (A), (C), and (E), the error bars denote the standard error of the mean (SEM) (* $p < 0.05$; ** $p < 0.01$; n.s. not significantly different).

Furthermore, the N-terminus is close to the C-terminal A42 of another $A\beta(1-42)$ monomer in the adjacent protofilament,⁸ which appears to be largely protonated at pH 2. A change to higher pH values has the following effects on the three residues involved in this triad interaction:

- The protonation/deprotonation of the terminal carboxyl group of A42 exhibits a slow exchange between two states (Figure 2B): The carboxyl group of A42 (intrinsic pK_a value for C-terminal Ala in a tripeptide: 3.55³²), protonated and β -strand-like at pH 2 and pH 4, adopts a deprotonated random coil-like conformation (with a CO shift of 182 ppm, in agreement with Wälti and Coworkers⁷) at pH 6 and pH 7 (Table S1). Both states can be observed at pH 5 (with a splitting for the deprotonated random coil-like resonances, Figure 2B). These findings can also be observed for NCA spectra (Figure S10A). Fitting the peak intensities of NCA and PDS peaks to the Henderson–Hasselbalch equation (Figure 2F and Figure S10B,C) leads to a pK_a value of 5.4 for C-terminal A42. The random-coil-like chemical shifts of deprotonated A42 are very similar to those obtained from solution NMR at pH 7 (Figure S11).
- Likewise, for K28 the pH shift to pH 7 induces a splitting of the K28 $C\beta$ resonance typical for slow exchange (Figure 2C, Figure S6I). One resonance signal has the chemical shift of 35.6 ppm, as already observed at lower pH values, while a second signal is detected at a lower chemical shift of 35.3 ppm. Additional minor shifts occurred for K28 $C\epsilon$ resonances at pH 7. The signal splitting points toward the existence of a second alternative conformation of the K28 side chain. The relatively high $C\gamma$ chemical shift of the amino acid residue D1 is indicative of a predominantly deprotonated state (~ 90 – 95% deprotonation) already at pH 2. Comparing the D1 and D7 side chains at pH 2.0 and the slightly enhanced pH 2.6, we can already observe the following tendencies: D7 shows an increased degree of deprotonation with increasing pH values, whereas for D1, the degree of deprotonation seems to even slightly decrease with increasing pH values (Figure S5B). The detailed degree of deprotonation of all residues at pH 2 and pH 7 is shown in Table S1.

These observations can be explained as follows: At acidic pH values, the intermolecular salt bridge between the (almost) fully deprotonated D1 and the positively charged K28 stabilizes the fibril structure, whereas for the C-terminal carboxyl group the protonated state seems to be favored up to its pK_a of 5.4. In fact, a shift of the pK_a value by +1.9 pH units with respect to the intrinsic pK_a of 3.55 can easily be explained by the high density of negative charges³⁸ present in an amyloid fibril, and similar effects have already been described for fibrillary self-assembly of FMoc-diphenylalanine³⁹ and fibril

formation of α -synuclein.⁴⁰ With increasing pH, the progressive deprotonation of A42 favors an additional interaction between the negatively charged C-terminal carboxyl group with K28, weakening the salt bridge between D1 and K28. This could explain the slightly enhanced protonation of D1 at pH 7 and the intensity losses for residues in the N-terminus at higher pH values, which are indicative of higher mobility. Matching our observations, MD simulations by Yin and co-workers⁴¹ indicated that the salt bridge between K28 and A42 leads to enhanced stability of the C-terminal deprotonated $A\beta(1-42)$ protofilaments compared to the protonated counterpart. The rearrangement of salt bridges in the triad between D1, K28, and A42 also influences the adjacent residues S26 (Figure S6D) and may also be responsible for conformational changes around the G37/G38 glycine pair (Figure 2E).

These findings are also supported by water-edited NMR spectroscopy,^{42–44} where 1H polarization transfer from water onto the protein is used to probe the water accessibility of amino acid residues on a residue-specific level (Figure 3, Figure S12): With an initial 1H spin echo (duration 2.5 ms), transverse magnetization on the solid protein but not the mobile water molecules is dephased irreversibly by strong dipolar couplings. In a subsequent longitudinal mixing step (1 to 100 ms), 1H polarization is transferred back to the protein and can then be detected in standard 1D or 2D solid-state NMR experiments (Figure S12A). As seen in Figure 3A and 3B, the overall magnetization transfer dynamics from water to the fibril is only slightly enhanced for pH 7 compared to pH 2. The small magnitude of this enhancement can be accounted for by faster chemical exchange at neutral pH,^{45,46} while the relative fibril–water interface remains the same (as seen for the Ile residues, Figure S12B,C). For one Asp residue (D1, Figure S12D), however, the exchange with water is strongly accelerated at pH 7, indicating that the carboxyl group is more water-exposed, a finding that supports a weakened salt bridge. In contrast, A42 is still highly water protected at pH 7, as expected for the deprotonated carboxyl group of A42 forming a salt bridge to K28 (Figure 3D).

Monomers in Solution. Acetonitrile is not a routinely used cosolvent in protein NMR spectroscopy. To obtain a reliable reference for the solvent and pH effects on random coil chemical shifts and the conformational ensemble of monomeric $A\beta(1-42)$, we also investigated [$U-^{13}C,^{15}N$] $A\beta(1-42)$ monomers in 28% acetonitrile at pH 2.0 (similar to the conditions used for fibril preparation), without acetonitrile at pH 2.0, and in citrate/phosphate buffer at pH 7.0 using solution NMR spectroscopy. Sequence-specific assignment of the 1H , ^{13}C , and ^{15}N backbone resonances of these three samples (BMRB access codes 51321, 51323, and 51322, respectively) reveals that $A\beta(1-42)$ monomers are highly disordered, with chemical shifts close to random coil values (Figure S11A) and low propensities for regular secondary structure elements under all these conditions. Accordingly, 2D ^{13}C – ^{13}C total correlation (TOCSY) spectra of $A\beta(1-42)$ monomers in 28% acetonitrile at pH 2.0 are very similar to those in Tris/HCl buffer at pH 7.2 (Figure S11B), except for the chemical shift changes expected due to different side chain protonation for Asp, Glu, and His residues. Thus, the influence of the pH on the fibril structure seems to be caused by the stabilization of the end product rather than any significant differences in monomer conformation.

MD Simulations for $A\beta(1-42)$ Fibrils with Different Protonation Degrees. We next performed MD simulations

of 1 μ s length of the A β (1–42) fibril with protonation states according to pH 2 and 7, with 10 replicas for each system (Figure S13) to provide a structural explanation at atomic resolution. The simulations revealed a shift in inter- and intramolecular polar interactions when going from pH 2 to pH 7, which preserves the structure and stability of the A β (1–42) fibril.

Mean backbone root-mean-square differences (RMSD) < 4 Å indicate only minor structural changes among the differently protonated states, which are not significant (Figure 4A). Without the terminal A β peptides, the mean RMSD decreases by $\sim$ 1 Å (Figure 4A), indicating higher mobility of the terminal A β peptides, as corroborated by a peptide-wise RMSD analysis (Figure S14). Hence, the fibril core remains structurally stable at acidic and neutral pH, agreeing with our NMR experiments. However, as the fibril in our simulations is much smaller than in NMR experiments, our simulations likely overestimate the structural changes between fibrils of different protonation states.⁴⁷

Next, we investigated the changes in the fibril's interaction network between pH 2 and pH 7. The interactions among D1, K28, and the carboxyl group of A42 connect three A β peptides in the two protofilaments (Figure 4B). In line with our previous findings,⁸ changes in the protonation state of the C-terminus of A42 (Figure 4B) do not lead to a collapse of the fibril. Our MD simulations suggest that the interactions between D1, K28, and A42 are significantly different at pH 2 and pH 7 (Figure 4C). In particular, hydrogen bond interactions between the N-terminus of D1 and the C-terminus of A42 are significantly more frequent at pH 7 (Figure 4C). This can be explained by attractive forces between the oppositely charged N- and C-termini at pH 7. At pH 2, on the other hand, the A42 C-terminus is neutral after protonation, which reduces the likelihood for interactions with the still positively charged D1 N-terminus. On the other hand, the interaction between the side chain of D1 and K28 is reduced substantially at pH 7, but this loss is compensated by a strong interaction between A42 and K28 (Figure 4C). Overall, our MD simulations suggest that interactions involving D1, K28, and A42 occur in the fibril at pH 7. At pH 2, however, when the C-terminus of A42 is protonated, D1 and K28 likely form salt bridges between the protofilaments, which agrees with the results obtained by cryo-EM.⁸ That way, interactions between protofilaments are formed in an A42-independent manner.

At pH 2, the L-shaped N-terminus is stabilized by salt bridges between the positively charged H6 and H13 and the negatively charged E11⁸ (Figure 4D). These interactions are present at pH 2 and 7 but with a different frequency of occurrence. At pH 2, E11 preferentially interacts with H6 (Figure 4E). As H6 is harbored on the small leg of the L-shaped N-terminus (Figure 4D), this interaction is likely essential for stabilizing the whole N-terminal region. At pH 7, E11 preferentially interacts with H13 on the large β -strand, which may weakly destabilize the N-terminus (Figure 4E).

To conclude, our simulations revealed a rebalancing of intra-subunit (H6/E11/H13) and inter-subunit (D1/K28/A42) interactions between fibrils with protonation states at pH 2 and pH 7, providing a plausible explanation of why most of the structure of the LS-shaped fibril grown at pH 2 remains unchanged between the two pH conditions.

CONCLUSION

In summary, we showed that the structure of the A β (1–42) fibril remains unchanged when grown at acidic pH and adjusted to neutral pH values afterward. However, upon pH adjustment, changes in the amino acid protonation states are observed for the residues A42, D1, and K28 as well as for E11 and H6/H13, as summarized in Figure 5. Although intra-

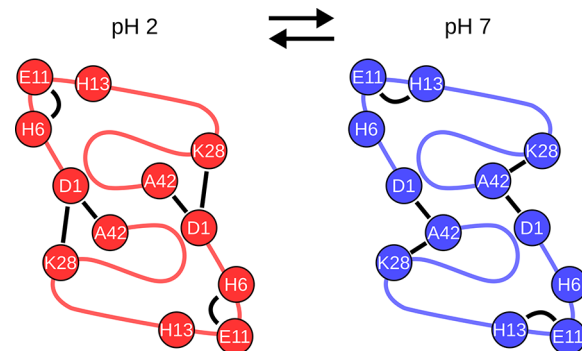


Figure 5. Model of interactions essential for fibril stability at acidic and neutral pH. Schematic of the A β (1–42) fibril investigated in the present study (pH conditions shown above). Amino acids identified to be essential for structural stability are depicted by circles and one-letter amino acid codes. Interactions between amino acids are shown as black bars. The A β (1–42) backbone is traced by a line.

subunit and inter-subunit salt bridges are rearranged as a consequence of deprotonation, fibrils remained stable up to pH 7. Performing a pH titration, we excluded any changes until pH 4 but observed a few specific changes in protonation at pH 6 and more pronounced at pH 7, with protonation states being populated at pH 2 at the respective sites. Our study strongly suggests that the A β (1–42) fibril grown at acidic pH⁸ conserves its structure and remains stable at physiological pH conditions. Thus, A β (1–42) fibrils grown at pH 2 can be used over the full pH range from pH 2 to pH 7 for binding and interaction studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.2c09231>.

Additional experimental details, materials and methods, and additional results (AFM, CD, ThT fluorescence intensities, NMR spectra, and chemical shifts) (PDF)

Accession Codes

Sequence-specific assignment of the ¹H, ¹³C, and ¹⁵N backbone resonances of A β (1–42) monomers in 28% ACN at pH 2.0, without ACN at pH 2.0, and in citrate/phosphate buffer at pH 7.0 using solution NMR spectroscopy are deposited in the Biological Magnetic Resonance Data Bank (BMRB) under the access codes 51321, 51323, and 51322, respectively. Sequence-specific solid-state NMR resonance assignments of ¹⁵N and ¹³C resonances of A β (1–42) fibrils shifted to pH 7.0 are deposited in the BMRB under the access code 51584.

AUTHOR INFORMATION

Corresponding Authors

Henrike Heise – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center

for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0002-9081-3894; Email: henrike.heise@uni-duesseldorf.de

Holger Gohlke – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; John von Neumann Institute for Computing (NIC), Jülich Supercomputing Centre (JSC), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany; Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0001-8613-1447; Email: gohlke@uni-duesseldorf.de

Authors

Nina Becker – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0002-6617-2775

Benedikt Frieg – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; John von Neumann Institute for Computing (NIC), Jülich Supercomputing Centre (JSC), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany; orcid.org/0000-0002-7877-0262

Lothar Gremer – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0001-7065-5027

Tatsiana Kupreichyk – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0002-5151-6424

Luis Gardon – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0003-0871-6487

Patrick Freiburg – Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany

Philipp Neudecker – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0002-0557-966X

Dieter Willbold – Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany; Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0002-0065-7366

Complete contact information is available at:

<https://pubs.acs.org/10.1021/jacs.2c09231>

Author Contributions

N.B., B.F., and L.G. contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge experimental assistance by Dr. Ü. Akbey and Dr. D. Schölzel, access to the Jülich-Düsseldorf Biomolecular NMR Center jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU), computational support and infrastructure provided by the “Zentrum für Informations- und Medientechnologie” at HHU, and computing time provided by the John von Neumann Institute for Computing (NIC) to H.G. and B.F. on the supercomputer JUWELS at Jülich Supercomputing Centre (JSC) (user IDs: HKF7, VSK33). This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (HE 3243/4-1, INST 208/771-1 FUGG, and INST 208/620-1 FUGG).

REFERENCES

- (1) Selkoe, D. J.; Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO molecular medicine* **2016**, 8 (6), 595–608.
- (2) Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J.-C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. β -Secretase Cleavage of Alzheimer's Amyloid Precursor Protein by the Transmembrane Aspartic Protease BACE. *Science* **1999**, 286 (5440), 735–741.
- (3) Chiti, F.; Stefani, M.; Taddei, N.; Ramponi, G.; Dobson, C. M. Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* **2003**, 424 (6950), 805–8.
- (4) Meisl, G.; Yang, X.; Hellstrand, E.; Frohm, B.; Kirkegaard, J. B.; Cohen, S. I.; Dobson, C. M.; Linse, S.; Knowles, T. P. Differences in nucleation behavior underlie the contrasting aggregation kinetics of the A β 40 and A β 42 peptides. *Proc. Nat. Acad. Sci. U.S.A.* **2014**, 111 (26), 9384–9.
- (5) Xiao, Y.; Ma, B.; McElheny, D.; Parthasarathy, S.; Long, F.; Hoshi, M.; Nussinov, R.; Ishii, Y. A β (1–42) fibril structure illuminates self-recognition and replication of amyloid in Alzheimer's disease. *Nat. Struct. Mol. Biol.* **2015**, 22, 499.
- (6) Colvin, M. T.; Silvers, R.; Ni, Q. Z.; Can, T. V.; Sergeyev, I.; Rosay, M.; Donovan, K. J.; Michael, B.; Wall, J.; Linse, S.; Griffin, R. G. Atomic Resolution Structure of Monomorphic A β 42 Amyloid Fibrils. *J. Am. Chem. Soc.* **2016**, 138 (30), 9663–9674.
- (7) Wälti, M. A.; Ravotti, F.; Arai, H.; Glabe, C. G.; Wall, J. S.; Böckmann, A.; Güntert, P.; Meier, B. H.; Riek, R. Atomic-resolution structure of a disease-relevant A β (1–42) amyloid fibril. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, 113 (34), E4976–E4984.
- (8) Gremer, L.; Schölzel, D.; Schenk, C.; Reinartz, E.; Labahn, J.; Ravelli, R. B. G.; Tusche, M.; Lopez-Iglesias, C.; Hoyer, W.; Heise, H.; Willbold, D.; Schröder, G. F. Fibril structure of amyloid- β (1–42) by cryo-electron microscopy. *Science* **2017**, 358 (6359), 116–119.
- (9) Yang, Y.; Arseni, D.; Zhang, W.; Huang, M.; Lövestam, S.; Schweighauser, M.; Kotecha, A.; Murzin, A. G.; Peak-Chew, S. Y.; Macdonald, J.; Lavenir, I.; Garringer, H. J.; Gelpi, E.; Newell, K. L.; Kovacs, G. G.; Vidal, R.; Ghetti, B.; Ryskeldi-Falcon, B.; Scheres, S. H. W.; Goedert, M. Cryo-EM structures of amyloid- β 42 filaments from human brains. *Science* **2022**, 375 (6577), 167–172.
- (10) Qiang, W.; Yau, W. M.; Lu, J. X.; Collinge, J.; Tycko, R. Structural variation in amyloid- β fibrils from Alzheimer's disease clinical subtypes. *Nature* **2017**, 541 (7636), 217–221.
- (11) Ghosh, U.; Yau, W.-M.; Collinge, J.; Tycko, R. Structural differences in amyloid- β fibrils from brains of nondemented elderly

individuals and Alzheimer's disease patients. *Proc. Nat. Acad. Sci. U. S. A.* **2021**, *118* (45), No. e2111863118.

(12) Wickramasinghe, A.; Xiao, Y.; Kobayashi, N.; Wang, S.; Scherpelz, K. P.; Yamazaki, T.; Meredith, S. C.; Ishii, Y. Sensitivity-Enhanced Solid-State NMR Detection of Structural Differences and Unique Polymorphs in Pico- to Nanomolar Amounts of Brain-Derived and Synthetic 42-Residue Amyloid- β Fibrils. *J. Am. Chem. Soc.* **2021**, *143* (30), 11462–11472.

(13) Schütz, A. K.; Vagt, T.; Huber, M.; Ovchinnikova, O. Y.; Cadalbert, R.; Wall, J.; Güntert, P.; Böckmann, A.; Glockshuber, R.; Meier, B. H. Atomic-Resolution Three-Dimensional Structure of Amyloid β Fibrils Bearing the Osaka Mutation. *Angew. Chem., Int. Ed.* **2015**, *54* (1), 331–335.

(14) Willbold, D.; Strodel, B.; Schröder, G. F.; Hoyer, W.; Heise, H. Amyloid-type Protein Aggregation and Prion-like Properties of Amyloids. *Chem. Rev.* **2021**, *121* (13), 8285–8307.

(15) Shammas, S. L.; Knowles, T. P. J.; Baldwin, A. J.; MacPhee, C. E.; Welland, M. E.; Dobson, C. M.; Devlin, G. L. Perturbation of the Stability of Amyloid Fibrils through Alteration of Electrostatic Interactions. *Biophys. J.* **2011**, *100* (11), 2783–2791.

(16) Tipping, K. W.; Karamanos, T. K.; Jakhria, T.; Iadanza, M. G.; Goodchild, S. C.; Tuma, R.; Ranson, N. A.; Hewitt, E. W.; Radford, S. E. pH-induced molecular shedding drives the formation of amyloid fibril-derived oligomers. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (18), 5691–5696.

(17) Maji, S. K.; Perrin, M. H.; Sawaya, M. R.; Jessberger, S.; Vadodaria, K.; Rissman, R. A.; Singru, P. S.; Nilsson, K. P. R.; Simon, R.; Schubert, D.; Eisenberg, D.; Rivier, J.; Sawchenko, P.; Vale, W.; Riek, R. Functional Amyloids As Natural Storage of Peptide Hormones in Pituitary Secretory Granules. *Science* **2009**, *325* (5938), 328–332.

(18) Nespovitaya, N.; Gath, J.; Barylyuk, K.; Seuring, C.; Meier, B. H.; Riek, R. Dynamic Assembly and Disassembly of Functional β -Endorphin Amyloid Fibrils. *J. Am. Chem. Soc.* **2016**, *138* (3), 846–856.

(19) Röder, C.; Vettore, N.; Mangels, L. N.; Gremer, L.; Ravelli, R. B. G.; Willbold, D.; Hoyer, W.; Buell, A. K.; Schröder, G. F. Atomic structure of PI3-kinase SH3 amyloid fibrils by cryo-electron microscopy. *Nat. Commun.* **2019**, *10* (1), 3754.

(20) Duysens, L. N. The flattening of the absorption spectrum of suspensions, as compared to that of solutions. *Biochim. Biophys. Acta* **1956**, *19* (1), 1–12.

(21) Gordon, D. J.; Holzwarth, G. Artifacts in the measured optic activity of membrane suspensions. *Arch. Biochem. Biophys.* **1971**, *142* (2), 481–8.

(22) Miles, A. J.; Wallace, B. A. Circular dichroism spectroscopy of membrane proteins. *Chem. Soc. Rev.* **2016**, *45* (18), 4859–4872.

(23) Hackl, E. V.; Darkwah, J.; Smith, G.; Ermolina, I. Effect of acidic and basic pH on Thioflavin T absorbance and fluorescence. *Eur. Biophys. J.* **2015**, *44* (4), 249–261.

(24) LeVine, H. Thioflavine T interaction with amyloid β -sheet structures. *Amyloid* **1995**, *2* (1), 1–6.

(25) Groenning, M.; Norrman, M.; Flink, J. M.; van de Weert, M.; Bukrinsky, J. T.; Schluckebier, G.; Frokjaer, S. Binding mode of Thioflavin T in insulin amyloid fibrils. *J. Struct. Biol.* **2007**, *159* (3), 483–97.

(26) Krebs, M. R. H.; Bromley, E. H. C.; Donald, A. M. The binding of thioflavin-T to amyloid fibrils: localisation and implications. *J. Struct. Biol.* **2005**, *149* (1), 30–37.

(27) Frieg, B.; Gremer, L.; Heise, H.; Willbold, D.; Gohlke, H. Binding modes of thioflavin T and Congo red to the fibril structure of amyloid- β (1–42). *Chem. Commun.* **2020**, *56* (55), 7589–7592.

(28) Spera, S.; Bax, A. Empirical correlation between protein backbone conformation and C_{α} and C_{β} ^{13}C nuclear magnetic resonance chemical shifts. *J. Am. Chem. Soc.* **1991**, *113* (14), 5490–5492.

(29) Wishart, D. S.; Case, D. A. Use of chemical shifts in macromolecular structure determination. *Nuclear Magnetic Resonance of Biological Macromolecules, Pt A* **2002**, *338*, 3–34.

(30) Oldfield, E. Chemical shifts in amino acids, peptides, and proteins: From quantum chemistry to drug design. *Annu. Rev. Phys. Chem.* **2002**, *53*, 349–378.

(31) Berjanskii, M. V.; Wishart, D. S. Unraveling the meaning of chemical shifts in protein NMR. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2017**, *1865* (11), 1564–1576.

(32) Platzer, G.; Okon, M.; McIntosh, L. P. pH-dependent random coil ^1H , ^{13}C , and ^{15}N chemical shifts of the ionizable amino acids: a guide for protein pK_a measurements. *J. Biomol. NMR* **2014**, *60* (2), 109–129.

(33) Andronesi, O. C.; Becker, S.; Seidel, K.; Heise, H.; Young, H. S.; Baldus, M. Determination of Membrane Protein Structure and Dynamics by Magic-Angle-Spinning Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2005**, *127* (37), 12965–12974.

(34) Grimsley, G. R.; Scholtz, J. M.; Pace, C. N. A summary of the measured pK values of the ionizable groups in folded proteins. *Protein Sci.* **2008**, *18* (1), 247–251.

(35) Keim, P.; Vigna, R. A.; Morrow, J. S.; Marshall, R. C.; Gurd, F. R. N. Carbon ^{13}C Nuclear Magnetic Resonance of Pentapeptides of Glycine Containing Central Residues of Serine, Threonine, Aspartic and Glutamic Acids, Asparagine, and Glutamine. *J. Biol. Chem.* **1973**, *248* (22), 7811–7818.

(36) van der Cruisen, E. A.; Prokofyev, A. V.; Pongs, O.; Baldus, M. Probing Conformational Changes during the Gating Cycle of a Potassium Channel in Lipid Bilayers. *Biophys. J.* **2017**, *112* (1), 99–108.

(37) Waudby, C. A.; Ramos, A.; Cabrita, L. D.; Christodoulou, J. Two-Dimensional NMR Lineshape Analysis. *Sci. Rep.* **2016**, *6* (1), 24826.

(38) Jeppesen, M. D.; Westh, P.; Otzen, D. E. The role of protonation in protein fibrillation. *FEBS Lett.* **2010**, *584* (4), 780–784.

(39) Tang, C.; Smith, A. M.; Collins, R. F.; Ulijn, R. V.; Saiani, A. Fmoc-Diphenylalanine Self-Assembly Mechanism Induces Apparent pK_a Shifts. *Langmuir* **2009**, *25* (16), 9447–9453.

(40) Pálmadóttir, T.; Malmendal, A.; Leiding, T.; Lund, M.; Linse, S. Charge Regulation during Amyloid Formation of α -Synuclein. *J. Am. Chem. Soc.* **2021**, *143* (20), 7777–7791.

(41) Yin, X.; Liu, S.; Perez-Aguilar, J. M.; Zhou, H.; Shao, Q.; Yang, Z.; Zhou, R. Different protonated states at the C-terminal of the amyloid-beta peptide modulate the stability of S-shaped protofibril. *J. Chem. Phys.* **2019**, *150* (18), 185102.

(42) Lesage, A.; Böckmann, A. Water-Protein Interactions in Microcrystalline Crh Measured by ^1H - ^{13}C Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2003**, *125* (44), 13336–13337.

(43) Ader, C.; Schneider, R.; Seidel, K.; Eitzkorn, M.; Becker, S.; Baldus, M. Structural Rearrangements of Membrane Proteins Probed by Water-Edited Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2009**, *131* (1), 170–176.

(44) Williams, J. K.; Hong, M. Probing membrane protein structure using water polarization transfer solid-state NMR. *J. Magn. Reson.* **2014**, *247*, 118–127.

(45) Mandala, V. S.; Loftis, A. R.; Shcherbakov, A. A.; Pentelute, B. L.; Hong, M. Atomic structures of closed and open influenza B M2 proton channel reveal the conduction mechanism. *Nat. Struct. Mol. Biol.* **2020**, *27* (2), 160–167.

(46) Gelenter, M. D.; Mandala, V. S.; Niesen, M. J. M.; Sharon, D. A.; Dregni, A. J.; Willard, A. P.; Hong, M. Water orientation and dynamics in the closed and open influenza B virus M2 proton channels. *Commun. Biol.* **2021**, *4* (1), 338.

(47) König, C.; Skånberg, R.; Hotz, I.; Ynnerman, A.; Norman, P.; Linares, M. Binding sites for luminescent amyloid biomarkers from non-biased molecular dynamics simulations. *Chem. Commun.* **2018**, *54* (24), 3030–3033.

Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-shaped A β (1-42) Amyloid Fibrils

Nina Becker^{1,2+}, Benedikt Friege^{1,3+}, Lothar Gremer^{1,2+}, Tatsiana Kupreichyk^{1,2}, Luis Gardon^{1,2}, Patrick Freiburg², Philipp Neudecker^{1,2}, Dieter Willbold^{1,2}, Holger Gohlke^{1,3,4*}, Henrike Heise^{1,2*}

¹Institute of Biological Information Processing (IBI-7: Structural Biochemistry) and JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52425 Jülich, Germany

²Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany

³John von Neumann Institute for Computing (NIC), Jülich Supercomputing Centre (JSC), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

⁴Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany

Supporting Information

Table of contents

Experimental Methods

- A β (1-42) sample and fibril preparation
- pH adjustment of [U- ^{13}C , ^{15}N]-A β (1-42) fibrils
- Atomic force microscopy
- Thioflavin T fluorescence intensity measurements
- Circular dichroism spectroscopy
- Solution NMR spectroscopy
- Filling the MAS rotor
- Solid-state NMR measurements
- Computational Methods

Supplementary results

- Figure S1: Reproducibility of the A β (1-42) fibrils.
- Figure S2: A β (1-42) fibrils morphology and their detectability by ThT in dependence on pH.
- Figure S3: A β (1-42) fibrils morphology and their detectability by ThT in presence of various ACN concentrations.
- Figure S4: pH titration of ThT monitored by NMR spectroscopy.
- Figure S5: Testing the influence of the citrate-phosphate buffer on the A β (1-42) fibril structure.
- Figure S6: Comparing the ^{13}C - ^{13}C spectra of A β (1-42) fibrils at pH 2 and pH 7 showing that the global fibril structure remains unchanged upon pH shift.
- Figure S7: The A β (1-42) fibril structure does not show any highly mobile parts at both extreme pH values.
- Figure S8: 2D NCOCX spectra at different pH values.
- Figure S9: Atomic model of the A β (1-42) fibrils.
- Figure S10: Influence of the pH shift on A β (1-42) in the NCa spectrum.
- Figure S11: Solution NMR spectra of A β (1-42) monomers at different pH and solvent conditions.
- Figure S12: Water-accessibility of A β (1-42) fibrils at different pH conditions.
- Figure S13: Assigned protonation states considered for molecular dynamics simulations.
- Figure S14: Subunit-wise conformational analyses of A β (1-42) fibril.
- Table S1: Chemical shift changes and calculated protonation states (%) based on ^{13}C chemical shifts

References

Experimental Methods

A β (1-42) sample and fibril preparation

Recombinantly produced [U- ^{13}C , ^{15}N]-A β (1-42) (95 % purity) was purchased from Isoloid GmbH (Düsseldorf, Germany). The fibrils were prepared as described previously.¹ Briefly, fibrils were grown after a final reverse-phase high-performance liquid chromatography (RP-HPLC) purification step leading to 98.5 % purity from the eluted monomeric [U- ^{13}C , ^{15}N]-A β (1-42) over weeks under quiescent incubation at room temperature directly in the RP-HPLC mobile phase at pH 2 (30 % v/v acetonitrile (ACN), 0.1 % (v/v) trifluoroacetic acid (TFA) in water).

pH adjustment of [U- ^{13}C , ^{15}N]-A β (1-42) fibrils

[U- ^{13}C , ^{15}N]-A β (1-42) fibrils grown at pH 2 in 30 % (v/v) ACN, 0.1 % (v/v) TFA were adjusted to different pH values in the range from 2.6 to 7.5 by addition of 20 vol % citrate-phosphate buffer² stocks, for each pH value independently. Buffer stocks were prepared by mixing aqueous solutions of 0.25 M citric acid and 0.5 M Na $_2$ HPO $_4$ as follows: pH 2.6 (89.10/10.90), pH 3.0 (79.45/20.55), pH 4.0 (61.45/38.55), pH 5.0 (48.50/51.50), pH 6.0 (36.85/63.15), pH 6.5 (29.00/71.00), pH 7.0 (17.65/82.35), pH 7.5 (7.75/92.25); values are vol % of 0.25 M citric acid and 0.5 M Na $_2$ HPO $_4$ solutions in the buffer stocks, respectively. pH adjusted [U- ^{13}C , ^{15}N]-A β (1-42) fibril samples contained 24 % (v/v) ACN, 0.08 % (v/v) TFA. To verify that the pH values in the pH-adjusted fibril samples do not deviate from the pH values of the added citrate-phosphate buffer, we recorded ^{13}C -detected MAS INEPT spectra of all citrate-phosphate buffer solutions (24 % ACN, 0.08 % TFA) without protein as well as for the pH shifted A β fibril samples (Figure S7A and B). ^{13}C chemical shifts of the CH $_2$ groups of citrate are pH-sensitive with higher deprotonation degrees of citrate resulting in a shift to higher frequencies. Chemical shift changes for the CH $_2$ groups range between 0.25 ppm (40 Hz at 600 MHz) in the pH interval from pH 2.6 to pH 4.0, and 0.7 ppm (110 Hz at 600 MHz) in the pH interval from pH 5 to pH 6. Chemical shift differences between the citrate CH $_2$ peaks of the protein-free buffer solutions and the fibril samples were between 0.03 ppm (5.0 Hz at 600 MHz) for the pH 5 samples and 0.06 ppm (9 Hz at 600 MHz) for the pH 2.6 samples, which is more than one order of magnitude smaller than the chemical shift change observed for a pH change of one unit. Therefore, the deviation of the pH value in the fibril samples from the buffer pH value is less than 0.1 in all samples.

Atomic force microscopy (AFM)

For morphological investigations of [U- ^{13}C , ^{15}N]-A β (1-42) fibrils by AFM, fibril samples at the original pH 2.0 and after pH adjustment to pH 2.6, 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, and 7.5, each at a concentration of 80.4 μM (monomer equivalents) were used. 2 μl of each fibril solution were applied onto freshly cleaved muscovite mica and left to dry at room temperature. Then samples were washed three times with 100 μl 24 % (v/v) ACN, followed by drying with a stream of N $_2$ gas.

To examine the effect of different ACN concentrations, [U- ^{13}C , ^{15}N]-A β (1-42) fibrils grown in 30 % (v/v) ACN, 0.1 % (v/v) TFA were diluted to 3 % (v/v) or 24 % (v/v) ACN % (v/v), 0.0125 % (v/v) TFA and a fibril concentration of 10 μM (monomer equivalents). Then, 2 μl of each sample were placed on freshly cleaved muscovite mica surface, left to dry, and additionally dried with the steam of N $_2$ gas, with no washing steps.

AFM imaging was performed in intermittent contact mode (AC mode) in a NanoWizard 3 atomic force microscope (JPK, Berlin) using a silicon cantilever with a silicon tip (OMCL-AC160TS-R3, Olympus) with a typical tip radius of 9 $\pm$ 2 nm, a force constant of 26 Nm $^{-1}$ and a resonance frequency of approximately 300 kHz. The images were processed using JPK data processing software (version spm-

5.0.84). For background flattening, a polynomial fit was subtracted from each scan line, first independently and then using a limited data range, for all height images presented.

Thioflavin T (ThT) fluorescence intensity measurements

ThT fluoresce intensity values were measured in sealed 96-well half-area polystyrene microplates (non-binding surface, black, flat transparent bottom, type 3881, Corning) in a microplate reader (FLUOstar Omega and CLARIOstar, BMG LABTECH, Ortenberg) as endpoint measurements with orbital averaging over 3 mm with 20 flashes per well. Experiments were conducted as independent triplicates.

pH variations: ThT fluorescence of pH-adjusted [U-¹³C, ¹⁵N]-Aβ(1-42) fibrils at pH 2.6, 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, and 7.5 was measured at 4 μM concentration (monomer equivalents) in citrate-phosphate buffered solutions containing 10 μM ThT, 24 % (v/v) ACN, 0.08 % (v/v) TFA, and 20 (v/v) % citrate-phosphate buffer stocks (see above); the original pH 2.0 sample contained no buffer. For comparison, ThT fluorescence was also measured in the same solutions without fibrils.

ACN concentration variations: Samples of [U-¹³C, ¹⁵N]-Aβ(1-42) fibrils were diluted to various final ACN concentrations (1.5 %, 3 %, 6 %, 12 %, 18 %, or 24 % (v/v)), 0.08 % (v/v) TFA, without (pH 2.0) or buffered with citrate-phosphate to pH 7.0 and measured at 4 μM concentration (monomer equivalents) containing 10 μM ThT. For comparison ThT fluorescence was also measured in the same solutions without fibrils.

Circular dichroism (CD) spectroscopy

Far-UV circular dichroism (CD) spectra of [U-¹³C, ¹⁵N]-Aβ(1-42) fibrils in 30 % (v/v) ACN, 0.1 % (v/v) TFA pH 2 (40 μM monomer equivalents) or buffered by addition of 20 % (v/v) citrate-phosphate buffer to pH 7 (see above) (32 μM monomer equivalents) were recorded on a Jasco J-1100 spectropolarimeter from 320 to 188 nm with 0.1 nm step size, 50 nm/min scan speed, and 1 nm bandwidth in 1 mm path-length cuvettes at 20°C. 10 scans were accumulated per sample. Background correction was done by subtraction of corresponding buffer spectra.

Solution NMR spectroscopy

[U-¹³C, ¹⁵N] Aβ(1-42) fibrils were disassembled into monomers by HPLC, aliquoted, frozen in liquid N₂ and lyophilized. Three samples were prepared for solution NMR spectroscopy: 91 μM [U-¹³C, ¹⁵N] Aβ(1-42) in 28 % (v/v) acetonitrile, 0.1 % (v/v) TFA, 8 % (v/v) D₂O at pH 2.0, 74 μM [U-¹³C, ¹⁵N] Aβ(1-42) without acetonitrile in 0.1 % (v/v) TFA, 8 % (v/v) D₂O at pH 2.0, and 73 μM [U-¹³C, ¹⁵N] Aβ(1-42) with 8.8 mM citrate/82.4 mM Na₂HPO₄ in 0.1 % (v/v) TFA, 8 % (v/v) D₂O at pH 7.0. For sequence-specific backbone resonance assignments, the following experiments were recorded on all three samples at 5.0 °C on a Bruker AVANCE III HD 600 MHz NMR spectrometer equipped with a cryogenically cooled inverse triple resonance probe with z-axis pulsed-field gradient capabilities: 2D ¹H-¹⁵N HSQC,³ 3D HNCO⁴ or BEST-TROSY-HNCO,⁵ and 3D HNCACB⁶ or BEST-TROSY-HNCO.⁵ In the case of the sample without acetonitrile at pH 2.0, we also recorded a 3D ¹H-¹⁵N TOCSY-HSQC³ with a 78 ms 10.0 kHz DIPSI-2rc isotropic mixing scheme,⁷ and a 3D ¹H-¹⁵N NOESY-HSQC³ with a mixing time of 150 ms for assignment of side-chain ¹H resonances. Aliphatic side-chain ¹³C resonances in 28 % acetonitrile at pH 2.0 were obtained from a 2D ¹³C-¹³C TOCSY spectrum with a 15.1 ms

15.6 kHz FLOPSY-16 isotropic mixing scheme⁸ recorded at 5.0°C on a Bruker AVANCE III HD 800 MHz NMR spectrometer equipped with a cryogenically cooled ¹³C/¹⁵N observe triple resonance probe; a corresponding 2D ¹³C-¹³C TOCSY spectrum of [U-¹³C, ¹⁵N] Aβ(1-42) at pH 7.2 has been reported previously.⁹ The sample temperature was calibrated using methanol-d₄.¹⁰ The ¹H₂O resonance was suppressed by gradient coherence selection with water flip-back,¹¹ with quadrature detection in the indirect dimensions achieved by States-TPPI¹² and the echo-antiecho method.¹³⁻¹⁴

All solution NMR spectra were processed with NMRPipe¹⁵ software and analyzed with NMRViewJ¹⁶ and CCPN.¹⁷ ¹H chemical shifts were referenced with respect to external DSS in the same buffer, ¹³C and ¹⁵N chemical shifts were referenced indirectly.¹⁸ For the sake of consistency, we followed the same DSS-based chemical shift referencing protocol for samples containing acetonitrile; alternatively, the ¹H resonance of acetonitrile itself has also been proposed as a suitable reference standard for acetonitrile/water mixtures.¹⁹ Random Coil Index (RCI)²⁰ backbone order parameters, S_{RCI}², were calculated from the backbone chemical shifts using TALOS-N²¹ with the default parameters. The presence of 28 % (v/v) acetonitrile in one of the samples causes a non-uniform solvent effect with average deviations from random coil chemical shifts²² of -0.333 ppm for ¹HN, -1.156 ppm for ¹⁵N, and -0.616 ppm for ¹³CO (where the negative sign indicates chemical shift values that are lower in 28 % (v/v) acetonitrile); these average deviations were corrected for in the input for TALOS-N. By contrast, the solvent effect on ¹³Cα and ¹³Cβ, with average deviations from random coil values of -0.142 ppm and +0.033 ppm, respectively, is very small, as reported in the literature.²³

To investigate the pH dependence of 500 μM ThT in citrate/phosphate buffer²⁴ with 24 % (v/v) acetonitrile, 0.08 % (v/v) TFA and 7.5 % (v/v) D₂O, a series of 1D ¹H and 2D ¹H-¹³C HSQC²⁵ spectra was recorded at 20.0 °C on a Bruker AVANCE III 750 MHz NMR spectrometer equipped with a cryogenically cooled inverse triple resonance probe with z-axis pulsed-field gradient capabilities. The ¹H₂O resonance in the 1D ¹H spectra was suppressed by excitation sculpting²⁶ with water flip-back,¹¹ the acetonitrile ¹H resonance by 1.5 s off-resonance presaturation; residual baseline effects were removed using a median baseline correction algorithm²⁷ during processing. The ¹H₂O resonance in the 2D ¹H-¹³C HSQC spectra was suppressed by gradient coherence selection, with quadrature detection in the indirect dimension achieved by the echo-antiecho method.¹³⁻¹⁴ ¹H chemical shifts were referenced with respect to 5 mM internal DSS, ¹³C chemical shifts were referenced indirectly.¹⁸

pH shift and filling the MAS rotor

All solid-state NMR experiments were performed from a single batch of [U-¹³C, ¹⁵N]-Aβ(1-42) fibrils grown in 30 % (v/v) ACN, 0.1 % (v/v) TFA, pH 2 (see above). Fibrils were harvested from the growth solution by a 16.000 xg centrifugation step for ~1 h, and the remaining supernatant was discarded from settled fibrils.

For the pH 2.0 sample, a ~50 μl fibril sample was loaded into a 3.2 mm Bruker zirconium oxide rotor by centrifugation for 1 min at 4000 xg. Solid-state NMR samples of fibrils adjusted to pH values of 2.6, 4.0, 5.0, 6.0, and 7.0 were prepared by addition of 10 μl respective citrate-phosphate buffer stocks (see above) to 40 μl pelleted fibrils grown at pH 2 and thorough mixing. Afterwards, the fibril slurries were placed into 3.2 mm Bruker rotors by centrifugation. Each 50 μl rotor sample contains ~1.7 to 2.1 mg Aβ(1-42) fibrils, equivalent to 380 to 460 nmol monomer equivalents of Aβ(1-42), which corresponds to final Aβ(1-42) concentrations of 7.5 to 9.3 mM. Thus, the fibril preparations contain ~96 % solvent and are highly hydrated.

Solid-state NMR measurements

Solid-state ^{13}C - ^{13}C and ^{13}C - ^{15}N magic-angle spinning (MAS) NMR spectra were acquired on a 18.8 T Bruker Avance III spectrometer (800 MHz ^1H resonance frequency) and on a 14.1 T Bruker Avance NEO spectrometer (600 MHz ^1H resonance frequency), both equipped with a 3.2 mm triple channel HCN Bruker probe. All experiments were conducted at a thermocouple-reported temperature of 263 K. The sample temperature was estimated to be around 10 K higher due to heating because of the magic angle spinning. It was tested that the water in the sample was not frozen. The MAS frequency was set to 11 kHz and 12.5 kHz for the proton driven spin diffusion (PDS) experiments and at 12.5 kHz for NCA experiments at 800 MHz and to 11 kHz for the NCA CX and NCOCX experiments at 600 MHz. Typical radiofrequency field strengths were 83 to 100 kHz for ^1H , 55 to 61 kHz for ^{13}C , and 45 to 50 kHz for ^{15}N . During all acquisition and evolution times, high-power broadband proton decoupling with a SPINAL phase modulation (rf intensity 83 to 93 kHz) was used.²⁸

For 2D ^{13}C - ^{13}C correlation experiments PDS spectra with 20 ms mixing time were recorded. To gain additional information about ^{15}N chemical shifts, ^{15}N - ^{13}C correlation experiments were performed by using frequency selective polarization transfer from amide ^{15}N to ^{13}C CA by SPECIFIC CP.²⁹ The error bars for the A42 intensities were estimated from the signal-to-noise ratio of the NCA spectra. The mixing time for the assignment experiments was 40 ms (NCA CX) and 50 ms (NCOCX).

All ^{13}C chemical shifts were externally referenced using adamantane by setting its CH signal to 31.4 ppm (corresponding to the DSS reference scale). The ^{15}N chemical shifts were indirectly referenced to liquid NH_3 based on the ^{13}C chemical shifts. All spectra were processed using TopSpin 3.5 and 4.0.9 (Bruker). The analysis was performed using CcpNMR Analysis 2.4¹⁷ based on the assignment of the A β (1-42) fibril structure published by Gremer and al.¹

For both water-edited 1D ^{13}C and 2D ^{13}C - ^{13}C , a ^1H T_2 filter of 2.5 ms was applied to suppress the magnetization of the protein. The ^1H - ^1H mixing time for the water-edited 1D ^{13}C experiments was set to 0.5, 1, 2, 3, 4, 5, 7.5, 10, 20, 30, 50, and 100 ms and to 3 ms and 100 ms for 2D ^{13}C - ^{13}C experiments before ^1H - ^{13}C transfer. The thermocouple-reported temperature was adjusted to 270 K, and the spinning frequency was 11 kHz.

The relative spacing between subsequent contour levels in plots of 2D spectra is 1.2, except for Figure 2A, where it is 1.4. The lowest contour level was set slightly above the noise level.

Computational Methods

System preparation, simulation protocol, and analysis procedure

To investigate the conformational dynamics of the A β (1-42) fibril at different pH conditions, we performed unbiased molecular dynamics (MD) simulations of the A β (1-42) fibril with amino acid protonation states mimicking either pH 2 or pH 7. Therefore, we prepared two distinct fibril protonation states; one with protonation states according to pH 7 and one for pH 2 (Figure S8). Note that we adapted the protonation states of all titratable residues of the fibril at pH 2 as resolved previously by NMR spectroscopy.¹ A detailed preparation protocol can be found in ref.³⁰; this protocol was also used to prepare the other systems. In short, the initial coordinates for the A β (1-42) fibril were taken from the cryo-EM structure (PDB ID 5OQV¹). We prepared an A β (1-42) fibril composed of 20 single A β (1-42) peptides, which are organized into two non-covalently attached protofilaments. The protonation states of all titratable residues of the fibril were resolved by NMR spectroscopy (see above) and incorporated into our setup. That way, the protonation states in the simulations match with *in vitro* experiments, allowing for direct comparison between computational and experimental results. The systems were neutralized and solvated with TIP3P³¹ water, using chloride (for pH 2) or sodium (for pH 7) counterions, respectively, applying LEaP³² of Amber16.³³ We used the Amber ff14SB force field³⁴ to describe the A β (1-42) fibril. For the A42-H residue, atomic partial charges were derived according to the RESP procedure.³⁵⁻³⁶

The exact minimization, thermalization, and equilibration protocol is reported in ref.³⁷, which was already applied previously.^{30, 38-39} In short, all systems were initially subjected to three rounds of energy minimization to resolve bad contacts. Subsequently, systems were heated to 300 K, and the pressure was adapted such that a density of 1 g/cm³ was obtained. During thermalization and density adaptation, we kept the solute fixed by positional restraints of 1 kcal mol⁻¹ Å⁻², which were gradually removed. For all systems, we performed ten replicas of MD simulations of 1 μ s length each of the A β (1-42) fibrils. The replica simulations were prepared following the protocol in ref.³⁷.

To evaluate the structural integrity of the fibril, we calculated the backbone root mean square deviation (RMSD) relative to the cryo-EM structure¹ that served as the starting structure. The results are expressed as average RMSD $\pm$ SEM (standard error of the mean). The hydrogen bond interactions were determined using a distance of 3.5 Å between the two donor and acceptor atoms and an angle (donor atom, H, acceptor atom) of 120° as cutoff criteria.⁴⁰ The results are expressed as average hydrogen bond occurrence $\pm$ SEM. As the SEM are almost equal in all cases, also the variances can be considered equal, such that a *t*-test was performed on two groups, and *p*-values < 0.05 were considered significant.

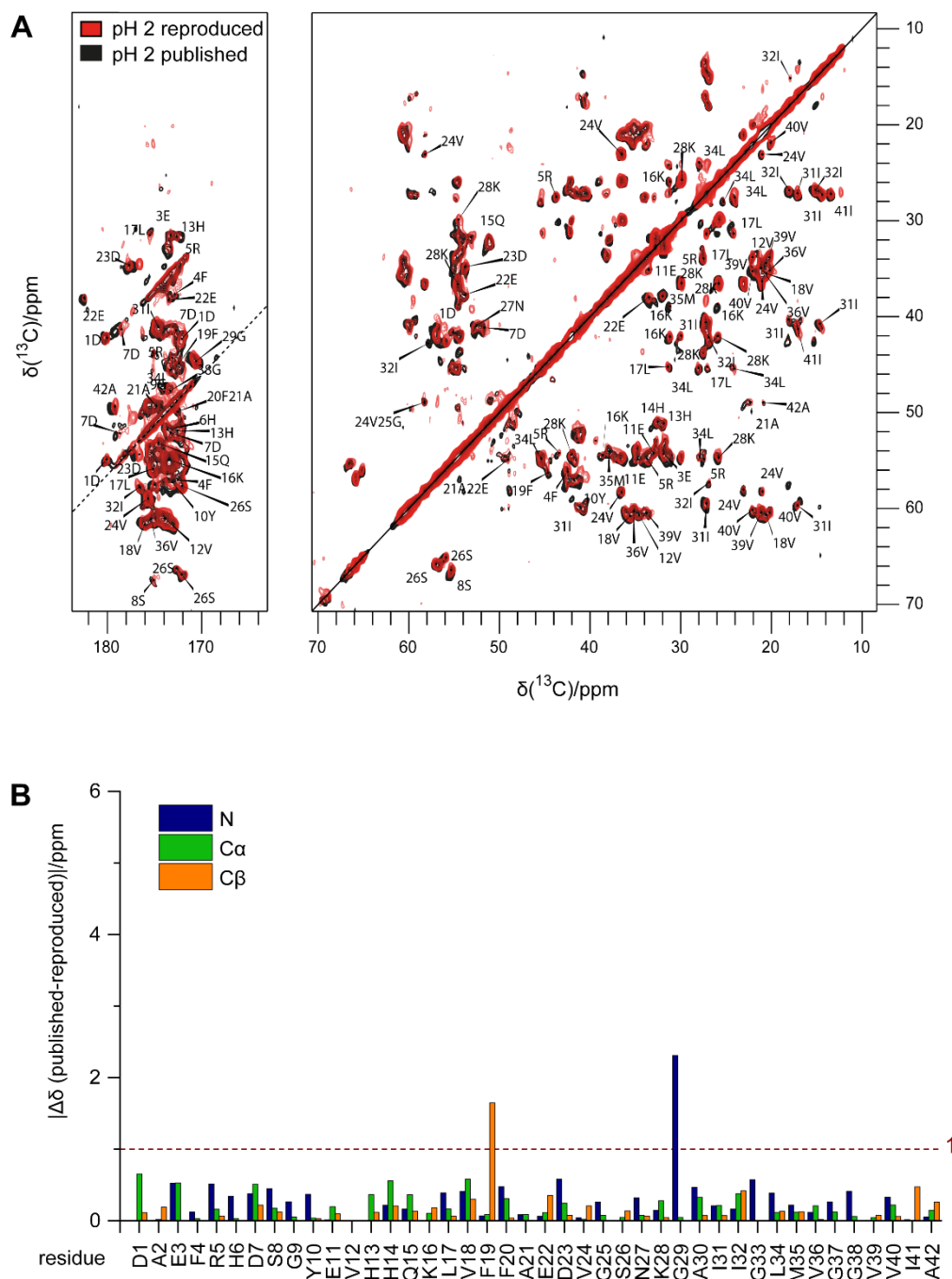


Figure S1: Reproducibility of the A β (1-42) fibrils.

A) 2D ^{13}C - ^{13}C MAS Proton-Driven Spin Diffusion (PDS) spectra of A β (1-42) fibrils grown at acidic pH published by Gremer et al.¹ (black) and reproduced under identical conditions and used for this study (red). The K16 side chain and H6 C α -C β correlation signals appear weaker in the spectrum of the reproduced sample. The lower D7 and D23 C γ chemical shift of the reproduced sample shows a slightly higher degree of protonation. A fifth Ala C α -C β correlation signal appeared for the reproduced sample. Additionally, the peak doubling of F20 and A21 was not observed in the second sample.

B) Chemical shift perturbation plot for A β (1-42) fibrils grown at acidic pH published by Gremer et al.¹ and reproduced under identical conditions and used for this study.

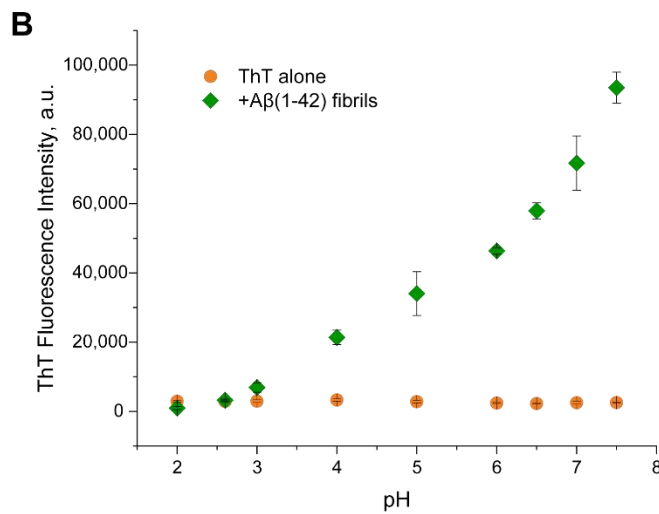
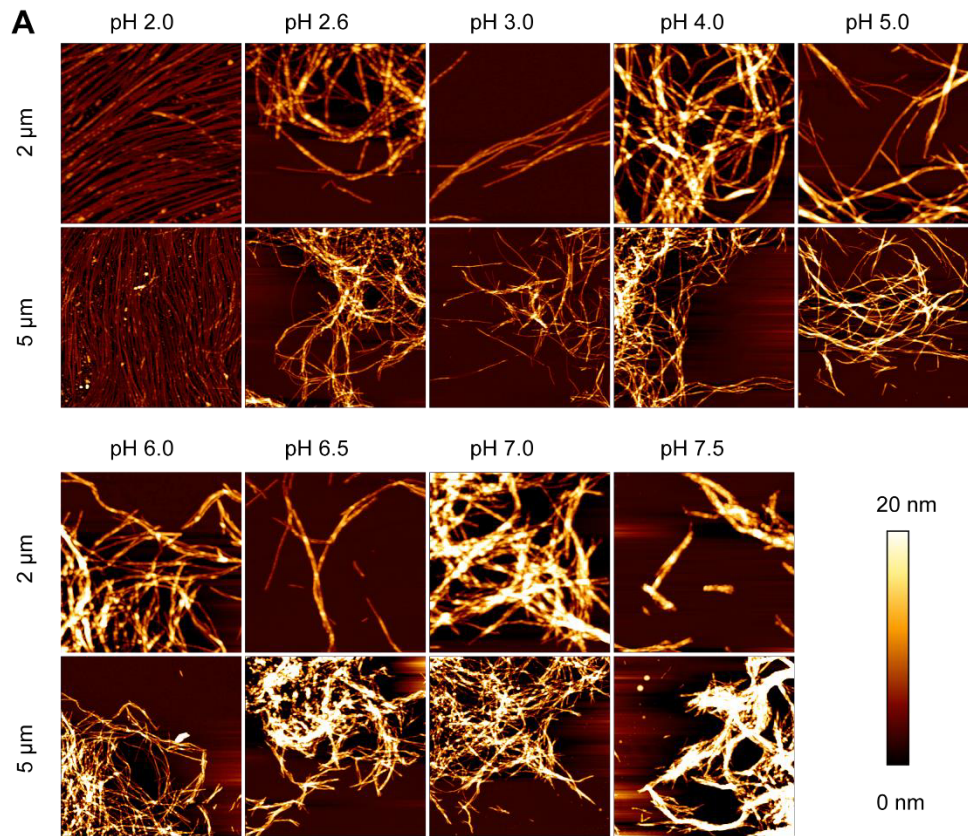
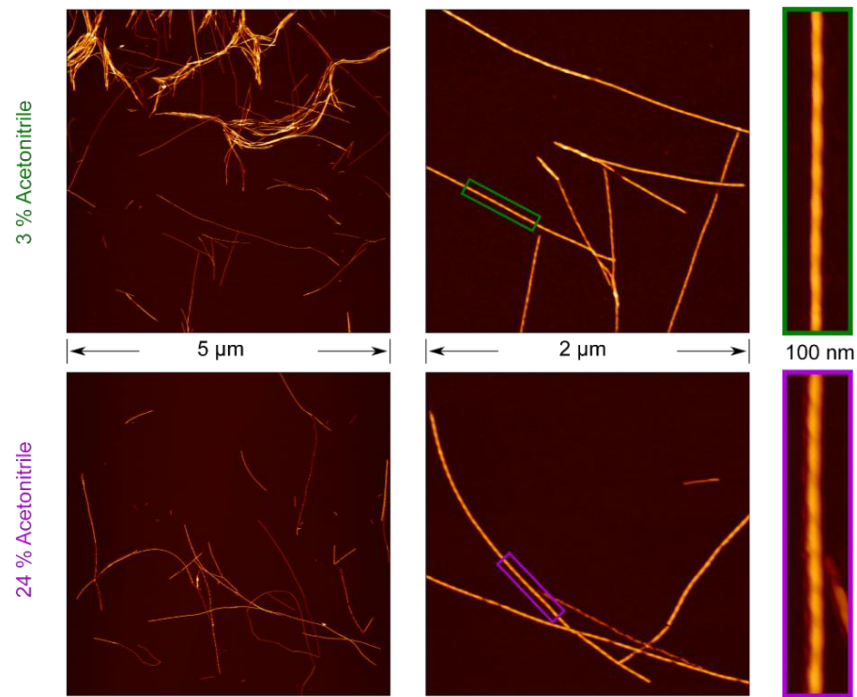


Figure S2: A β (1-42) fibrils morphology and their detectability by ThT in dependence on pH.

A) AFM images of A β (1-42) fibrils under various pH conditions. Height profiles of the fibrils at original pH 2.0 and shifted to pH 2.6, 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, and 7.5 are shown: 2 μm x 2 μm close-ups (top rows) and 5 μm x 5 μm overview images (bottom rows). The color scale represents the heights from 0 nm (dark brown) to 20 nm (white). While clearly visible single fibrils are present at pH 2.0, increasing the pH results in network formation and even gathering the fibrils into bigger clusters at neutral pH. However, individual fibrils featuring the characteristic pitch are distinguishable in the broad pH range.

B) pH-dependence of ThT fluorescence intensity in the absence (yellow circles) and presence of A β (1-42) fibrils (green diamonds) at a concentration of 4 μM (monomer equivalents).

A



B

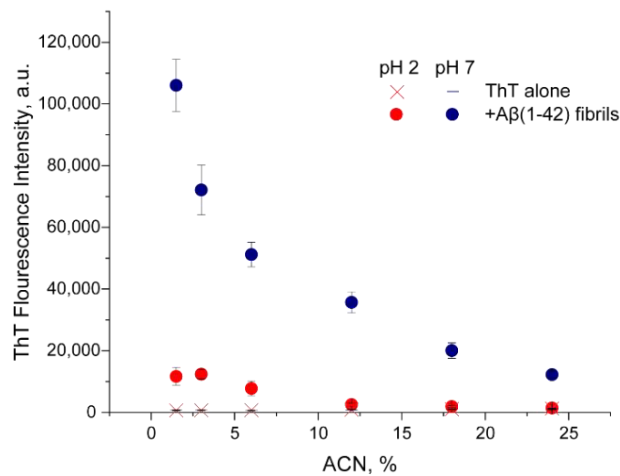


Figure S3: Aβ(1-42) fibrils morphology and their detectability by ThT in presence of various ACN concentrations.

A) Aβ(1-42) fibrils in the presence of 3 % and 24 % ACN. AFM height images show the fibrils at pH 2.0 in the presence of 3 % (top row) and 24 % (bottom row) ACN. Left: 5 μm x 5 μm overview images, middle: 2 μm x 2 μm close-ups, and right: 100 nm x 500 nm single fibril cut-outs. The colors correspond to the heights from 0 nm (dark brown) to 10 nm (white). It is noticeable that also at comparably low ACN concentration, the original morphology of the fibrils is preserved. B) ThT fluorescence intensity highly depends on ACN concentration. In the graph, the data collected from samples containing Aβ(1-42) fibrils are plotted as circles, and those with no protein as thin lines, barely visible due to their close proximity to zero. Measurements at pH 2.0 and pH 7.0 are shown in red and blue accordingly.

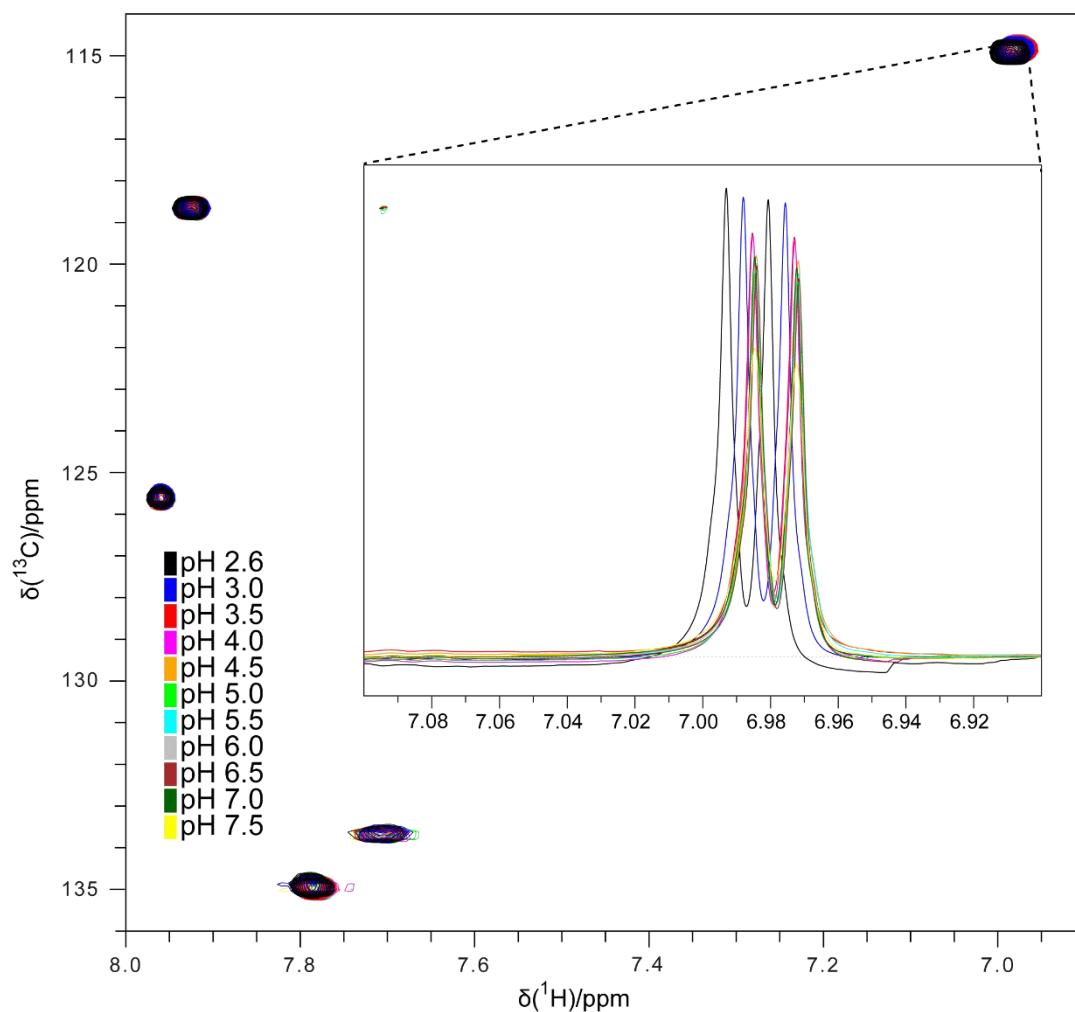


Figure S4: pH titration of ThT monitored by NMR spectroscopy.

From pH 7.5 to pH 3.5, the benzene ring resonances of ThT in the 2D ^1H - ^{13}C HSQC spectra superimpose within experimental error. At pH 3.0 (blue) and pH 2.6 (black), the benzene ring resonance adjacent to the dimethylamino group at 6.98 ppm (^1H) / 114.83 ppm (^{13}C) (designated B4 in ref.⁴¹) exhibits small but significant chemical shift changes of +4 ppb/+37 ppb and +8 ppb/+75 ppb, respectively. This change is also observed in the 1D ^1H spectra, where this resonance gives rise to a doublet (inset). Relative to the ^1H chemical shift change of +1.03 ppm reported in the literature,⁴¹ this change would correspond to a minute degree of protonation of the dimethylamino group of 0.4 % of the ThT molecules at pH 3.0 and 0.8 % of the ThT molecules at pH 2.6.

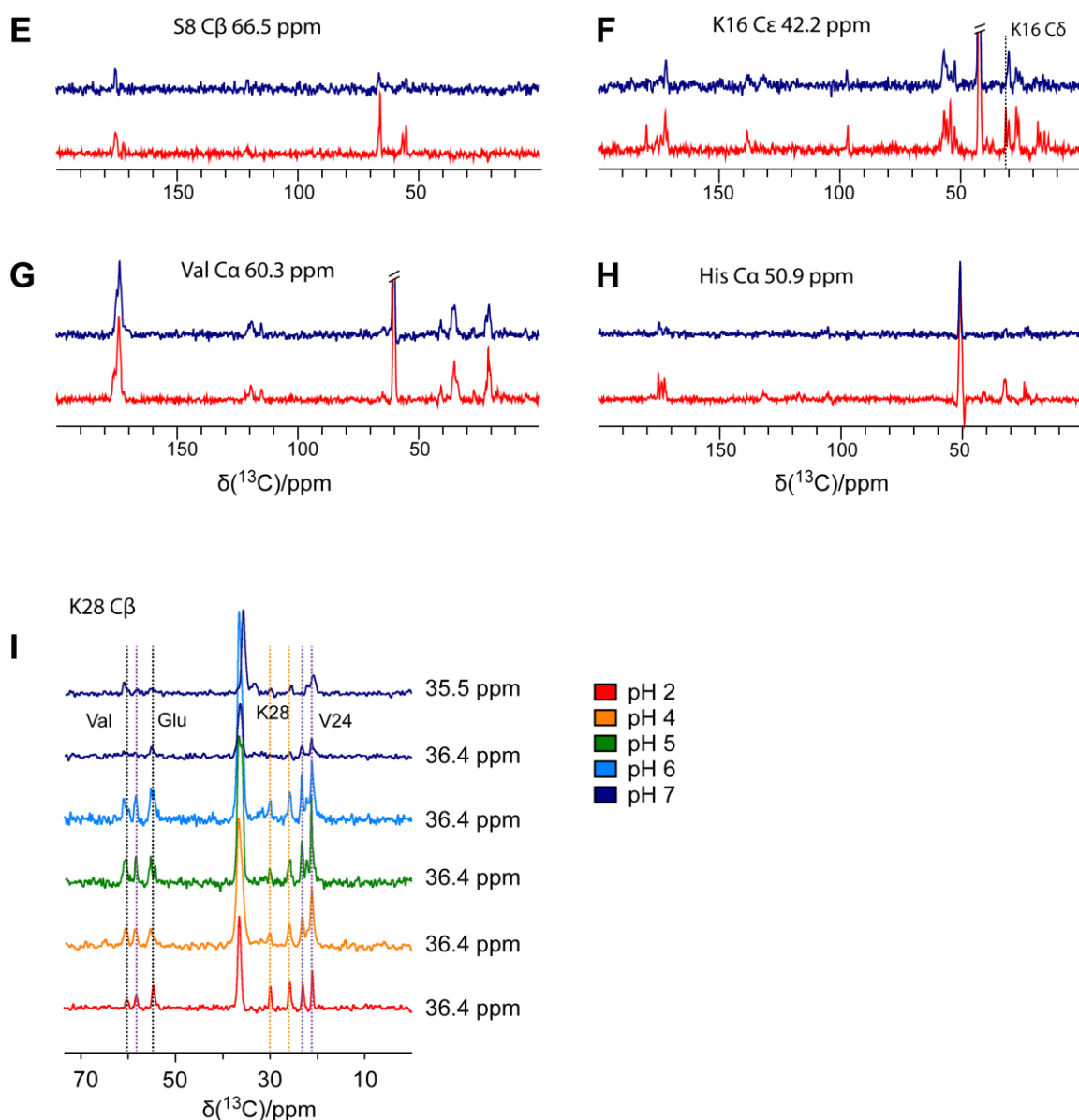


Figure S6: Comparing the ^{13}C - ^{13}C spectra of A β (1-42) fibrils at pH 2 and pH 7 showing that the global fibril structure remains unchanged upon pH shift.

A) 2D ^{13}C - ^{13}C MAS Proton-Driven Spin Diffusion (PDSD) spectra of A β (1-42) fibrils at pH 2 (red) and pH 7 (navy). B) Zoom of (A). No significant changes occur for, e.g., Val and Ile side-chain resonances. C) 1D cross-section of (A). The intensity of the R5 C β -C γ correlation is reduced; no significant intensity changes occur for all Val resonances. D) Zoom of the overlay of the aliphatic regions of the 2D ^{13}C - ^{13}C MAS Proton-Driven Spin Diffusion (PDSD) spectra of A β (1-42) fibrils at pH 2 (red), pH 4 (orange), pH 5 (green), pH 6 (blue) and pH 7 (navy). The S26 C α -C β correlation shows two signals, and the intensity of the signal at 56.1 and 65.4 ppm decreased with increasing pH value. The S8 C α -C β correlation shows reduced intensities for increasing pH values. E-H) Representative 1D slices from spectrum A). I) Representative 1D slices from Figure 2C.

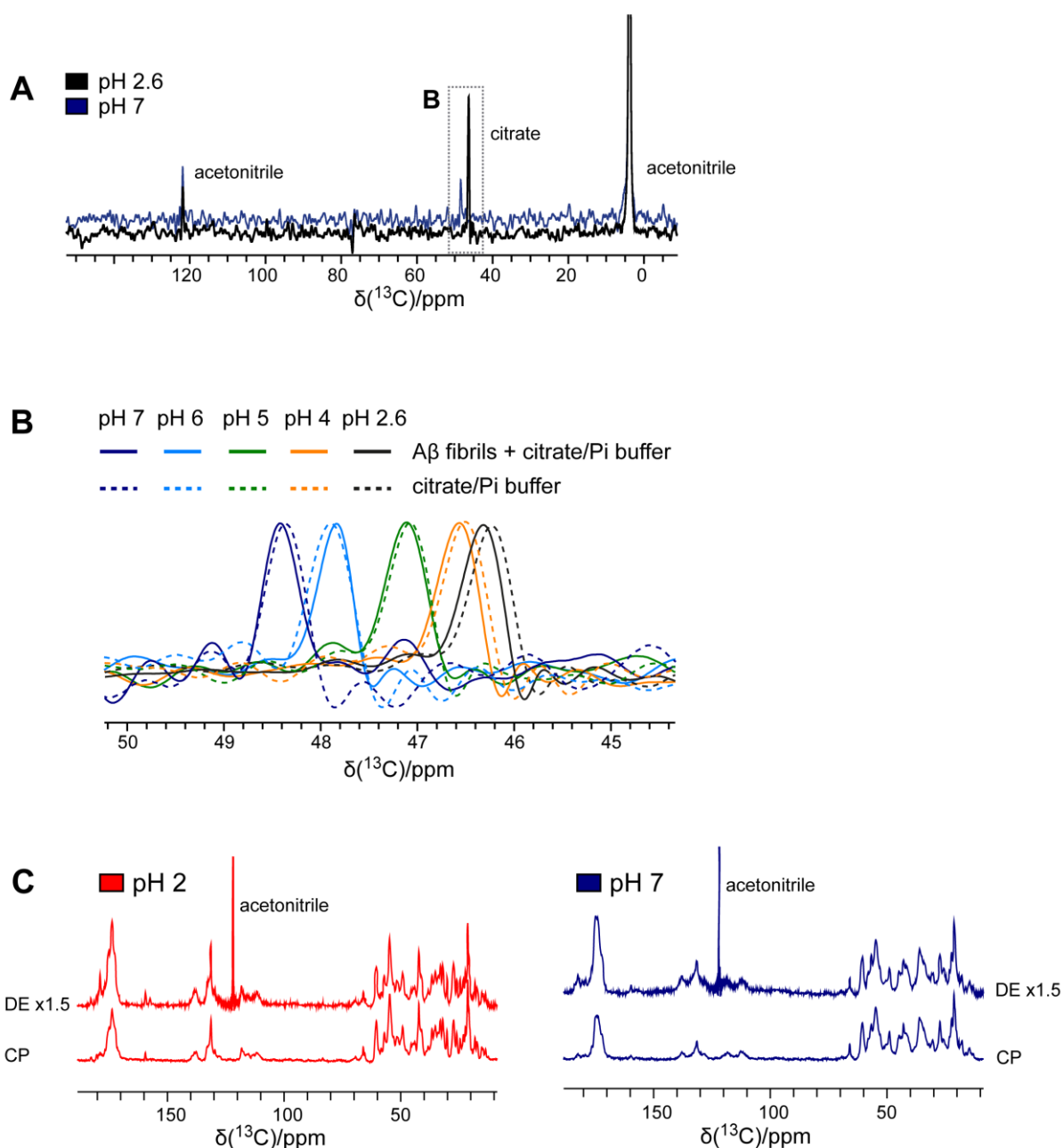


Figure S7: The A β (1-42) fibril structure does not show any highly mobile parts at both extreme pH values, and the pH values of the fibril samples do not deviate from the pH values of the citrate-phosphate buffer solutions.

A) ^{13}C detected refocused INEPT spectra of A β (1-42) fibrils at pH 2.6 (black) and pH 7 (blue) are devoid of protein signals, only solvent and buffer signals are visible. B) The chemical shift of the signal of the CH_2 groups of citrate is pH sensitive and can be used as an internal pH sensor of the fibril samples. Chemical shift differences between the fibril sample (full line) and the buffer solution (24 % ACN, 0.08 % TFA, dashed line) for a given pH value are one order of magnitude smaller than the shift differences observed between different pH steps, thus, the deviation of the pH value from that of the buffer is less than 0.1. C) Comparison of the 1D ^{13}C cross-polarization (CP) and the direct excitation (DE) spectra at pH 2 and pH 7. The acetonitrile peaks were cut off. No additional protein signals were detected for direct excitation compared to the CP spectrum, so any highly mobile or flexible parts can be excluded.

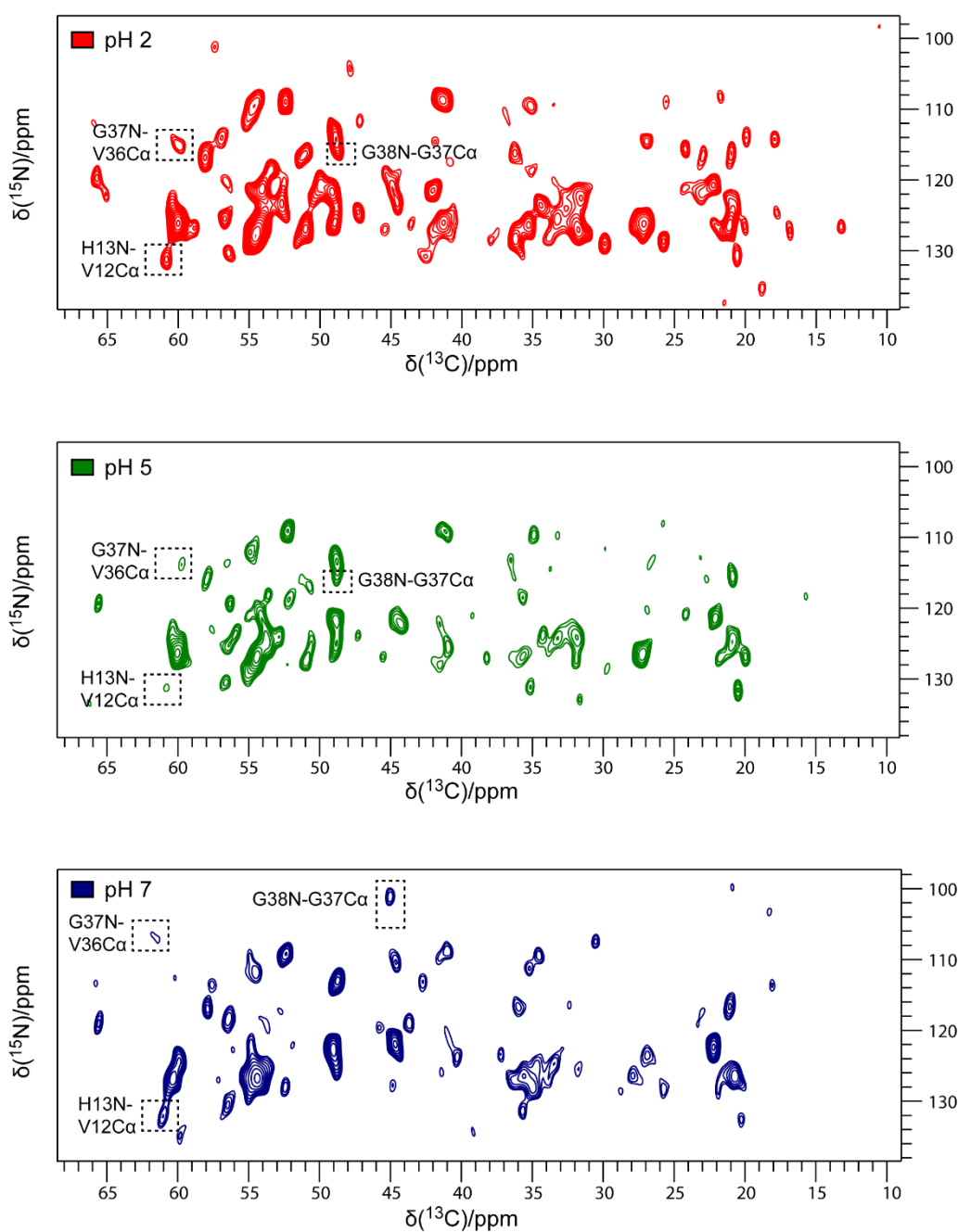


Figure S8: 2D NCOX spectra at different pH values. The changes for the C-terminal Gly residues (G37 and G38) are highlighted using boxes. For all pH values the strong V12-H13 correlation is visible (highlighted using boxes), which was also already present, but which has not been described in detail before.¹

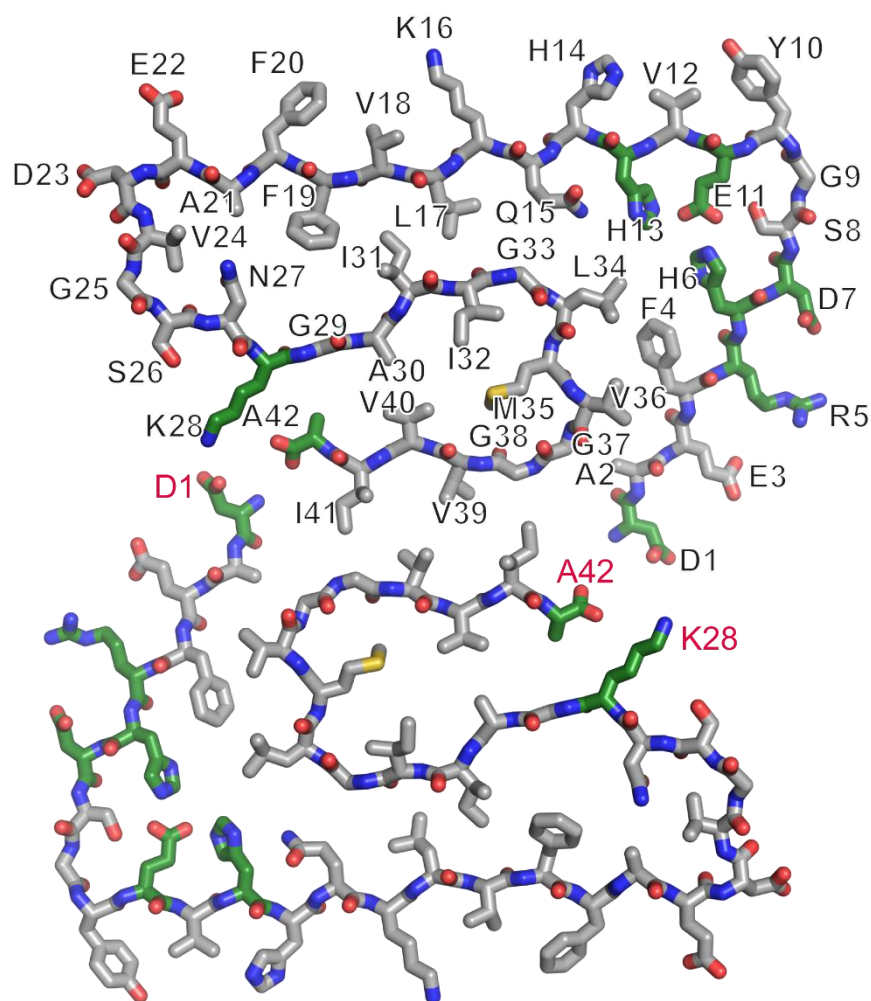


Figure S9: Atomic model of the A β (1-42) fibrils.

The atomic model of the A β (1-42) (PDB: 5OQV Gremer et al., 2017¹) is shown as a stick model. Amino acids essential for stabilizing the protofilament are colored green. The residues of the triad D1-K28-A42, important for the stabilization of the fibril structure, on the other protofilament are labeled in red.

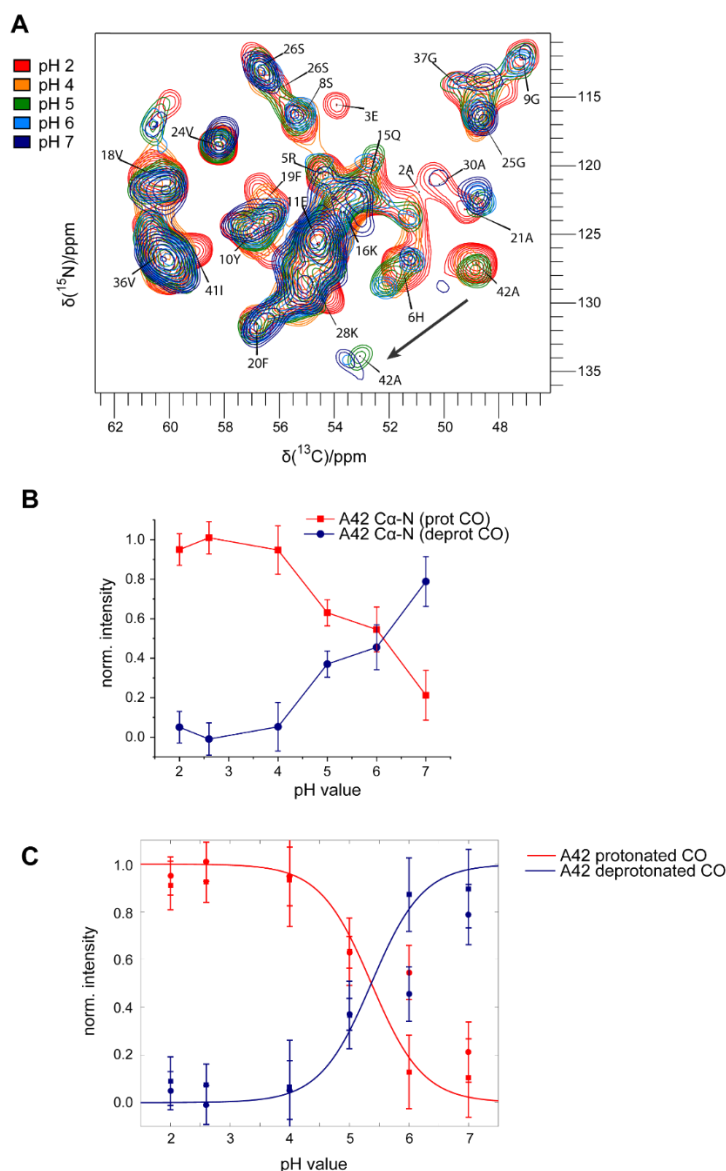


Figure S10: Influence of the pH shift on Aβ(1-42) in the NCa spectrum.

A) Overlay of 2D ^{15}N - ^{13}C MAS NCa spectra of Aβ(1-42) fibrils at different pH conditions (pH 2.0 (red), pH 4.0 (orange), pH 5.9 (green), pH 6.0 (blue) and pH 7.0 (navy)). The chemical shift change (slow exchange) of the A42 Cα-N resonance signal is shown with a black arrow. The normalized intensities for each pH value are shown in Figure 2F. B) Normalized intensities for the Cα-N (Figure 2F) correlation signals (in the protonated and deprotonated state) from the 2D and NCa experiments are plotted against the pH value. The intensities were normalized using the following equations: $I_{\text{norm}}(\text{prot}) = I(\text{prot}) / (I(\text{prot}) + I(\text{deprot}))$ and $I_{\text{norm}}(\text{deprot}) = 1 - I_{\text{norm}}(\text{prot})$. The C-terminal A42 carboxyl group is completely protonated until pH 4. For pH 5 and 6, both states can be observed, while the carboxyl group is to a high degree deprotonated at pH 7. Further details with all A42 chemical shifts can be found in Table S1. C) Plot of the Henderson-Hasselbalch equation: data derived from the NCa spectra (circles Figure S9B) and from the PDS spectra (squares, Figure 2F) were fit together using non-linear regression as implemented in Matlab R2015b based on the following equation: $I_{\text{norm}}(\text{prot}) = \exp(-\ln(10) \cdot \text{pH}) / [\exp(-\ln(10) \cdot \text{pH}) + \exp(-\ln(10) \cdot \text{pK}_a)]$ resulting in a pK_a of 5.37 ± 0.13 .

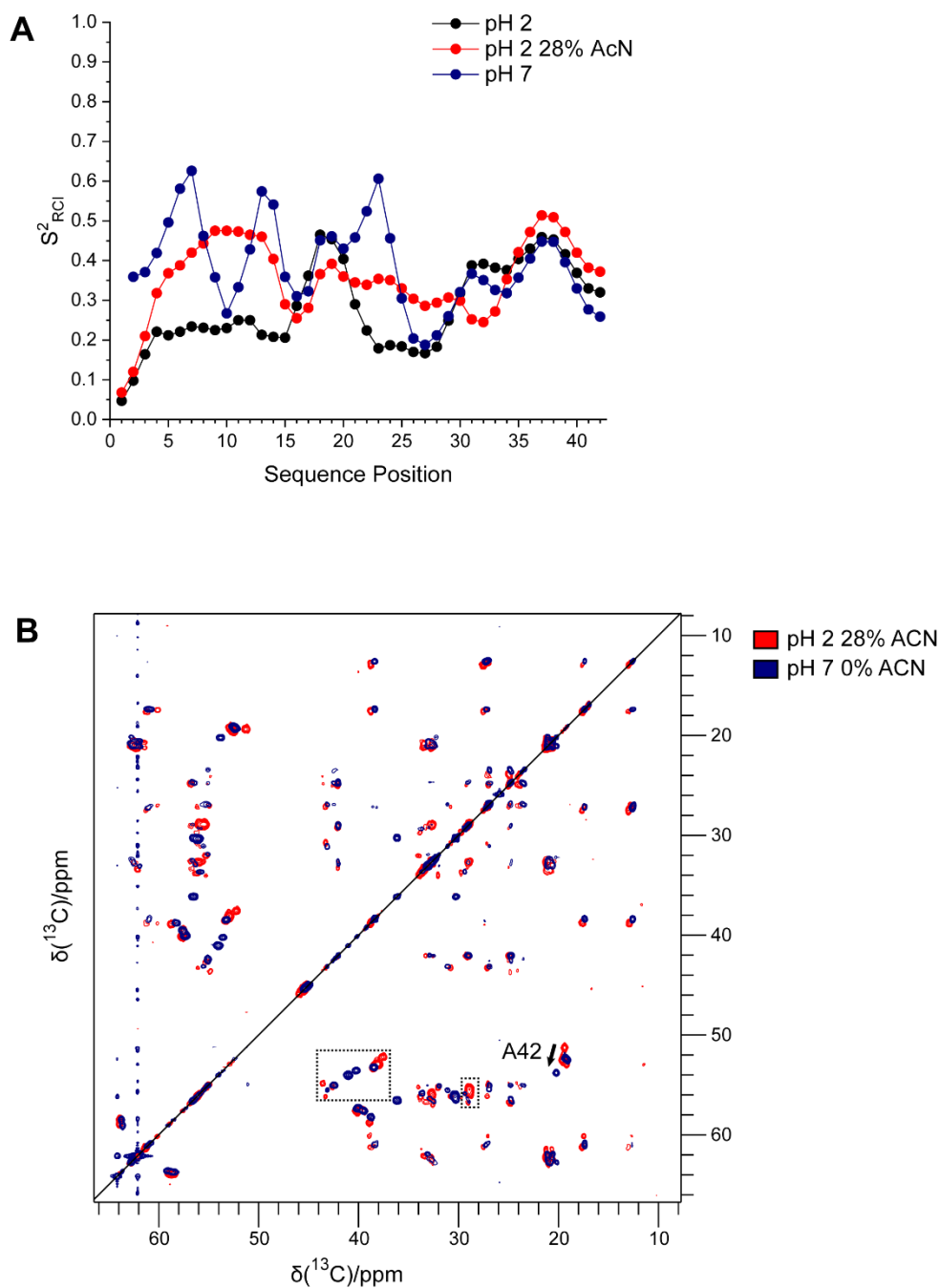


Figure S11: Solution NMR spectra of A β (1-42) monomers at different pH and solvent conditions.

A) Backbone order parameters predicted from the Random Coil Index (RCI), S_{RCI}^2 , for A β (1-42) monomers in 28 % (v/v) acetonitrile at pH 2.0 (red), without acetonitrile at pH 2.0 (black), and in citrate/phosphate buffer at pH 7.0 (navy blue), as calculated by TALOS-N²³ with the default parameters. A β (1-42) monomers are highly disordered at all these conditions, with chemical shifts close to random coil values (Figure S10B) and concomitantly low S_{RCI}^2 values below about 0.6 for all residues. B) Comparison of the 2D ^{13}C - ^{13}C TOCSY spectrum of A β (1-42) monomers at pH 2.0 in 28 % (v/v) acetonitrile (red) with the 2D ^{13}C - ^{13}C TOCSY at pH 7.2 in 30 mM Tris-HCl buffer reported previously⁹ (navy blue). Changes between pH 2 and pH 7 are observed primarily for the titratable residues (Asp, Glu and His residues) and for A42 (change from a protonated to a deprotonated C-terminal carboxyl group).

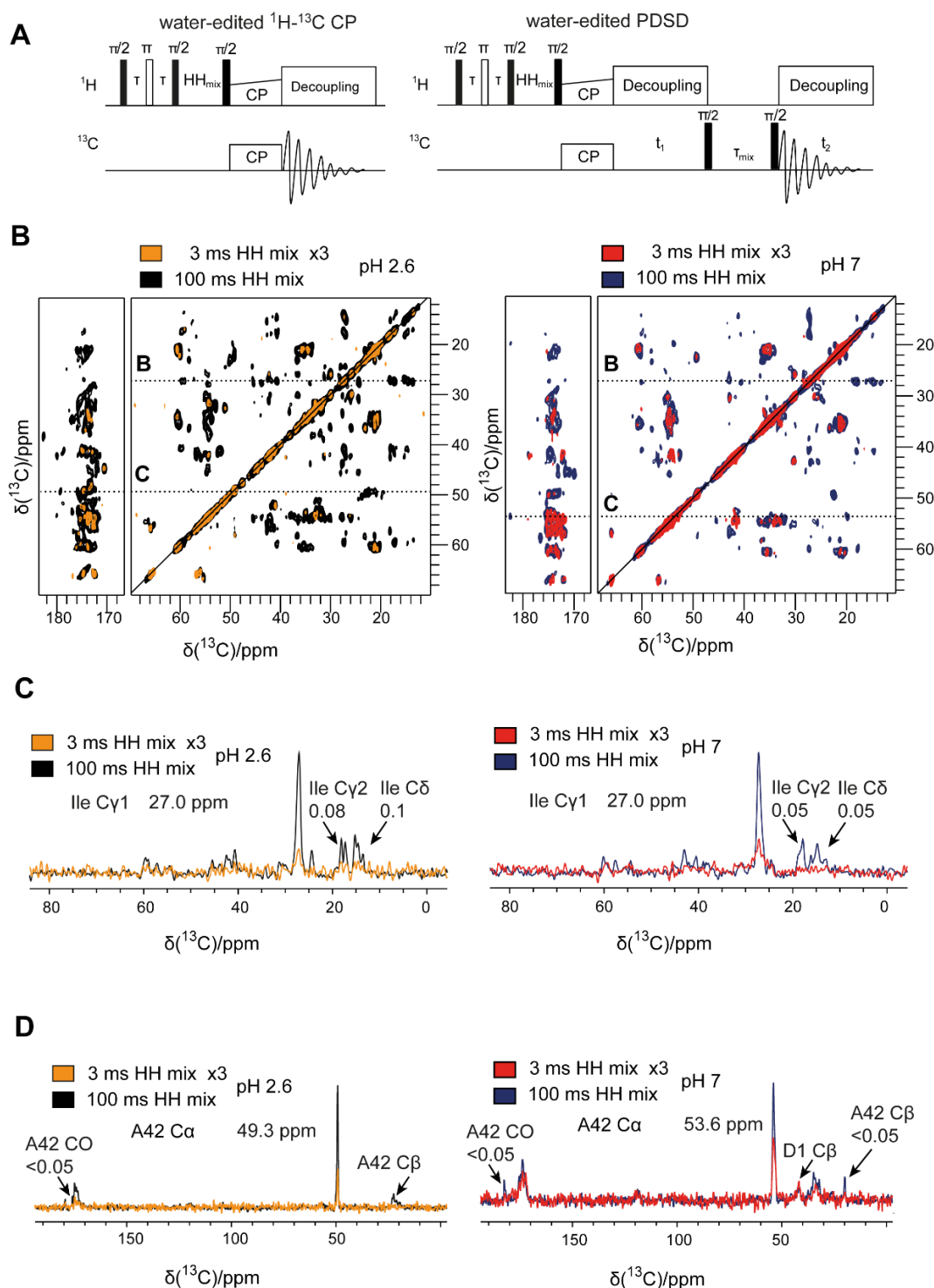


Figure S12: Water-accessibility of A β (1-42) fibrils at different pH conditions.

A) Pulse sequences for water-edited 1D CP and 2D PDSD spectra. B) Water-edited PDSD spectra of A β (1-42) fibrils at pH 2.6 and pH 7 with ^1H - ^1H mixing times of 3 ms and 100 ms. C) 1D ^{13}C cross-sections taken from the Ile C γ 1 chemical shift at 27.0 ppm. The Ile C γ 1 resonances show low water-transferred intensities of less than 0.1. D) Complete 1D ^{13}C cross-sections taken from the A42 C α shifts. Spectra with a short mixing time (3 ms) were recorded with a three times higher number of scans than the spectra with a mixing time of 100 ms.

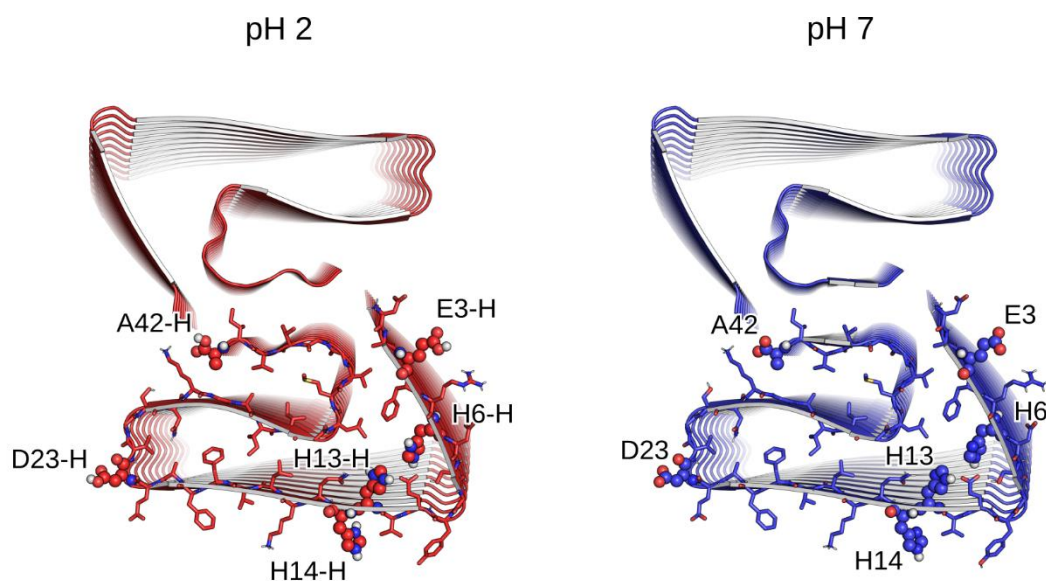


Figure S13: Assigned protonation states considered for molecular dynamics simulations.

Top view of the A β (1-42) structural models as used for multi-microsecond length molecular dynamics (MD) simulations. All systems were prepared on the basis of the A β (1-42) structure resolved by cryo-EM at pH 2 (PDB ID 5OQV¹). Residues with changing protonation states, according to our NMR experiments, are shown as a sphere model and labeled explicitly. Residues with a constant protonation state are shown as a stick model. Amino acid names with “-H” extension denote protonated amino acids at pH 2 conditions.

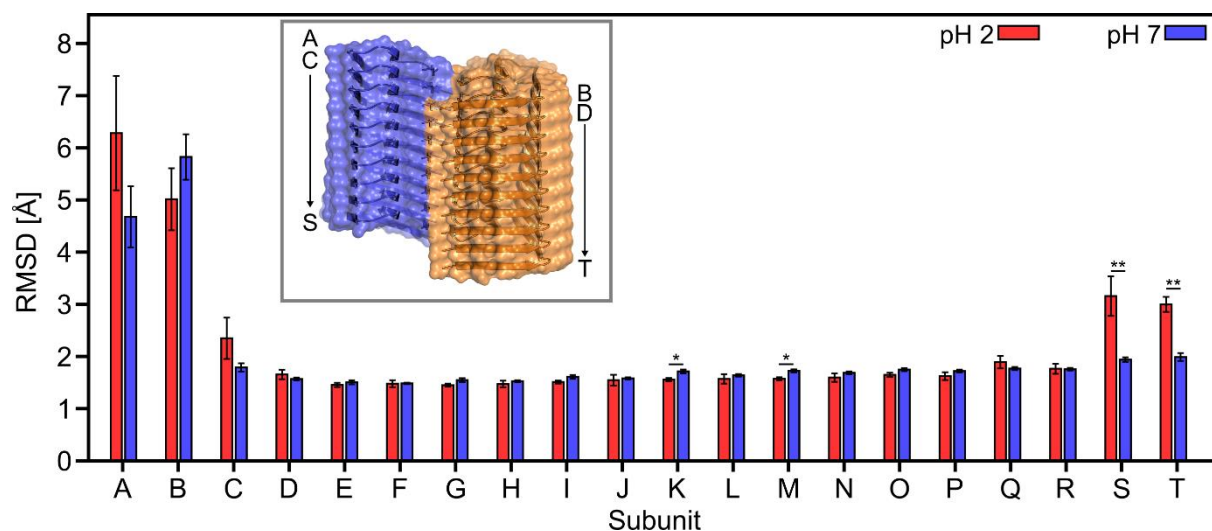


Figure S14: Subunit-wise conformational analyses of Aβ(1-42) fibril.

The mean backbone root mean-square deviation (RMSD, $n = 10$) was calculated for each Aβ(1-42) peptide individually. The molecular dynamics simulations were performed on an Aβ(1-42) fibril composed of 20 single Aβ(1-42) peptides (labeled A-T, see inset for details), which are organized into two non-covalently attached protofilaments (blue and orange domains in the inset). The error bars denote the standard error of the mean (SEM) (* $p < 0.05$; ** $p < 0.01$).

Table S1: Chemical shift changes and calculated protonation states (%) based on ^{13}C chemical shifts (see Keim et al.⁴² and Platzer et al.⁴³) of different amino acid residues of pH-shifted A β (1-42) fibrils.

Assignment based on the similarity in the CC correlation spectra between different pH values

		N	CO	Ca	C β	C γ	C γ 2	C δ	C ϵ	Prot %
D1	pH 2		172.2	54.3	42.0	180				5
	pH 7		171.9	54.4	41.3	178.8				40
						180.0				5
	Δ		0.3	-0.1	0.7	1.2				
D7	pH 2		172.8	51.5	41.0	178.5				45
	pH 7		171.9	52.6	42.2	180.1				10
	Δ		0.9	-1.1	-1.2	-1.6				
E22	pH 2		173.4	54.4	33.1	38.1		182.5		33
	pH 7		173.2	54.6	33.4	36.8		182.6		23
						40.3		183.1		
	Δ		0.2	-0.2	-0.3	1.3		-0.1		
D23	pH 2			53.7	35.0	176.6				85
					34.7	177.9				
	pH 7			53.3	34.5	177.1				75
					35.1	178.0				
	Δ			0.4	0.5	-0.5				
K28	pH 2	128.7		54.6	36.4	25.8		30	42.1	
	pH 7	128.1		54.6	36.6	26		30.3	42.4	
		128.1		54.9	35.3	25.5		30	42.7	
	Δ	0.6		0	-0.2	-0.2		-0.3	-0.3	
	Δ	0.6		-0.3	1.1	0.3		0	-0.6	
A42	pH 2	126.9	179.2	49.2	21.5					100
	pH 7	134.0	182.1	53.5	19.7					0
	Δ	-7.1	-2.9	-4.3	1.8					

References

1. Gremer, L.; Schölzel, D.; Schenk, C.; Reinartz, E.; Labahn, J.; Ravelli, R. B. G.; Tusche, M.; Lopez-Iglesias, C.; Hoyer, W.; Heise, H.; Willbold, D.; Schröder, G. F., Fibril structure of amyloid- β (1–42) by cryo-electron microscopy. *Science* **2017**, *358* (6359), 116-119.
2. McIlvaine, T. C., A buffer solution for colorimetric comparison. *J. Biol. Chem* **1921**, *49*, 183-186.
3. Zhang, O.; Kay, L. E.; Olivier, J. P.; Forman-Kay, J. D., Backbone ^1H and ^{15}N resonance assignments of the N-terminal SH3 domain of drk in folded and unfolded states using enhanced-sensitivity pulsed field gradient NMR techniques. *J. Biomol. NMR* **1994**, *4* (6), 845-858.
4. Ikura, M.; Kay, L. E.; Bax, A., A novel approach for sequential assignment of proton, carbon-13, and nitrogen-15 spectra of larger proteins: heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry* **1990**, *29* (19), 4659-4667.
5. Solyom, Z.; Schwarten, M.; Geist, L.; Konrat, R.; Willbold, D.; Brutscher, B., BEST-TROSY experiments for time-efficient sequential resonance assignment of large disordered proteins. *J. Biomol. NMR* **2013**, *55* (4), 311-321.
6. Wittekind, M.; Mueller, L., HNCACB, a High-Sensitivity 3D NMR Experiment to Correlate Amide-Proton and Nitrogen Resonances with the Alpha- and Beta-Carbon Resonances in Proteins. *J. Magn. Reson., Ser B* **1993**, *101* (2), 201-205.
7. Cavanagh, J.; Rance, M., Suppression of cross-relaxation effects in TOCSY spectra via a modified DIPSI-2 mixing sequence. *J. Magn. Reson.* **1992**, *96* (3), 670-678.
8. Kovacs, H.; Gossert, A., Improved NMR experiments with ^{13}C -isotropic mixing for assignment of aromatic and aliphatic side chains in labeled proteins. *J. Biomol. NMR* **2014**, *58* (2), 101-112.
9. König, A. S.; Rösener, N. S.; Gremer, L.; Tusche, M.; Flender, D.; Reinartz, E.; Hoyer, W.; Neudecker, P.; Willbold, D.; Heise, H., Structural details of amyloid β oligomers in complex with human prion protein as revealed by solid-state MAS NMR spectroscopy. *J. Biol. Chem.* **2021**, *296*, 100499.
10. Findeisen, M.; Brand, T.; Berger, S., A ^1H -NMR thermometer suitable for cryoprobes. *Magn. Reson. Chem.* **2007**, *45* (2), 175-178.
11. Grzesiek, S.; Bax, A., The importance of not saturating water in protein NMR. Application to sensitivity enhancement and NOE measurements. *J. Am. Chem. Soc.* **1993**, *115* (26), 12593-12594.
12. Marion, D.; Ikura, M.; Tschudin, R.; Bax, A., Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J. Magn. Reson.* **1989**, *85* (2), 393-399.
13. Kay, L.; Keifer, P.; Saarinen, T., Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc.* **1992**, *114* (26), 10663-10665.
14. Schleucher, J.; Sattler, M.; Griesinger, C., Coherence Selection by Gradients without Signal Attenuation: Application to the Three-Dimensional HNCO Experiment. *Angew. Chem. Int. Ed.* **1993**, *32* (10), 1489-1491.
15. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A., NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **1995**, *6* (3), 277-293.
16. Johnson, B. A.; Blevins, R. A., NMR View: A computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* **1994**, *4* (5), 603-614.
17. Vranken, W. F.; Boucher, W.; Stevens, T. J.; Fogh, R. H.; Pajon, A.; Llinas, M.; Ulrich, E. L.; Markley, J. L.; Ionides, J.; Laue, E. D., The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins* **2005**, *59* (4), 687-696.
18. Markley, J. L.; Bax, A.; Arata, Y.; Hilbers, C. W.; Kaptein, R.; Sykes, B. D.; Wright, P. E.; Wüthrich, K., Recommendations for the presentation of NMR structures of proteins and nucleic acids (IUPAC Recommendations 1998). *Pure Appl. Chem.* **1998**, *70* (1), 117-142.

19. Keifer, P. A., Chemical-shift referencing and resolution stability in gradient LC–NMR (acetonitrile:water). *J. Magn. Reson.* **2009**, *199* (1), 75-87.
20. Berjanskii, M. V.; Wishart, D. S., A Simple Method To Predict Protein Flexibility Using Secondary Chemical Shifts. *J. Am. Chem. Soc.* **2005**, *127* (43), 14970-14971.
21. Shen, Y.; Bax, A., Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *J. Biomol. NMR* **2013**, *56* (3), 227-241.
22. Schwarzing, S.; Kroon, G. J. A.; Foss, T. R.; Chung, J.; Wright, P. E.; Dyson, H. J., Sequence-Dependent Correction of Random Coil NMR Chemical Shifts. *J. Am. Chem. Soc.* **2001**, *123* (13), 2970-2978.
23. Thanabal, V.; Omecinsky, D. O.; Reily, M. D.; Cody, W. L., The ¹³C chemical shifts of amino acids in aqueous solution containing organic solvents: Application to the secondary structure characterization of peptides in aqueous trifluoroethanol solution. *J. Biomol. NMR* **1994**, *4* (1), 47-59.
24. McIlvaine, T. C., A buffer solution for colorimetric comparison. *J. Biol. Chem.* **1921**, *49* (1), 183-186.
25. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletsky, O.; Glaser, S. J.; Sørensen, O. W.; Griesinger, C., A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J. Biomol. NMR* **1994**, *4* (2), 301-306.
26. Hwang, T. L.; Shaka, A. J., Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. *J. Magn. Reson., Ser A* **1995**, *112* (2), 275-279.
27. Friedrichs, M. S., A model-free algorithm for the removal of baseline artifacts. *J. Biomol. NMR* **1995**, *5* (2), 147-153.
28. Fung, B. M.; Khitrin, A. K.; Ermolaev, K., An Improved Broadband Decoupling Sequence for Liquid Crystals and Solids. *J. Magn. Reson.* **2000**, *142* (1), 97-101.
29. Baldus, M.; Petkova, A. T.; Herzfeld, J.; Griffin, R. G., Cross polarization in the tilted frame: assignment and spectral simplification in heteronuclear spin systems. *Mol. Phys.* **1998**, *95* (6), 1197-1207.
30. Friege, B.; Gremer, L.; Heise, H.; Willbold, D.; Gohlke, H., Binding modes of thioflavin T and Congo red to the fibril structure of amyloid-β(1–42). *Chem. Commun.* **2020**, *56* (55), 7589-7592.
31. Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L., Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **1983**, *79* (2), 926-935.
32. Schafmeister, C. E. A. F.; Ross, W. S.; Romanowski, V., *LEaP*. University of California, San Francisco, 1995.
33. D. A. Case; R. M. Betz; W. Botello-Smith; D. S. Cerutti; T. E. Cheatham III; T. A. Darden; R. E. Duke; T. J. Giese; H. Gohlke; A. W. Goetz; N. Homeyer; S. Izadi; P. Janowski; J. Kaus; A. Kovalenko; T. S. Lee; S. LeGrand; P. Li; C. Lin; T. Luchko; R. Luo; B. Madej; D. Mermelstein; K. M. Merz; G. Monard; H. Nguyen; H. T. Nguyen; I. Omelyan; A. Onufriev; D. R. Roe; A. Roitberg; C. Sagui; C. L. Simmerling; J. Swails; R. C. Walker; J. Wang; R. M. Wolf; X. Wu; L. Xiao; D. M. York; Kollman, P. A., *Amber 16*. University of California, San Francisco, 2016.
34. Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C., ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theor. Comp.* **2015**, *11* (8), 3696-3713.
35. Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Kollman, P. A., Application of RESP charges to calculate conformational energies, hydrogen bond energies, and free energies of solvation. *J. Am. Chem. Soc.* **1993**, *115* (21), 9620-9631.
36. Bayly, C. I.; Cieplak, P.; Cornell, W.; Kollman, P. A., A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* **1993**, *97* (40), 10269-10280.
37. Friege, B.; Görg, B.; Homeyer, N.; Keitel, V.; Häussinger, D.; Gohlke, H., Molecular Mechanisms of Glutamine Synthetase Mutations that Lead to Clinically Relevant Pathologies. *PLOS Comput. Biol.* **2016**, *12* (2), e1004693.
38. Bhatia, S.; Diedrich, D.; Friege, B.; Ahlert, H.; Stein, S.; Bopp, B.; Lang, F.; Zang, T.; Kröger, T.; Ernst, T.; Kögler, G.; Krieg, A.; Lüdeke, S.; Kunkel, H.; Rodrigues Moita, A. J.; Kassack, M. U.;

- Marquardt, V.; Opitz, F. V.; Oldenburg, M.; Remke, M.; Babor, F.; Grez, M.; Hochhaus, A.; Borkhardt, A.; Groth, G.; Nagel-Steger, L.; Jose, J.; Kurz, T.; Gohlke, H.; Hansen, F. K.; Hauer, J., Targeting HSP90 dimerization via the C terminus is effective in imatinib-resistant CML and lacks the heat shock response. *Blood* **2018**, *132* (3), 307-320.
39. Frieg, B.; Häussinger, D.; Gohlke, H., Towards restoring catalytic activity of glutamine synthetase with a clinically relevant mutation. In *Proceedings of the NIC Symposium 2016*, Binder, K.; Müller, M.; Kremer, M.; Schnurpfeil, A., Eds. Jülich, 2016; pp 97-104.
40. G. R. Desiraju; Steiner, T., *Weak hydrogen bond*. Oxford University Press New York: 2001.
41. Hackl, E. V.; Darkwah, J.; Smith, G.; Ermolina, I., Effect of acidic and basic pH on Thioflavin T absorbance and fluorescence. *European Biophysics Journal* **2015**, *44* (4), 249-261.
42. Keim, P.; Vigna, R. A.; Morrow, J. S.; Marshall, R. C.; Gurd, F. R. N., Carbon 13 Nuclear Magnetic Resonance of Pentapeptides of Glycine Containing Central Residues of Serine, Threonine, Aspartic and Glutamic Acids, Asparagine, and Glutamine. *J. Biol. Chem.* **1973**, *248* (22), 7811-7818.
43. Platzner, G.; Okon, M.; McIntosh, L. P., pH-dependent random coil ¹H, ¹³C, and ¹⁵N chemical shifts of the ionizable amino acids: a guide for protein pK_a measurements. *J. Biomol. NMR* **2014**, *60* (2), 109-129.

Isoleucine Side Chains as Reporters of Conformational Freedom in Protein Folding Studied by DNP-Enhanced NMR

Leonardo Levorin, Nina Becker, Boran Uluca-Yazgi, Luis Gardon, Mirko Kraus, Marc Sevenich, Athina Apostolidis, Kai Schmitz, Neomi Rüter, Irina Apanasenko, Dieter Willbold, Wolfgang Hoyer, Philipp Neudecker, Lothar Gremer, and Henrike Heise*



Cite This: *J. Am. Chem. Soc.* 2025, 147, 15867–15879



Read Online

ACCESS |



Metrics & More

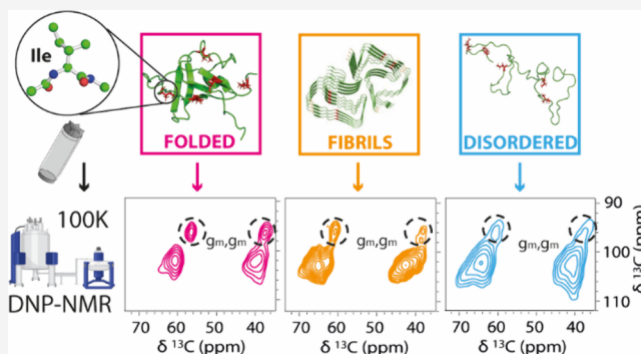


Article Recommendations



Supporting Information

ABSTRACT: Conformations of protein side chains are closely linked to protein function. DNP-enhanced solid-state NMR (ssNMR), which operates at cryogenic temperatures (<110 K), can be used to freeze-trap protein conformations, including the side chains. In the present study, we employed two-dimensional DNP-enhanced ssNMR to get detailed insights into backbone and side chain conformations of isoleucine. We used different amino acid selectively labeled model proteins for intrinsically disordered proteins (IDPs), denatured and well-folded proteins, and amyloid fibrils. ^{13}C chemical shifts are closely correlated with secondary structure elements and χ_1 and χ_2 angles in isoleucine side chains. Thus, line shape analysis by integration of representative peak areas in 2D spectra provides an accurate overview of the distribution of backbone and side chain conformations. For the well-folded proteins GABARAP and bovine PI3-kinase (PI3K) SH3 domain, most Ile chemical shifts in frozen solution are well resolved and similar to those observed in solution. However, line widths of individual Ile residues are directly linked to residual mobility, and line broadening or even signal splitting appears for those Ile residues, which are not part of well-defined secondary structure elements. For unfolded PI3K SH3 and the IDP α -synuclein (α -syn), all Ile side chains have full conformational freedom, and as a consequence, inhomogeneous line broadening dominates the cryogenic spectra. Moreover, we demonstrate that conformational ensembles of proteins strongly depend on solvent and buffer conditions. This allowed different unfolded structures for chemical and acidic pH denaturation of the PI3K SH3 domain to be distinguished. In amyloid fibrils of α -syn and PI3K SH3, chemical shifts typical for β -strand like secondary structure dominate the spectra, whereas Ile residues belonging to the fuzzy coat still add the IDP-type line shapes. Hence, DNP-enhanced ssNMR is a useful tool for investigating side chain facilitated protein functions and interactions.



INTRODUCTION

Protein side chain conformations are closely related to protein function since they play a key role in enzyme active site chemistry and interaction with small molecules or other ligands.^{1,2} At physiological temperature in solution, side chains of amino acid residues in certain regions of a protein may still exhibit substantial conformational freedom, which results in rapid averaging of an extensive conformational ensemble in many spectroscopic experiments. In crystal structures, on the other hand, most side chains are confined to one energetic minimum, which does not necessarily represent the full conformational space sampled by a residue. Likewise, NMR structures of proteins in solution are often based on averaged J-couplings, which reflect average values of torsional angles instead of the actual rotamer distribution, which may also distort the representation of side chains in solution NMR structures. Dynamics and flexibility of protein domains can be mapped by residual dipolar couplings and NMR relaxation,

and combined with MD simulations to obtain conformational ensembles at ambient temperatures.^{3–6} Recently, several approaches have been developed to experimentally determine conformational ensembles directly in fully or partly disordered proteins in frozen solution^{7–12} or in lyophilized form¹³ by solid-state NMR-spectroscopy, using conformation-dependent chemical shifts^{14,15} as reporters for secondary structure. Upon freezing in a cryoprotectant medium, the exchange between different conformations is halted, and all conformations sampled by each nucleus are present with their respective probability.^{7,8,16–19} Furthermore, the combination of frozen

Received: March 10, 2025

Revised: April 13, 2025

Accepted: April 15, 2025

Published: April 26, 2025



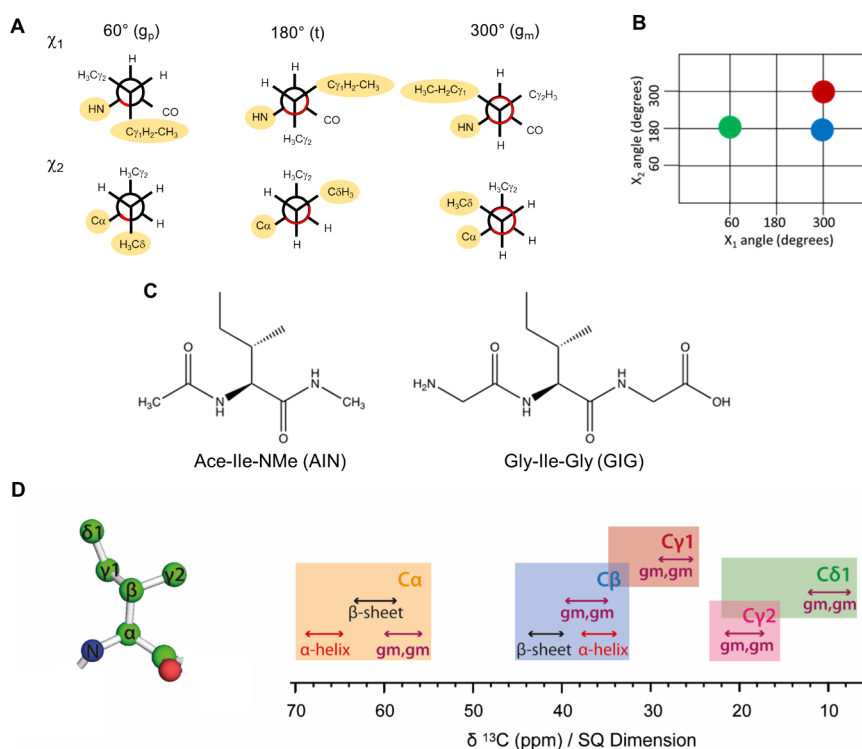


Figure 1. (A) Newman projections of the χ_1 and χ_2 dihedral angles for isoleucine. Correlation between χ_1 and χ_2 dihedral angles. (B) The most populated three rotameric states (g_m,t), (g_m,g_m), and (g_p,t) are highlighted with a red, green, and blue circle, respectively (right). (C) Structure of the random coil mimic peptides AIN and GIG. (D) Ile side chain structure (left; heavy atoms only) and typical chemical shift regions for each Ile side chain ^{13}C (right).

solution NMR with dynamic nuclear polarization (DNP) makes it possible to study distributions of conformations in large proteins with high sensitivity.^{9,12,20,21} After focusing on the backbone conformations in a previous study,⁹ we started to investigate the conformational space sampled by isoleucine side chains. The chemical shifts of isoleucine (Ile) side chain carbons strongly reflect the side chain torsion angles χ_1 and χ_2 , which each have three energetic minima at $\sim 60^\circ$, $\sim 180^\circ$, and $\sim 300^\circ$, referred to as gauche+ (g_p), trans (t), and gauche- (g_m), respectively.

Density functional theory (DFT) calculations of rotameric states from ^{13}C chemical shifts as well as from scalar couplings have shown that three of the nine possible rotameric states. χ_1 and χ_2 angles in the combinations (g_m,t), (g_p,t), and (g_m,g_m), represent over 95% of all rotameric states sampled by the side chains in solution at ambient temperature (Figure 1A,B).²⁰

In our previous work, the rotamer distributions of isoleucine in two random coil peptide mimetics, Ace-Ile-NMe (AIN) and Gly-Ile-Gly (GIG) (Figure 1C), were calculated from the experimental chemical shifts and scalar couplings for two peptides. ^{13}C chemical shift distributions obtained from DNP-enhanced solid-state NMR spectra of these peptides in frozen solution supported the finding that the random coil sampling of AIN and GIG is significantly different from that obtained from the average of all isoleucine side chains in a large set of high-resolution crystal structures.²⁰ In particular, the $\text{C}\delta 1$ chemical shift is highly sensitive to the χ_2 torsion angle; in the g_m conformation the $\text{C}\delta 1$ methyl group has two γ -gauche substituents (Figure 1A), which reduces the chemical shift by ~ 5.5 ppm.²² Typical NMR chemical shift values for Ile ^{13}C are shown in Figure 1D.

In this contribution, we adopt the approach to exploit isoleucine residues as structural reporters for backbone and side chain conformational freedom in proteins recombinantly expressed with uniform ^{13}C , ^{15}N isotope labeling exclusively for isoleucine residues.²³ We studied three different proteins representing well-folded, intrinsically disordered, misfolded and fibrillar states by DNP-enhanced solid-state NMR spectroscopy in frozen solution, under different conditions.

As an example of a stable, well-folded protein with a defined secondary and tertiary structure, we studied the γ -aminobutyric acid type A receptor-associated protein (GABARAP), a 117 amino acid residue protein playing an important role in autophagy.^{24,25}

In contrast, intrinsically disordered proteins (IDPs) can readily adopt different secondary structures under different conditions.^{26–29} One member of the group of IDPs is the so-called ‘protein-chameleon’ α -synuclein (α -syn),³⁰ and its aggregation is associated with various synucleinopathies including Parkinson’s Disease, dementia with Lewy bodies and multiple system atrophy.³¹ In its monomeric form α -syn is fully disordered, upon fibrillation the extreme N- and C-termini stay disordered while the middle region adopts a cross β structure forming the fibril core.³² Therefore, Ile-labeled α -syn was chosen as a model protein to study a natively unfolded monomeric as well as a fibrillar state.

The bovine PI3-kinase (PI3K) SH3 domain is a well-studied model protein for the investigation of protein folding, unfolding and aggregation.³³ The structure of natively folded PI3K SH3, a globular domain consisting of 86 amino acids, from bovine PI3K³⁴ and its human homologue^{35–37} has been well-characterized by X-ray crystallography and NMR spectroscopy. At low pH, the protein unfolds and forms amyloid

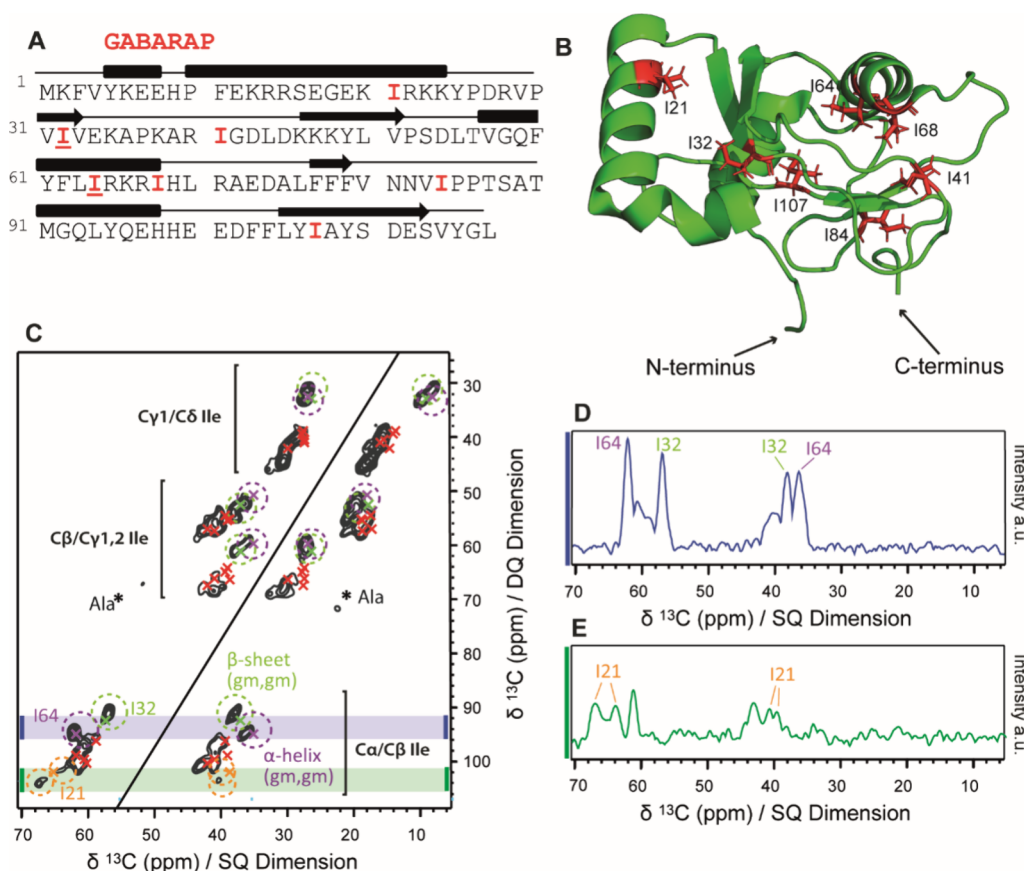


Figure 2. (A) Primary sequence of GABARAP with the positions of isoleucine residues highlighted in red. The secondary structure elements α -helix and β -strand are depicted in rectangles and arrows, respectively. (B) 3D structure of GABARAP with Ile residues labeled and marked in red (1KOT).⁴² (C) 2D ^{13}C – ^{13}C correlation DQ/SQ spectrum of GABARAP recorded at cryogenic temperatures in frozen solution. Green and purple circles show typical regions for β -strand and α -helical (g_m, g_m) states, respectively; green (I32), purple (I64), orange (I21), and red (all other Ile residues) crosses mark the experimental chemical shifts from solution NMR.⁴³ The splitting of the I21 Ca – $\text{C}\beta$ correlation in frozen solution is highlighted using orange circles. Ala cross peaks are marked with asterisks. The spectrum was Fourier transformed without apodization of the FIDs in both dimensions. (D, E) Significant 1D projections extracted from the marked areas of the original 2D data set shaded in gray in panel (B).

fibrils,^{33,38,39} whose structure was characterized by solid-state NMR spectroscopy⁴⁰ and more recently in high resolution by cryoEM.⁴¹ Here, by using Ile residues as structural reporters, we investigate the different globular, unfolded and fibrillar conformations of PI3K SH3 as a third model system.

RESULTS

Rotational Freedom in a Folded Protein: GABARAP.

The globular protein GABARAP was selected as a candidate for studying residual dynamics in a well-folded protein by comparing solution NMR data measured at room temperature to DNP-enhanced data recorded in frozen solution at 100 K. As the seven isoleucine residues are distributed all over the sequence, they represent all parts of the protein (Figure 2A,B). Three isoleucine residues are located in α -helices (I21, I64, and I68), two in β -strands (I32 and I107), and two in loop regions (I41 and I84) of the protein.

We recorded a 2D double quantum/single quantum (DQ/SQ) spectrum for selectively Ala- and Ile-labeled GABARAP in frozen solution (Figure 2C). DQ/SQ experiments were preferred over standard proton driven spin diffusion spectra (PDS, Figure S1B), since they show only one bond correlations and, therefore, allow a full discrimination between $\text{C}\gamma_2$ and $\text{C}\delta_1$ resonances, which partially overlap in PDS spectra due to their similar shift range. In this folded protein,

the ^{13}C resonances are well-dispersed at ambient and cryogenic temperatures, and a good resolution with ^{13}C line widths below 1.5 ppm for most resonances was obtained even at 100 K. Ala cross peaks, which are outside of the relevant spectral area for Ile cross peaks, are relatively weak, possibly due to isotopic scrambling, and are not further evaluated in this study.

As shown in Figure 2C, most of the well-separated DNP-NMR peaks in frozen solution match the solution NMR chemical shift values, allowing the identification of the spin systems for all seven isoleucine residues (Figure S1).⁴³ As ^{13}C line widths in frozen solution reflect the degree of residual conformational disorder of the side chains, the rather high overall resolution in this spectrum is explained by the well-defined rotamers for most Ile residues of GABARAP,⁴ which is typical for a globular protein, in contrast to an IDP.

However, the line widths for different Ile residues in GABARAP in frozen solution vary significantly (Table S1). Residues I32 and I64 exhibit extremely narrow lines (full width at half-maximum (fwhm) of 1.3 ± 0.1 and 1.4 ± 0.1 ppm, respectively) for all five side chain ^{13}C atoms, which indicate particularly high conformational restriction for both residues. These two residues also exhibit low $\text{C}\delta_1$ chemical shifts <10 ppm, which are typical for (g_m, g_m) rotameric conformations for both dihedral angles χ_1 and χ_2 . Indeed, these conformations are also confirmed in the PDB structure 1KOT⁴² (Table 1).

Table 1. Torsion Angles of the Isoleucine Side Chains in GABARAP, Measured in PyMOL Using the PDB File 1KOT⁴²

Ile	χ_1 angle (°)	state	χ_2 angle (°)	state
21	−72.2	g_m	151.7	trans
32	−44.4	g_m	−53.4	g_m
41	35.9	g_p	177.1	trans
64	−64.6	g_m	−55.3	g_m
68	17.1	g_p	162.9	trans
84	−56.6	g_m	−152.9	trans
107	−59.1	g_m	−160.6	trans

Both residues are located in well-defined regions of the protein: I32 in a β -sheet and I64 in an α -helix. Such a conformational restriction of the backbone in a defined secondary structure element may be a necessary but not sufficient prerequisite for the confinement of the side chain to the (g_m/g_m) rotamer. In contrast, for I21, located in an α -helix lining one of the two hydrophobic binding pockets, the single $Ca-C\beta$ correlation visible at room temperature (Figure S1A) is split into two distinct $Ca-C\beta$ cross-peaks at cryogenic temperatures (Figure 2C). One of these two signals has

random-coil like $^{13}C\alpha$ shifts (63 ppm), while the other is shifted strongly toward α -helical chemical shifts (67 ppm). By integrating over the areas of I32, I64 and the split I21 peaks and relating them to the full signal volume (see Methods), we measured a fraction of the total signal volume of around 15% for each of them (Table S2). This result is in agreement with seven Ile residues ($1/7 \cong 0.14$) contributing to the total integral. The I21 signal is split into two peaks, with an integral of about 7% each. This suggests that the $^{13}C\alpha$ chemical shift observed in solution at room temperature (64.9 ppm) represents an average between two rapidly exchanging states with roughly equal populations.

Isoleucine in an Intrinsically Disordered Protein: α -Syn. To study the distribution of Ile side chain ^{13}C chemical shifts in an IDP we selected α -syn. The primary sequence of α -syn has two Ile residues; one in the central amyloidogenic (formerly known as NAC) region (aa 61–95) at position 88, the other in the highly negatively charged C-terminus at position 112 (Figure 3A).

The DQ/SQ spectrum of monomeric α -syn shows relatively broad peaks (average fwhm of 3.6 ppm), indicating a very high degree of conformational freedom for the two Ile residues I88 and I112. The overlay of a DQ/SQ spectra of α -syn with that

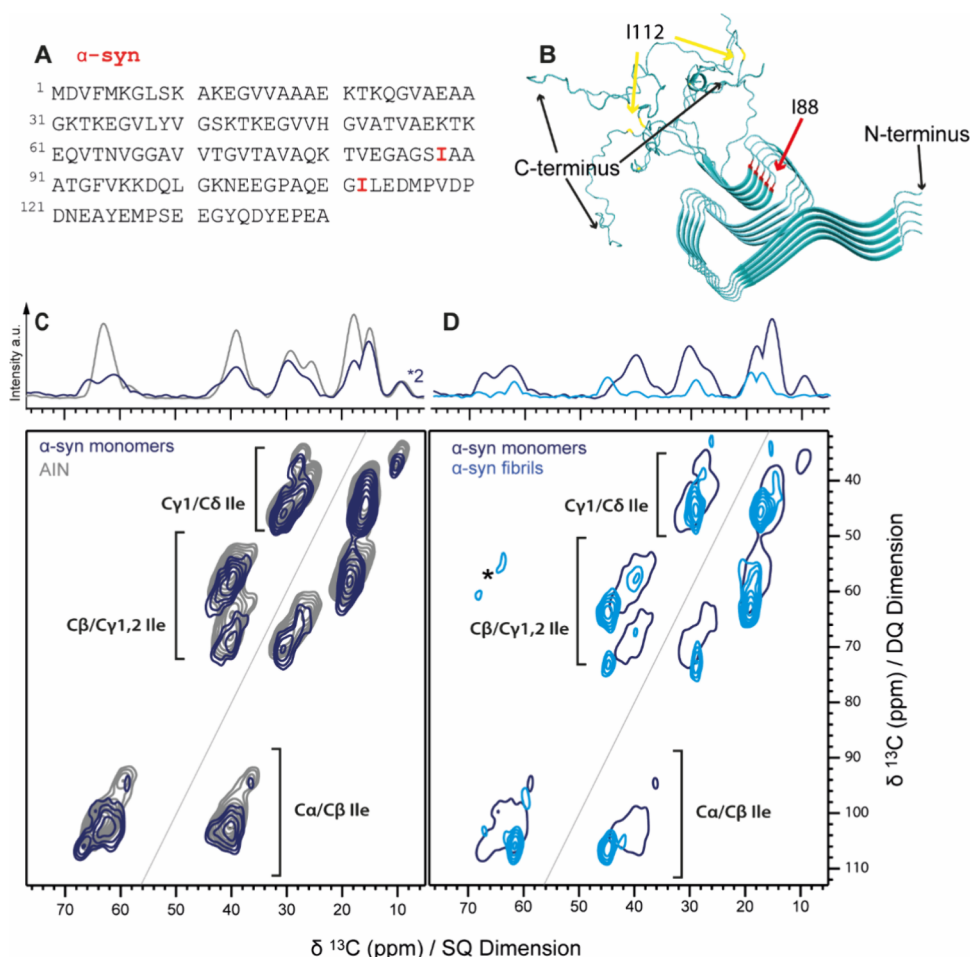


Figure 3. (A) Primary sequence of α -syn with both isoleucine I88 and I112 residues highlighted in red. (B) 3D structure of α -syn fibrils (PDB 2N0A) with the positions of I88 (cross- β core region) marked in red and I112 (flexible and therefore not confined region) in yellow.⁴⁴ (C) 2D ^{13}C - ^{13}C correlation DQ/SQ spectra of AIN (gray) and isoleucine-labeled monomeric α -syn (navy) recorded at cryogenic temperatures in frozen solution. (D) Isoleucine-labeled α -syn fibrils (blue) and the lowest contour level of α -syn monomers shown in (C) for comparison. Spinning sidebands of glycerol are marked with asterisks. 1D projections of the full spectra are shown on top of the spectra in (C) and (D).

of the random coil mimetic AIN²⁰ (Figure 3C) shows that the chemical shift range for Ile residues in α -syn roughly covers the full rotameric ensemble of an unfolded peptide mimetic. While chemical shift distributions for side chain carbon atoms agree well in both molecules, $C\alpha$ chemical shifts of α -syn exhibit a larger dispersion than in AIN, with two distinct maxima around 66 and 62 ppm instead of one very broad maximum at 64 ppm, as observed for AIN. This finding reflects differences in the energy landscape for backbone conformations between a small peptide mimetic and a full protein. To quantify the amount of (g_m/g_m) conformation in Ile side chains and to obtain an estimate of the relative amount of α -helix like backbone conformations, respective peak areas were integrated and related to the full signal volume (see Methods). These integrated peak volumes from the α -syn DQ/SQ spectrum are in agreement with the peak volumes measured for AIN (Table 2 and Table S3) for (g_m/g_m) conformations, while the preference for α -helical conformations is slightly higher in monomeric α -syn.

Table 2. Populations of Different Conformations and Average Line Widths of Signals in Spectra of α -Syn and the Random Coil Peptide Mimetic AIN

	g_m/g_m population (%)	estimate of α -helical population (%)	range of fwhm (ppm)
AIN	14 $\pm$ 1	15 $\pm$ 1	2.2–3.2
α -syn (native)	15 $\pm$ 2	18 $\pm$ 2	2.7–4.9
α -syn (fibrils)	17 $\pm$ 6	9 $\pm$ 6	1.6–3.5

We thus could show that the side chains of both Ile residues in α -syn cover a broad conformational space, which is close to the conformational space in the random coil Ile mimetic AIN, while the backbone conformational space showed more pronounced conformational preferences toward either α -helical or extended conformations in α -syn. Overall, these results confirm the IDP character of α -syn.

For comparison, we recorded a DQ/SQ spectrum of Ile-labeled α -syn fibrils (Figure 3B) in frozen suspension (Figure 3D). The resonance pattern of the two Ile residues in the fibrillar form is dominated by peaks that are significantly narrower (see Table S4) than those of the monomeric IDP state and have chemical shifts typical for β -sheet secondary structure (Figure 3D). However, in addition, weak and broad signals covering a significant part of the typical random coil line shape pattern can also be observed. Even without knowing the exact fibril structure, we expect high β -sheet content for I88, as it is part of the fibril core in most fibril structures known so far.^{44,45} I112 on the other hand is expected to be disordered, as it is not inside the fibril core (Figure 3B) and thus gives rise to strongly inhomogeneously broadened signals. Although precise quantification of the signal patterns is challenging, the overall pattern is in line with a superposition of a well-defined I88 spin system with β -sheet secondary structure and a spin system from a disordered I112 residue.

Different Stages in Protein Folding: PI3K SH3–Folded, Unfolded, and Fibrillar State. To investigate different stages of protein folding, unfolding and misfolding, we chose the bovine PI3K SH3 domain (Figure 4A), which differs from its human homologue p85 α PI3K SH3 by only one residue in position 49. This SH3 domain is a promising model system, as it can adopt many different (meta)stable states

depending on pH, buffer conditions and temperature. At neutral pH, the protein adopts the stable five-stranded antiparallel β -sandwich fold typical of SH3 domains^{34–37} (Figure 4B).

Compared to most SH3 domains from the src tyrosine kinase family, however, the n-src loop connecting strands β 2 and β 3 is approximately 15 residues longer in the PI3K SH3 and somewhat mobile, as reflected in reduced⁴⁶ ¹⁵N NOE values.³⁴ At acidic pH the PI3K SH3 domain (partially) unfolds, and in this unfolded state, it is also prone to fibril formation.³³ The structure of the amyloid fibrils formed at pH 2.5 has recently been determined by cryoEM.⁴¹ In this fibril type, almost the entire protein is part of the well-defined parallel in-register β -sheet core (Figure 5A). We used the five isoleucine residues in the PI3K SH3 domain (Figure 4A) as reporters for backbone and side chain flexibility in the native, unfolded and fibrillar state in frozen solution.

Native State and Unfolded States. The PI3K SH3 domain is well-ordered at neutral pH. In the native form, two isoleucines are located in β -strands β 2 (I29) and β 5 (I77) (Figure 4A). I22 is located in the relatively rigid hairpin-like RT-src loop connecting strands β 1 and β 2 (Figure 4B). I53 is located in a helical loop at the end of the somewhat mobile n-src loop, while I82 is part of the C-terminal tail, which is disordered.³⁴ The DNP spectrum recorded in frozen buffer solution containing 60% glycerol is shown in Figure 4C and agrees well with the chemical shifts obtained by solution NMR spectroscopy (Figure S2), which in turn are similar to the chemical shifts published previously for bovine PI3K SH3.⁴⁷ However, in frozen solution, the chemical shift dispersion of the five PI3K SH3 isoleucine residues is insufficient to resolve individual resonances due to inhomogeneous line broadening which is larger than the shift dispersion for most of the residues. Consequently, most signals overlap in the DNP spectrum, forming a composite set of cross-peaks. This observation aligns with the domain's structure, as PI3K SH3 contains isoleucine residues (e.g., I83) within less ordered regions. Notably, I29, located within a β -sheet (strand β 2), exhibits a characteristic low $C\delta$ chemical shift and distinctive $C\alpha$ and $C\beta$ shifts in solution NMR, indicative of the (g_m/g_m) side chain conformation, consistent with that observed in most PI3K SH3 PDB structures (Table 3). These shifts are clearly visible in both solution (Figure S2) and frozen solution spectra (Figure 4C). To confirm this assignment, we expressed a PI3K SH3 sequence variant where I29 is replaced by the also β -branched amino acid valine (Figure S3A), a point mutation, which does not perturb the protein structure, as confirmed by solution NMR (Figure S3C). The absence of the well-resolved (g_m/g_m) signals in the corresponding spectrum in frozen solution of this variant unambiguously identifies these signals as originating from I29 (Figure S4A).

To unambiguously identify the signals of I53, which is located in a helical turn in the mobile n-src loop (Figure 4B), we expressed a second sequence variant, where I22, I77 and I82 were replaced with valine (Figure S3B). Also in this variant, the overall structure of the PI3K SH3 domain was conserved (Figure S3D). The DNP spectrum of this mutant displays two distinct $C\alpha/C\beta$ cross peak signals for I53, in addition to the (g_m/g_m) peak of I29 (Figure 4C and Figure S5A). Thus, I53, like I21 in GABARAP, exhibits a doubling of the $C\alpha$ resonance. This observation is consistent with I53's location within a helical turn in the mobile n-src loop (Figure 4B).

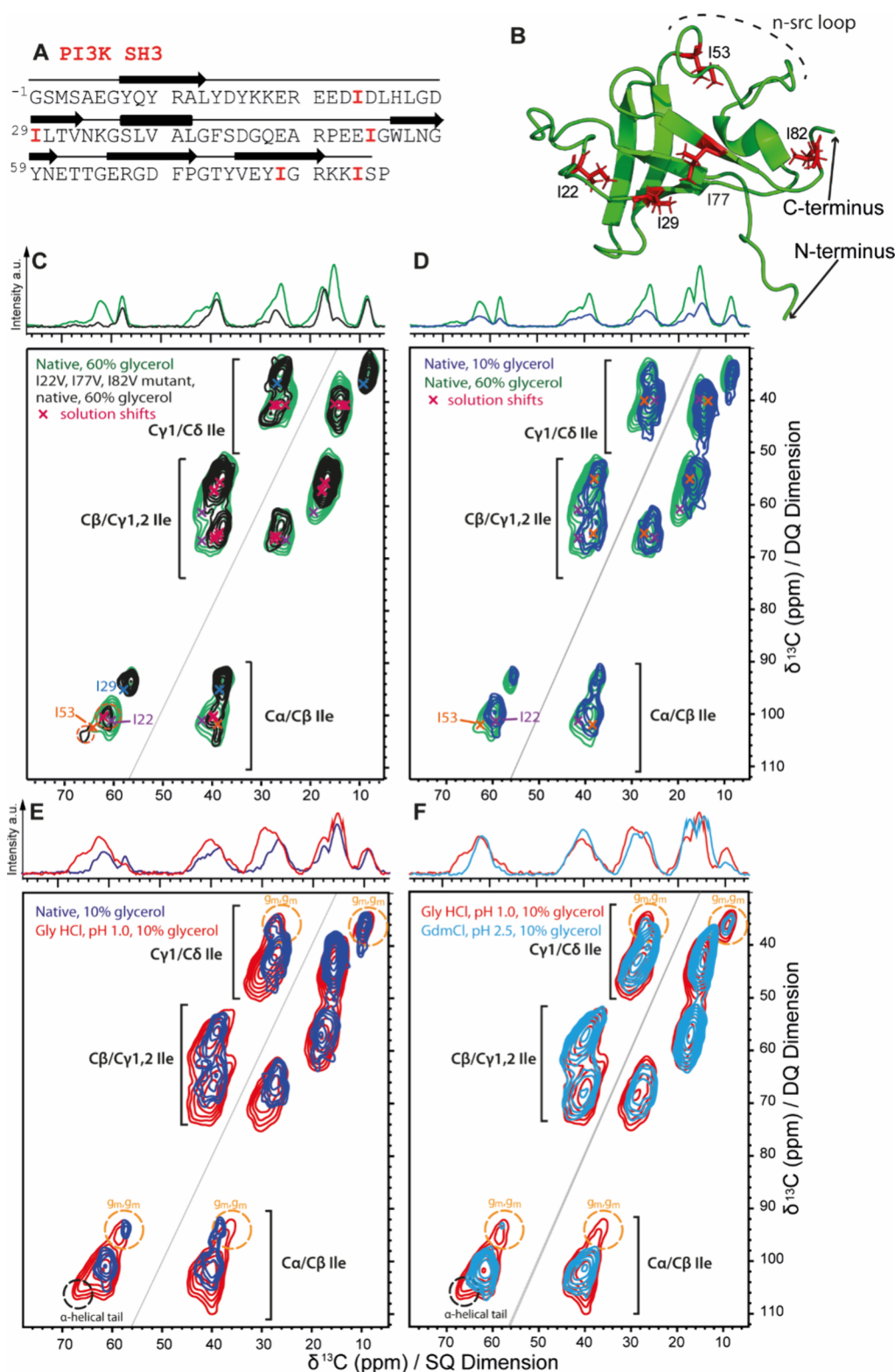


Figure 4. (A) Primary sequence of PI3K SH3 with the positions of isoleucine residues highlighted in red. The secondary structure elements α -helix and β -strand in the native structure are depicted as rectangles and arrows, respectively. (B) 3D structure of PI3K SH3 in its native form (1PNJ).³⁴ The five Ile residues are labeled in red. (C) 2D ^{13}C – ^{13}C correlation DQ/SQ spectra of isoleucine-labeled PI3K SH3 WT (green) and its I22V/I77V/I82V variant (black) in its native form recorded at cryogenic temperatures in frozen solution with 60% glycerol. The NMR chemical shifts of the natively folded protein in solution (Figure S2) are indicated by crosses. The peak doubling experienced by I53 is highlighted with orange circles. (D) Comparison between 2D ^{13}C – ^{13}C correlation DQ/SQ spectra of isoleucine-labeled PI3K SH3 in its native form recorded at cryogenic temperatures in frozen solution with 60% glycerol (green) and 10% glycerol (navy blue). (E) Comparison of isoleucine-labeled PI3K SH3 in its native form in 10% glycerol (navy blue) with its unfolded state at pH 1.0 (red). (F) Comparison between DQ/SQ spectra of Ile-labeled PI3K SH3 at pH 1.0 (red) and at pH 2.5 in the presence of 6 M GdmCl (light blue). 1D projections of the 2D spectra are shown on top.

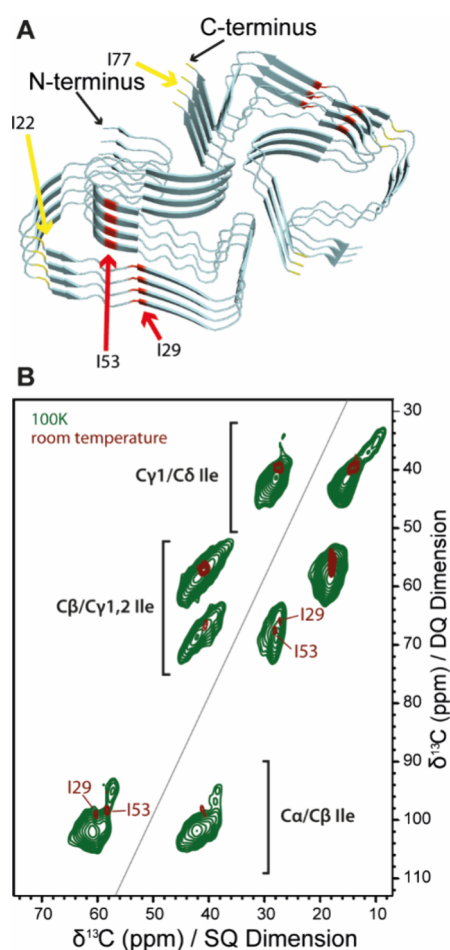


Figure 5. (A) 3D structure of PI3K SH3 fibrils (6R4R)⁴¹ spanning the sequence region from M1 to I77, with the isoleucine residues highlighted in red (fixed region) and yellow (flexible region). The disordered C-terminus including I82 is not part of the structural model deposited with the PDB. (B) 2D ¹³C–¹³C DQ/SQ spectrum of isoleucine-labeled PI3K SH3 in its fibrillar form in frozen solution (green) overlaid with that of fibrillar PI3K SH3 recorded at ambient temperature (brown).

Since some of the five Ile residues are located in regions with enhanced flexibility, the range of backbone as well as of side chain conformations are expected to be sensitive reporters on the effect of changes in solution conditions.

To study the effect of pH and solvent conditions on the conformational ensemble adopted by PI3K SH3 in more detail, we also performed a series of DNP experiments in different buffers containing only 10% glycerol as a cryoprotectant.

Decreasing the glycerol concentration in the solvent from 60% to 10% significantly narrows the chemical shift range of inhomogeneously broadened signals in phosphate buffer at pH 6.8 (Figure 4D). Notably, the minor peak characteristic of α -helical propensity, previously observed for the $Ca/C\beta$ cross-peak (64–66 ppm of SQ and 100–110 ppm DQ) at 60% glycerol, is greatly diminished at 10% glycerol. This reduction in α -helical signal is also evident in the I22V/I77V/I82V variant for Ile53 (Figure S5A), for which the estimate of α -helical propensity is reduced (Table S5).

Moreover, in the spectra of the WT-PI3K SH3 domain, the range of $C\beta$ and $C\gamma 1$ shifts is reduced at 10% glycerol concentration. In particular, the $C\beta$ and $C\gamma 1$ chemical shifts of I22 in native solution (indicated as crosses in Figure 4D) are no longer covered by the inhomogeneous peak shapes at 10% glycerol, indicating that an amount of 60% glycerol indeed helps to stabilize native conformations in proteins at cryogenic temperature. Therefore, 10% glycerol were used to study unfolded states of PI3K SH3.

In frozen solution in a pH 2.5 buffer with 10% glycerol, the overall appearance of the spectrum does not differ substantially from that obtained at pH 6.8 (Figure S6), suggesting that the native fold is not affected by this pH value at temperatures close to the freezing point. By contrast, in liquid solution at pH 2.5 the PI3K SH3 domain is in a temperature-dependent equilibrium between the native state and a denatured state, the latter becoming the dominant form at temperatures above 40 °C (99% denatured form).³³ Lowering the pH further to a value of 1.0 leads to a substantial broadening of all lines in the low-temperature spectrum (Figure 4E; average fwhm of 4.09 ppm), suggesting that the backbone conformational space is enhanced due to the lack of secondary structure restraints. At pH 1.0, the $Ca/C\beta$ cross peak covers the typical chemical shift range of IDPs (see Figure 3C), including even α -helical chemical shifts. Furthermore, the reduced intensity of peaks characteristic of the ($g_m g_m$) conformation, compared to folded PI3K SH3, indicates a loss of structural constraint for I29. This suggests that, at this pH, single conformation restrictions are abolished, allowing each residue to explore its entire conformational landscape. This conclusion is supported by analyses of the I29V (Figure S4B) and I22V/I77V/I82V (Figure S5B) variants (Table S5).

The PI3K SH3 can alternatively be unfolded by addition of 6 M guanidine hydrochloride (GdmCl) at pH 2.5 (Figure 4F). While the overall line-width for most resonances is as broad as for unfolded PI3K SH3 at pH 1.0, some significant differences are observed: (i) The $Ca/C\beta$ cross peaks are devoid of any α -helical population, and (ii) the ($g_m g_m$) population is lower than for PI3K SH3 at pH 1.0. This finding indicates that

Table 3. Rotameric States of the Isoleucine Side Chains in the Human PI3K SH3 Determined in PyMOL Using the PDB Files 2PNI,³⁴ 3ISS,³⁷ 1PKT,³⁵ 1PHT,³⁶ and 6R4R^{41a}

	2PNI (NMR, 26 models)		3ISS (X-ray, 4 subunits)		1PKT (NMR, 30 models)		1PHT (X-ray)		6R4R (fibril, CryoEM)	
residue	$\chi 1$	$\chi 2$	$\chi 1$	$\chi 2$	$\chi 1$	$\chi 2$	$\chi 1$	$\chi 2$	$\chi 1$	$\chi 2$
I22	g_p	t	g_p	t	g_p	n.d.	g_p	t	g_m	t
I29	g_m	n.d.	g_m	g_m	g_m	n.d.	g_m	g_m	g_m	t
I53	g_m	t	g_m	t	g_m	t	g_m	t	g_p	t
I77	g_p	t	g_p	t	n.d.	n.d.	g_p	t	g_m	t
I82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	g_m^*	g_m^*	n.d.	n.d.

^aDihedral angles whose circular variance over the ensemble of models/subunits exceeds 0.1 were considered ill-defined (n.d.). I82 is not included in models 3ISS, 1PKT, and 6R4R; in the model 1PHT, it is constrained by an artificial crystal contact (indicated by *).

GdmCl shifts the equilibrium of backbone conformations toward extended conformations.

We quantified the relative amounts of Ile in (g_m/g_m) conformation and estimate the population with α -helical backbone conformation by integrating the indicative peak areas (Figure S7 and Tables S3 and S6) for PI3K SH3 in all states. Once again, we employed the random coil mimic AIN for comparison. In AIN, the (g_m/g_m) population as determined from the $Ca/C\beta$ and $C\gamma1/C\delta1$ cross peaks is between 15% and 11%, respectively, the estimate of α -helical population determined from the $Ca/C\beta$ cross peak is roughly 15% (Table 2). Interestingly, the populations of the (g_m/g_m) state as well as the α -helix population of the combined signals of all five Ile residues in unfolded PI3K SH3 at pH 1.0 are very similar to those of AIN, suggesting that backbone torsion angles as well as side chain rotamers of all five Ile residues of denatured PI3K SH3 at pH 1.0 cover the same conformational space as in the random coil peptide mimic AIN.

For natively folded PI3K SH3 at pH 6.8, the α -helical population is close to 0%, reflecting the fact that no Ile residue is located in an α -helix. The population of the (g_m/g_m) conformation is between 23% and 25% and thus higher than in random coil ($\sim 15\%$). I29 adopts the (g_m/g_m) conformation in solution (see above) and is therefore expected to adopt the (g_m/g_m) conformation with a population close to 100% in frozen solution as well. The contribution of I29 to the (g_m/g_m) area of the combined signal of all five Ile residues is therefore estimated to be 20%. The observation that the (g_m/g_m) signal contribution is higher than 20% is thus an indication that other Ile residues may as well in part adopt the (g_m/g_m) conformation. In particular, I82 in the disordered C-terminus should have sufficient conformational freedom to populate the (g_m/g_m) rotamer to some degree.

Upon lowering the pH to 2.5, the (g_m/g_m) population decreases to $\sim 20\%$. Since restriction of I82 and I22 to (g_p/t) or (t/t) rotamers is implausible, the lower overall population of (g_m/g_m) likely reflects more extensive conformational freedom of the side chain of I29 to also populate other rotamers at pH 2.5 as the native hydrophobic core packing is destabilized. This is also supported by the rise of the α -helical population to 7% (Table 4), suggesting increased flexibility of the protein at pH 2.5. This trend continues and culminates in full conformational flexibility in the denatured protein at pH 1.0, where α -helical as

well as (g_m/g_m) populations resemble those in an IDP (Tables 2 and 4).

For comparison, we also recorded and analyzed PDSD spectra of PI3K SH3 at different conditions (see Figure S8 and Table S7). Evaluation of respective $Ca/C\beta$ cross peaks gave similar results within error margins ($C\gamma1/C\delta1$ cross peaks could not be evaluated as they overlap with $C\gamma1/C\gamma2$ relay cross peaks). For the estimate of α -helical populations, a similar trend is observed.

PI3K SH3 Fibrils. Ile-labeled PI3K SH3 amyloid fibrils formed at pH 2.5 (Figure 5A) were studied both at room temperature and in frozen suspension. The comparison between DQ/SQ spectra of Ile-labeled PI3K SH3 fibrils recorded in the different conditions is shown in Figure 5B. At room temperature, only I29 and I53 give rise to cross peaks, while I77 and I82 at the disordered C-terminus of the protein and I22 in a stretch with enhanced mobility (unpublished results) are apparently undetectable. Secondary chemical shifts of I29 and I53 in PI3K SH3 fibrils are typical for β -sheet backbone conformation and their $C\delta1$ shifts are typical for a χ^2 angle in trans orientation (Figure 5B).

At cryogenic temperatures, the mobility of these three residues is frozen out, and the five Ile residues give rise to overlapping heterogeneously broadened signals (Figure 5B). The intensities of the $C\gamma1/C\delta1$ cross peaks typical for the (g_m/g_m) rotamer are clearly visible, with intensities of $\sim 11\%$ of the total signal, indicating that the (g_m/g_m) state is still accessible for at least some of the Ile residues in the fibrils (in particular I77 and I82 and possibly also I22), but the percentage is significantly reduced compared to natively folded and unfolded monomeric PI3K SH3 states. Moreover, the α -helical conformation represents about 6% of the population. With an α -helical propensity of 15% for an Ile residue in an IDP (Table 2), this result is in agreement with two out of five Ile residues (I77 and I82) being completely disordered.

CONCLUSIONS

In this study, we introduce a novel NMR-based approach to assess the conformational dynamics of proteins by analyzing backbone conformational and rotameric ensembles of isoleucine side chains. To this end, we selected several proteins that cover a variety of different stages in protein folding, from disordered and denatured states to well-folded native states and to misfolded amyloid fibrillar states.

In well-folded proteins, rigid structural constraints limit backbone and side chain motion, resulting in well-resolved NMR signals for individual isoleucine residues at cryogenic temperatures.⁴⁸

For the protein GABARAP in frozen solution, we could discriminate individual signals for most of the seven Ile residues, whereas for well-folded bovine PI3K SH3 the signal overlap in frozen solution was larger, as three out of five Ile residues in this domain are located at the flexible C-terminus or in coil regions lacking well-defined secondary structure. However, with the help of two variants with one and three Ile to Val point mutations, we could identify individual signals also in this protein. In both proteins, individual line shapes of different Ile residues differ strongly between the residues, and the narrowest lines could be observed for those residues, which are confined in (g_m/g_m) conformation, i.e. I32 and I64 in GABARAP and I29 in PI3K SH3. In both proteins, these residues are located in well-defined secondary structure elements, which is linked to steric confinement of the side

Table 4. Populations of Different Conformations and Average Line Widths of Signals in Spectra of Bovine PI3K SH3 in Different Solvents

PI3K SH3	g_m/g_m population (%)	index of α -helical population (%)	range of fwhm (ppm)
native, 60% glycerol (pH 6.8)	25 $\pm$ 1	4 $\pm$ 1	2.0–3.6
native, 10% glycerol (pH 6.8)	23 $\pm$ 1	1 $\pm$ 1	2.0–4.6
fibrils, 60% glycerol (pH 2.5)	11 $\pm$ 4	6 $\pm$ 4	1.6–3.5
intermediate, 10% glycerol (pH 2.5)	20 $\pm$ 2	7 $\pm$ 2	3.3–5.8
unfolded, 10% glycerol (pH 1.0)	16 $\pm$ 2	16 $\pm$ 2	2.8–6.2
unfolded, GdmCl, 10% glycerol (pH 2.5)	8 $\pm$ 1	0 $\pm$ 1	2.9–5.2

chains. In both proteins, for one Ile residue each located in an α -helix a signal doubling for C α was observed (I21 in GABARAP, I53 in PI3K SH3) at low temperature. This indicates that the chemical shifts observed for these positions at room temperature are the result of residual conformational averaging, which takes place even in well-defined globular proteins.

In unfolded and intrinsically disordered proteins backbone and side chain conformations can rapidly interchange at ambient temperature, thus leading to an averaging of secondary chemical shifts or the characteristic low-frequency shift of C δ 1 of Ile in the (g_m, g_m) conformation. Therefore, the chemical shift dispersion between different Ile residues in unfolded proteins is usually low. In frozen solution, the large distribution of chemical shifts leads to substantial inhomogeneous line broadening which dominates the line shape. We could demonstrate for the monomeric IDP state of α -syn that the two Ile residues I88 and I112 in the unfolded monomer adopt the same conformational space as in random coil peptide mimetics. The typical IDP-like spectrum is obtained for PI3K SH3 at pH 1.0, when the protein is completely unfolded. Under these acidic conditions, the conformational freedom for Ile side chains is similar to that in an IDP and in the random coil peptide mimetics. A different unfolded state of PI3K SH3 is obtained when the protein is denatured by a strong unfolding agent such as GdmCl in high concentration. This denaturant appears to stabilize the backbone in an extended conformation where, despite of the protein's high backbone flexibility, helical conformations are prevented.

In the fibrillar state, in α -syn as well as in PI3K SH3 most Ile residues are conformationally restricted as part of the β -sheet fibril core, which is reflected by chemical shifts typical for β -sheet secondary structure dominating the resonance pattern in the spectra, as well as a reduced population of the (g_m, g_m) conformation. However, in both fibril types, not all Ile residues are part of the β -sheet core. In α -syn the C-terminal I112 residue, and in PI3K SH3 fibrils I77 and I82 remain disordered as part of the fuzzy coat outside the fibril core, and give rise to inhomogeneously broadened signals covering the typical random coil line shape.

For each protein, by integrating the peaks' volumes, we were able to quantify the prevalence of selected conformations. This approach matched with the respective PDB files and provided further information. In particular by successfully elucidating different unfolded states of PI3K SH3, we demonstrated that unfolded proteins can cover different conformational spaces in different solvents.

Future outlooks of this study include the extension of the investigation to amino acids other than isoleucine. Moreover, the presumed impact of glycerol on the conformation of the protein as well as the possible structural reorganization during the sample freezing process should be further inspected.

In conclusion, our study highlights the strength of NMR in tackling one of the major challenges in the structural investigation of (partially) disordered biomolecules. By cooling the sample to cryogenic temperatures, we stop the physiological conformational exchange and take a snapshot of the investigated molecules. Thus, all conformations sampled by each nucleus are preserved in line with their respective probability rather than average values.

MATERIAL AND METHODS

Expression and Purification of Ala and Ile-Labeled GABARAP. Expression and purification of GABARAP protein were carried out essentially as described.⁴ However, in case of selectively Ile-labeled protein, the expression protocol had minor changes. GABARAP protein was expressed recombinantly in *E. coli* BL21-(DE3)-T1R in 1 L M9 minimal medium. The medium contained [¹³C,¹⁵N]-Ile and [¹³C,¹⁵N]-Ala as the only labeled amino acids (Cortecnet) at a concentration of 150 mg/L. The other 18 proteinogenic amino acids (Sigma-Aldrich) were added in natural isotope abundance at concentrations of 150 mg/L. The cultures were induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) after reaching an OD₆₀₀ of 0.8 and grown for further 15 h at 20 °C before harvesting.

Expression and Purification of Ile-Labeled α -Syn. Selectively isoleucine-labeled N-terminal acetylated α -syn_{1–140} was expressed in *Escherichia coli* BL21(DE3) carrying codon-optimized α -syn in pT7 vector and the pNatB vector with the N-terminal acetylation enzyme from *Schizosaccharomyces pombe*.^{49,50} M9 medium was supplemented with all 19 proteinogenic nonlabeled amino acids and 150 mg/L [¹³C,¹⁵N]-labeled isoleucine, similar to a protocol described previously.⁵¹ Protein purification was performed as described previously.⁵² The culture was grown at 37 °C with 120 rpm shaking, at OD₆₀₀ of 1.2 α -syn expression was induced with 1 mM IPTG, and cells were harvested after 6 h at 37 °C. Cell pellets were resuspended in 25 mL 20 mM Tris pH 8.0 and boiled at 95–100 °C for 2 $\times$ 15 min. After centrifugation at 20,000g for 30 min at 4 °C recombinant α -syn was precipitated from the supernatant using a final concentration of 0.45 g/mL ammonium sulfate and centrifuged at 20,000g for 30 min. The pellet was dissolved in 50 mL 20 mM Tris-HCl pH 8.0 and loaded on a HiPrep Q Fast flow 16/10 anion exchange column 16/10 (Cytiva, CV = 20 mL). Elution was performed by applying a linear NaCl gradient of 20-fold CV from 0 mM to 800 mM NaCl in 20 mM Tris-HCl pH 8.0. α -syn was eluted at a conductivity of 28–32 mS/cm and fractions containing α -syn were pooled and precipitated using ammonium sulfate, as described previously. The protein pellet was resuspended in 5 mL 50 mM Tris-HCl pH 7.4 and loaded on a HiLoad Superdex 60/75 pg SEC column (Cytiva, CV = ~120 mL). α -syn eluted at ~60 mL with a final yield of ~8 mg/L culture and >97% purity.

Expression and Purification of Ile-Labeled PI3K SH3. The Ile-labeled WT-PI3K SH3 domain from *Bos taurus* was expressed in *E. coli* BL21 (DE3) ROSETTA with an additional His₆-tag for purification followed by a thrombin protease cleavage site.⁴¹ Cells were grown in M9 medium supplemented with all 19 proteinogenic nonlabeled amino acids and 150 mg/L [¹³C,¹⁵N]-labeled isoleucine for 24 h. Gene expression was induced by 1 mM IPTG at an OD₆₀₀ of 0.6. Cells were harvested by centrifugation and resuspended in 50 mM HEPES/NaOH buffer, pH 7.6, containing 100 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/L DNase I and 70 mg/L lysozyme. After sonication on a Bandelin sonopuls sonicator using a VS 70T sonotrode (60% amplitude, 3 $\times$ 5 min, 3 s 'on', 5 s 'off') on ice and ultracentrifugation in a Beckman Optima XPN-80 ultracentrifuge equipped with a 70Ti rotor at 4 °C and 42,000 rpm for 1 h. The supernatant was loaded on 5 mL Protino Ni-NTA column (Macherey-Nagel, Düren, Germany) equilibrated with 50 mM HEPES/NaOH, 100 mM NaCl and 20 mM imidazole, pH 7.6. The protein was eluted in a linear imidazole gradient (from 20 mM to 500 mM imidazole in 50 mM HEPES/NaOH, 100 mM NaCl, pH 7.6 within 20 CV). The fractions containing the eluted protein were pooled, concentrated and the His₆-tag was cleaved off with thrombin using 5 U thrombin (SERVA Electrophoresis GmbH, Heidelberg, Germany) per mg protein for 2 days at 4 °C under mild shaking conditions. The protein solution was loaded on a SEC HiLoad 16/60 Superdex 75 column (GE Healthcare Europe GmbH, Freiburg, Germany) equilibrated with 5 mM ammonium acetate, pH 7.7. Fractions containing PI3K SH3 were pooled and lyophilized before further usage. As described previously [29], the sequence of the bovine PI3K SH3 construct contains a glycine-serine overhang at the

N-terminus from the thrombin cleavage site and has the amino-acid sequence GS MSAEGYQYRA LYDYKKEREE DIDLHLGDIL TVNKGSLVAL GFSDGQEAKEP EEIGWLNLYN ETTGERGDFF GTYVEYIGRK KISP.

The bovine PI3K SH3 variants were expressed and purified essentially as described in ref 39. Briefly, bovine PI3K SH3 domain was expressed in *E. coli* BL21 (DE3) as a GST-fusion protein using the pGEX-4T vector. Cells were grown in isotope-labeled M9 medium containing all 19 proteinogenic nonlabeled amino acids and 150 mg/L [^{13}C , ^{15}N]-labeled isoleucine, lysed, and the fusion protein was purified using GST-affinity chromatography. The GST tag was cleaved with thrombin and the cleaved PI3K SH3 domain was further purified via an additional GST-affinity step to remove residual GST and size exclusion chromatography. The final [^{13}C , ^{15}N]-labeled protein was lyophilized before further usage.

The amino acid sequence of the I29V variant is GS MSAEGYQYRA LYDYKKEREE DIDLHLGDVL TVNKGSLVAL GFSDGQEAKEP EEIGWLNLYN ETTGERGDFF GTYVEYIGRK KISP, and the amino acid sequence of the I22V/I77V/I82V variant GS MSAEGYQYRA LYDYKKEREE DVDLHLGDIL TVNKGSLVAL GFSDGQEAKEP EEIGWLNLYN ETTGERGDFF GTYVEYVGRK KVSP.

Fibril Formation of Ile-Labeled α -Syn. α -Syn fibril formation was performed by incubating 100 μM purified monomeric α -syn in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM NaH_2PO_4), 0.05% (w/v) NaN_3 , pH 7.4 at 37 $^\circ\text{C}$ and 900 rpm continuous shaking in a sealed 2 mL low-bind surface reaction tube (Eppendorf, GE). For induction of aggregation one borosilicate bead was added ($d = 3$ mm, Hilgenberg, GE). Mature α -syn fibrils were harvested after five days incubation at $100.000 \times g$ for 30 min at 4 $^\circ\text{C}$ and the total α -syn fibril mass was determined by subtracting the concentration of the soluble protein fraction found in the supernatant. The α -syn fibril pellet was washed several times with a stock solution of $\text{H}_2\text{O}/\text{D}_2\text{O}$ buffer mixture in a 1:3 ratio after ultracentrifugation using a TLA-55 Fixed-Angle Rotor (100.000g).

Fibril Formation of Ile-Labeled PI3K SH3. 100 nmol lyophilized Ile-labeled PI3K SH3 were dissolved in 1 mL of 10 mM glycine/HCl buffer (pH 2.5) prepared from 20 μL 500 mM glycine/HCl buffer (pH 2.5) and 980 μL D_2O . Unlabeled fibril seeds were added to a final concentration of 5 μM (monomer concentration and the solution was incubated at 50 $^\circ\text{C}$ overnight under quiescent conditions in an Eppendorf tube, as described before.⁴¹

DNP Sample Preparation. AIN, GIG, Ala-Ile-labeled GABARAP and Ile-labeled α -syn samples for DNP-enhanced NMR prepared in d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ solutions (60:30:10 volume ratio) with 2.5 mM AMUPol or 5 mM M-TinyPol. As a first step, a buffer containing 150 mM NaCl, 100 mM NaPi, and 30 mM NaN_3 in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture at a ratio of 1:3 ratio was prepared, and each protein sample was buffer-exchanged to this buffer using Amicon centrifugal filter devices with a 3 kDa cutoff. The sample was then concentrated to 10–12 μL . d8-glycerol was added to a final concentration of 60% (v/v). Finally, 2.5 mM AMUPol or 5 mM M-TinyPol was added from a 100 mM stock solution.

For the DNP sample preparation of α -syn fibrils, after the buffer exchange and concentration of the sample via ultracentrifugation, d8-glycerol was added to a final concentration of 60% (v/v). Finally, 2.5 mM AMUPol or 5 mM M-TinyPol was added from a 100 mM stock solution.

For Ile-labeled PI3K SH3 in its native state, lyophilized protein (100 nmol) was dissolved in either (i) 75 μL 60:30:10, d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ v/v/v, containing 25 mM NaPi, pH 6.8, or (ii) 67 μL 10:80:10, d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ containing 25 mM NaPi, pH 6.8. For unfolded conditions, the protein was dissolved in (iii) 10:80:10, d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ v/v/v, containing 50 mM glycine/HCl, pH 2.5 or 1.0, or (iv) 10:80:10, d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ v/v/v containing 6 M GdmCl, pH 2.5. M-TinyPol was added to a final concentration of 5 mM, yielding to a final protein concentration of ~ 1.4 mM.

For the fibrillar sample of PI3K SH3 the fibril solution was centrifuged at $186.000 \times g$ in an OptimaTM MAX-XP ultracentrifuge equipped with a TLA-55 rotor for 1 h at RT. The supernatant was

removed and the fibril pellet (~ 15 – 20 μL for 100 nmol monomer equivalent) was resuspended 60:30:10, d8-glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$ v/v/v, containing 10 mM glycine/HCl and 5 mM M-TinyPol.

All samples were filled into 3.2 mm sapphire rotors, and measurements were performed at 100 K.

Solution NMR Spectroscopy. A sample of 0.50 mM [^{13}C , ^{15}N]-Ala/Ile *H. sapiens* GABARAP with 100 mM NaCl, 100 mM KCl, 0.1 mM EDTA, 25 mM sodium phosphate buffer in 10% (v/v) D_2O (pH 6.9) was used for solution NMR spectroscopy. Solution NMR measurements of the *B. taurus* PI3K SH3 at neutral pH were performed on samples containing 0.25 mM [^{13}C , ^{15}N] PI3K SH3 wt, 0.25 mM [^{13}C , ^{15}N]-Ile PI3K SH3 wt, or 0.25 mM [^{13}C , ^{15}N]-Ile PI3K SH3 I29V with 25 mM sodium phosphate buffer in 8% (v/v) D_2O (pH 6.8) at a temperature of 25.0 $^\circ\text{C}$. Acidic samples contained 0.28 mM [^{13}C , ^{15}N] or 0.40 mM [^{13}C , ^{15}N]-Ile *B. taurus* PI3K SH3 wt with 10 mM glycine/HCl buffer in 8% (v/v) D_2O (pH 2.5). 2D ^1H - ^{15}N HSQC spectra⁵³ were recorded to verify the integrity of the samples after lyophilization and dissolution in the respective buffer. Two 2D ^{13}C - ^{13}C TOCSY spectra covering either the aliphatic (bandwidth 70 ppm) or full (bandwidth 180 ppm) spectral region with a 13.6 ms (aliphatic) or 20.4 ms (full bandwidth) 13.9 kHz FLOPSY-16 isotropic mixing scheme⁵⁴ were recorded on the [^{13}C , ^{15}N]-Ala/Ile GABARAP sample on a Bruker AVANCE III HD 600 MHz NMR spectrometer equipped with a cryogenically cooled inverse quadrupole resonance probe. For each PI3K SH3 sample, aliphatic and full bandwidth (not recorded on [^{13}C , ^{15}N]-Ile PI3K SH3 at pH 2.5) 2D ^{13}C - ^{13}C TOCSY spectra with a 15.1 ms (aliphatic) or 21.1 ms (full bandwidth) 15.6 kHz FLOPSY-16 isotropic mixing scheme⁵⁴ were recorded on a Bruker AVANCE III HD 800 MHz NMR spectrometer equipped with a cryogenically cooled $^{13}\text{C}/^{15}\text{N}$ observe triple resonance probe. All probes had z axis pulsed field gradient capabilities. The sample temperature was calibrated using methanol- d_4 .⁴⁶ Quadrature detection in the indirect ^{13}C dimension was achieved by States-TPPI.⁵⁵ All solution NMR spectra were processed with NMRPipe⁵⁶ software and analyzed with NMRView⁵⁷ and CCPN.^{58,59} ^1H chemical shifts were referenced with respect to external DSS in D_2O , ^{13}C and ^{15}N chemical shifts were referenced indirectly.⁶⁰

DNP Experiments. All experiments were conducted on an 18.8 T (800 MHz ^1H Larmor frequency) spectrometer (Bruker Avance) connected to a 525 GHz gyrotron as a source of continuous microwaves for cross-effect DNP hyperpolarization. Enhancement factors obtained were between 19 and 64 (see Figure S3 and Tables S8 and S9). All samples were filled into 3.2 mm sapphire rotors. All experiments were performed at a temperature of 100 K. Spectra were recorded using a recycle delay of 5 s. The 2D ^{13}C - ^{13}C double quantum/single quantum (DQ/SQ) SPC5 spectra were recorded using a magic angle spinning frequency of 8.2 kHz and SPCS recoupling.⁶¹ SPC 5 recoupling time and reconversion times were set to 488 μs in all experiments, corresponding to four rotor periods. Proton Driven Spin Diffusion (PDS) spectra were recorded at MAS frequencies of 11 kHz (GABARAP) or 12 kHz (PI3K SH3) with mixing times between 10 and 50 ms. Contact times for cross-polarization were between 100 and 900 μs . High power proton decoupling with an rf power of ~ 83 kHz was applied during evolution and detection as well as DQ excitation and recoupling. During SPCS recoupling, CW proton decoupling was employed, during evolution and detection SPINAL-64 decoupling.⁶²

Experimental parameters for all samples, including number of scans, t_1 increments, maximum evolution time and total experimental time are provided in Tables S8 and S9. Spectra were referenced externally using adamantane by calibrating its CH peak to 31.4 ppm, corresponding to the DSS reference scale. All NMR spectra were processed in TopSpin 3.6-4.3 and analyzed using CCPN. Relevant processing parameters of the spectra are listed in Table S10. DQSQ spectra were plotted with contour lines at 1.31 incremental spacing, PDS spectra with an increment of 1.4.

Solid-State MAS NMR Spectroscopy at Ambient Temperature. The DQSQ spectrum of Ile-labeled PI3K SH3 fibrils was recorded on a Bruker 600 MHz spectrometer equipped with an

AVANCE NEO console and a 3.2 mm triple resonance probe head. VT gas was adjusted to $-10\text{ }^{\circ}\text{C}$, resulting in an effective sample temperature of $\sim 0\text{ }^{\circ}\text{C}$.

The 2D ^{13}C – ^{13}C double quantum/single quantum (DQ/SQ) SPC5 spectrum was recorded using a magic angle spinning frequency of 8.0 kHz and SPC5 recoupling.⁶¹

High power proton decoupling with an rf power of $\sim 83\text{ kHz}$ was applied during evolution and detection as well as DQ excitation and recoupling was applied. During SPC5 recoupling, CW proton decoupling was employed, during evolution and detection SPINAL-64 decoupling.⁶²

Conformational Quantification. The peaks in the proteins' DQ/SQ spectra, recorded at 800 MHz spectrometer, were assigned to each conformation according to their respective chemical shift. Each peak was volume integrated using a rectangular box integration (Topspin 4.0.9). Rotamer distributions in percentages (given in Tables 2 and 4 and Tables S3 and S7) were determined as follows.

For the g_m/g_m conformation, the volume of the rectangular boxes around respective $\text{Ca}/\text{C}\beta$ and $\text{C}\gamma 1/\text{C}\delta 1$ cross peaks (marked in yellow in Figure S7), was integrated and then divided by the full peak volume for the same side chain carbon (marked in blue in Figure S7) in order to get the population percentages (%). From the different g_m/g_m population percentages determined for each side chain carbon peak an average value was calculated.

For an estimate of the helical population (also called index) we have integrated a rectangular box around the typical helical tail visible on the $\text{Ca}/\text{C}\beta$ cross peak in the range 63–69 ppm (marked in black in Figure S7). The integrated volume was then compared to the full Ca peak integral in order to gain a measure for the relative population % shown in the tables. The detailed integrated areas used for each protein system are provided in Table S6.

$\text{Ca}/\text{C}\beta$ and $\text{C}\gamma 1/\text{C}\delta 1$ cross peaks for g_m/g_m conformations are clearly separated from the other cross peaks also in unfolded proteins and thus can easily be quantified. In contrast, the selection of the α -helical part of a heterogeneously broadened continuum of overlapping chemical shifts can only give an estimate for a general trend, but should not be regarded as a quantification of the backbone conformational ensemble.

The fwhm was also measured for every peak visible in the DQ/SQ spectrum, and in Table S4 all the values are shown. Those values were then averaged to the fwhm average value, which was used for the discussion.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.5c04159>.

Detailed experimental NMR acquisition and processing parameters and additional spectra (PDF)

NMR raw data are available under <https://bmrbig.org/released/bmrbig114>

■ AUTHOR INFORMATION

Corresponding Author

Henrike Heise – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0000-0002-9081-3894; Email: h.heise@fz-juelich.de

Authors

Leonardo Levorin – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0009-0006-5814-4367

Nina Becker – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Boran Uluca-Yazgi – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Luis Gardon – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0000-0003-0871-6487

Mirko Kraus – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Marc Sevenich – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Athina Apostolidis – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany

Kai Schmitz – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Neomi Rüter – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany

Irina Apanasenko – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Dieter Willbold – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0000-0002-0065-7366

Wolfgang Hoyer – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany

Philipp Neudecker – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0000-0002-0557-966X

Lothar Gremer – Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf 40225, Germany; Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich 52425, Germany; orcid.org/0000-0001-7065-5027

Complete contact information is available at:
<https://pubs.acs.org/doi/10.1021/jacs.5c04159>

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Nick Rähse, Christoph Hölbling, Anna Thimm, and Paula E. Philippsen for assistance in the laboratory and Prof. D. Flemming Hansen, Dr. Lucas Siemons, and Dr. Anna König for valuable discussions. We acknowledge access to the Jülich-Düsseldorf Biomolecular NMR Center jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU). This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (HE 3243/4-1, INST 208/771-1 FUGG, INST 208/620-1 FUGG, and SFB 1208/267205415).

■ REFERENCES

- (1) Miao, Z.; Cao, Y. Quantifying side-chain conformational variations in protein structure. *Sci. Rep.* **2016**, *6*, 37024.
- (2) Hong, M.; Mishanina, T. V.; Cady, S. D. Accurate Measurement of Methyl ^{13}C Chemical Shifts by Solid-State NMR for the Determination of Protein Side Chain Conformation: The Influenza A M2 Transmembrane Peptide as an Example. *J. Am. Chem. Soc.* **2009**, *131* (22), 7806–7816.
- (3) Adamski, W.; Salvi, N.; Maurin, D.; Magnat, J.; Milles, S.; Jensen, M. R.; Abyzov, A.; Moreau, C. J.; Blackledge, M. A Unified Description of Intrinsically Disordered Protein Dynamics under Physiological Conditions Using NMR Spectroscopy. *J. Am. Chem. Soc.* **2019**, *141* (44), 17817–17829.
- (4) Möckel, C.; Kubiak, J.; Schillinger, O.; Kühnemuth, R.; Della Corte, D.; Schröder, G. F.; Willbold, D.; Strodel, B.; Seidel, C. A. M.; Neudecker, P. Integrated NMR, Fluorescence, and Molecular Dynamics Benchmark Study of Protein Mechanics and Hydrodynamics. *J. Phys. Chem. B* **2019**, *123* (7), 1453–1480.
- (5) Sormanni, P.; Piovesan, D.; Heller, G. T.; Bonomi, M.; Kukic, P.; Camilloni, C.; Fuxreiter, M.; Dosztanyi, Z.; Pappu, R. V.; Babu, M. M.; Longhi, S.; Tompa, P.; Dunker, A. K.; Uversky, V. N.; Tosatto, S. C. E.; Vendruscolo, M. Simultaneous quantification of protein order and disorder. *Nat. Chem. Biol.* **2017**, *13* (4), 339–342.
- (6) Schneider, R.; Huang, J. R.; Yao, M. X.; Communie, G.; Ozenne, V.; Mollica, L.; Salmon, L.; Jensen, M. R.; Blackledge, M. Towards a robust description of intrinsic protein disorder using nuclear magnetic resonance spectroscopy. *Mol. Biosyst.* **2012**, *8* (1), 58–68.
- (7) Havlin, R. H.; Tycko, R. Probing site-specific conformational distributions in protein folding with solid-state NMR. *P Natl. Acad. Sci. USA* **2005**, *102* (9), 3284–3289.
- (8) Heise, H.; Luca, S.; de Groot, B. L.; Grubmüller, H.; Baldus, M. Probing conformational disorder in neurotensin by two-dimensional solid-state NMR and comparison to molecular dynamics simulations. *Biophys. J.* **2005**, *89* (3), 2113–2120.
- (9) Uluca, B.; Viennet, T.; Petrovic, D.; Shaykhalishahi, H.; Weirich, F.; Gönülalan, A.; Strodel, B.; Etzkorn, M.; Hoyer, W.; Heise, H. DNP-Enhanced MAS NMR: A Tool to Snapshot Conformational Ensembles of alpha-Synuclein in Different States. *Biophys. J.* **2018**, *114* (7), 1614–1623.
- (10) Jeon, J.; Yau, W.-M.; Tycko, R. Millisecond Time-Resolved Solid-State NMR Reveals a Two-Stage Molecular Mechanism for Formation of Complexes between Calmodulin and a Target Peptide from Myosin Light Chain Kinase. *J. Am. Chem. Soc.* **2020**, *142* (50), 21220–21232.
- (11) Jeon, J.; Blake Wilson, C.; Yau, W. M.; Thurber, K. R.; Tycko, R. Time-resolved solid state NMR of biomolecular processes with millisecond time resolution. *J. Magn. Reson.* **2022**, *342*, No. 107285.
- (12) Yi, X.; Fritzsche, K. J.; Rogawski, R.; Xu, Y.; McDermott, A. E. Contribution of protein conformational heterogeneity to NMR lineshapes at cryogenic temperatures. *Proc. Natl. Acad. Sci. U. S. A.* **2024**, *121* (8), No. e2301053120.
- (13) Burakova, E.; Vasa, S. K.; Linser, R. Characterization of conformational heterogeneity via higher-dimensionality, proton-detected solid-state NMR. *Journal of Biomolecular NMR* **2022**, *76* (5), 197–212.
- (14) Fritzsche, K. J.; Hong, M.; Schmidt-Rohr, K. Conformationally selective multidimensional chemical shift ranges in proteins from a PDB database purged using intrinsic quality criteria. *Journal of Biomolecular NMR* **2016**, *64* (2), 115–130.
- (15) Berjanskii, M. V.; Wishart, D. S. Unraveling the meaning of chemical shifts in protein NMR. *Biochim. Biophys. Acta, Proteins Proteomics* **2017**, *1865* (11, Part B), 1564–1576.
- (16) Siemer, A. B.; Huang, K. Y.; McDermott, A. E. Protein Linewidth and Solvent Dynamics in Frozen Solution NMR. *PLoS One* **2012**, *7* (10), No. e47242.
- (17) Siemer, A. B. Advances in studying protein disorder with solid-state NMR. *Solid state nuclear magnetic resonance* **2020**, *106*, No. 101643.
- (18) Concistre, M.; Carignani, E.; Borsacchi, S.; Johannessen, O. G.; Mennucci, B.; Yang, Y. F.; Geppi, M.; Levitt, M. H. Freezing of Molecular Motions Probed by Cryogenic Magic Angle Spinning NMR. *J. Phys. Chem. Lett.* **2014**, *5* (3), 512–516.
- (19) Kragelj, J.; Dumariéh, R.; Xiao, Y.; Frederick, K. K. Conformational ensembles explain NMR spectra of frozen intrinsically disordered proteins. *Protein Sci.* **2023**, *32* (5), No. e4628.
- (20) Siemons, L.; Uluca-Yazgi, B.; Pritchard, R. B.; McCarthy, S.; Heise, H.; Hansen, D. F. Determining isoleucine side-chain rotamer-sampling in proteins from C-13 chemical shift. *Chem. Commun.* **2019**, *55* (94), 14107–14110.
- (21) König, A.; Schölzel, D.; Uluca, B.; Viennet, T.; Akbey, U.; Heise, H. Hyperpolarized MAS NMR of unfolded and misfolded proteins. *Solid state nuclear magnetic resonance* **2019**, *98*, 1–11.
- (22) Hansen, D. F.; Neudecker, P.; Kay, L. E. Determination of Isoleucine Side-Chain Conformations in Ground and Excited States of Proteins from Chemical Shifts. *J. Am. Chem. Soc.* **2010**, *132* (22), 7589–7591.
- (23) Shewmaker, F.; Wickner, R. B.; Tycko, R. Amyloid of the prion domain of Sup35p has an in-register parallel beta-sheet structure. *P Natl. Acad. Sci. USA* **2006**, *103* (52), 19754–19759.
- (24) Mohrlüder, J.; Schwarten, M.; Willbold, D. Structure and potential function of gamma-aminobutyrate type A receptor-associated protein. *FEBS J.* **2009**, *276* (18), 4989–5005.
- (25) Shpilka, T.; Weidberg, H.; Pietrovski, S.; Elazar, Z. Atg8: an autophagy-related ubiquitin-like protein family. *Genome Biology* **2011**, *12* (7), 226.
- (26) Fink, A. L. Natively unfolded proteins. *Curr. Opin. Struct. Biol.* **2005**, *15* (1), 35–41.
- (27) Wright, P. E.; Dyson, H. J. Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. *J. Mol. Biol.* **1999**, *293* (2), 321–331.
- (28) Donne, D. G.; Viles, J. H.; Groth, D.; Mehlhorn, I.; James, T. L.; Cohen, F. E.; Prusiner, S. B.; Wright, P. E.; Dyson, H. J. Structure of the recombinant full-length hamster prion protein PrP(29–231): the N terminus is highly flexible. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (25), 13452–7.
- (29) Dyson, H. J.; Wright, P. E. Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell Bio* **2005**, *6* (3), 197–208.
- (30) Uversky, V. N. A protein-chameleon: Conformational plasticity of alpha-synuclein, a disordered protein involved in neurodegenerative disorders. *J. Biomol. Struct. Dyn* **2003**, *21* (2), 211–234.
- (31) Goedert, M. Alpha-synuclein and neurodegenerative diseases. *Nat. Rev. Neurosci* **2001**, *2* (7), 492–501.
- (32) Chiti, F.; Dobson, C. M. Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* **2006**, *75*, 333–366.
- (33) Zurdo, J.; Guijarro, J. I.; Jiménez, J. L.; Saibil, H. R.; Dobson, C. M. Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain. Edited by P. E. Wright. *J. Mol. Biol.* **2001**, *311* (2), 325–340.
- (34) Booker, G. W.; Gout, I.; Kristina; Downing, A.; Driscoll, P. C.; Boyd, J.; Waterfield, M. D.; Campbell, I. D. Solution structure and

ligand-binding site of the SH3 domain of the p85 α subunit of phosphatidylinositol 3-kinase. *Cell* **1993**, 73 (4), 813–822.

- (35) Koyama, S.; Yu, H.; Dalgarno, D. C.; Shin, T. B.; Zydowsky, L. D.; Schreiber, S. L. Structure of the PI3K SH3 domain and analysis of the SH3 family. *Cell* **1993**, 72 (6), 945–52.
- (36) Liang, J.; Chen, J. K.; Schreiber, S. L.; Clardy, J. Crystal Structure of PI3K SH3 Domain at 2.0 Å Resolution. *J. Mol. Biol.* **1996**, 257 (3), 632–643.
- (37) Batra-Safferling, R.; Granzin, J.; Mödder, S.; Hoffmann, S.; Willbold, D. Structural studies of the phosphatidylinositol 3-kinase (PI3K) SH3 domain in complex with a peptide ligand: role of the anchor residue in ligand binding. *Biol. Chem.* **2010**, 391 (1), 33–42.
- (38) Guijarro, J. I.; Sunde, M.; Jones, J. A.; Campbell, I. D.; Dobson, C. M. Amyloid fibril formation by an SH3 domain. *P Natl. Acad. Sci. USA* **1998**, 95 (8), 4224–4228.
- (39) Gardon, L.; Becker, N.; Rähse, N.; Hölbling, C.; Apostolidis, A.; Schulz, C. M.; Bochinsky, K.; Gremer, L.; Heise, H.; Lakomek, N.-A. Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques. *Front. Mol. Biosci.* **2023**, 10, No. 1254721.
- (40) Bayro, M. J.; Maly, T.; Birkett, N. R.; MacPhee, C. E.; Dobson, C. M.; Griffin, R. G. High-Resolution MAS NMR Analysis of PI3-SH3 Amyloid Fibrils: Backbone Conformation and Implications for Protofilament Assembly and Structure. *Biochemistry* **2010**, 49 (35), 7474–7484.
- (41) Röder, C.; Vettore, N.; Mangels, L. N.; Gremer, L.; Ravelli, R. B. G.; Willbold, D.; Hoyer, W.; Buell, A. K.; Schröder, G. F. Atomic structure of PI3-kinase SH3 amyloid fibrils by cryo-electron microscopy. *Nat. Commun.* **2019**, 10 (1), 3754.
- (42) Stangler, T.; Mayr, L. M.; Willbold, D. Solution structure of human GABA(A) receptor-associated protein GABARAP: implications for biological function and its regulation. *J. Biol. Chem.* **2002**, 277 (16), 13363–6.
- (43) Stangler, T.; Mayr, L. M.; Dingley, A. J.; Luge, C.; Willbold, D. Sequence-specific ¹H, ¹³C and ¹⁵N resonance assignments of human GABA receptor associated protein. *J. Biomol. NMR* **2001**, 21 (2), 183–4.
- (44) Tuttle, M. D.; Comellas, G.; Nieuwkoop, A. J.; Covell, D. J.; Berthold, D. A.; Kloepper, K. D.; Courtney, J. M.; Kim, J. K.; Barclay, A. M.; Kendall, A.; Wan, W.; Stubbs, G.; Schwieters, C. D.; Lee, V. M. Y.; George, J. M.; Rienstra, C. M. Solid-state NMR structure of a pathogenic fibril of full-length human [alpha]-synuclein. *Nat. Struct. Mol. Biol.* **2016**, 23 (5), 409–415.
- (45) Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O. C.; Riedel, D.; Baldus, M. Molecular-level secondary structure, polymorphism, and dynamics of full-length α -synuclein fibrils studied by solid-state NMR. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, 102 (44), 15871–15876.
- (46) Findeisen, M.; Brand, T.; Berger, S. A ¹H-NMR thermometer suitable for cryoprobes. *Magn. Reson. Chem.* **2007**, 45 (2), 175–178.
- (47) Hsu, S. T. NMR assignments of PI3-SH3 domain aided by protonless NMR spectroscopy. *Biomolecular NMR assignments* **2014**, 8 (2), 291–5.
- (48) Fricke, P.; Mance, D.; Chevelkov, V.; Giller, K.; Becker, S.; Baldus, M.; Lange, A. High resolution observed in 800 MHz DNP spectra of extremely rigid type III secretion needles. *Journal of Biomolecular NMR* **2016**, 65 (3), 121–126.
- (49) Johnson, M.; Coulton, A. T.; Geeves, M. A.; Mulvihill, D. P. Targeted amino-terminal acetylation of recombinant proteins in *E. coli*. *PLoS One* **2010**, 5 (12), No. e15801.
- (50) Wördehoff, M. M.; Shaykhalishahi, H.; Groß, L.; Gremer, L.; Stoldt, M.; Buell, A. K.; Willbold, D.; Hoyer, W. Opposed Effects of Dityrosine Formation in Soluble and Aggregated α -Synuclein on Fibril Growth. *J. Mol. Biol.* **2017**, 429 (20), 3018–3030.
- (51) Blanco, F. J.; Hess, S.; Pannell, L. K.; Rizzo, N. W.; Tycko, R. Solid-state NMR data support a helix-loop-helix structural model for the N-terminal half of HIV-1 Rev in fibrillar form. *J. Mol. Biol.* **2001**, 313 (4), 845–859.
- (52) Hoyer, W.; Antony, T.; Cherny, D.; Heim, G.; Jovin, T. M.; Subramaniam, V. Dependence of alpha-synuclein aggregate morphology on solution conditions. *J. Mol. Biol.* **2002**, 322 (2), 383–93.
- (53) Zhang, O.; Kay, L. E.; Olivier, J. P.; Forman-Kay, J. D. Backbone ¹H and ¹⁵N resonance assignments of the N-terminal SH3 domain of drk in folded and unfolded states using enhanced-sensitivity pulsed field gradient NMR techniques. *Journal of Biomolecular NMR* **1994**, 4 (6), 845–858.
- (54) Kovacs, H.; Gossert, A. Improved NMR experiments with ¹³C-isotropic mixing for assignment of aromatic and aliphatic side chains in labeled proteins. *Journal of Biomolecular NMR* **2014**, 58 (2), 101–112.
- (55) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *Journal of Magnetic Resonance* (1969) **1989**, 85 (2), 393–399.
- (56) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **1995**, 6 (3), 277–293.
- (57) Johnson, B. A.; Blevins, R. A. NMR View: A computer program for the visualization and analysis of NMR data. *Journal of Biomolecular NMR* **1994**, 4 (5), 603–614.
- (58) Vranken, W. F.; Boucher, W.; Stevens, T. J.; Fogh, R. H.; Pajon, A.; Llinas, P.; Ulrich, E. L.; Markley, J. L.; Ionides, J.; Laue, E. D. The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins* **2005**, 59 (4), 687–696.
- (59) Skinner, S. P.; Fogh, R. H.; Boucher, W.; Ragan, T. J.; Mureddu, L. G.; Vuister, G. W. CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. *Journal of Biomolecular NMR* **2016**, 66 (2), 111–124.
- (60) Markley, J. L.; Bax, A.; Arata, Y.; Hilbers, C. W.; Kaptein, R.; Sykes, B. D.; Wright, P. E.; Wüthrich, K. Recommendations for the presentation of NMR structures of proteins and nucleic acids (IUPAC Recommendations 1998). *Pure Appl. Chem.* **1998**, 70 (1), 117–142.
- (61) Hohwy, M.; Rienstra, C. M.; Jaroniec, C. P.; Griffin, R. G. Fivefold symmetric homonuclear dipolar recoupling in rotating solids: Application to double quantum spectroscopy. *J. Chem. Phys.* **1999**, 110 (16), 7983–7992.
- (62) Fung, B. M.; Khitrin, A. K.; Ermolaev, K. An improved broadband decoupling sequence for liquid crystals and solids. *J. Magn. Reson.* **2000**, 142 (1), 97–101.

Supporting Information

Isoleucine Side Chains as Reporters of Conformational Freedom in Protein Folding Studied by DNP-Enhanced NMR

Leonardo Levorin^{1,2}, Nina Becker^{1,2}, Boran Uluca-Yazgi^{1,2}, Luis Gardon^{1,2}, Mirko Kraus^{1,2}, Marc Sevenich^{1,2}, Athina Apostolidis¹, Kai Schmitz^{1,2}, Neomi Rüter¹, Irina Apanasenko^{1,2}, Dieter Willbold^{1,2}, Wolfgang Hoyer^{1,2}, Philipp Neudecker^{1,2}, Lothar Gremer^{1,2}, Henrike Heise^{1,2*}

¹Institute of Physical Biology,
Heinrich-Heine-Universität Düsseldorf
40225 Düsseldorf, Germany

²Institute of Biological Information Processing (IBI-7: Structural Biochemistry)
Forschungszentrum Jülich
52425 Jülich, Germany

* Email: h.heise@fz-juelich.de

NMR RAW DATA

NMR raw data are available under <https://bmrbig.org/released/bmrbig114>

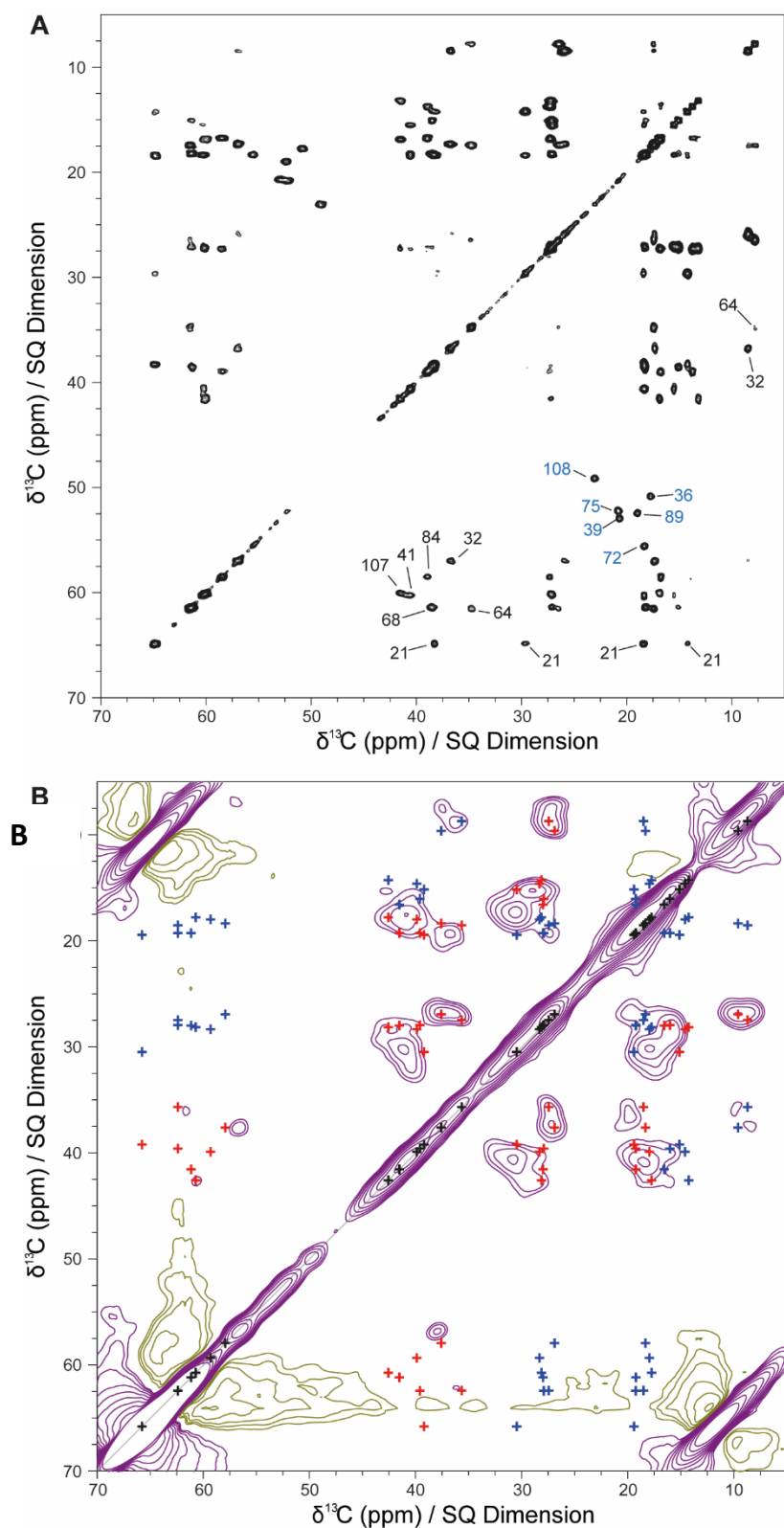
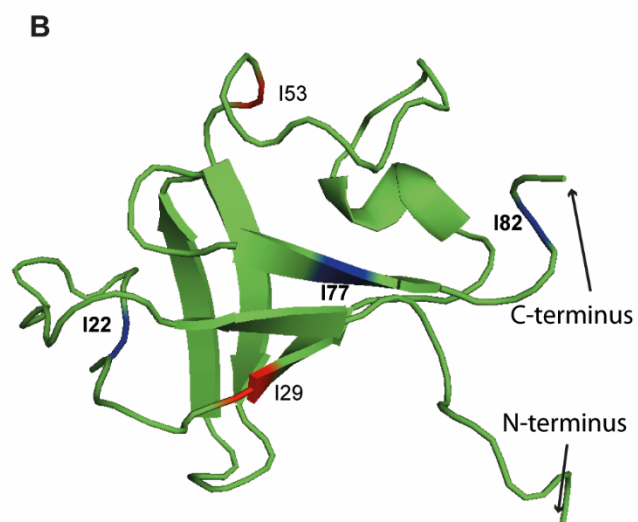
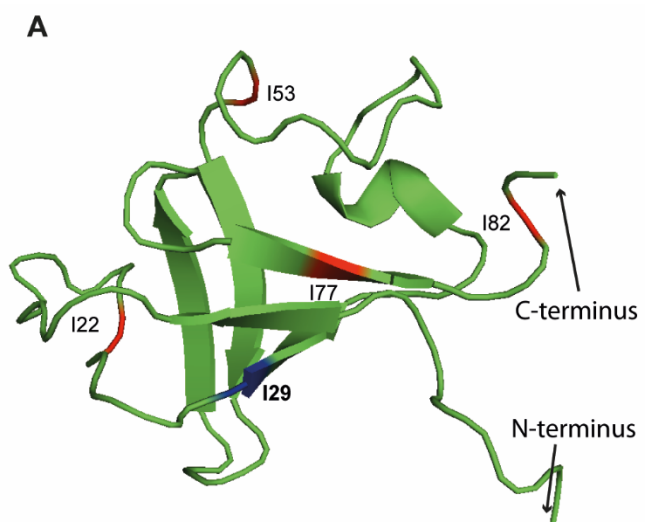


Figure S1: Aliphatic 2D ^{13}C - ^{13}C -TOCSY spectrum of $[\text{}^{13}\text{C}, \text{}^{15}\text{N}]$ -Ala/Ile labeled GABARAP in solution at 25.0°C recorded with 13.6 ms FLOPSY-16 mixing at 600 MHz. Selected TOCSY cross peaks for Ile (black) and Ala (blue) are labeled according to their residue number. B) 2D PDSD of $[\text{}^{13}\text{C}, \text{}^{15}\text{N}]$ -Ala/Ile labeled GABARAP in frozen solution (see Table S9) at a temperature of 100 K recorded at 800 MHz with a spinning speed of 11 kHz. The spectrum was processed with an unshifted squared cosine function. Positive contour lines are displayed in purple, negative in green. Simulated one-bond and two-bond cross peaks from solution NMR chemical shifts are indicated as red and blue crosses, respectively ($\text{C}\alpha/\text{C}\beta$ cross peaks are affected by baseline perturbations from the strong glycerol diagonal signal).



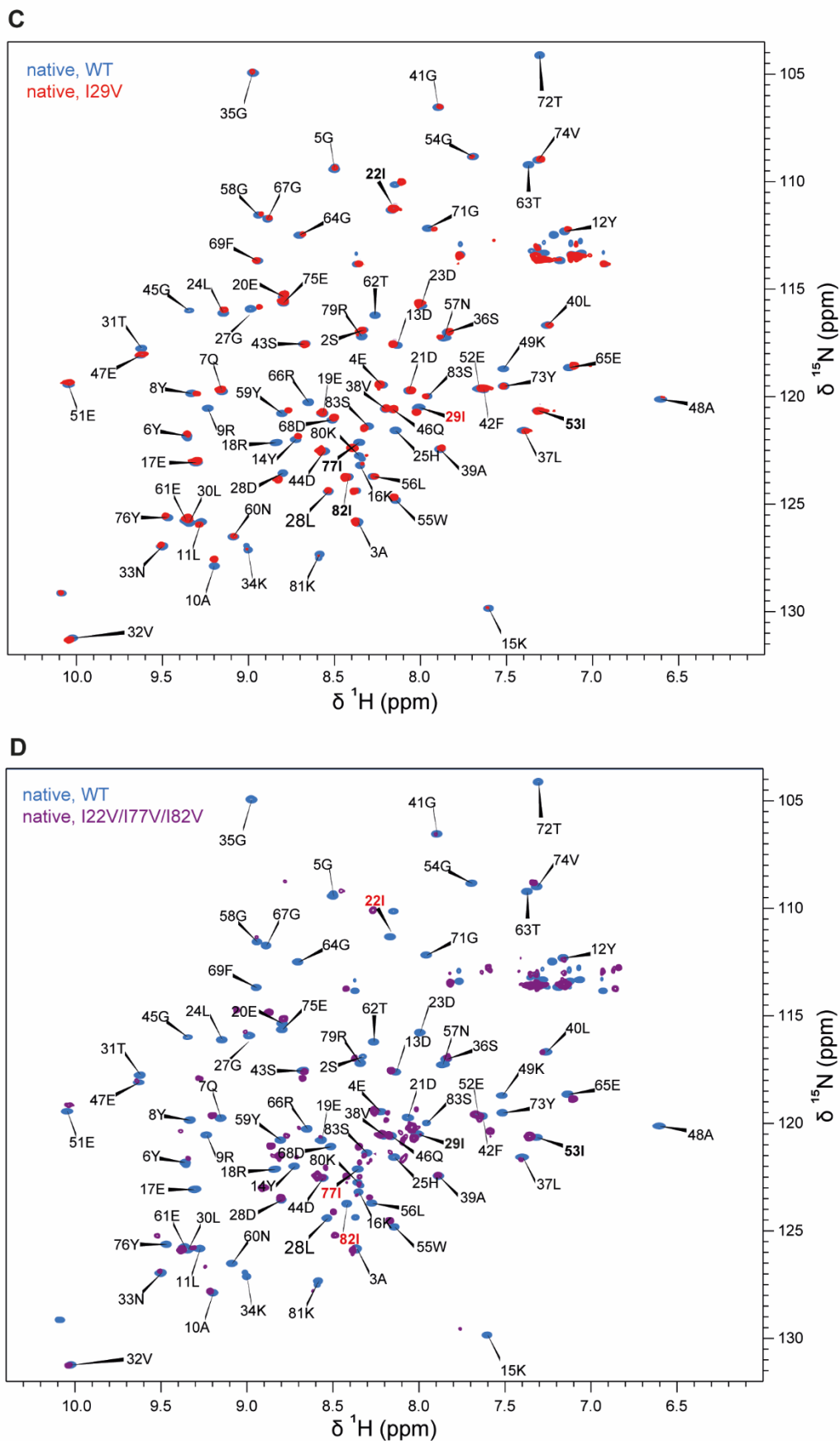


Figure S3: I29V (A) and I22V/I77V/I82V (B) mutants with the respective substitution marked in blue in the 3D structure of PI3K SH3 in its native form (1PNJ). (C-D) Comparison of their respective HSQC spectra with the one recorded for the wildtype protein in the native form.

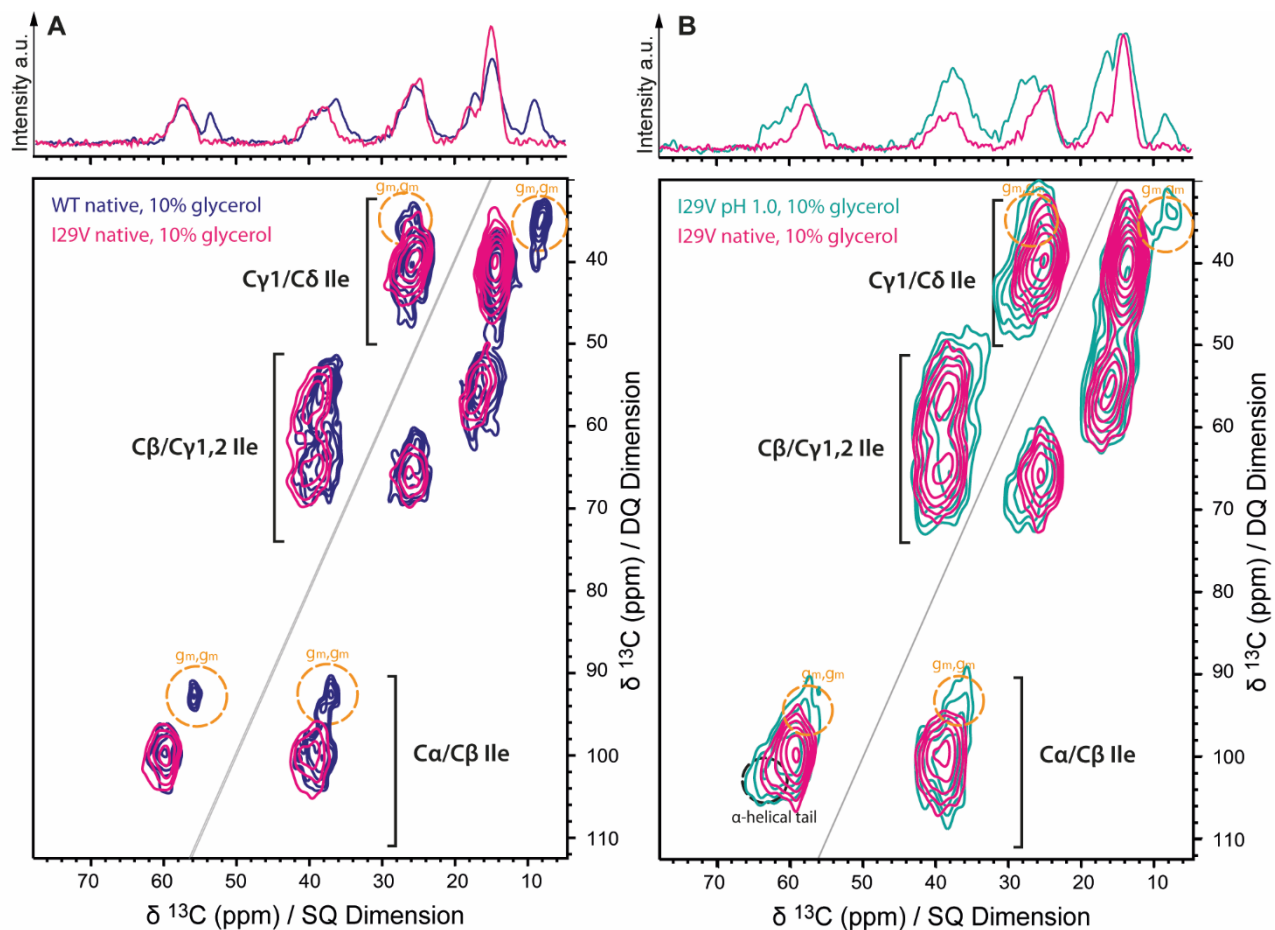


Figure S4: 2D ^{13}C - ^{13}C correlation DQ/SQ spectra of Ile-labeled PI3K SH3 I29V variant in its native form recorded at cryogenic temperatures in frozen solution with 10% glycerol (blue), overlaid with the wildtype Ile-labelled PI3K SH3 spectra recorded in the same condition (pink) (A) and with the unfolded I29V variant spectra recorded at pH 1.0, 10% glycerol (cyan) (B). Spectra were processed with squared cosine functions in both dimensions using effective maximum acquisition times of 5.6 ms in the direct dimension and 2.9 ms (both spectra overlaid in A) or 1.4 ms (both spectra overlaid in B), respectively, in the indirect dimensions.

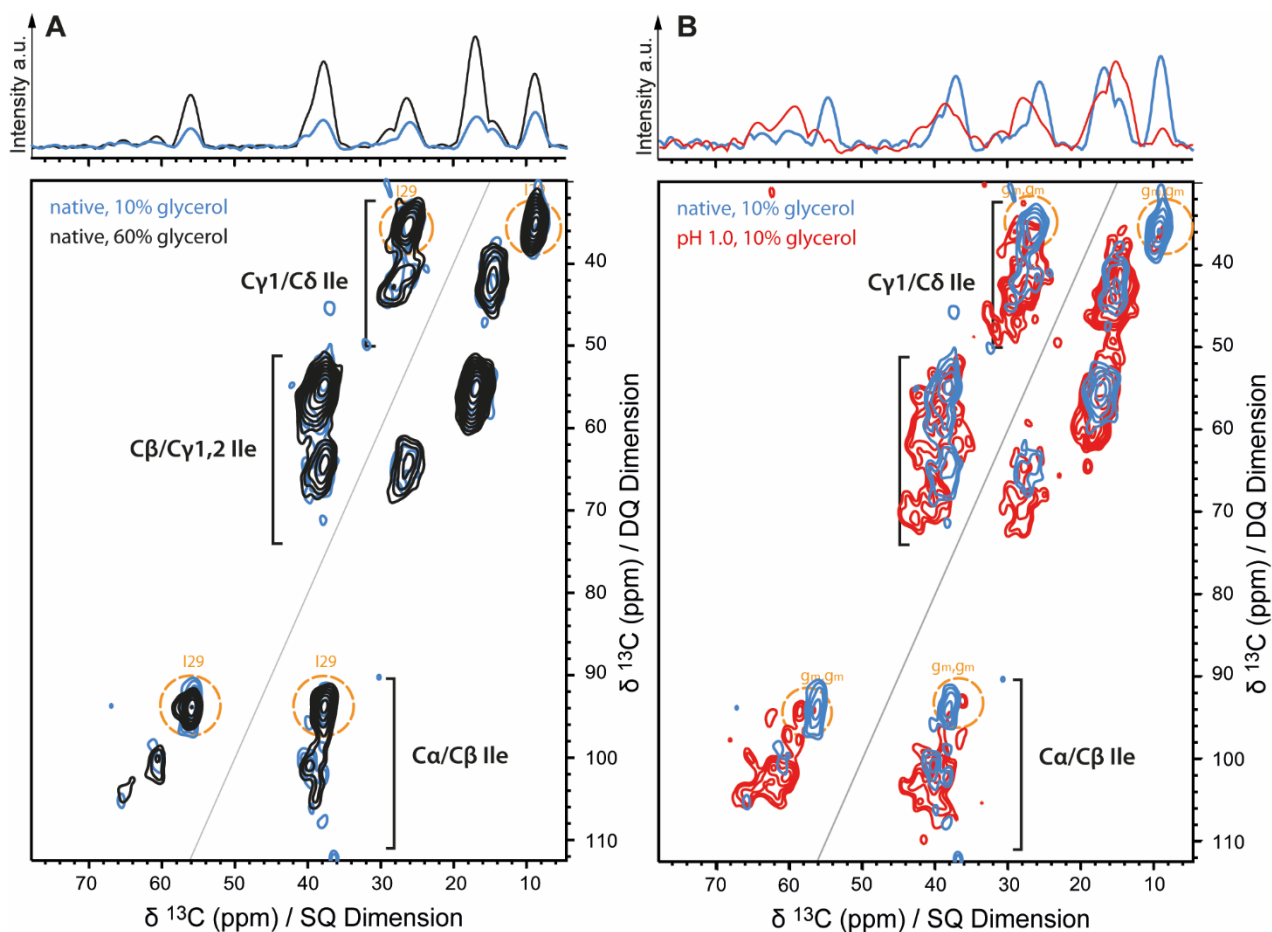


Figure S5: 2D ^{13}C - ^{13}C correlation DQ/SQ spectra of Ile-labeled PI3K SH3 I22V/I77V/I82V variant in its native form recorded at cryogenic temperatures in frozen solution with 60% (black) and 10% glycerol (cyan) (A). Comparison of the same isoleucine-labeled PI3K SH3 variant in its native form in 10% glycerol (blue) with its unfolded state at pH 1.0 (red) (B).

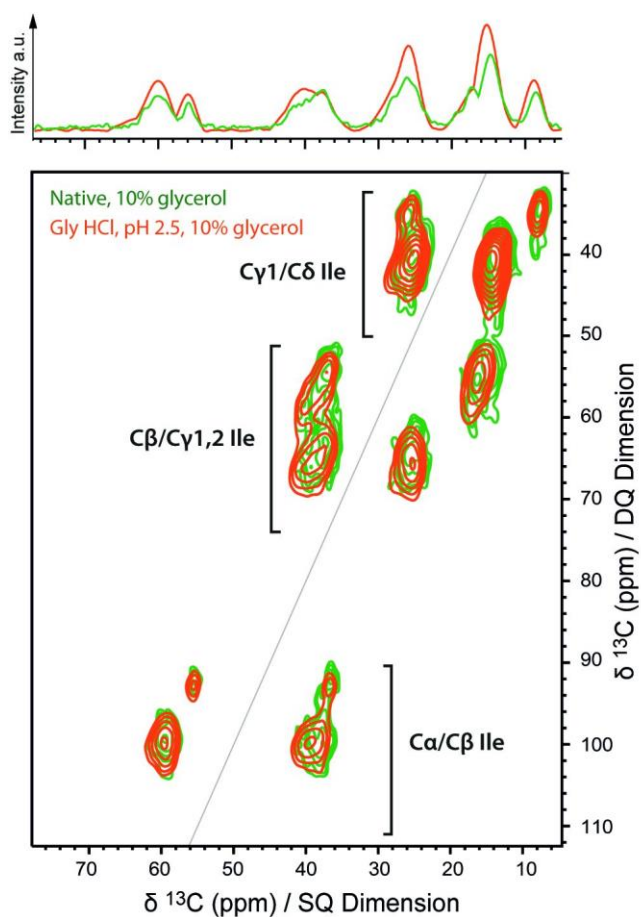


Figure S6: $2D^{13}\text{C}$ - ^{13}C correlation DQ/SQ spectra of isoleucine-labeled PI3K SH3 in its native form recorded at cryogenic temperatures in frozen solution with 10% glycerol (green) and in Gly HCl, pH 2.5 with 10% glycerol (orange).

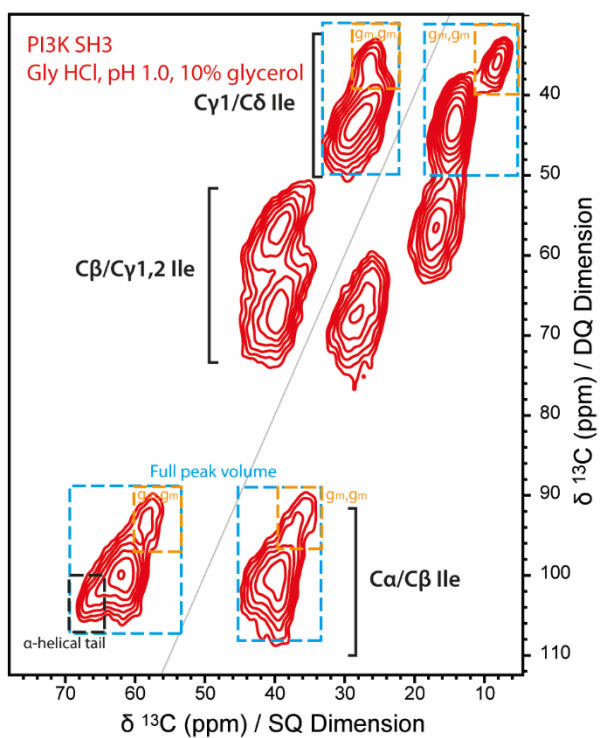
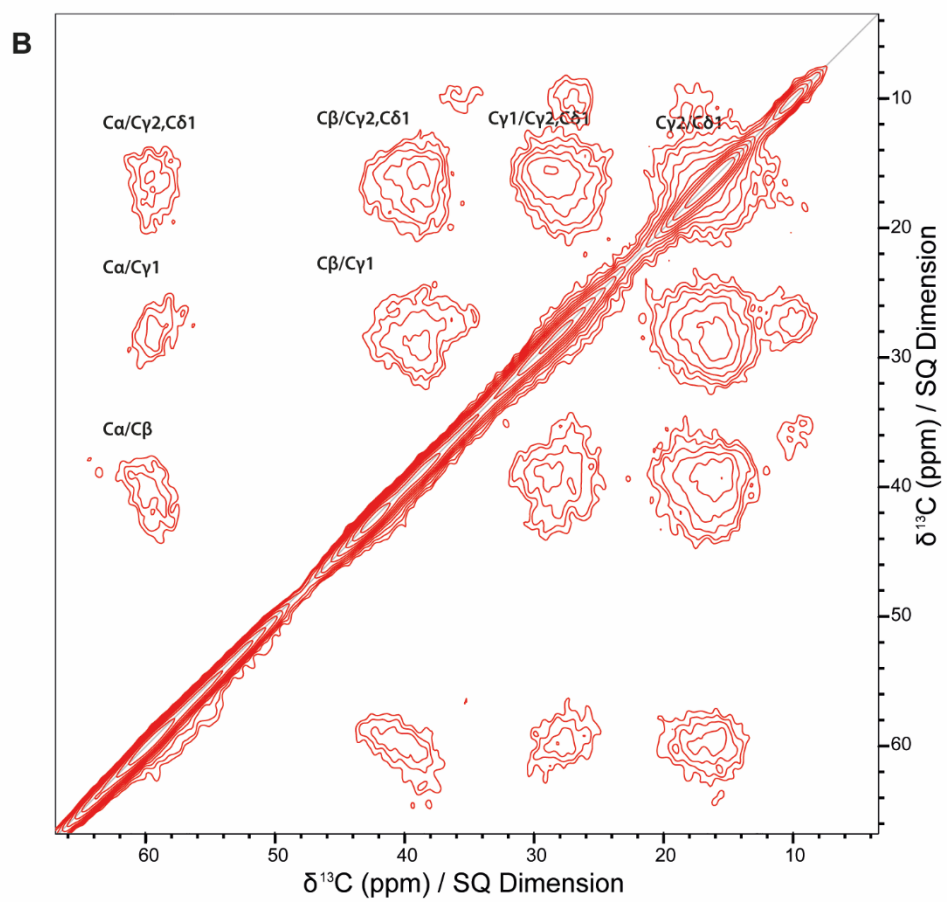
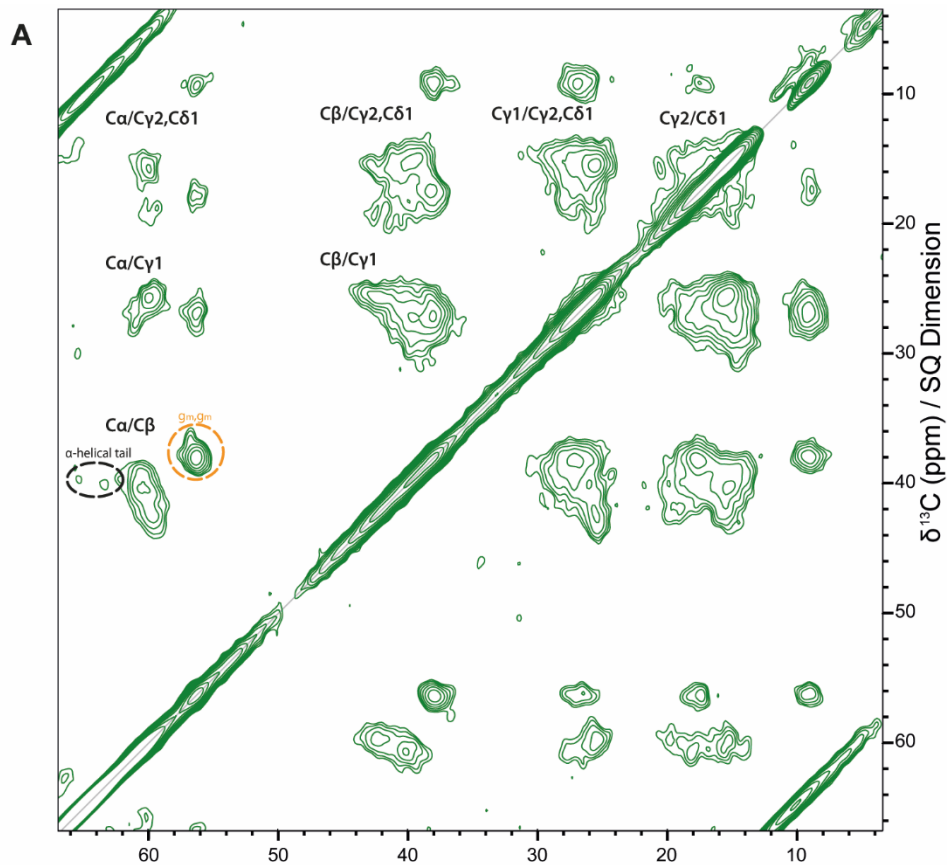


Figure S7: 2D ^{13}C - ^{13}C correlation DQ/SQ spectra of isoleucine labeled PI3K SH3 in its unfolded state in 25mM glycine HCl, pH 1.0. The areas for the volume integration are highlighted in yellow (g_m, g_m state), black (α -helical), and blue (full peak).



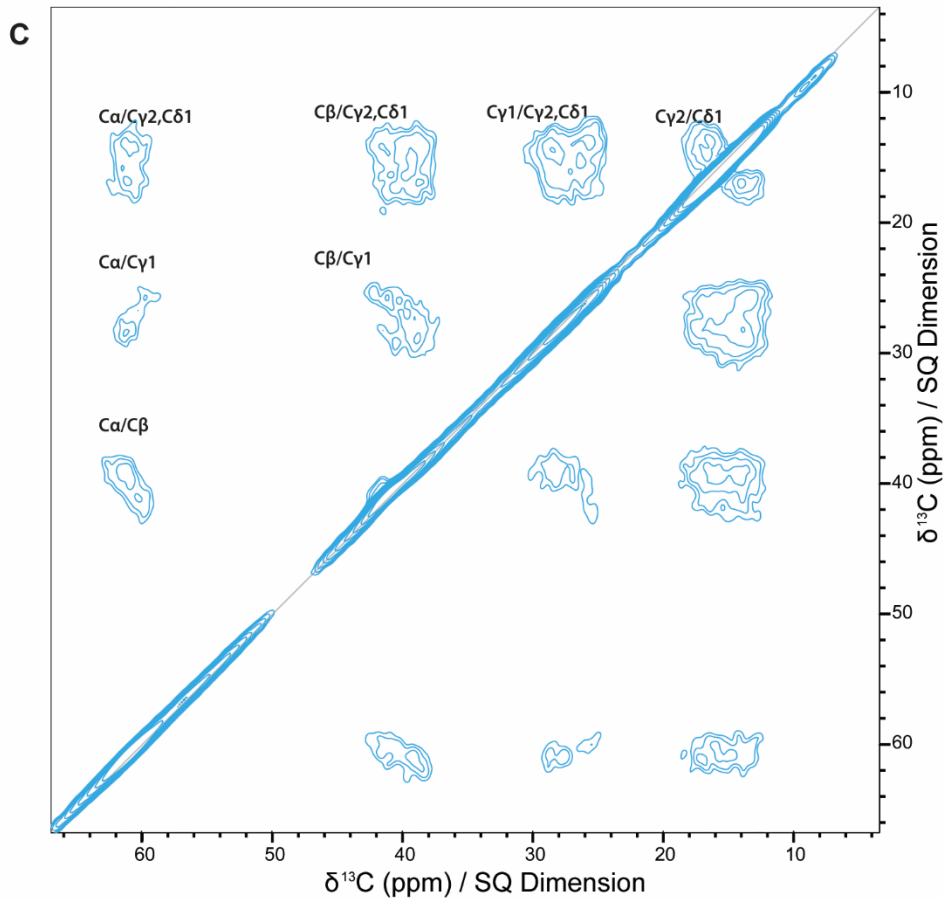


Figure S8: PDSD spectra of PI3K-SH3 in its native form at pH 6.8 with 10% glycerol (A), in 25 mM Gly HCl, pH 1.0 with 10% glycerol (B) and in GdmCl, pH 2.5 with 10% glycerol (C). The areas of the g_m, g_m and α -helical signals for the Ca/C β crosspeak are marked with circles. PDSD mixing times were 20 ms (A) or 50 ms (B and C). Spectra were processed with squared sine bell functions with sine bell shifts of 3 in each dimension. (Contour levels are displayed at positive multipliers of 1.41).

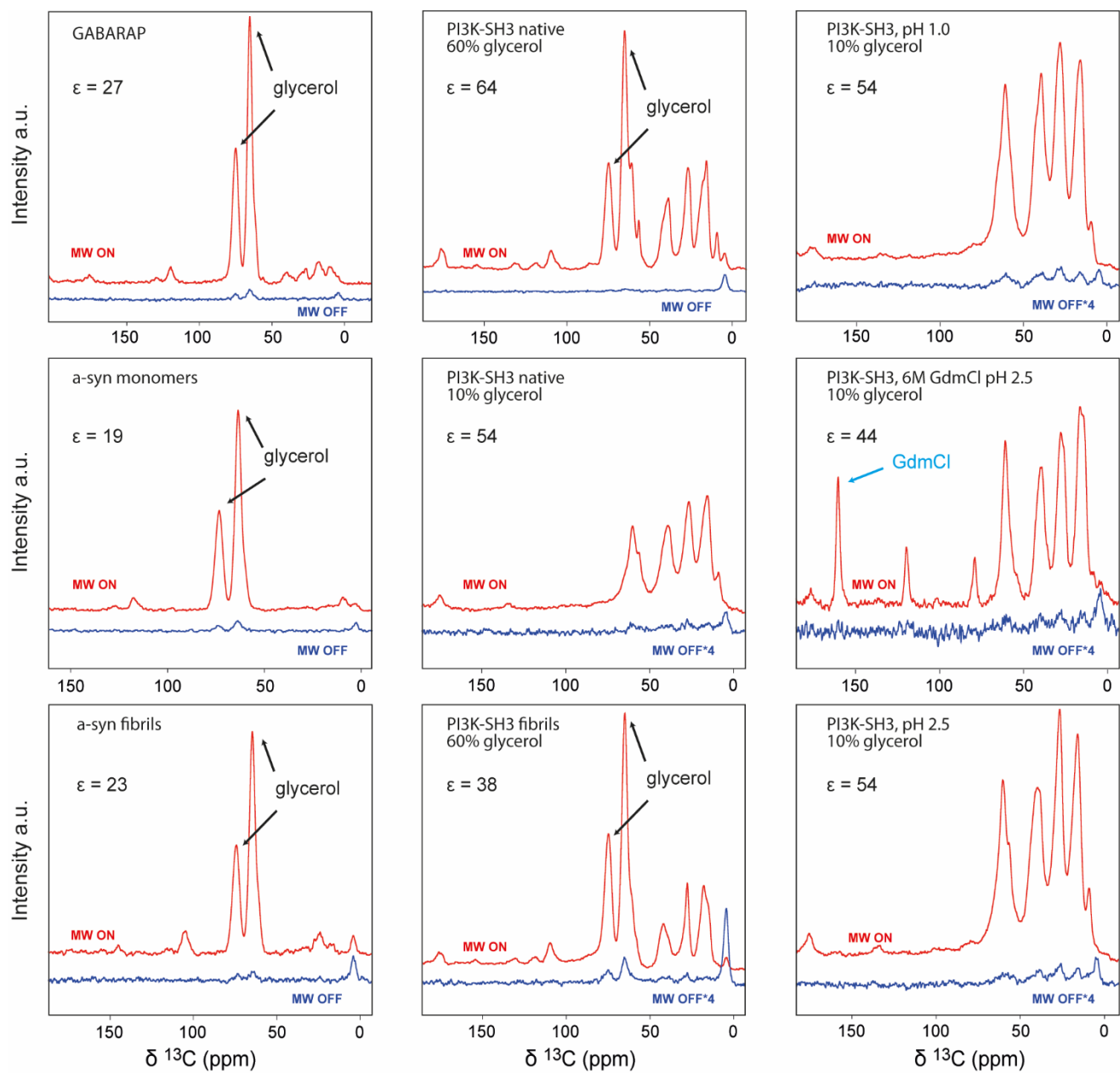


Figure S9: ^{13}C ssNMR spectra of all the samples recorded with and without microwave irradiation, with the respective DNP enhancement (ϵ) indicated.

Table S1: *line widths of signals in the spc5 spectrum of GABARAP in ppm.*

C α /C β	C α /C β (I32 g _m ,g _m)	C α /C β (I64 g _m ,g _m)	C β /C α	C β /C α (I32 g _m ,g _m)	C β /C α (I64 g _m ,g _m)	C γ /C δ	C δ /C γ	C δ /C γ (g _m ,g _m)	CG(g _m ,g _m)
2.8	1.3	1.4	3.6	1.4	1.6	2.3	1.7	1.7	2.0

Table S2: *Populations of different conformations measured in GABARAP spc5 spectrum.*

I32 (g _m ,g ^m)	I64 (g _m ,g _m)	I21 total	I21 (helical)	I21 (coil)
16±2%	16±2%	15±2%	8±2%	7±2%

Table S3: *Populations of different conformations measured in AIN, α -syn and PI3K SH3 spc5 spectra.*

Sample	C α /C β (g _m ,g _m)	C β /C α (g _m ,g _m)	C γ /C δ (g _m ,g _m)	C δ /C γ (g _m ,g _m)	Average value (g _m ,g _m) (%)	Index for C α /C β α -helical population (%)
AIN	15 $\pm$ 1	14 $\pm$ 1	14 $\pm$ 1	11 $\pm$ 1	14 $\pm$ 1	15 $\pm$ 1
α -syn monomers	14 $\pm$ 2	17 $\pm$ 2	14 $\pm$ 2	15 $\pm$ 2	15 $\pm$ 2	18 $\pm$ 2
α -syn fibrils	24 $\pm$ 6	11 $\pm$ 6	17 $\pm$ 6	14 $\pm$ 6	17 $\pm$ 6	9 $\pm$ 6
PI3K SH3 native, pH 6.8, 60% glycerol	26 $\pm$ 1	23 $\pm$ 1	26 $\pm$ 1	26 $\pm$ 1	25 $\pm$ 1	4 $\pm$ 1
PI3K SH3 native, pH 6.8, 10% glycerol	23 $\pm$ 1	22 $\pm$ 1	23 $\pm$ 1	23 $\pm$ 1	23 $\pm$ 1	1 $\pm$ 1
PI3K SH3, pH 2.5, 10% glycerol	20 $\pm$ 1	21 $\pm$ 1	20 $\pm$ 1	19 $\pm$ 1	20 $\pm$ 1	7 $\pm$ 1
PI3K SH3, 6M GdmCl, pH 2.5, 10% glycerol	8 $\pm$ 1	7 $\pm$ 1	9 $\pm$ 1	9 $\pm$ 1	8 $\pm$ 1	0 $\pm$ 1
PI3K SH3, pH 1.0, 10% glycerol	16 $\pm$ 1	16 $\pm$ 1	14 $\pm$ 1	19 $\pm$ 1	16 $\pm$ 1	16 $\pm$ 1
PI3K SH3 fibrils	11 $\pm$ 3	11 $\pm$ 3	9 $\pm$ 3	11 $\pm$ 3	11 $\pm$ 3	6 $\pm$ 3

Table S4: *line widths of signals in spc5 spectra of AIN, α -syn and PI3K SH3 in ppm.*

Sample	C α /C β (g _m ,g _m)	C α /C β	C β /C α	C β /C α (g _m ,g _m)	C γ /C δ	C γ /C δ (g _m ,g _m)	C δ /C γ	C δ /C γ (g _m ,g _m)
AIN	2.7	2.9	2.6	2.3	3.2	2.2	2.2	2.6
α -syn fibrils	2.0	3.5	2.6	1.7	1.7	1.6	2.1	1.8
α -syn monomers	4.9	3.3	4.9	4.1	2.7	2.7	3.6	3.1
PI3K SH3 native, pH 6.8, 60% glycerol	2.8	3.6	3.2	3.1	2.5	2.4	2.2	2.0
PI3K SH3 native, pH 6.8, 10% glycerol	4.6	1.7	4.4	2.0	4.0	4.0	2.8	2.3
PI3K SH3, pH 2.5, 10% glycerol	4.0	5.5	5.8	4.0	3.9	4.2	3.3	3.4
PI3K SH3, 6 M GdmCl pH 2.5, 10% glycerol	4.0	4.4	5.2	5.2	3.9	3.1	4.0	3.0
PI3K SH3, pH 1.0, 10% glycerol	6.0	5.4	4.2	4.7	3.5	3.2	3.0	2.8
PI3K SH3 fibrils, pH 2.5, 60 % glycerol	4.2	4.3	2.5	2.1	3.3	2.5	2.1	2.5

Table S5: Population values for each conformation detected for I29 and I53 in the isoleucine-labeled PI3K SH3 I22V/I77V/I82V variant, from the spectrum shown in Fig. S6

60% glycerol	g _m ,g _m (%)	Helical (%)	Coil (%)
Cα/Cβ	53±1	19±1	28±1
Cβ/Cα	50±1	22±1	28±1
Cγ/Cδ	54±1	46±1	
Cδ/Cγ	50±1	50±1	
10% glycerol	g _m ,g _m (%)	Helical (%)	Coil (%)
Cα/Cβ	48±1	14±1	38±1
Cβ/Cα	46±1	16±1	38±1
Cγ/Cδ	46±1	54±1	
Cδ/Cγ	46±1	54±1	
pH 1.0	g _m ,g _m (%)	Helical (%)	Coil (%)
Cα/Cβ	29±2	23±2	48±2
Cβ/Cα	26±2	24±2	50±2
Cγ/Cδ	34±2	66±2	
Cδ/Cγ	33±2	67±2	

Table S6: Detailed integrated areas of each peak for α -syn, AIN and PI3K SH3.

	C α /C β			C β /C α		C γ /C δ		C δ /C γ	
	g _m ,g _m	helical	total	g _m ,g _m	total	g _m ,g _m	total	g _m ,g _m	total
α -syn	F2: 60.0-55.0 ppm F1: 88.0-97.5 ppm	F2: 69.2-64.9 ppm F1: 103.1-108.9 ppm	F2: 69.2-55.0 ppm F1: 88.0-108.9 ppm	F2: 38.4-33.1 ppm F1: 89.1-97.3 ppm	F2: 46.1-33.1 ppm F1: 89.1-111.2 ppm	F2: 29.7-25.2 ppm F1: 33.5-39.6 ppm	F2: 33.7-23.2 ppm F1: 33.5-50.2 ppm	F2: 11.8-6.17 ppm F1: 30.5-40.0 ppm	F2: 20.0-6.17 ppm F1: 30.5-50.3 ppm
AIN	F2: 60.7-55.3 ppm F1: 88.0-97.5 ppm	F2: 68.0-64.2 ppm F1: 98.5-109.6 ppm	F2: 68.0-55.3 ppm F1: 88.0-109.6 ppm	F2: 39.8-33.3 ppm F1: 87.8-96.8 ppm	F2: 46.2-33.3 ppm F1: 87.8-114.1 ppm	F2: 29.5-24.4 ppm F1: 30.5-36.8 ppm	F2: 33.6-21.5 ppm F1: 30.5-51.1 ppm	F2: 11.5-6.5 ppm F1: 30.0-41.5 ppm	F2: 19.5-6.5 ppm F1: 30.0-49.1 ppm
PI3K SH3	F2: 59.0-53.5 ppm F1: 88.0-97.5 ppm	F2: 69.0-64.3 ppm F1: 97.5-108.5 ppm	F2: 69.0-53.5 ppm F1: 88.0-108.5 ppm	F2: 40.0-34.0 ppm F1: 87.5-96.8 ppm	F2: 45.5-34.0 ppm F1: 87.5-111.1 ppm	F2: 29.5-23.5 ppm F1: 30.3-37.5 ppm	F2: 33.5-23.5 ppm F1: 30.3-48.3 ppm	F2: 11.8-6.0 ppm F1: 28.0-40.0 ppm	F2: 18.8-6.0 ppm F1: 28.0-49.0 ppm

Table S7: *Populations of different conformations measured in PI3K SH3 PDSD spectrum.*

Sample	C α /C β (g _m ,g _m) (%)	C β /C α (g _m ,g _m) (%)	Average value (g _m ,g _m) (%)	Index for C α /C β α -helical population (%)
PI3K SH3 native, pH 6.8, 60% glycerol	27 $\pm$ 2	28 $\pm$ 2	28 $\pm$ 2	8 $\pm$ 2
PI3K SH3, 6M GdmCl pH 2.5, 10% glycerol	9 $\pm$ 1	7 $\pm$ 1	8 $\pm$ 1	4 $\pm$ 1
PI3K SH3, pH 1.0, 10% glycerol	15 $\pm$ 2	13 $\pm$ 2	14 $\pm$ 2	18 $\pm$ 2

Table S8: Experimental details for *spc5* spectra. SPC5 double quantum excitation and reconversion times were 488 μ s. Full data available here: <https://bmrbig.org/released/bmrbig114>

#	sample	Buffer	Polarizing agent	# t ₁ incr.	t ₁ max (ms)	number of scans per increment	total exp. time (h)	Enhancement factor ϵ
1	GABARAP	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	2.5 mM Amupol	394	6	48	36.93	27
2	AIN	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	2.5 mM Amupol	316	6	32	19.75	64
3	α -syn monomers	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	2.5 mM Amupol	100	1.5	240	46.85	19
4	α -syn fibrils	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	2.5 mM Amupol	200	3	112	43.73	23
5	PI3K SH3	60/30/10 d ₈ , glycerol/D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8,	5 mM M-Tinypol	200	3	112	43.73	64
6		10/80/10 d ₈ , glycerol/D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8,	5 mM M-Tinypol	200	3	112	43.73	54
7		50 mM glycine/HCl, pH 2.5, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	190	2.9	112	41.55	54
8		6M GdmCl, pH 2.5, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	190	2.9	112	41.55	44
9		50 mM glycine/HCl, pH 1.0, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	200	3	112	43.73	54
10	PI3K SH3 fibrils	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	5 mM M-Tinypol	302	4.6	32	18.88	38
11	I29V PI3K SH3	10/80/10 d ₈ , glycerol/D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8,	5 mM M-Tinypol	200	3	112	43.73	64
12		50 mM glycine/HCl, pH 1.0, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	93	1.4	112	20.3	44

13	122V/I77V /I82V PI3K SH3	60/30/10 d ₈ , glycerol/ D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8,	5 mM M- Tinypol	200	3	112	43.73	64
14		10/80/10 d ₈ , glycerol/ D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8,	5 mM M- Tinypol	200	3	112	43.73	64
15		50 mM glycine/HCl, pH 1.0, 10% d ₈ , ¹² C ₃ glycerol	5 mM M- Tinypol	200	3	112	43.73	54

Table S9: *Experimental details for pdsd spectra. Full data available here:*
<https://bmrbig.org/released/bmrbig114>

#	sample	Buffer	Polarizing agent	# t1 incr.	t ₁ max (ms)	mixing time (ms)	number of scans	total exp. time (h)	Enhancement factor ϵ
100	GABARAP	60/30/10 d ₈ glycerol/D ₂ O/H ₂ O	2.5 mM Amupol	576	6.5	10	64	51	27
101	PI3K SH3	60/30/10 d ₈ , glycerol/ D ₂ O/25 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ , pH 6.8	5 mM M-Tinypol	220	5	20	16	4.92	64
102		50 mM glycine/HCl, pH 1.0, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	480	6.7	50	64	43.22	54
103		6M GdmCl, pH 2.5, 10% d ₈ , ¹² C ₃ glycerol	5 mM M-Tinypol	480	6.7	50	64	43.22	44

Table S10: *Detailed processing parameters for spc5 spectra.*

#	sample	Window function	Sine bell shift SSB	AQeff SQ/DQ (msec)	Td _{eff}	Coutour level
1	GABARAP	-	-	4.5/2.1	400/140	1.31
2	AIN	QSINE	2/2	5.6/1.5	500/100	1.31
3	α -synuclein (monomeric)	QSINE	2/2	5.6/1.5	500/100	1.31
4	α -synuclein (fibrils)	QSINE	2/2	4.5/1.5	500/100	1.31
5	PI3K SH3	QSINE	2/2	5.6/2.9	500/190	1.31
6		QSINE	2/2	5.6/2.9	500/190	1.31
7		QSINE	2/2	5.6/2.9	500/190	1.31
8		QSINE	2/2	5.6/2.9	500/190	1.31
9		QSINE	2/2	5.6/2.9	500/190	1.31
10	PI3K SH3 fibrils	QSINE	2/2	5.6/2.4	500/160	1.31
11	I29V PI3K SH3	QSINE	2/2	5.6/2.9 5.6/1.4	500/190 500/93	1.31
12		QSINE	2/2	5.6/1.4	500/93	1.31
13	122V, I77V,	QSINE	2/2	5.6/2.9	500/190	1.31
14	I82V PI3K	QSINE	2/2	5.6/2.9	500/190	1.31
15	SH3	QSINE	2/2	5.6/2.9	500/190	1.31

Surface Mapping of Bovine PI3K-SH3 Domain using Gadoteric Acid for Solvent PRE effect

Luis Gardon, Nina Becker, Melinda Jaspert, Leonardo Levorin, Athina Apostilidis, Philipp Neudecker, Nils-Alexander Lakomek, Lothar Gremer, Henrike Heise

Abstract

Amyloid fibril formation is related to numerous neurodegenerative diseases. PI3K-SH3 serves as a well-established model system for investigating the mechanisms underlying amyloid fibril formation. Here, we employ a combination of solution and solid-state NMR spectroscopy combined with solvent Paramagnetic Relaxation Enhancement (sPRE) using meglumine gadoteric acid (Dotarem) to map the surface of both bovine PI3K-SH3 in all stages of fibrillation. We detect a high accessibility at low concentrations of Dotarem in regions of the globular monomeric state at neutral pH corresponding to the binding site of PI3K-SH3 to proline rich sequences. In PI3K-SH3 fibrils the fibril core appears shielded from the solvent, whereas the homodimer interface remains solvent-accessible. Notably, a few residues stay shielded at elevated Dotarem concentrations in both the monomeric and fibrillar state. These results demonstrate that sPRE is a straightforward and effective method for probing protein surface accessibility. Moreover, it might present a valuable complementary approach for characterizing aggregation interfaces and surfaces of amyloidogenic proteins in different states.

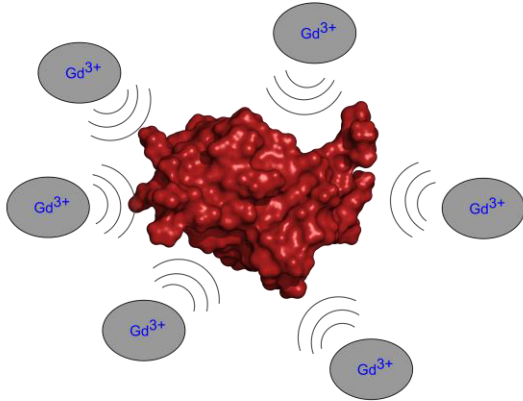
Introduction

Paramagnetic relaxation enhancement (PRE) in NMR spectroscopy leads to accelerated nuclear spin relaxation rates for nuclei in proximity to a paramagnetic center ¹. Spectral resonances in NMR spectra are, therefore, broadened or suppressed in the spatial presence of paramagnetic agents. Through site-directed mutagenesis, paramagnetic probes can be introduced at defined solvent-exposed residues, where they are covalently attached via spin labeling. This can be used as a tool to assist structure elucidation and measure distances in proteins in EPR and NMR ^{2, 3}. However, the paramagnetic agent can also be added to the solvent to cause a solvent PRE effect to probe the solvent accessible surface of proteins ⁴. Solvent PRE was used on folded proteins supporting structure determination ⁵, intrinsically disordered proteins ^{6, 7}, large biomolecular complexes ^{8, 9} and fibrils ¹⁰ using liquid-or solid-state NMR experiments ¹¹. For structural studies, a solvent PRE agent must be inert and soluble.

In this study we probe the surface of monomeric, globular and fibrillar PI3K-SH3 domain, a model system for amyloid fibril formation for years ¹². Under neutral pH conditions SH3 domains adopt a well-folded beta-sandwich shape ^{13, 14}. At acidic pH, however, the protein unfolds and forms fibrils^{12, 15}. High-resolution structures at the atomic level have been obtained ^{16, 17}. To elucidate the structural characteristics of SH3 in both its native and fibrillar forms, we utilize Dotarem to induce solvent paramagnetic relaxation enhancement (PRE) effects, as shown in Figure 1. Although it was shown for other Gd containing agents to interact with carboxylic groups of proteins ^{18, 19}, gadoteric acid of Dotarem is an inert substance used for MRI with a high stability and no cross effects^{20, 21}. We record ¹H-¹⁵N HSQC spectra for the monomer and ¹³C-¹³C correlation spectra for fibrils at different DOTAREM concentrations and compare our results to known structures of both.

Solvent PRE

SH3 Monomer



SH3 Fibrils

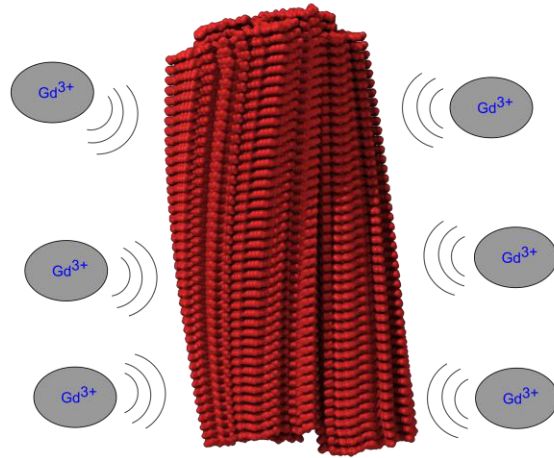


Figure 1: Solvent PRE effect. Inert gadoteric acid in the solvent distributes homogenously around the protein (PDB for SH3 monomer: 1PNJ, for SH3 fibrils: 6R4R). Gd^{3+} in the center of Gadoteric acid is paramagnetic and leads to faster spin relaxation and therefore line broadening of nearby nuclei resonances in NMR spectra.

Results

sPRE effect on the native state of PI3K-SH3 at neutral pH reveals a hot spot of solvent accessibility at low Dotarem concentrations. To study the PI3K-SH3 monomer in its globular fold 1H - ^{15}N HSQC spectra at different Dotarem concentrations were recorded. Due to the folded nature of the PI3K-SH3 monomer the peaks in the 1H - ^{15}N HSQC spectrum are well separated. Peak intensities were integrated for the different Dotarem concentrations and divided by the integral in the reference spectrum in absence of Dotarem (Figure 2, top) and plotted on the surface representation of the native fold of PI3K-SH3 (PDB:1PNJ¹⁴) (Figure 2, bottom). No peak shifting is observed excluding structural changes of PI3K-SH3 in presence of Dotarem in the solvens (Figure S1). An assignment of the globular folded monomer at neutral pH was carried out (Fig. S2) and is in good agreement with Hsu et al ²² (BMRB accession number 19110). Proline residues and L58 are not assigned in 1H - ^{15}N HSQC spectra and residues G69/80G and 81R/4S are overlapping, respectively, and couldn't be distinguished perfectly. At 2 mM Dotarem concentration, line broadening become visible and reduce several spectral intensities including most significantly the regions 11L-15K and 72T-74V which are far apart in sequence but close in space (highlighted in Figure 2). These residues were also found to be near the binding pocket found for a 12-residue proline-rich peptide PD1R ¹³. This was observed independently by Booker et al. with the binding of a

synthetic peptide (dynamin) at this site¹⁴. At increasing Dotarem concentrations, the line broadening/reduced integral of all resonances eventually increases (Figure S2-S8) with a dominance in the described region, whereas the other part of the protein surface is not as affected. Interestingly, highly charged regions with a high abundance of charged amino acid residues of the protein (e.g., residues 19-25 or 48-54) are not as affected. Negatively charges could also be shielded by the positively charged counterion meglumine and don't allow the Gadoteric acid to come close.

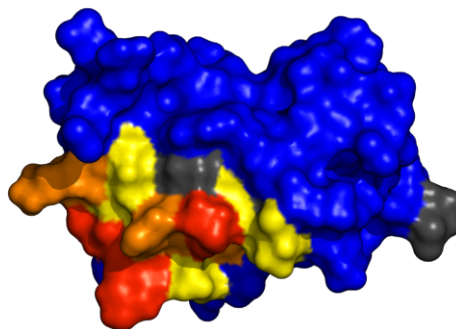
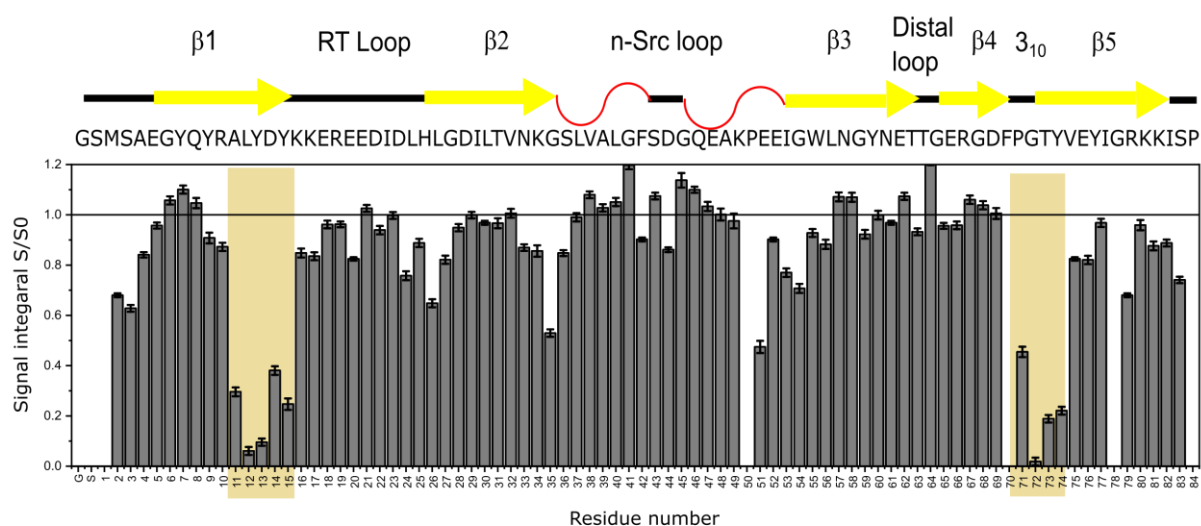


Figure 2: Solvent PRE effect on native SH3 at 2 mM Dotarem concentration. Top: Primary sequence and secondary structure elements of PI3K-SH3 derived from TALOSN. Bar plot showing the integral of the respective residue resonance divided by the integral of the same residue in the reference spectrum in the absence of Dotarem. Bottom: The most effected residues are located at one epitope when plotted on the structure (1PNJ). Color code: Red: high exposure ($I/I_0 \leq 0.1$), Orange: medium exposure ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposure ($0.25 < I/I_0 \leq 0.5$), Blue: minimal exposure ($I/I_0 > 0.5$), Grey: not assigned.

(We can choose the thresholds).

Residues in the fibril core are shielded from the solvent. We use seeded fibril formation at pH 2.5 with glycine HCl buffer¹⁶. Dotarem was added to the solvent after fibril growth, followed by incubation for few hours. To prevent dissolving the fibrils¹⁶, the Dotarem solution was pH adjusted. For every Dotarem concentration a new fibril solution was placed in a rotor.

A resonance assignment of PI3K-SH3 fibrils allowed 43 of the 86 residues to be assigned (Figure 3). Residues Y12 to D28 appear to be absent in the spectra which might be due to flexibility of this region. Our obtained shifts differ from those reported by Bayro et al.¹⁷, likely due to variations in fibril growth conditions and therefore different fibril polymorphs. Due to limitations in the handling of fibrillar samples we simplified the spectral analysis to visible, i.e. solvent shielded, and not visible, i.e. solvent accessible residues.

In the presence of 2 mM Dotarem many resonances in the ¹³C-¹³C PDSD correlation spectrum vanish (Figure 3, bottom marked in red). Nevertheless, certain resonances remain visible in the spectra (Figure 3 Top). When mapped onto the surface representation of the fibril structure (6R4R) solved by cryo-EM, these signals predominantly correspond to residues of the fibril core (indicated in blue). This includes the side chains for several aliphatic amino acids since they are also detected and observed in the PDSD spectrum at 50 ms mix (e.g. A48, E49, I53, T62, T63).

Interestingly, the fibril protomer interface seems solvent accessible suggesting that a fibril type is present which is not a homodimer (also seen in Röder but not predominant (in AFM)). AFM micrographs show a different fibril polymorph present in the spectra with 10 mM Dotarem approximately half thickness of the one present without Dotarem (Figure S10, indicated with red arrows). Moreover, minor chemical shift perturbations are observed for a few resonances at 1 mM and 2 mM Dotarem concentrations (Figure 3 and Figure S11, marked with red squares). These shifts differ between the two concentrations, potentially indicating the presence of different predominant polymorphs. Despite the high concentration, residues comprising the fibril core remain detectable even at 10 mM Dotarem (Figure S13). However, some resonances visible in the 10 mM Dotarem spectra remain unassigned, limiting a complete assessment of solvent exposure.

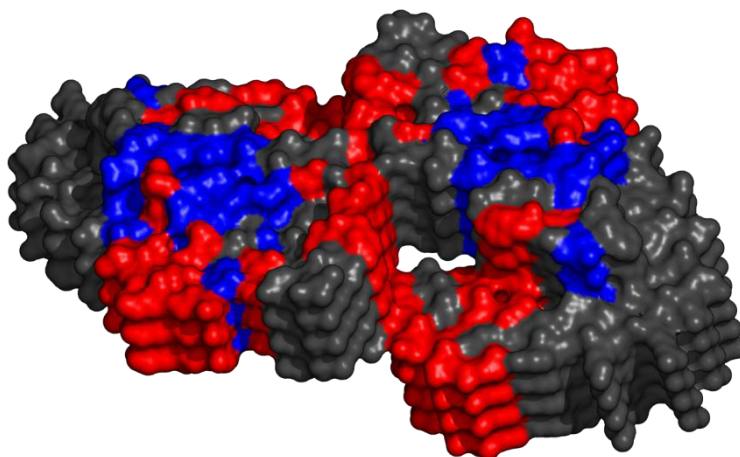
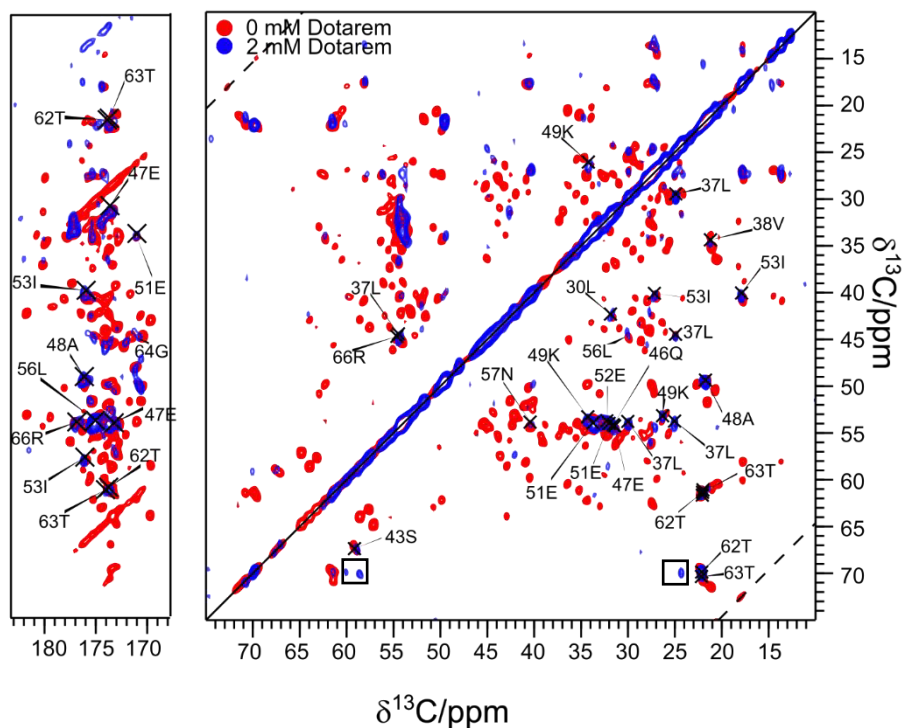


Figure 3: Solvent PRE effect on PI3K-SH3 fibrils at 2 mM Dotarem concentration. A) Overlay of ^{13}C - ^{13}C proton driven spin diffusion (PDS) spectra at 11 kHz and 50 ms mixing time of SH3 fibrils without (red) and 2 mM Dotarem (blue) concentration. B) Surface representation of SH3 fibrils (PDB:6R4R); Resonances for residues that are present in the 2 mM Dotarem spectrum are marked in blue. Resonances that vanish are marked in red and unassigned residues are marked in grey.

Conclusion

Solvent PRE was used to map the surface of PI3K-SH3 in both its native and fibrillar state. In the native state a “hot spot” at low Dotarem was observed at a region known to bind peptides^{13, 14} (Figure 4, top) which corresponds to the region of protein-protein interaction in the SH3 superfamily domain²³. This observation might be caused by a transient interaction with Dotarem in the binding site of SH3 or self-association of SH3 proteins with a surface exposition in this region at the protein concentration used (250 μ M). A comparison with a hydrophobicity plot shows that the region 11L-15K, which is close to the most solvent-exposed part of the protein, does not align perfectly with the plot (Figure S13). Another solvent-exposed region, 72T-74V, is not predicted to be solvent-exposed in the plot.

For fibrillar SH3 the core region of a subunit of the homodimer is shielded from the solvent (i.e. visible in the spectra) at the Dotarem concentrations used (Figure 3, bottom). Interestingly, the interface of the homodimer is not shielded, which could be explained by a monomeric nature of one fibril morph. Some peaks remain visible but could not be assigned. The most solvent exposed residues at low Dotarem concentration for the globular fold are close in space (Figure 1). These Residues near to N- and C-terminus are also close in space in the fibrils. Additionally, residues 71-74 are exposed to Dotarem at 1 mM in the monomer and the fibrils (the other solvent exposed region in the monomer is not assigned in the fibril).

Comparison solvent accessibility between native and fibrillar state shows that residues E47 to K49 stay shielded in the globular monomer and in fibrils at 10 mM Dotarem. Interestingly, at 25 mM Dotarem Residues 37-49 are still over 10 % of the integral of the reference spectrum in the monomeric state. These residues resemble some of those comprising the fibril core (S43-F69) and may remain spatially close upon unfolding and forming amyloid fibrils at low pH. PI3K-SH3 undergoes a transition from a folded to an unfolded structure at low pH^{24, 25} with both a folded and unfolded state being present at room temperatures (and 10 mM glycine HCl concentration). Experiments with varying Dotarem concentrations on the low pH state of PI3K-SH3 show a linear decrease in signal intensities with higher solvent PRE effect at lower Dotarem concentrations compared to the neutral pH state (Figure S9). However, an assignment is missing for this state. This study demonstrates how solvent PRE data can be effectively used to analyze the surface properties of proteins, even in different conformational states of an amyloidogenic protein.

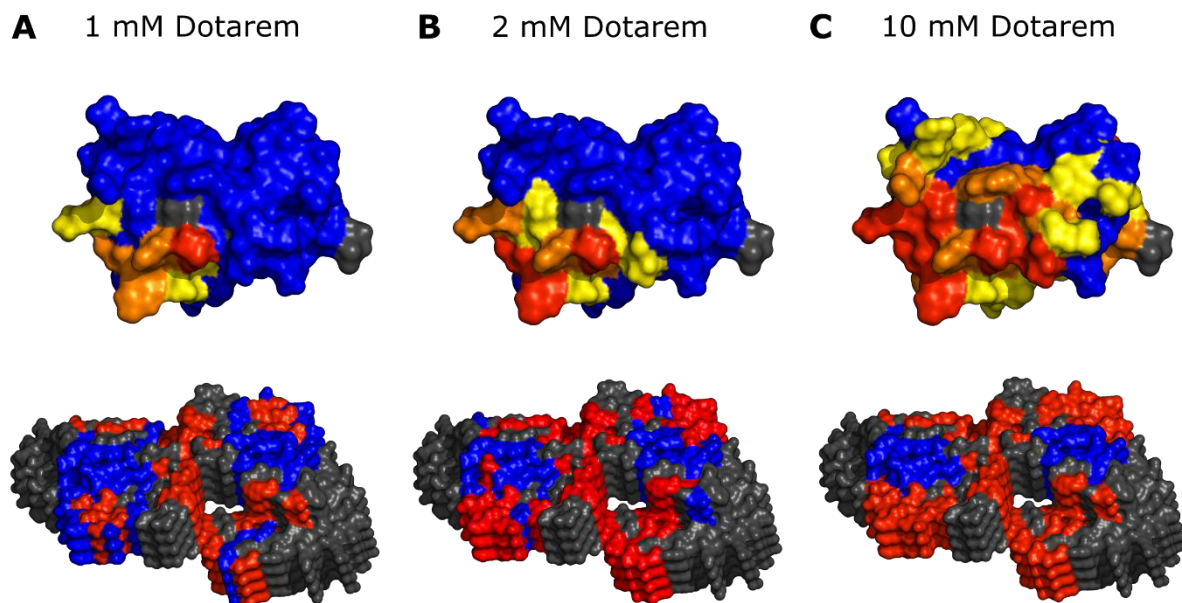


Figure 4: Surface representation of the solvent PRE effect on the structure of the monomer (1PNJ, top) and fibrils (6R4R, bottom) of PI3K-SH3. Plotted is the effect of different Dotarem concentrations on the protein with A) 1 mM, B) 2 mM and C) 10 mM Dotarem. Color code top: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey not assigned. Color code bottom: visible in spectra containing Dotarem are marked in blue, vanished resonances in red and unassigned residues in grey.

Methods

Purification and sample preparation

PI3K-SH3 was expressed in *E.coli* and purified as described previously²⁵ using a GST-tag. In short, M9 Medium containing 3.2 g ¹³C glucose and 2 g ¹⁵N ammonium chloride was inoculated by BL21 (DE3) pre culture. Plasmid gene expression was induced using 1 mM IPTG at a cell density of 0.6 OD600. After incubation overnight at 25 °C cells were harvested, and the pellets were lysated and sonicated. GST-affinity, thrombin cleavage and size exclusion chromatography were used to isolate the SH3 domain from the mixture. SH3 was aliquoted and lyophilized for further usage. A glycine-serine overhang at the N-terminus is present due to the thrombin cleavage. Sequence:

Solution-state NMR sample preparation:

For solvent PRE on the native state 50 nmol of lyophilized SH3 were dissolved in 200 µl buffer containing 25 mM sodium phosphate, pH 6.8 and the desired Dotarem concentration. A Dotarem stock of 0.5 M and 0.05 M were used to adjust the Dotarem concentration to 1,2,5,10 and 25 mM final.

For solvent PRE on the low pH state 50 nmol of lyophilized SH3 were dissolved in 200 µl buffer containing 10 mM glycine hydrochloride, pH 2.5, and the desired Dotarem concentration. A Dotarem stock of 100 mM, adjusted with HCl to pH 2.5 (6 mM) was used to concentrations of a final Dotarem concentration of 0.5, 1, 2 and 5 mM Dotarem in the sample.

Solid-state NMR sample preparation:

Fibrillar samples were prepared by dissolving lyophilized PI3K-SH3 in 10 mM glycine HCl, pH 2.5 and 5 % equivalent monomer mass of seeds²⁵ and incubating the solution for 1 day at 50 °C to form amyloid fibrils (confirmed by AFM). After fibril growth Dotarem from a 100 mM stock, that was adjusted to pH 2.5 with HCl, was added to the fibril solution and incubated for at least 1 h. Afterwards, a 3.2 mM rotor was filled with approx. 2 mg of fibrillar PI3K-SH3 with homemade rotor filling devices.

NMR Spectroscopy

Solution NMR measurements were conducted on a Bruker 600 MHz Avance III HD spectrometer equipped with a ¹H, ¹³C, ¹⁵N triple resonance TCI cryoprobe for the native state of PI3K-SH3. For the low pH state NMR measurements were conducted on a Bruker 800 MHz Avance III HD spectrometer equipped with ¹H, ¹³C, ¹⁵N triple resonance TXO probe. For analysis two-dimensional solution state NMR ¹H-¹⁵N HSQC spectra were recorded. Detailed parameters are listed in table S1 below. The temperature was set to 20 °C (no calibration with methanol?).

Table S1: Experimental parameters of 2D ^1H - ^{15}N HSQC experiments:

	Native state	Low pH state
Field (T)	14.1	18.8
^1H Larmor frequency	600 MHz	800 MHz
Sample volume	200 μl (3 mm tube)	200 μl (3 mm tube)
Interscan recovery delay (s)	1	1.2
Number of scans	16	8
Dummy scans	2	8
Total experimental time	56 min 36 sec	56 min 39 sec
Direct dimension (^1H)		
^1H carrier (ppm)	4.7	4.698
Spectral width (ppm)	16.0293	16.0293
Data points	2048	2048
Acquisition time (ms)	106.5	79.9
Indirect dimension (^{15}N)		
^{15}N carrier (ppm)	117	117
Spectral width (ppm)	35	35
Data points	256	256
Acquisition time (ms)	60.2	44.8

Solid-state NMR experiments were conducted on an 18.8 T (800 MHz ^1H frequency) Avance III spectrometer equipped with triple resonance HCN 3.2 mm MAS Efree probe. Typical radiofrequency field strengths were ~ 90 kHz for ^1H and 55 kHz for ^{13}C . The VT gas temperature was set to 263 K (thermocouple reported temperature); the sample temperature was estimated to be around 5–10 K higher due to frictional heating under MAS. Spinal64 ^1H decoupling (rf field of 83–85 kHz) was applied during ^{13}C acquisition. The MAS frequency was set to 11 kHz for the Proton Driven Spin Diffusion (PDSD) and the mixing time for short mainly intraresidual transfer was set to 50 ms. Detailed experimental parameters can be found in Table S2 for every Dotarem concentration. ^{13}C chemical shifts were externally referenced using adamantane by setting its CH signal to 31.4 ppm (corresponding to the DSS reference scale). The ^{15}N chemical shifts were indirectly referenced to liquid NH_3 .

Table S2: Experimental parameters of 2D ^{13}C - ^{13}C PDSD experiments:

	Referenz	1 mM Dotarem	2 mM Dotarem	5 mM Dotarem	10 mM Dotarem
Field (T)	18.8	18.8	18.8	18.8	18.8
^1H Larmor frequency	800 MHz	800 MHz	800 MHz	800 MHz	800 MHz
Rotor size	3.2 mm (Thin wall, 46.7 μl)	3.2 mm (Thick wall, 32.1 μl)	3.2 mm (Thick wall, 32.1 μl)	3.2 mm (Thick wall, 32.1 μl)	3.2 mm (Thin wall, 46.7 μl)
Interscan recovery delay (s)	2	2	2	2	2
^1H-^{13}C CP	ZQ ($2n \omega_{\text{rot}}$)	ZQ ($1n \omega_{\text{rot}}$)	ZQ ($3n \omega_{\text{rot}}$)	ZQ ($1n \omega_{\text{rot}}$)	ZQ ($2n \omega_{\text{rot}}$)
Contact time	800	250	300	200	700
^1H field (kHz)	73	61	83	61	73
^{13}C field (kHz)	51	50	50	50	51
^1H carrier (ppm)	6.2	6.2	6.2	6.2	6.2
^{13}C carrier (ppm)	100	100	100	100	100
t1 increments	480	500	440	450	480
t1 spectral width	200	218	218	218	200
t1 acquisition time (ms)	6	6	5	5	6
T2 acquisition time (ms)	10	9	9	9	10
Number of scans	160	216	232	280	160
^1H Decoupling power (kHz) during acquisition	85	83	83	83	85
Window function FID	Qsine (4,4)	Qsine (4,4)	Qsine (4,4)	Qsine (4,4)	Qsine (4,4)
Contour level spacing	1.2	1.2	1.2	1.2	1.2

Spectra analysis

All spectra were processed using the Bruker Topspin 4 software (Bruker, Billerica, MA, United States). Two-dimensional spectra were plotted using the CCPN Analysis 2.4 software²⁶. Surface and Cartoon 3 D representation were presented using PyMOL(TM) 3.1.3²⁷.

Integration of the peaks in two-dimensional solution NMR spectra for analysis of the Dotarem effect on the native state of PI3K-SH3 was performed using Topspin 4. Peak areas were quantified by integrating over a square of 0.2 ppm in the ¹H and 1 ppm in the ¹⁵N dimension. Noise levels were estimated by integrating a square region of the identical dimensions in an empty region of each spectrum recorded at a given Dotarem concentration. These values were then referenced to the corresponding peak in the reference spectrum to obtain residue-specific error bars across Dotarem concentrations.

Atomic force microscopy (AFM)

For AFM measurements, 100 μM fibril solution of PI3K-SH3 was diluted in 10 mM glycine-hydrochloride buffer, pH 2.5, to a final protein concentration of 2 μM (equivalent to monomer concentration). A volume of 10 μL of the solution was pipetted on freshly cleaved mica surface and incubated for 15 minutes. The mica surface was then washed four times with 100 μL Milli-Q water and dried under a gentle nitrogen gas flush. AFM micrographs were acquired in ScanAsyst mode using the peak-force tapping mechanism on a Bruker Multimode 8 (Billerica, Massachusetts, United States) using OMCL-AC160TS cantilevers (Shinjuku, Tokyo, Japan) at 1024 × 1024 pixel resolution. The images were processed with Gwyddion 2.61²⁸.

Supplementary information

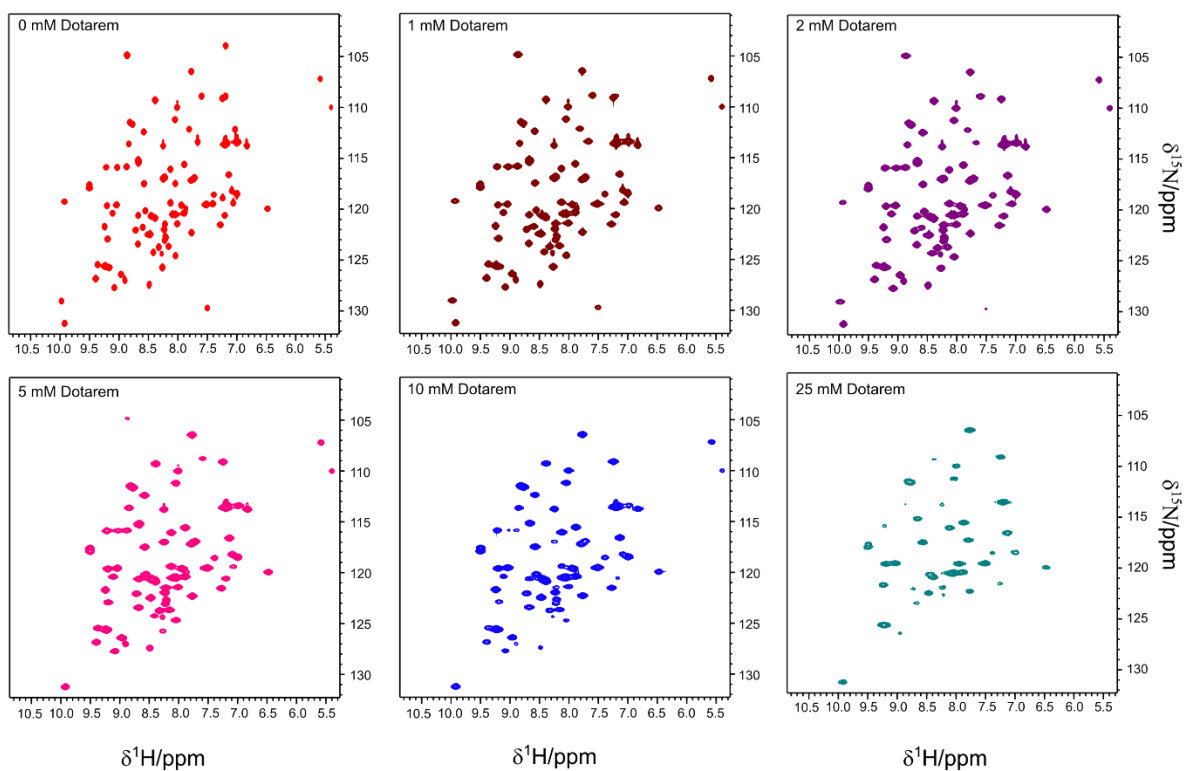


Figure S1: ^1H - ^{15}N HSQC spectra of PI3K-SH3 (250 μM) in 25 mM NaPi, pH 6.8, at 25 $^\circ\text{C}$ and varying (0, 1, 2, 5, 10 and 25 mM) Dotarem concentration in the solvents. Line broadening and peak suppression is increased with increasing Dotarem concentrations.

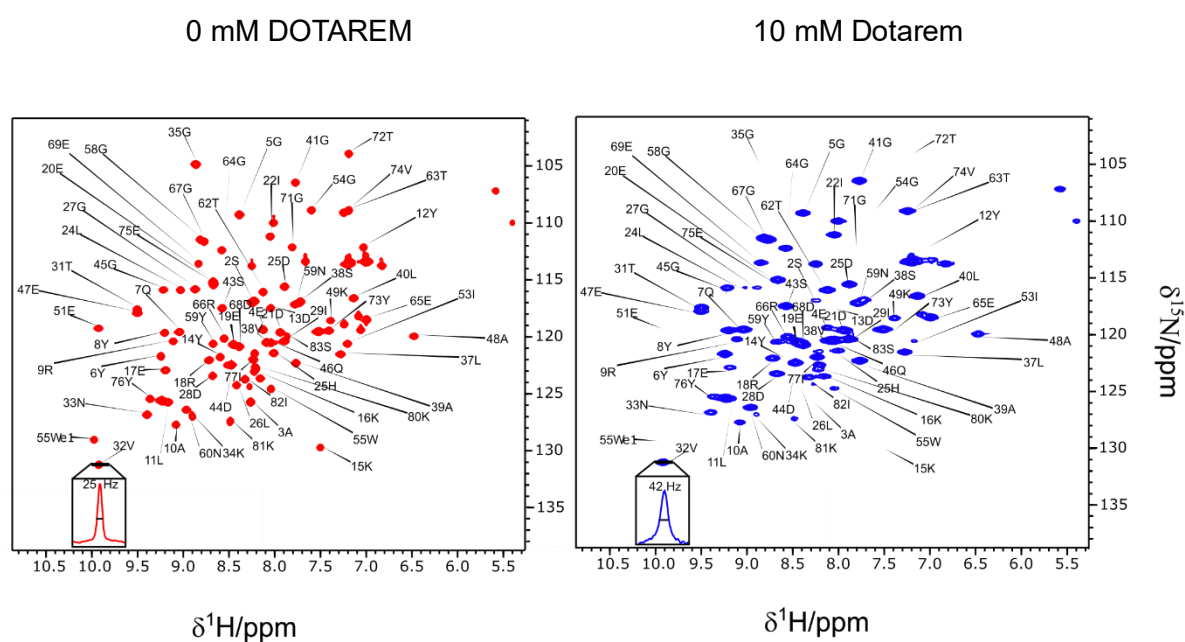


Figure S2: Assignment and solvent PRE effect in ^1H - ^{15}N HSQC spectra of PI3K-SH3 (250 μM) in 25 mM NaPi, pH 6.8, at 25 $^\circ\text{C}$. A) ^1H ^{15}N HSQC spectra for SH3 monomer at the absence of Dotarem (reference, red) and 10 mM Dotarem concentration. Line broadening is shown for V34 as an example.

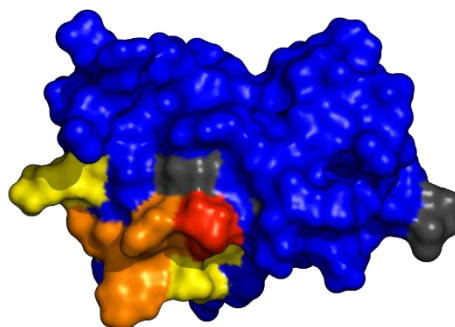
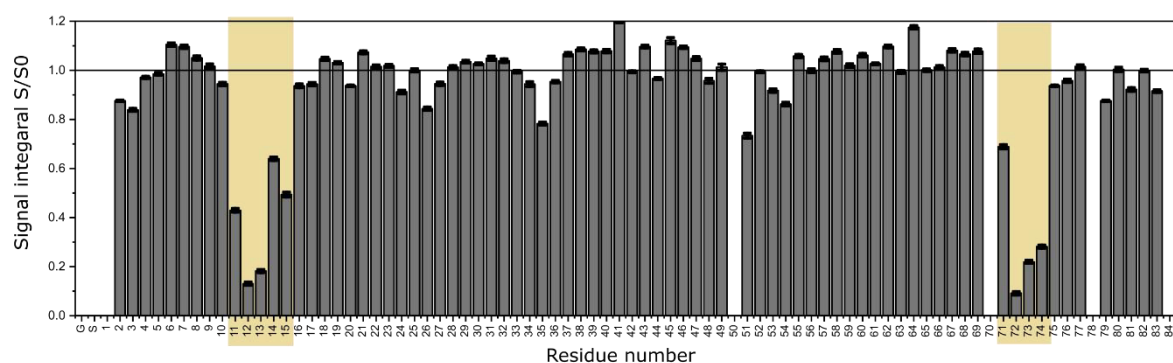


Figure S3: Top: Bar plot of integral changes of residue resonances in ^1H - ^{15}N HSQC spectrum for 1 mM Dotarem compared to 0 mM Dotarem as I/I_0 . Error bars were estimated integrating the noise region for each Dotarem concentration dependent spectra and normalizing it to the reference spectrum. Bottom: Surface representation of SH3 Monomer (PDB:1PNJ) with Integrals of (top). Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

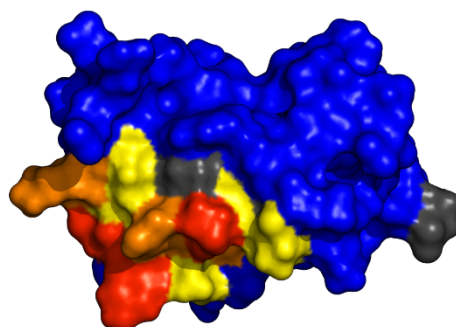
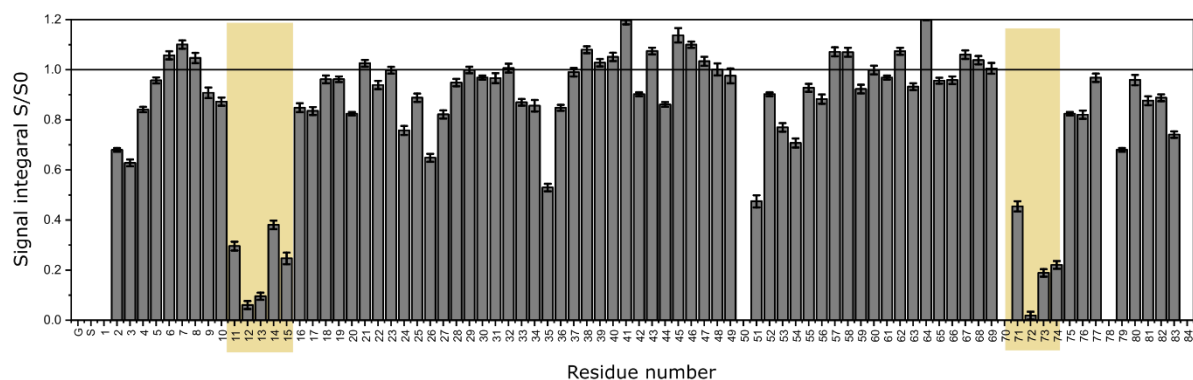


Figure S4: Top: Barplot of integral changes of residue resonances in ^1H - ^{15}N HSQC spectrum for 2 mM Dotarem compared to 0 mM Dotarem as I/I_0 . Error bars were estimated integrating the noise region for each Dotarem concentration dependent spectra and normalizing it to the reference spectrum. Bottom: Surface representation of SH3 Monomer (PDB:1PNJ) with Integrals of (top). Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

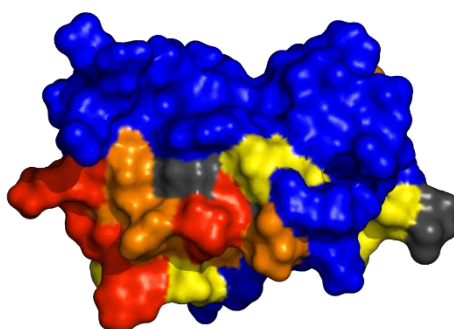
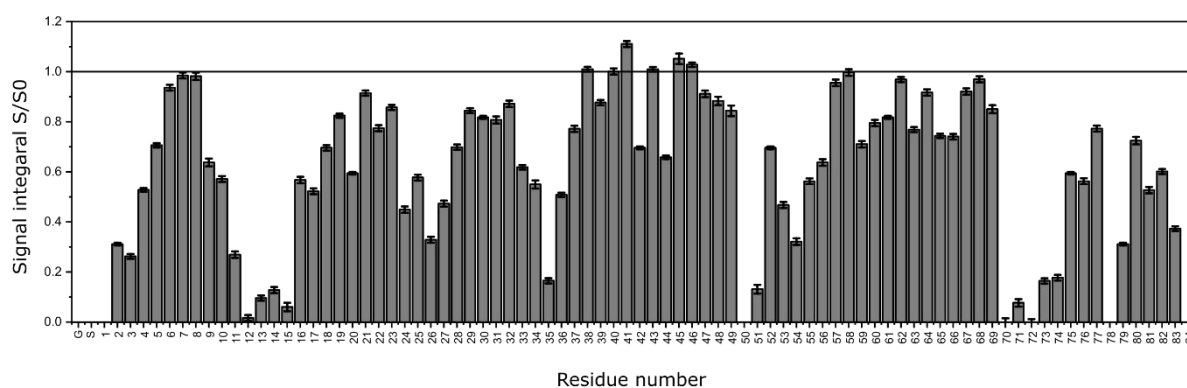


Figure S5: Top: Barplot of integral changes of residue resonances in ^1H - ^{15}N HSQC spectrum for 5 mM Dotarem compared to 0 mM Dotarem as I/I_0 . Error bars were estimated integrating the noise region for each Dotarem concentration dependent spectra and normalizing it to the reference spectrum. Bottom: Surface representation of SH3 Monomer (PDB:1PNJ) with Integrals of (top). Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

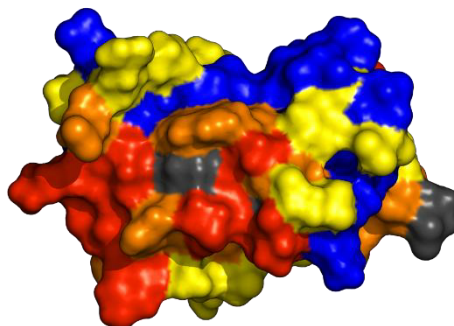
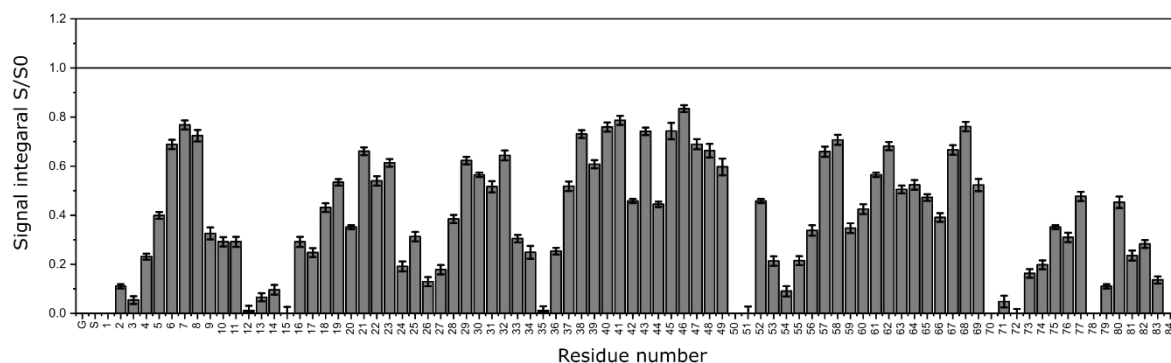


Figure S6: Top: Bar plot of integral changes of residue resonances in ^1H - ^{15}N HSQC spectrum for 10 mM Dotarem compared to 0 mM Dotarem as I/I_0 . Error bars were estimated integrating the noise region for each Dotarem concentration dependent spectra and normalizing it to the reference spectrum. Bottom: Surface representation of SH3 Monomer (PDB:1PNJ) with Integrals of (top). Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

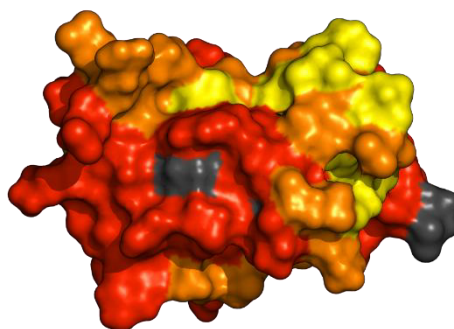
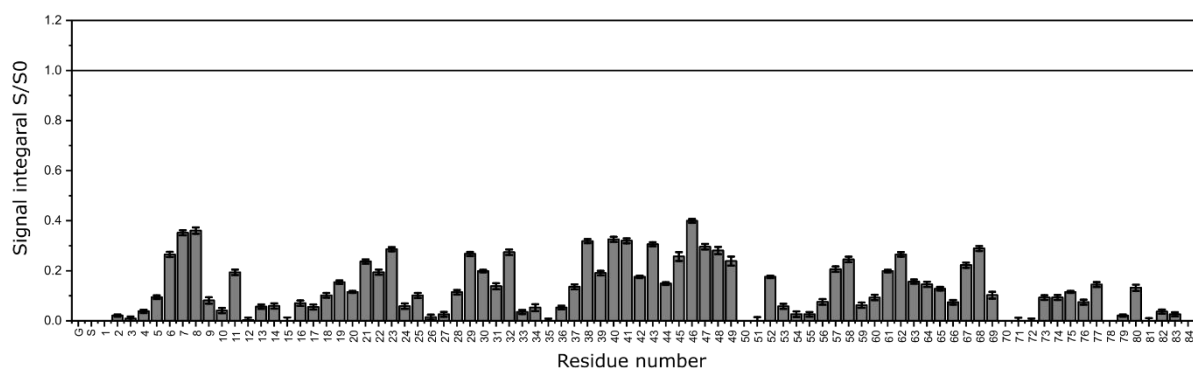


Figure S7: Top: Bar plot of integral changes of residue resonances in ^1H - ^{15}N HSQC spectrum for 25 mM Dotarem compared to 0 mM Dotarem as I/I_0 . Error bars were estimated integrating the noise region for each Dotarem concentration dependent spectra and normalizing it to the reference spectrum. Bottom: Surface representation of SH3 Monomer (PDB:1PNJ) with Integrals of (top). Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

SH3 solvent Dotarem effect plotted on structure (1PNJ) (surface representation)

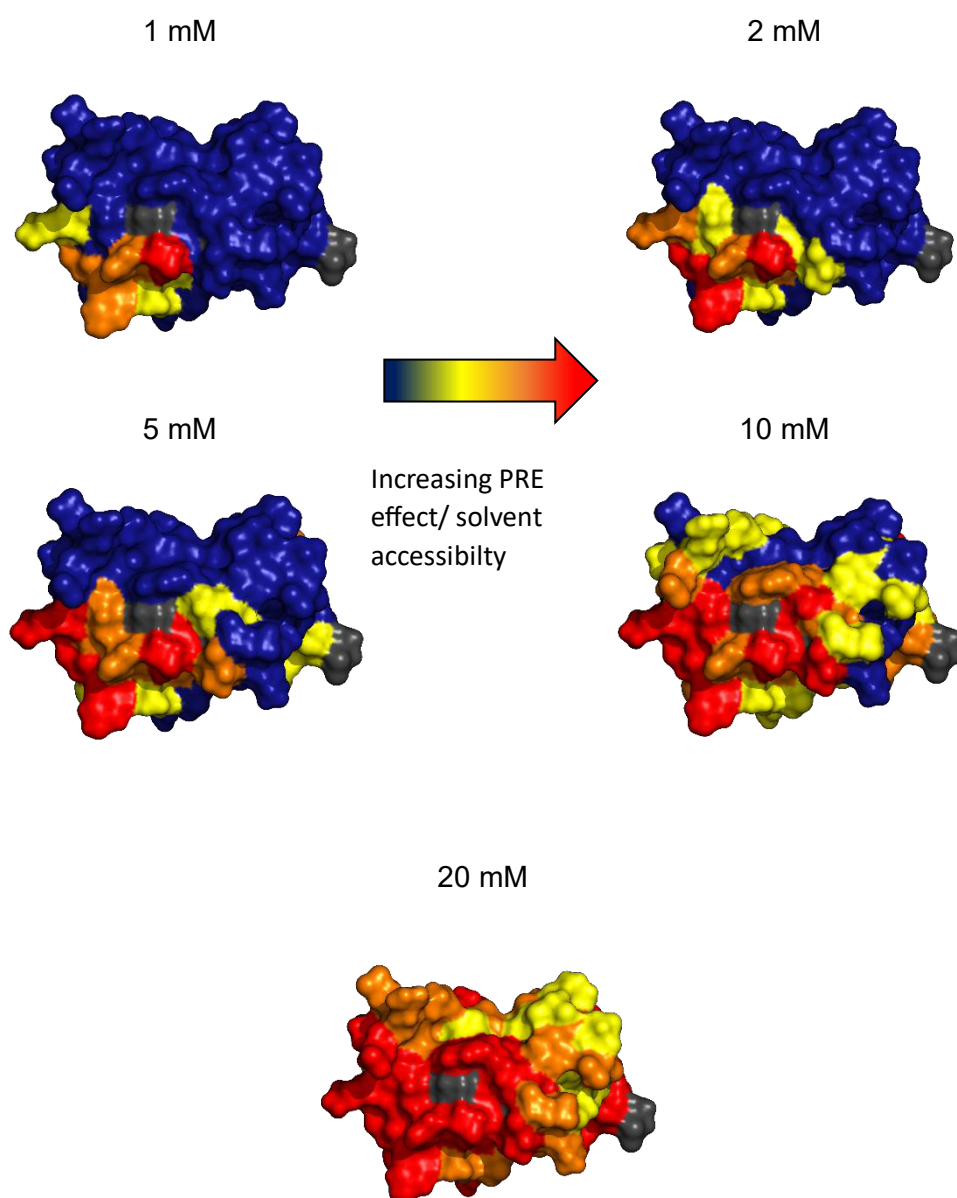


Figure S8: Surface representations of Figure S3-S7 of SH3 Monomer (PDB:1PNJ) with varying Dotarem concentrations. Color code: Red: highly exposed ($I/I_0 \leq 0.1$), Orange: medium exposed ($0.1 < I/I_0 \leq 0.25$), Yellow: low exposed ($0.25 < I/I_0 \leq 0.5$), Blue: less low exposed ($I/I_0 > 0.5$), Grey: not assigned.

Solvent PRE on low pH SH3 state 20 °C:

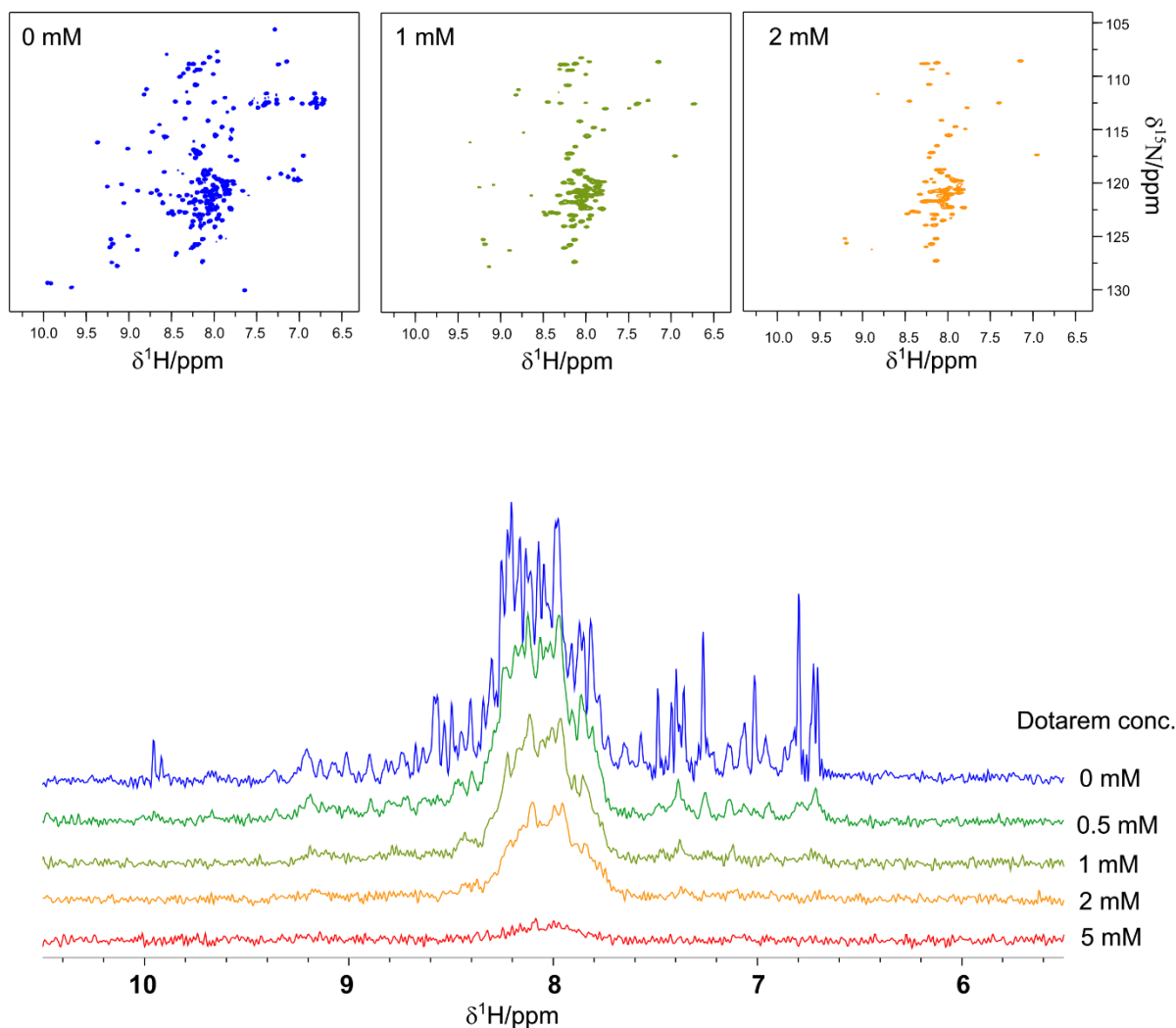


Figure S9: ^1H - ^{15}N HSQC spectra of PI3K-SH3 (250 μ M) in 10 mM Gly HCl, pH 2.5, at 20 °C and varying (0, 1, 2) Dotarem concentration in the solvents. Line broadening and peak suppression is increased with increasing Dotarem concentrations. Bottom: 1 D slices of the first FID of ^1H - ^{15}N HSQC spectra of PI3K-SH3 (250 μ M) in 10 mM NaPi, pH 2.5, at 20 °C and varying (0, 0.5, 1, 2, 5 mM) Dotarem concentration in the solvents.

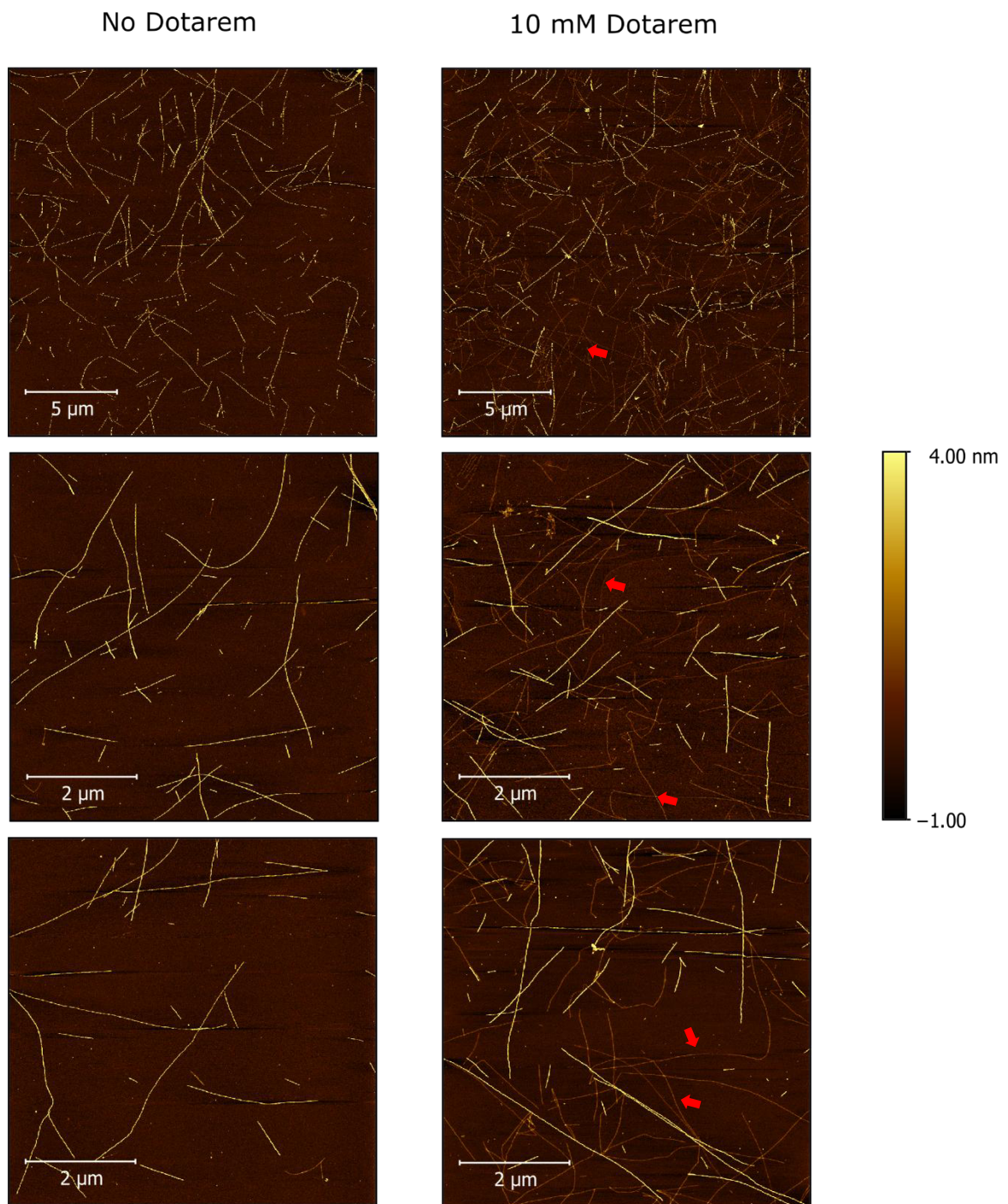


Figure S10: Atomic force microscopy micrographs of PI3K-SH3 fibrils grown in 10 mM Gly HCl, pH 2.5, at 50 °C, 5 % Seeds for one day. Left: Fibrils without addition of Dotarem after fibril growth. Right: Fibrils with addition to a final concentration of 10 mM Dotarem after fibril growth. In presence of Dotarem a second Polymorph is observed (marked by red arrows) ?

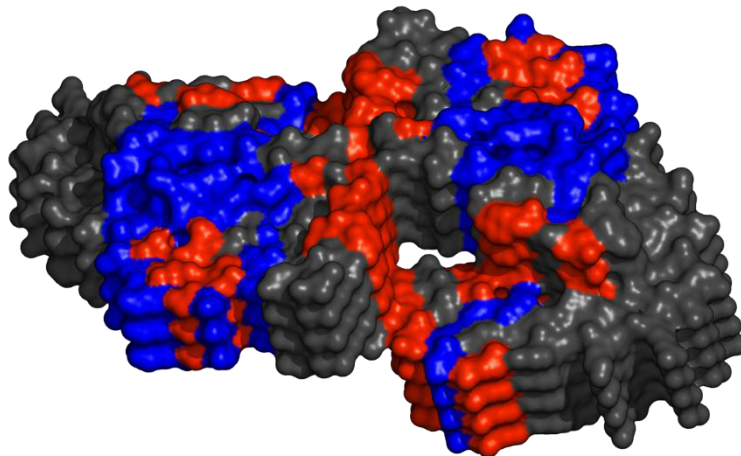
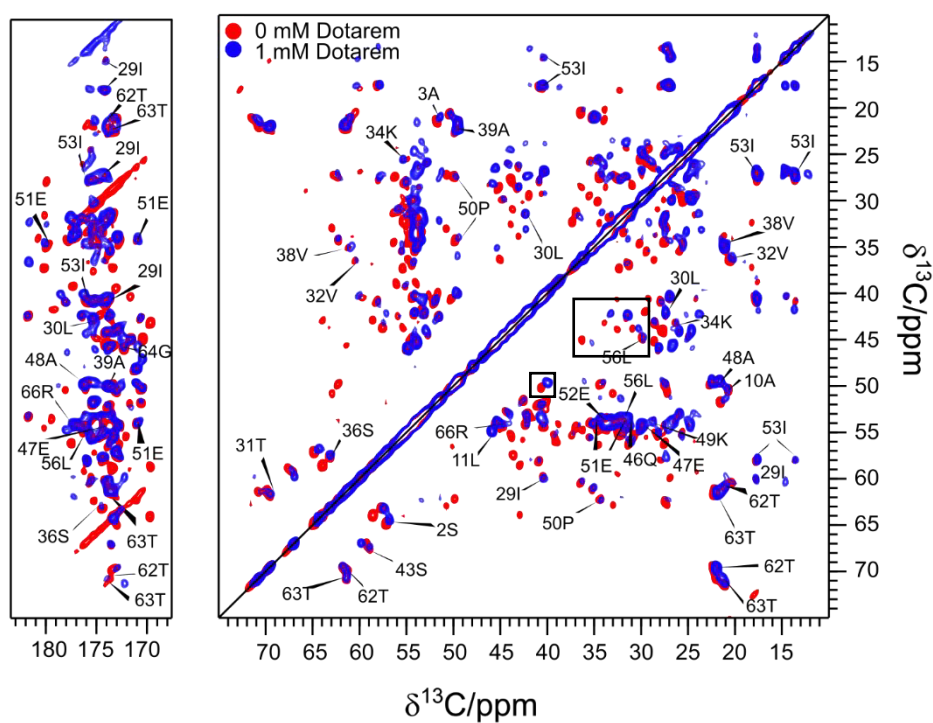


Figure S11: Solvent PRE effect on PI3K-SH3 fibrils at 1 mM Dotarem concentration. A) Overlay of ^{13}C - ^{13}C proton driven spin diffusion (PDSD) spectra at 11 kHz and 50 ms mixing time of SH3 fibrils without (red) and 1 mM Dotarem (blue) concentration. B) Surface representation of SH3 fibrils (PDB:6R4R); Resonances for residues that are present in the 1 mM Dotarem spectrum are marked in blue. Resonances that vanish are marked in red and unassigned residues are marked in grey.

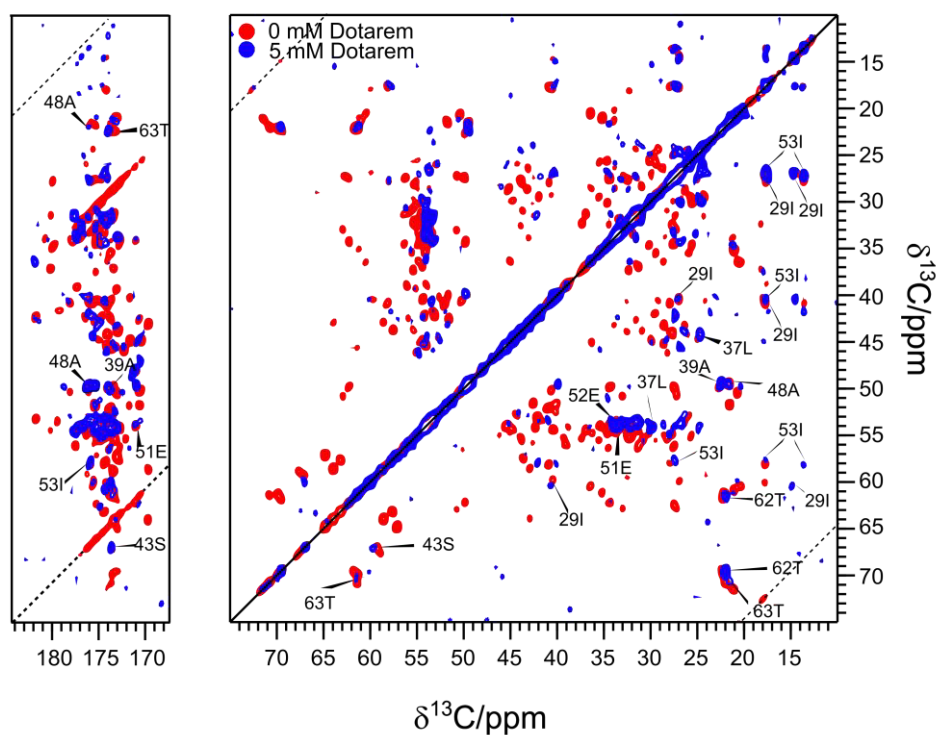


Figure S12: Solvent PRE effect on PI3K-SH3 fibrils at 5 mM Dotarem concentration. A) Overlay of ^{13}C - ^{13}C proton driven spin diffusion (PDS) spectra at 11 kHz and 50 ms mixing time of SH3 fibrils without (red) and 5 mM Dotarem (blue) concentration. B) Surface representation of SH3 fibrils (PDB:6R4R); Resonances for residues that are present in the 5 mM Dotarem spectrum are marked in blue. Resonances that vanish are marked in red and unassigned residues are marked in grey.

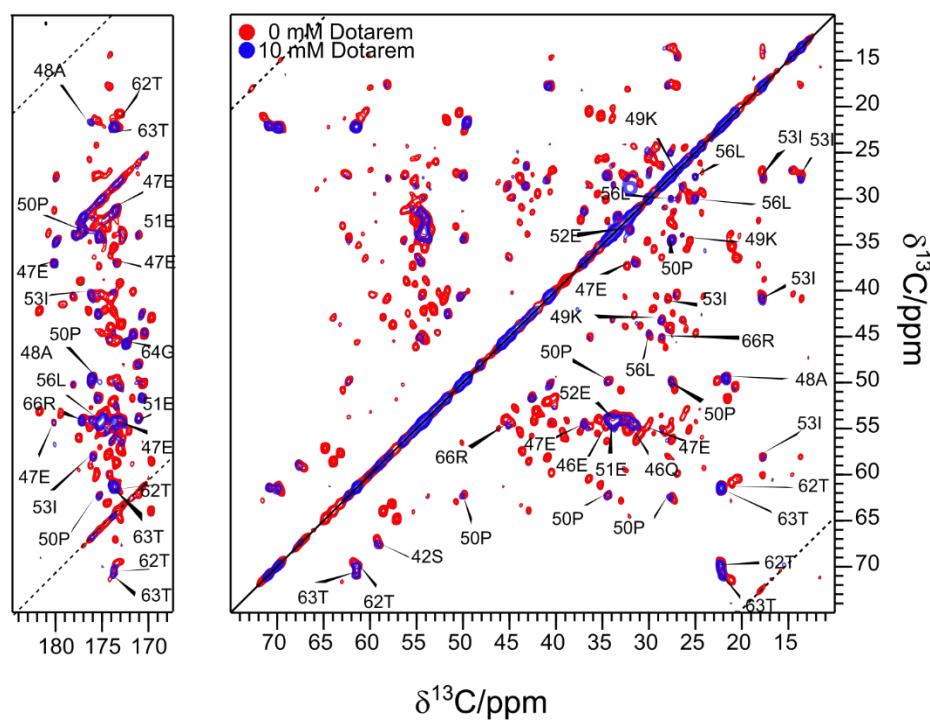


Figure S13: Solvent PRE effect on PI3K-SH3 fibrils at 10 mM Dotarem concentration. A) Overlay of ^{13}C - ^{13}C proton driven spin diffusion (PDS) spectra at 11 kHz and 50 ms mixing time of SH3 fibrils without (red) and 10 mM Dotarem (blue) concentration. B) Surface representation of SH3 fibrils (PDB:6R4R); Resonances for residues that are present in the 10 mM Dotarem spectrum are marked in blue. Resonances that vanish are marked in red and unassigned residues are marked in grey.

10 mM Dotarem comparison

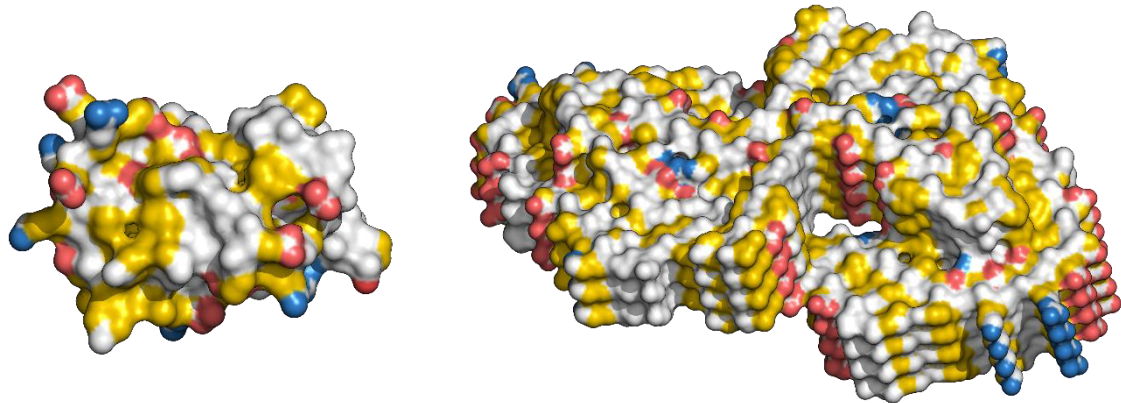


	1	11	21	31	41	
1PNJ	GS MSAEGYQYRA LYDYKKEREE DIDLHLGDI L TVNKGSLVAL GFSDGQEAKP					
6R4R	GS MSAEGYQYRA LYDYKKEREE DIDLHLGDI L TVNKGSLVAL GFSDGQEAKP					

	51	61	71	81	
1PNJ	EEIGWLNQYN ETTGERGDFP GTYVEYIGRK KISP				
6R4R	EEIGWLNQYN ETTGERGDFP GTYVEYIGRK KISP				



Figure S14: Comparison of secondary structure of both PDB structures used (1PNJ, 6R4R) (yellow: beta sheet, red: alpha helix). Top: Primary sequence. Bottom: Cartoon representation.



“RB highlighting visualizes hydrophobicity by highlighting all carbon atoms that are not bound to nitrogen and oxygen atoms. The charged oxygens of glutamate and aspartate are highlighted red and the charged nitrogens of arginine and lysine are highlighted blue.”

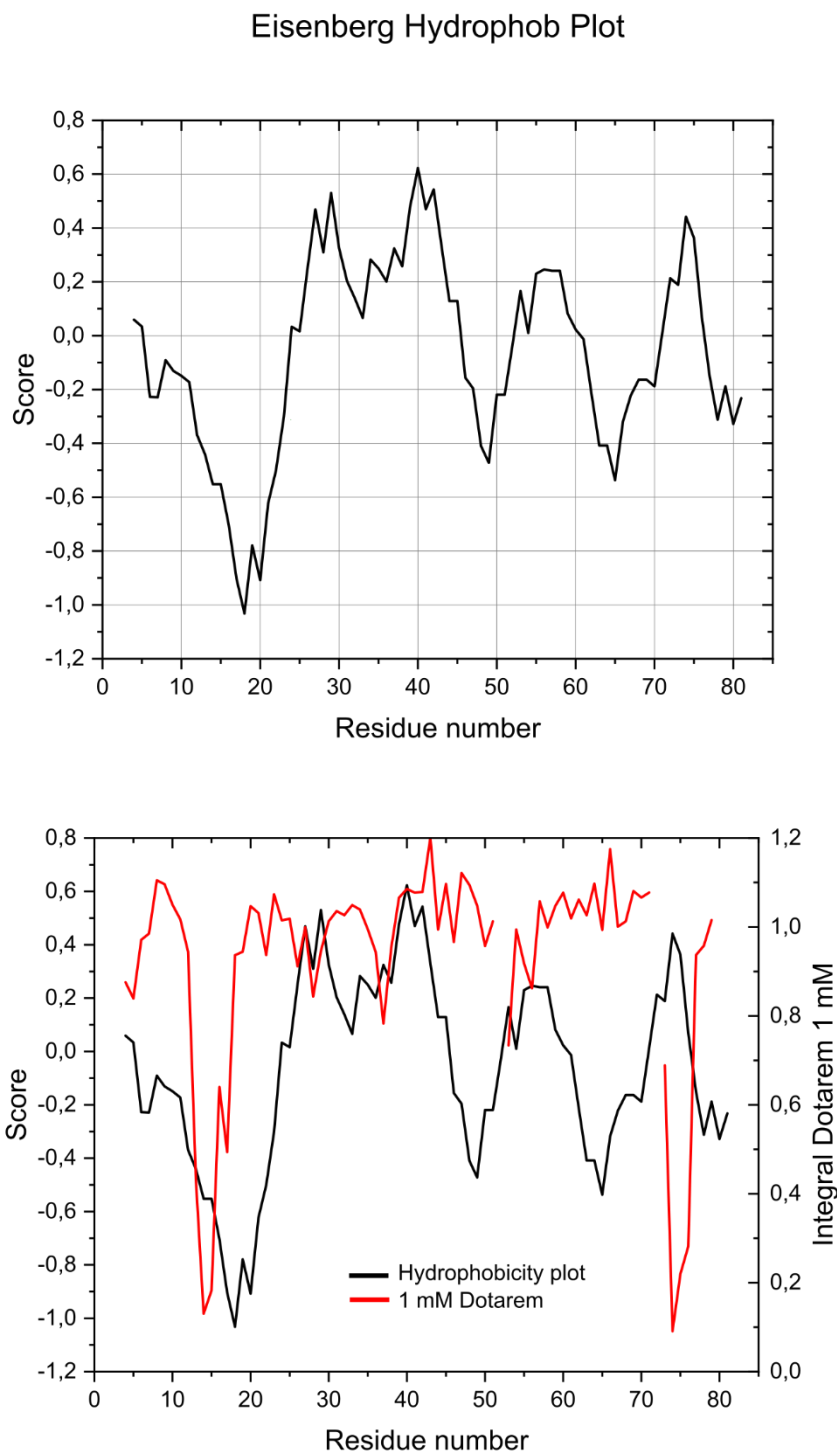


Figure S14: Top: Eisenberg Hydrophobicity plot³⁰ (expasy) of sequence of PI3K-SH3 (with GS overhang). Bottom: Overlay of Eisenberg Hydrophobicity plot and integrals of 1 mM Dotarem.

References

- (1) Solomon. Relaxation Processes in a System of Two Spins*. **1955**.
- (2) Zehnder, J.; Cadalbert, R.; Yulikov, M.; Kunze, G.; Wiegand, T. Paramagnetic spin labeling of a bacterial DnaB helicase for solid-state NMR. *J Magn Reson* **2021**, 332, 107075. DOI: 10.1016/j.jmr.2021.107075 From NLM Medline.
- (3) Liang, B.; Bushweller, J. H.; Tamm, L. K. Site-directed parallel spin-labeling and paramagnetic relaxation enhancement in structure determination of membrane proteins by solution NMR spectroscopy. *J Am Chem Soc* **2006**, 128 (13), 4389-4397. DOI: 10.1021/ja0574825 From NLM Medline.
- (4) Gong, Z.; Schwieters, C. D.; Tang, C. Theory and practice of using solvent paramagnetic relaxation enhancement to characterize protein conformational dynamics. *Methods* **2018**, 148, 48-56. DOI: 10.1016/j.ymeth.2018.04.006 From NLM Medline.
- (5) Madl, T.; Bermel, W.; Zangger, K. Use of relaxation enhancements in a paramagnetic environment for the structure determination of proteins using NMR spectroscopy. *Angew Chem Int Ed Engl* **2009**, 48 (44), 8259-8262. DOI: 10.1002/anie.200902561 From NLM Medline.
- (6) Oktaviani, N. A.; Risor, M. W.; Lee, Y. H.; Megens, R. P.; de Jong, D. H.; Otten, R.; Scheek, R. M.; Enghild, J. J.; Nielsen, N. C.; Ikegami, T.; et al. Optimized co-solute paramagnetic relaxation enhancement for the rapid NMR analysis of a highly fibrillogenic peptide. *J Biomol NMR* **2015**, 62 (2), 129-142. DOI: 10.1007/s10858-015-9925-8 From NLM Medline.
- (7) Hartlmüller, C.; Spreitzer, E.; Göbl, C.; Falsone, F.; Madl, T. NMR characterization of solvent accessibility and transient structure in intrinsically disordered proteins. *J Biomol NMR* **2019**, 73 (6-7), 305-317. DOI: 10.1007/s10858-019-00248-2 From NLM Medline.
- (8) Madl, T.; Guttler, T.; Gorlich, D.; Sattler, M. Structural analysis of large protein complexes using solvent paramagnetic relaxation enhancements. *Angew Chem Int Ed Engl* **2011**, 50 (17), 3993-3997. DOI: 10.1002/anie.201007168 From NLM Medline.
- (9) Oster, C.; Kosol, S.; Hartlmüller, C.; Lamley, J. M.; Iuga, D.; Oss, A.; Org, M. L.; Vanatalu, K.; Samoson, A.; Madl, T.; et al. Characterization of Protein-Protein Interfaces in Large Complexes by Solid-State NMR Solvent Paramagnetic Relaxation Enhancements. *J Am Chem Soc* **2017**, 139 (35), 12165-12174. DOI: 10.1021/jacs.7b03875 From NLM Medline.
- (10) Aucoin, D.; Xia, Y.; Theint, T.; Nadaud, P. S.; Surewicz, K.; Surewicz, W. K.; Jaroniec, C. P. Protein-solvent interfaces in human Y145Stop prion protein amyloid fibrils probed by paramagnetic solid-state NMR spectroscopy. *J Struct Biol* **2019**, 206 (1), 36-42. DOI: 10.1016/j.jsb.2018.04.002 From NLM Medline.
- (11) Jaroniec, C. P. Structural studies of proteins by paramagnetic solid-state NMR spectroscopy. *J Magn Reson* **2015**, 253, 50-59. DOI: 10.1016/j.jmr.2014.12.017 From NLM Medline.
- (12) Guijarro, J. I.; Sunde, M.; Jones, J. A.; Campbell, I. D.; Dobson, C. M. Amyloid fibril formation by an SH3 domain. *Proc Natl Acad Sci U S A* **1998**, 95 (8), 4224-4228. DOI: 10.1073/pnas.95.8.4224 From NLM Medline.
- (13) Batra-Safferling, R.; Granzin, J.; Modder, S.; Hoffmann, S.; Willbold, D. Structural studies of the phosphatidylinositol 3-kinase (PI3K) SH3 domain in complex with a peptide ligand: role of the anchor residue in ligand binding. *Biol Chem* **2010**, 391 (1), 33-42. DOI: 10.1515/BC.2010.003 From NLM Medline.
- (14) Booker, G. W.; Gout, I.; Downing, A. K.; Driscoll, P. C.; Boyd, J.; Waterfield, M. D.; Campbell, I. D. Solution structure and ligand-binding site of the SH3 domain of the p85 alpha subunit of phosphatidylinositol 3-kinase. *Cell* **1993**, 73 (4), 813-822. DOI: 10.1016/0092-8674(93)90259-s From NLM Medline.
- (15) Bader, R.; Bamford, R.; Zurdo, J.; Luisi, B. F.; Dobson, C. M. Probing the mechanism of amyloidogenesis through a tandem repeat of the PI3-SH3 domain suggests a generic model for protein aggregation and fibril formation. *J Mol Biol* **2006**, 356 (1), 189-208. DOI: 10.1016/j.jmb.2005.11.034 From NLM Medline.

- (16) Roder, C.; Vettore, N.; Mangels, L. N.; Gremer, L.; Ravelli, R. B. G.; Willbold, D.; Hoyer, W.; Buell, A. K.; Schroder, G. F. Atomic structure of PI3-kinase SH3 amyloid fibrils by cryo-electron microscopy. *Nat Commun* **2019**, *10* (1), 3754. DOI: 10.1038/s41467-019-11320-8 From NLM Medline.
- (17) Bayro, M. J.; Maly, T.; Birkett, N. R.; Macphee, C. E.; Dobson, C. M.; Griffin, R. G. High-resolution MAS NMR analysis of PI3-SH3 amyloid fibrils: backbone conformation and implications for protofilament assembly and structure. *Biochemistry* **2010**, *49* (35), 7474-7484. DOI: 10.1021/bi100864t From NLM Medline.
- (18) Aime, S.; D'Amelio, N.; Fragai, M.; Lee, Y. M.; Luchinat, C.; Terreno, E.; Valensin, G. A paramagnetic probe to localize residues next to carboxylates on protein surfaces. *J Biol Inorg Chem* **2002**, *7* (6), 617-622. DOI: 10.1007/s00775-002-0340-8 From NLM Medline.
- (19) Petros, A. M.; Mueller, L.; Kopple, K. D. NMR identification of protein surfaces using paramagnetic probes. *Biochemistry* **1990**, *29* (43), 10041-10048. DOI: 10.1021/bi00495a005 From NLM Medline.
- (20) Lenard, A. J.; Mulder, F. A. A.; Madl, T. Solvent paramagnetic relaxation enhancement as a versatile method for studying structure and dynamics of biomolecular systems. *Prog Nucl Magn Reson Spectrosc* **2022**, *132-133*, 113-139. DOI: 10.1016/j.pnmrs.2022.09.001 From NLM Medline.
- (21) Bousquet, J. C.; Saini, S.; Stark, D. D.; Hahn, P. F.; Nigam, M.; Wittenberg, J.; Ferrucci, J. T., Jr. Gd-DOTA: characterization of a new paramagnetic complex. *Radiology* **1988**, *166* (3), 693-698. DOI: 10.1148/radiology.166.3.3340763 From NLM Medline.
- (22) Hsu, S. T. NMR assignments of PI3-SH3 domain aided by protonless NMR spectroscopy. *Biomol NMR Assign* **2014**, *8* (2), 291-295. DOI: 10.1007/s12104-013-9503-5 From NLM Medline.
- (23) Saksela, K.; Permi, P. SH3 domain ligand binding: What's the consensus and where's the specificity? *FEBS Lett* **2012**, *586* (17), 2609-2614. DOI: 10.1016/j.febslet.2012.04.042 From NLM Medline.
- (24) Zurdo, J.; Guijarro, J. I.; Jimenez, J. L.; Saibil, H. R.; Dobson, C. M. Dependence on solution conditions of aggregation and amyloid formation by an SH3 domain. *J Mol Biol* **2001**, *311* (2), 325-340. DOI: 10.1006/jmbi.2001.4858 From NLM Medline.
- (25) Gardon, L.; Becker, N.; Rahse, N.; Holbling, C.; Apostolidis, A.; Schulz, C. M.; Bochinsky, K.; Gremer, L.; Heise, H.; Lakomek, N. A. Amyloid fibril formation kinetics of low-pH denatured bovine PI3K-SH3 monitored by three different NMR techniques. *Front Mol Biosci* **2023**, *10*, 1254721. DOI: 10.3389/fmolb.2023.1254721 From NLM PubMed-not-MEDLINE.
- (26) Vranken, W. F.; Boucher, W.; Stevens, T. J.; Fogh, R. H.; Pajon, A.; Llinas, M.; Ulrich, E. L.; Markley, J. L.; Ionides, J.; Laue, E. D. The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* **2005**, *59* (4), 687-696. DOI: 10.1002/prot.20449 From NLM Medline.
- (27) Schrodinger, LLC. The PyMOL Molecular Graphics System, Version 1.8. 2015.
- (28) Nečas, D.; Klapetek, P. Gwyddion: an open-source software for SPM data analysis. *Open Physics* **2012**, *10* (1), 181-188. DOI: 10.2478/s11534-011-0096-2.
- (29) Hagemans, D.; van Belzen, I. A.; Moran Luengo, T.; Rudiger, S. G. A script to highlight hydrophobicity and charge on protein surfaces. *Front Mol Biosci* **2015**, *2*, 56. DOI: 10.3389/fmolb.2015.00056 From NLM PubMed-not-MEDLINE.
- (30) Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol* **1984**, *179* (1), 125-142. DOI: 10.1016/0022-2836(84)90309-7 From NLM Medline.