

The investigation of structural dynamics of intrinsically disordered proteins

Inaugural-Dissertation

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

vorgelegt von

Tobias Stief

aus Düsseldorf

Düsseldorf, Dezember 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

Berichterstatter:

1. Dr. habil. Nils-Alexander Lakomek

2. Prof. Dr. Henrike Heise

Tag der mündlichen Prüfung: 03.07.2026

Contents

1	List of Publications and Manuscripts	1
2	Abstract	3
3	Introduction	5
4	NMR	11
5	Motivation	21
6	Summary	25
7	Intrinsic Disorder of SNAP25a	35
8	Improved R_1 and $R_{1\rho}$ Relaxation Experiments	59

9 Userfriendly Relaxation Experiments	101
10 Internal Backbone Dynamics of SNAP25a	151
11 R2Hahn Echo Experiment for Detection of ms Dynamics	173
12 Conclusion and Outlook	185
List of Abbreviations	191
Bibliography	194
Eidesstattliche Versicherung	215
Danksagung	217
Appendix	219

1 List of Publications and Manuscripts

This PhD work resulted in the following publications and manuscripts:

Publications

Stief, T., Gremer, L., Pribicevic, S., Espinueva, D. F., Vormann, K., Biehl, R., Jahn, R., Perez-Lara, A., Lakomek, N. A. (2023). Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its Pre-fusion Conformation. *Journal of molecular biology*, 435(10).

Stief, T., Vormann, K., Lakomek, N. A. (2024). Sensitivity-enhanced NMR ^{15}N R1 and R1 ρ relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields. *Methods (San Diego, Calif.)*, 223, 115.

Stief, T., Vormann, K., Lakomek, N. A. (2024). NMR ^{15}N Relaxation Experiments for the Investigation of Picosecond to Nanoseconds Structural Dynamics of Proteins. *Journal of visualized experiments : JoVE*, (213).

Manuscripts

Stief, T., Vormann, K., Lakomek, N. A. (2025). Picosecond and nanosecond dynamics in the backbone of the intrinsically disordered protein SNAP25a.

Stief, T., Vormann, K., Kraus, M., Werner, S., Lakomek, N. A. (2025). Sensitivity enhanced ^{15}N Hahn echo R2 experiment to visualize chemical exchange contribution.

Vormann, K., Stief, T., Tucholski, FT., Cukkemane, A., Nagel-Steger, L., Bartenschlager, R., Biehl, R., Lakomek, N.A. (2025). Integrative NMR pipeline: From an AlphaFold model to the dynamic validation of SARS-CoV-2 NSP3 N-terminus.

Vormann, K. Stief, T., Tucholski, FT., Nagel-Steger, L., Biehl, R., Lakomek, N. A. (2025). Impact of ionic strength on the self-association of the SARS-CoV-2 non-structural protein 3 N-terminal domain.

Werner, S., Stief, T., Schulz, M., Vormann, K., Backer, R., Schützmann, M., Gremer, L., Hoyer, W., Lakomek, N. A. (2025) Direct NMR evidence for transient binding of the vesicular SNARE protein synaptobrevin-2 / VAMP2 to the α -synuclein C-terminus.

BMRB entries

BMRB entry 51598: SNAP25a (Rattus norvegicus)

BMRB entry 53437: ¹H, ¹⁵N and ¹³C Chemical Shift Assignment of Synaptobrevin 2 at 5degC.

2 Abstract

For information processing in the brain, spatially separated neurons need a mechanism to exchange information over the synaptic cleft. Essential for this mechanism is the neuronal exocytosis, which involves the fusion of the pre-synaptic plasma membrane with the vesicle membrane for the release of neurotransmitters. These neurotransmitters can cross the synaptic cleft and activate receptors at the post-synaptic neuron. Key proteins of the neuronal exocytosis, such as Synaptobrevin-2, SNAP25a (Synaptosomal-associated protein 25 a) and Syntaxin-1A, exhibit extensive regions of intrinsic disorder.

Intrinsic disorder is a widespread feature of the human proteome, with approximately 60% of proteins containing intrinsically disordered regions (IDRs) and about 5% being nearly complete intrinsically disordered (IDPs). In my PhD work, I used solution NMR spectroscopy to study the structural and dynamic properties of IDPs. Widely used structural biology methods, such as X-ray crystallography and electron microscopy generate contrast based on electron density and therefore cannot extract relevant biophysical information about the protein backbone of IDPs and IDRs, as these regions exhibit diffuse electron density due to their high internal flexibility. NMR spectroscopy provides secondary-structure information about the protein, because carbon chemical shifts are sensitive to its backbone structure. ^{15}N relaxation rates reveal the dynamics of the protein backbone on the nanosecond and picosecond time scales, providing a more detailed and comprehensive picture of protein dynamics.

In this work, I aimed to develop a robust, reliable, and user-friendly NMR workflow to investigate the structure and the dynamics of IDPs and IDRs. We optimized and tailored ^{15}N relaxation experiments for magnetic fields ranging from 600 MHz to 1200 MHz. Furthermore, we published a step-by-step protocol for setting up and evaluating the experiments, including an instructional video.

These ^{15}N relaxation rate experiments, as well as NMR triple-resonance experiments and other biophysical methods such as CD-spectroscopy and X-ray scattering, were applied to the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein SNAP25a, which revealed large regions of intrinsic disorder and an N-terminus with increased α -helical propensity. Three independent approaches were used to extract amplitudes and timescales of protein backbone motion from ^{15}N relaxation rates, yielding to consistent dynamic modes at 50 ps, hundreds of ps, roughly 1 ns, and 5 ns to 20 ns.

A ^{15}N Hahn-echo-based R_2 experiment was optimized to enable a fast yet reliable assessment of microsecond- and millisecond-scale dynamics in protein backbones.

3 Introduction

Due to the high internal flexibility of intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs)^{1,2,3}, these proteins and regions are characterized by a diffusive electron density, which challenges structural biology methods as X-ray crystallography⁴. Nuclear magnetic resonance (NMR) spectroscopy however can measure ensemble-averaged signals of fast fluctuating IDPs^{5,6,7,8}. It provides structural and dynamic information with residue-specific resolution.

The secondary structure propensity and the dynamic of proteins are closely coupled with the functionality of the protein⁹. They also impact the interactions with ligands and consequently the biological interactivity with ligands⁹ and consequently the biological functions of the IDPs^{10,11,12,13,14}. This also holds true for proteins involved in complex mechanisms as the neuronal exocytosis. Multiple IDPs and proteins with IDRs are involved in neuronal exocytosis^{15,16}, which is on molecular level not fully understood yet. Dynamic information would help to achieve a deeper understanding of the functionality of soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins that drive synaptic vesicle membrane fusion. For this study highly accurate NMR spectroscopy experiments are necessary, which are free of systematic errors. Recently advances in magnetic technology have led to higher available magnetic fields in NMR spectroscopy (up to 1200 MHz). This high field strength offers unprecedented resolution, particularly needed for IDPs because of their narrow dispersion in the proton dimension. But predominantly established pulse sequences need to be tailored and tested for this high field strength.

In my PhD work I addressed the following questions: Which structural preferences and structural dynamics characterize the SNARE protein SNAP25a (Synaptosomal-associated protein 25)? How can we detect ¹⁵N relaxation rates more accurately and with high signal-to-noise at multiple magnetic fields? How do we make NMR ¹⁵N relaxation experiments accessible for a wider scientific community beyond trained NMR experts? Which dynamic regimes exist in the backbone dynamics of IDPs and IDRs and which method can reveal the most credible information from the relaxation rates? These guiding questions resulted into a number of publications and manuscripts (see page 1).

3.1 SNARE proteins driving neuronal exocytosis

In 2025 most people are using generative artificial intelligence tools, often in form of large language models. These models are usually based on the technique of neuronal networks, which are inspired by the information exchange processes in the human brain. The communication between neurons is achieved by the Ca^{2+} -triggered release of neurotransmitters from the presynaptic neuron into the synaptic cleft, which subsequently activate the postsynaptic receptors. Before communication takes place, the neurotransmitters are packed in vesicles located in the axon terminal of the presynaptic neuron. The vesicle membrane has to fuse with the plasma membrane for the release of the neurotransmitters into the synaptic cleft. This fusion process of lipid bilayers is described by a complex energy landscape with multiple energy barriers resulting from the hydrophobic effect, as well as electrostatic, solvation and steric forces¹⁵. The theoretically calculated energy required for the fusion is approximately between 40 $k_B T$ and 100 $k_B T$ ^{17,16}, meaning that the fusion of lipid membranes does not happen spontaneously. For passing these energy barriers, fusion proteins are needed that can undergo complex conformational changes to form fusion complexes providing the necessary energy and overcome the hindering forces¹⁵. These fusion proteins are called soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs) and form a protein super family that is essential for almost all fusion processes along the secretory pathway¹⁵. SNARE proteins are characterized by an approximately 65 residue long sequence, called SNARE motif^{18,19}. Four different SNARE motifs form a bundle of four parallel helices^{20,21} in a composite and highly stable SNARE complex²². The SNARE four-helix bundle consists of layers of four polar residues, with a central polar layer characterized by one arginine (R) and three glutamines (Q) contributed by the SNARE motifs²³. We therefore classify four SNARE motifs: Q_a , Q_b , Q_c and R, which are specified by their embedded position in the SNARE complex^{22,23}.

In neuronal exocytosis, three SNARE proteins play a key role in the formation of the neuronal SNARE complex^{15,16} (Figure 2.1). Synaptobrevin-2 (also known as VAMP2) contributes an R-motif that is connected to a transmembrane domain (TMD) via a short linker domain. Syntaxin-1A contains a Q_a -motif, which is also connected to a TMD via a short linker, as well as an additional N-terminal Habc domain²⁴, which folds into a three-helix bundle, that is able to bind to the SNARE motif forming a closed inactive conformation²⁵. SNAP25 contributes two SNARE motifs, Q_b and Q_c , which are connected by a linker domain that acts as a palmitoylation site²⁶. Synaptobrevin-2 is anchored in the vesicle membrane with its TMD and is therefore classified as a vSNARE (vesicle SNARE). Syntaxin-1A is anchored in the plasma membrane with its TMD, categorized as a target membrane SNARE (tSNARE). SNAP25 binds to the plasma membrane via palmitoylation (tSNARE)^{27,23,28}.

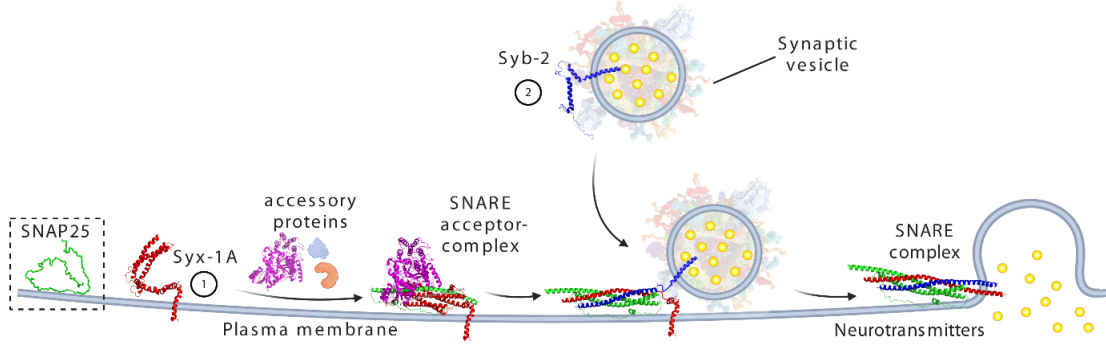


Figure 3.1: Scheme of SNARE assembly driving neuronal exocytosis. (Syx-1A, syntaxin-1A, modified from PDB ID: 1BR0 and 2M8R, and syb-2, synaptobrevin-2, modified from PDB ID: 2KOG. The accessory protein Munc18 appears in magenta, modified from PDB ID: 3C98, the SNARE acceptor complex is modified from the AlphaFold model AF-P60881-F1-model_v4 and PDB ID: 3C98, and the SNARE complex is modified from PDB ID: 3HD7). The figure and caption is taken from Stief et al. 2023²⁹

The SNARE complex assembly begins at the N-terminus of the SNARE proteins and proceeds along the backbone towards the C-terminus. This process is called *zippering*, during which the membranes get pulled close together. Before zippering is finalized, the SNARE proteins assemble to a so-called *trans-complex*. After zippering, the fully fused SNARE complex, in which the linker domains and TMDs α -helices extend the four-helix bundle into the membrane³⁰, is formed and called *cis-complex*. The assembly of the SNARE complex provides energy of approximately $35 k_B T$ up to $68 k_B T$ ^{31,32}. It is therefore assumed that several SNARE complexes are necessary for the vesicle fusion, but this is still under discussion^{33,34,35}.

Beside the SNARE proteins, also several accessory proteins are important for the vesicle fusion. These accessory proteins are Munc18-1^{36,37}, Munc13-1^{38,39}, synaptotagmin and complexin. Because of its binding to Syntaxin-1A⁴⁰ and debated binding to synaptobrevin-2⁴¹, Munc18-1 supports SNARE complex forming as a template⁴², but also hindering SNARE complex assembly by stabilizing the closed conformation of syntaxin-1A⁴³. Munc13-1 supports the opening of syntaxin-1A, and accelerates the transition from the assembly of the SNARE acceptor complex (syntaxin-1A:SNAP25:Munc18-1)⁴⁴ to the SNARE complex^{45,46,47}. Further studies are needed to clarify how Munc13-1 and Munc18-1 organize the assembly of SNARE proteins.

Complexin is a small soluble protein that both inhibits and promotes the SNARE complex assembly by binding tightly to the SNARE complex^{48,49,50,51,32,52,53,54}. Synaptotagmin has Ca^{2+} sensors⁵⁵ for detection of the Ca^{2+} influx that triggers the neurotransmitter release⁵⁶.

It is still unclear how the SNARE proteins interact with those accessory proteins and how the SNARE complex assembly transfer its energy to the membrane to initiate the fusion. The structure and the dynamics of the transition states between the pre-fusion states of all participating proteins and the fully assembled cis-complex are still largely unclear and must be reconciled with the complex energy landscape of lipid bilayer fusion^{15,16}.

3.2 IDRs and IDPs

Approximately 70 percent of the human proteome consists of proteins with sections of disordered residues that are longer than 30 amino acids^{57,58}. Therefore, human proteins mostly contain both, structured building blocks and disordered building blocks (intrinsically disordered regions). The nomenclature disordered was introduced 2001 by Dunker et al. to describe the nonrigid conformation of a polypeptide chain^{4,59}. Most disordered regions in proteins are found in linker regions (37%) which connect two folded regions and in terminally tails at the N-terminus (34%) as well as at the C-terminus (25%) of proteins⁵⁷.

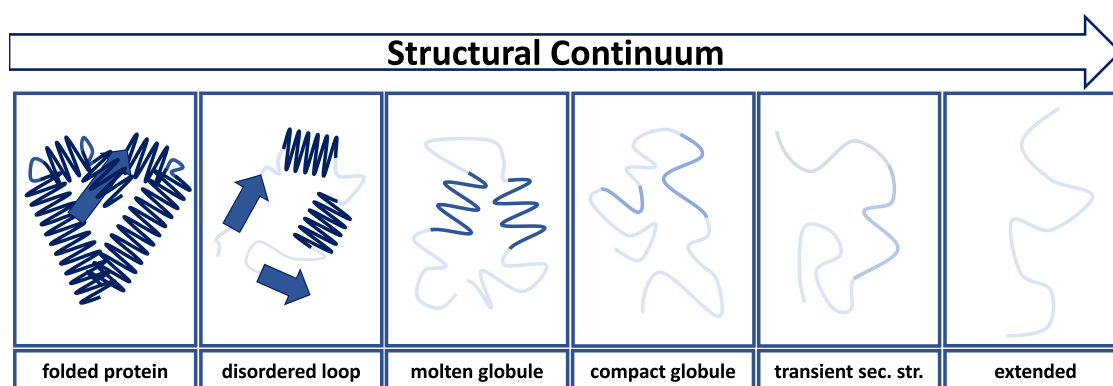


Figure 3.2: Structural continuum of proteins ranging from globular proteins with a fixed tertiary structure (left) to complete intrinsically disordered proteins without a tertiary structure, but containing residues that occur in a random coil secondary structure (right). Spirals illustrate the secondary structure type helix, the arrows illustrate sheets. Dynamic disordered residues are sketched by lines. The degree of disorder is color-coded from dark blue for residues in a fixed secondary structure to light blue for residues resembling in a random coil. This figure is adapted from Lee et al. 2014⁶⁰.

Proteins that does not include intrinsically disordered regions are called structured or globular proteins. Proteins whose polypeptide chains are almost completely disordered and which lack a tertiary structure are defined as intrinsically disordered proteins (IDPs). In addition, between these two contrasting states lies a continuum of protein conformations⁶¹. Previous characterizations of this continuum were the protein trinity⁴ or the protein quartet⁵⁹, which structural subdivisions such as the molten globule are also represented in the continuum⁶¹. Figure 2.2 shows more subdivisions between tightly folded domains with no disorder and a completely disordered protein backbone that is almost random coil: multi-domain proteins connected by disordered linker domains, molten globule forming almost clear secondary structure, collapsed globule staying in an extended shape due to polar interactions and unfolded states with a low propensity for secondary structure (transient secondary structure)⁶⁰. Higher disorder of the polypeptide chain correlates with an increase in the hydrodynamic radius⁵⁹.

The polypeptide chain of an IDP resembles a random coil with the property that all backbone dihedral angles are randomly distributed within the allowed Ramachandran regions^{62,4,63}. This ideal case is not observable in vivo, even highly unfolded proteins show small propensities to form local secondary structures or hydrophobic clusters^{62,64,65,61}. Nevertheless, IDPs does not stay in shape, due to their height internal flexibility^{1,13,2}. They are described by an ensemble of rapidly interconverting structural conformations^{6,5,7,8}. These conformations exist on a relatively flat energy landscape, explaining the rapid interconversion between these states^{66,67,68,69}. For IDR and IDPs the term flexibility describes the speed of conversion between the different conformations of the ensemble and not the degree of mobility of the atoms from their equilibrium position, as flexibility means for globular proteins⁶¹.

The property of intrinsic disorder is anchored as specific signatures in the amino acid sequence of the protein. Typical are the bias of polar and charged amino acids (Gln, Ser, Pro, Lys, Gly, Ala), the absence of bulky hydrophobic amino acids (Val, Leu, Ile, Met, Phe, Trp, Tyr), as well as a low sequence complexity. These properties result in a weak hydrophobic effect, which normally cause the spontaneously folding of the polypeptide chain into well-organized tertiary structures^{70,71,72,61,60}.

The high flexibility of IDPs or IDRs offers significant advantages for functional roles and interaction⁵⁹. Therefore, a broad range of functions for IDRs and IDPs were found in eukaryotic cells⁹. A rough classification of IDRs and IDPs according to their function is made by dividing them into six classes: entropic chains, display sites, chaperones, effectors, assemblers, and scavengers^{73,74,75}. Entropic chains, such as linker domains, fulfill their function due to their essential property of disorder and flexibility, which they do not lose during their function^{76,77,78}. The loss of disorder would mean the loss of functionality. IDPs and IDRs are enriched of short peptide motifs which are highly accessible for post-translational

modifications (PTMs)^{79,80,81,82} and the flexibility of IDRs also favor PTMs as phosphorylation^{83,84,79,85,86}.

Since PTMs are essential for many proteins with regulatory and signaling function most IDRs and IDPs, that contain display sites contribute to such cell functions^{87,61,88,89,90}. Chaperons (support folding of proteins and RNA^{91,92}) often show intrinsic disorder within IDRs gaining the advantage to interact with multiple interaction partners and executing fast macromolecular interactions^{60,59,93}. Effectors that are completely IDP or consist of IDRs often undergo so-called disorder-to-order conformational transitions (coupled folding and binding)^{94,95} in order to bind permanently to other proteins and modify their activity^{85,96}. Due to competitive interactions or allosteric modulation, IDRs also are able to modulate the activity of segments in the same protein^{97,98}. Assembler, rich of IDRs, are able to interact with multiple binding partners and assist the formation of higher-order protein complexes^{99,100}, e.g. the ribosome¹⁰¹. IDRs and IDPs storing and neutralizing small ligands are classified into the class of scavengers^{102,103}.

Due to the occurrence of IDRs and IDPs in an ensemble of rapidly interconverting conformations^{6,5,7,8} these regions show a diffuse electron density. For structural biology methods as electron microscopy or X-ray crystallography, its nearly impossible to observe structural information of IDRs, because of the low and diffuse electron density. Further, these proteins additionally does not tend to crystalize, basic requirement for X-ray crystallography and also important for electron microscopy. Solution NMR does not require high electron densities or crystallization. Although it measures an ensemble average over all populated conformations and provides valuable information about disordered proteins and disordered protein regions like secondary structure propensities of transient structures or residue-specific dynamic insights.^{104,105,106,107,108,109,110,111,112}

4 NMR

4.1 Basics

The foundation of NMR spectroscopy are the magnetic characteristics of atomic nuclei¹¹³. The protons have an angular momentum and because atomic nuclei are built out of protons and neutrons, atomic nuclei also have an angular momentum and a magnetic moment resulting from the angular momentum^{114,115,116,117}. Equation 2.1 shows the correlation between the magnetic moment and the angular momentum. The coefficient of proportionality is known as the nuclei specific gyromagnetic ratio γ . The intrinsic form of the nuclear angular momentum I is defined as the nuclear spin^{115,116,117,118,119}. The following equations are taken from ‘Protein NMR Spectroscopy’ by J. Cavanagh¹²⁰ and illustrate the basics of quantum mechanics of the nuclear spin and the related magnetic moment¹²¹.

$$\vec{\mu} = \gamma \cdot \vec{I} \quad (4.1)$$

In quantum mechanics the angular momentum and the magnetic moment are quantized. Therefore, classical physics can not describe these observables. Only specific states are allowed for the z-component of the nuclear spin I and these states are defined by the nuclear spin magnetic quantum number m_I specifying the z-axis component of the spin angular momentum.

$$I_z = \hbar \cdot m_I \quad (4.2)$$

The nuclear spin magnetic quantum number m_I defines the quantum state of the nuclear spin and can assume values between $-I$ and I ($-I, -I+1, \dots, I-1, I$). In a quantum system $2I + 1$ quantum states exist. The nuclear spin angular momentum quantum number I is an integer or a half-integer and depends on the atomic nucleus. Following correlation of the z-component of the magnetic moment results from equation 3.1 and equation 3.2:

$$\mu_z = \gamma \cdot I_z = \gamma \cdot \hbar \cdot m_I \quad (4.3)$$

In the following I is $\frac{1}{2}$, describing the most simple quantum system. This is valid for the most relevant nuclei in biomolecular NMR spectroscopy ^1H , ^{15}N , ^{13}C . From $I = \frac{1}{2}$ result two eigenstates of angular momentum with spin magnetic quantum numbers $m = \pm\frac{1}{2}$. These are written in Dirac-Notation, quoted from Spin Dynamics¹²²:

$$|\alpha\rangle = |I, m_I\rangle = |\frac{1}{2}, +\frac{1}{2}\rangle \quad (4.4)$$

$$|\beta\rangle = |I, m_I\rangle = |\frac{1}{2}, -\frac{1}{2}\rangle \quad (4.5)$$

$|\alpha\rangle$ and $|\beta\rangle$ are the eigenstates of the Zeeman Hamiltonian $\hat{\mathcal{H}}_0 = \omega_0 \hat{I}_z$ with eigenvalues $\pm\frac{1}{2}\omega_0$. Due to the multiplicity of spins in the system, in the quantum mechanics the density operator is introduced to represent the state of the entire spin ensemble, taken from Spin Dynamics by M. H. Levitt¹²²:

$$\hat{\rho} = |\psi\rangle\langle\psi| \quad (4.6)$$

The diagonal of the density operator represents the populations of the two spin states $|\alpha\rangle$ and $|\beta\rangle$. The off-diagonal elements show the present coherences. The propagation of the density operator describes the state of the entire spin ensemble during a pulse program¹²².

4.2 Relaxation Theory

In the case of an undisturbed quantum mechanical system and assuming the high-temperature limit, the population of spins can be described by the Boltzmann distribution and no coherences are present. We call this state thermal equilibrium. Radiofrequency pulses disturb this equilibrium by changing the spin populations and generating coherences. After the influence of radiofrequency pulses, the system is only influenced by the external magnetic field along the z-axis and returns to the thermal equilibrium. We call this process relaxation. The so-called spin-lattice relaxation (longitudinal relaxation) describes the return of the population to the Boltzmann equilibrium. The decrease of coherences is defined as the spin-spin relaxation (transversal relaxation)^{123,124}.

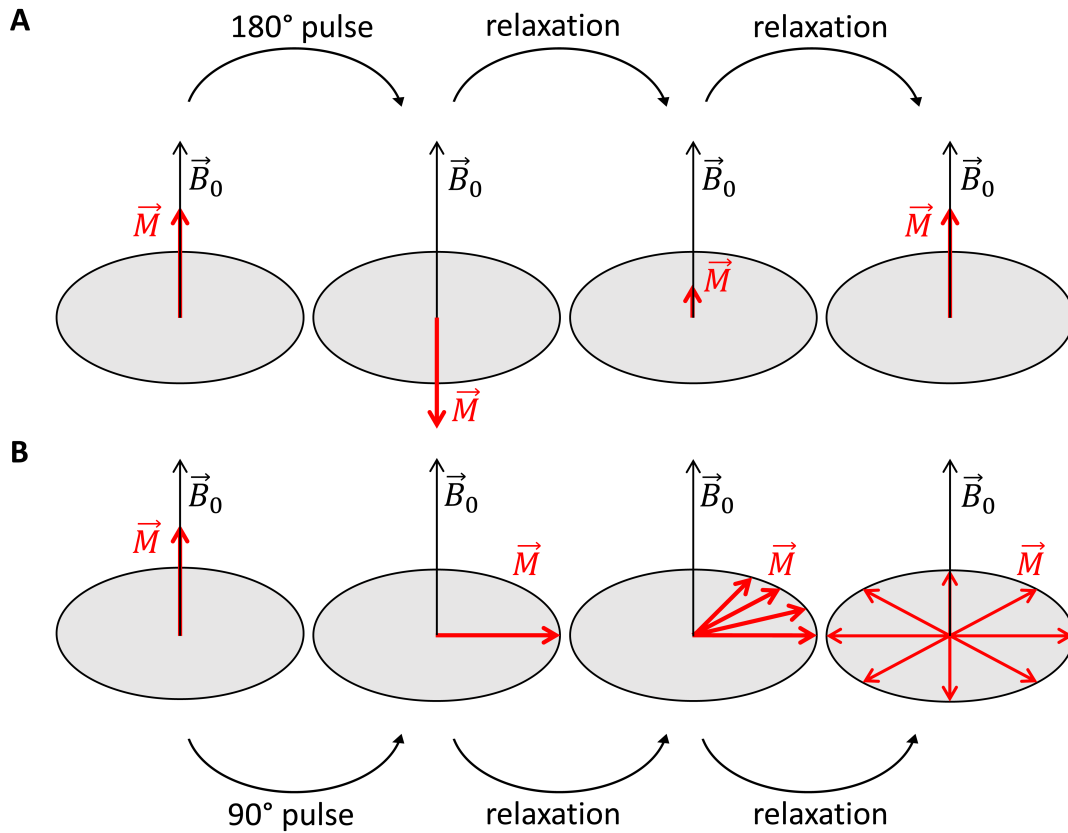


Figure 4.1: Sketch of longitudinal and transversal relaxation of a spin system using the vector model description. A: Spin-lattice relaxation after an 180 radio frequency (RF) pulse (Inversion recovery experiment). B: Spin-spin relaxation after a 90 RF pulse.

4.2.1 Random Fields

The following description of relaxation is based on the book ‘Spin Dynamics’ by M. H. Levitt¹²², the original work was published by N. Bloembergen, E. M. Purcell and R. V. Pound in 1948¹²⁴.

Thermal motion of the molecules induces fluctuating magnetic fields, which cause the drive of the system to return to the thermal equilibrium. Two important interactions are explaining the presence of fluctuating fields: dipolar coupling and chemical shift anisotropy (CSA).

Every nucleus with a spin $\neq 0$ act as a magnetic dipole and is surrounded by a magnetic field. This field influences the magnetic field of other spins in the quantum mechanical system ($B_{loc} = B_0 + B_{DD}$). Due to thermal motion, the dipole rotates and the local field of the spins in range of the rotating magnetic dipole is changing. This causes fluctuations in the local magnetic field of spins that are influenced by dipolar coupling.

The second reason for these fluctuations gives the CSA. Each nucleus in a protein is surrounded by an electron cloud. The static magnetic field B_0 induces electric currents in these electron clouds. Moving electric charges cause a magnetic field, which shields or de-shields the external magnetic field. The nuclei in the molecule feel a local magnetic field ($B_{loc} = B_0 + B_{induced}$). Due to the thermal motion of the molecule, the induced magnetic field changes and the local magnetic field of the nuclei starts fluctuating. For nuclei with a spin of $\frac{1}{2}$ the dipolar coupling has a stronger impact on the relaxation of a disturbed system than the CSA¹²².

From the idea of fluctuating local fields, the random field model can be derived to explain the relaxation in a quantum mechanical system. Because of the strong external magnetic field B_0 along z-direction every spin is influenced by the same B_z , but the magnetic field in x-direction distinguish between all spin and fluctuates due to thermal motion of the molecule. The time-averaged fluctuation $\langle B_x(t) \rangle$ is zero and the time-average of the square of fluctuations $\langle B_x^2(t) \rangle$ is defined as the magnitude of fluctuation. The fluctuations can be statistically described by an auto correlation functions (ACF):

$$G(\tau) = \langle B_x(t)B_x(t + \tau) \rangle \quad (4.7)$$

The correlation function depends on the magnetic field fluctuations, which depend on all interactions which cause field fluctuations due to thermal motions. In a simple model the correlation function can be described by a mono-exponential decay¹²².

For slow fluctuations the correlation function decays slowly and the exponential decay can be described by large correlation times. In the case of fast magnetic field fluctuations, the correlation function decays rapidly, which goes along with small correlation times (see Figure 3.2). For proteins, the correlation times are in the range of several nanoseconds. Performing a Fourier transformation on the auto correlation function, the so-called spectral density function is obtained. All relaxation rates occurring in a quantum mechanical system can be described by these density functions¹²².

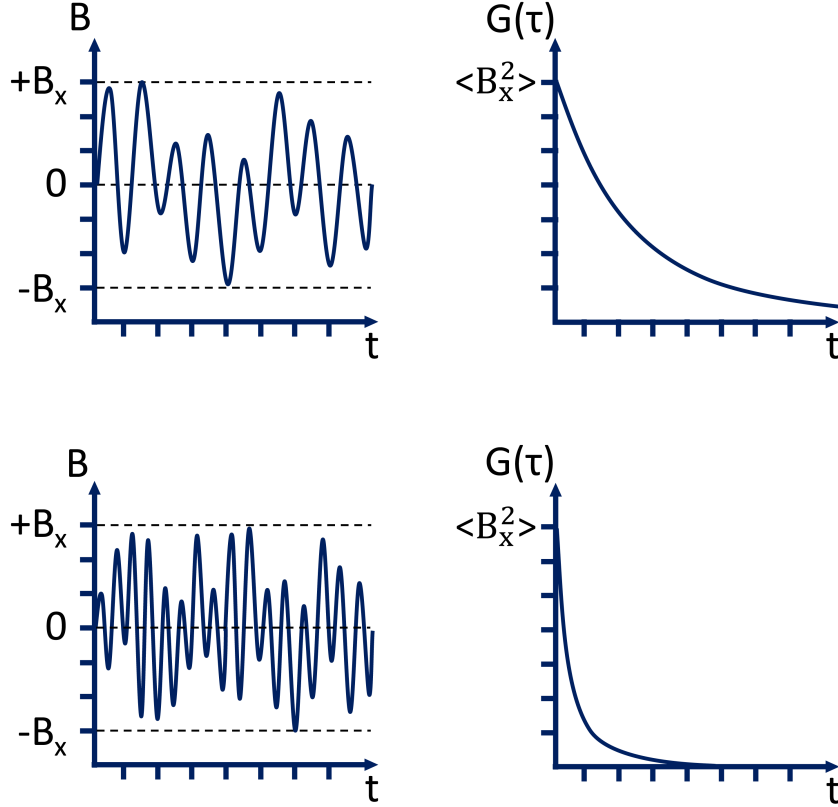


Figure 4.2: Illustration of magnetic field fluctuations on the left for two different correlation times τ . The diagrams on the right show the corresponding autocorrelation function $G(\tau)$. For slow fluctuations of the magnetic field, which are described by long correlation times, the autocorrelation function decreases slowly. For rapidly fluctuating magnetic fields, which are described by short correlation times, the autocorrelation function decreases rapidly.

4.2.2 Semi-classical theory of spin relaxation

The dependence of the relaxation rates present in a quantum mechanical system and the density function is described by a semiclassical theory of spin relaxation. Bloch, Wangsness and Redfield derived the BWR (Bloch-Wangsness-Redfield) theory in the 1950s and 1960s^{124,125,126,127,128,129,130,131,132,133}. Here, the system is described by a Hamiltonian $\mathcal{H}$, which has a deterministic (static) part $\mathcal{H}_0$ and a stochastic contribution $\mathcal{H}_1(t)$. The static part includes the isotropic terms: Zeeman term $\mathcal{H}_Z$, isotropic chemical shift $\mathcal{H}_{CS}$ and J-coupling $\mathcal{H}_J$. The stochastic part includes the second-rank terms of the Hamiltonian: dipolar coupling $\mathcal{H}_D$ and the chemical-shift anisotropy $\mathcal{H}_{CSA}$.

$$\mathcal{H}(t) = \mathcal{H}_0 + \mathcal{H}_1(t) \quad (4.8)$$

The Liouville-von Neumann equation¹³⁴ describes the time evolution of the density operator:

$$\frac{d}{dt}\hat{\sigma} = -i[\hat{\mathcal{H}}(t), \hat{\sigma}(t)] = -i[\hat{\mathcal{H}}_0 + \hat{\mathcal{H}}_1(t), \hat{\sigma}(t)] \quad (4.9)$$

After an interaction frame transformation, the application of second-order perturbation theory, an ensemble averaging, the application of the Redfield limit and the back transformation into the laboratory frame the master equation of relaxation is obtained:

$$\frac{d}{dt}\hat{\sigma} = -i[\hat{\mathcal{H}}_0, \hat{\sigma}(t)] - \hat{\Gamma}\{\hat{\sigma}(t) - \sigma_{eq}\} = -i\hat{\mathcal{H}}_0\hat{\sigma}(t) - \hat{\Gamma}\{\hat{\sigma}(t) - \sigma_{eq}\} \quad (4.10)$$

Γ is the so-called relaxation superoperator and is defined as:

$$\hat{\Gamma}\{\hat{Q}\} = \int_0^t \overline{[\hat{\mathcal{H}}_1(t), [e^{-i\hat{\mathcal{H}}_0\tau} \hat{\mathcal{H}}_1(t-\tau) e^{i\hat{\mathcal{H}}_0\tau}, \hat{Q}]]} d\tau \quad (4.11)$$

The operator $\hat{Q}$ is the operator on which the relaxation superoperator $\hat{\Gamma}$ acts on.

Every Hamiltonian can be written as the sum of the product of spatial spherical tensor terms A and spin tensor terms T:

$$\hat{\mathcal{H}} = \sum_{\mu, l, m} (-1)^m A_{l, -m}^{(\mu)} \hat{T}_{l, m}^{(\mu)} \quad (4.12)$$

μ describes the interaction (dipolar, CSA), l the rank of interaction and m the z-component.

Using this notation and additionally introducing the eigenoperators of the deterministic Hamiltonian superoperator $\hat{V}_p$ and the correlation function $C(\tau)$ as well as the spectral density function $J(\omega_p)$, the relaxation superoperator can be expressed as:

$$\hat{\Gamma}\{\hat{Q}\} = \frac{1}{2} \sum_{\mu, \mu', l, m, p} [\hat{T}_{l,m}^{(\mu)}, ([\hat{V}_p^{(\mu)}])^\dagger, \hat{Q}] J_{l,l,-m,-m}^{\mu,\mu}(\omega_p) \quad (4.13)$$

If $\mu = \mu'$ (interactions are equal) the relaxation is auto correlated, if $\mu \neq \mu'$ (interactions are unequal) the relaxation is called cross-correlated. The decay of coherence / polarization is called auto relaxation, the transfer of coherence or polarization to another one is called cross relaxation.

$$\Gamma_{rs} = \langle \hat{Q}_r | \hat{\Gamma} \hat{Q}_s \rangle / \langle \hat{Q}_r | \hat{Q}_r \rangle \quad (4.14)$$

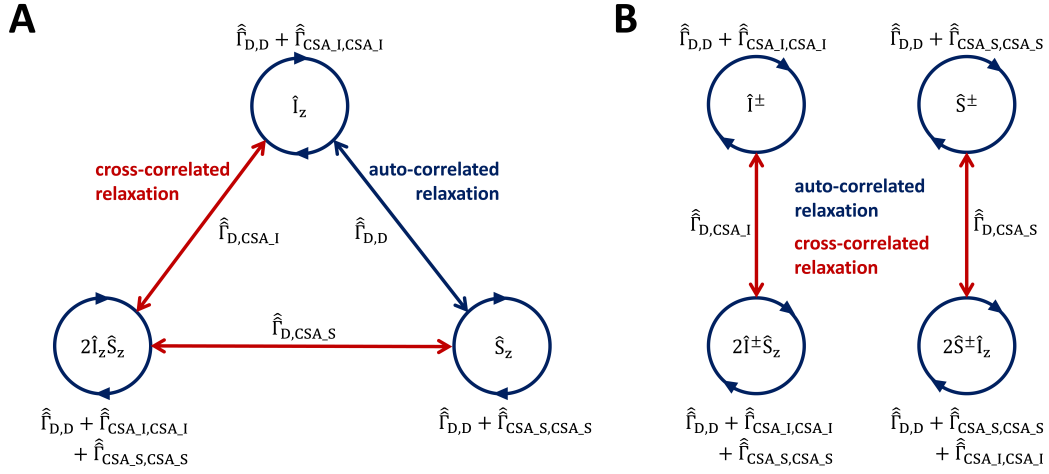


Figure 4.3: Relaxation pathways resulting from the Bloch-Wangsness-Redfield theory for a heteronuclear two-spin system under consideration of the secular approximation. Circles mark auto-relaxation, arrows mark cross-relaxation. In blue: auto-correlated relaxation, in red: cross-correlated relaxation. A: relaxation mechanisms for longitudinal relaxation. B: relaxation mechanisms for transverse relaxation.

The relaxation pathways illustrated in Figure 3.3 can be determined in a heteronuclear spin system considering the secular approximation, which prohibits the cross relaxation to zero-quantum terms and cross relaxation between the single-quantum coherences of two different spins. The associated rate constants can be calculated with equation 3.14. The relaxation causes a flux of magnetization from $\hat{Q}_s$ to $\hat{Q}_r$.

The equations for the relaxation rates R_1 (spin-lattice relaxation), R_2 (spin-spin relaxation) and σ_{IS} (dipole-dipole cross relaxation rate) are derived from equation 3.14 assuming isotropic rotational tumbling for the molecule motion^{128,135}.

$$R_1 = \frac{d^2}{4}[J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N)] + c^2 J(\omega_N) \quad (4.15)$$

$$R_2 = \frac{d^2}{8}[4J(0) + J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H) + 6J(\omega_H + \omega_N)] + \frac{c^2}{6}[3J(\omega_N) + 4J(0)] \quad (4.16)$$

$$NOE = 1 + \left(\frac{d^2}{4}\right)\left(\frac{\gamma_H}{\gamma_N}\right)[6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)]T_1 \quad (4.17)$$

$$\sigma_{HN} = \frac{d^2}{4}[6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)] \quad (4.18)$$

with

$$d = \frac{\mu_0 h \gamma_N \gamma_H}{8\pi^2} \frac{1}{r_{NH}^3}$$

$$c = \frac{\omega_N}{\sqrt{3}}(\sigma_{\parallel} - \sigma_{\perp})$$

ω_H is the Larmor frequency of the 1H nuclei, the Larmor frequency of the ^{15}N nuclei is ω_N . The constant μ_0 is the permeability of free space, γ_H and γ_N are the gyromagnetic ratios of 1H and ^{15}N . The Planck constant is h , r_{NH} is the length of the amide bond vector, and $\sigma_{\parallel}$ and $\sigma_{\perp}$ are the parallel and perpendicular components of the axially symmetric chemical shift tensor.

4.2.3 Interpretation of relaxation data

Different theoretical approaches lead to several appearance of the spectral density function.

Model-free:

1982 Lipari and Szabo developed the model-free formalism using the following spectral density function¹¹:

$$J(\omega) = \frac{2}{5} \left(\frac{S^2 \tau_M}{1 + (\tau_M \omega)^2} + \frac{(1 - S^2) \tau}{1 + (\tau \omega)^2} \right) \quad (4.19)$$

with

$$\tau^{-1} = \tau_M^{-1} + \tau_e^{-1}$$

The overall motion of the polypeptide chain is described by a single correlation time τ_M , measuring the rate of motion and by a generalized order parameter S^2 , which defines the degree of spatial restriction of the motion¹¹. The effective correlation time τ_e specifies the rate of internal motion.

Extended model-free:

The model-free of Lipari and Szabo approach was expanded 1990 for a more detailed description of internal protein dynamics¹³⁶, resulting in a fast internal correlation time τ_f and a slow internal correlation time τ_s :

$$J(\omega) = \frac{2}{5} \left(\frac{S^2 \tau_M}{1 + (\tau_M \omega)^2} + \frac{(1 - S_f^2) \tau_f'}{1 + (\tau_f' \omega)^2} + \frac{(S_f^2 - S^2) \tau_s'}{1 + (\tau_s' \omega)^2} \right) \quad (4.20)$$

with

$$\begin{aligned} \tau_f'^{-1} &= \tau_M^{-1} + \tau_f^{-1} \\ \tau_s'^{-1} &= \tau_M^{-1} + \tau_s^{-1} \end{aligned}$$

IMPACT:

The Interpretation of Motions by a Projection onto an Array of Correlation Times (IMPACT)¹³⁷ approach uses a correlation function that is composed of the sum of several correlation times τ_i weighted by coefficients A_i ($\sum_{i=1}^n A_i = 1$). The resulting spectral density has the following form:

$$J(\omega) = \frac{2}{5} \sum_{i=1}^n \frac{A_i \tau_i}{1 + (\omega \tau_i)^2} \quad (4.21)$$

DETECTORS:

The Detectors approach uses the general expression of the spectral density function resulting from a distribution of correlation times $\theta(z)$ ($\int_{-\infty}^{\infty} \theta(z) dz = 1$) on a logarithmic scale with $z = \log_{10}(\tau_c/1s)$ ¹³⁸. Every relaxation rate constant can be expressed as a linear combination of the spectral density function of the following form¹³⁹:

$$J(\omega) = \frac{2}{5} (1 - S^2) \int_{-\infty}^{\infty} \theta(z) \frac{10^z \cdot 1s}{1 + (\omega \cdot 10^z \cdot 1s)^2} dz \quad (4.22)$$

$$R_\zeta = \sum_p a_p^\zeta J(\omega_p) \quad (4.23)$$

5 Motivation

Due to the high internal flexibility of intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs)^{1,2,3}, which results in low and diffuse electron density, structural biology methods as X-ray crystallography and electron microscopy struggle to achieve high-resolution structural information for IDPs⁴. Nuclear magnetic resonance (NMR) spectroscopy, however, can measure ensemble-averaged signals of fast fluctuating IDPs^{5,6,7,8}. NMR spectroscopy can detect residue-specific insights along the polypeptide chain named protein. Many NMR spectroscopy methods have been developed over the years to obtain information about proteins across a broad dynamic timescale. This makes NMR spectroscopy a versatile toolbox that has led to several important insights into protein function, revealing a comprehensive picture of protein structure, protein dynamics, or protein interactions^{140,141,10,142,143,144,145,146,147,148,149,150}.

As both the structure and the dynamics of proteins are closely linked to their function and ligand interactions⁶⁵, they are of great interest to biologists^{10,11,12,13,14,151,108}. This is particularly true for proteins involved in complex signal transductions, and also essential in drug development¹⁴. IDPs, characterized by their high disorder, and proteins with IDRs are often involved in signal transduction^{4,61}. In neurodegenerative diseases like Parkinson's disease, IDPs play a central role in the fibrillation process, which leads to well-known symptomatic consequences^{152,153}. Furthermore, IDPs are involved in liquid-liquid phase separation and biomolecular condensate formation, which has been of great interest in recent years^{154,155,156}.

IDPs are also particularly important for neuronal exocytosis, which underlies neuronal information exchange by directing the flow of neurotransmitters across the synaptic cleft between two neurons. Several IDPs are involved in the neuronal soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) zippering, which enables vesicles packed with neurotransmitters to fuse with the plasma membrane of the neuron - a process that is not yet fully understood on molecular level. In 2013, the Nobel Prize in medicine was awarded for the discovery of the machinery regulating vesicle traffic, a major transport system in our cells¹⁵⁷. SNARE proteins are at the center of this process by driving synaptic vesicle fusion. This award has underscored the importance of this field of research and drawn also public attention to SNARE proteins. Structural and dynamic information about the protein backbone would deepen the understanding of SNARE zippering and justify observations of protein interactions involved in neuronal exocytosis.

In this PhD work, we discovered and characterized the ‘Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its monomeric Pre-fusion Conformation’ described in chapter 7 (Stief et al. 2023). In chapter 10, I further characterized the ‘Internal Backbone Dynamics of SNAP25a’.

The important requirements for such a dynamic analysis are the reproducibility and accuracy of relaxation rates derived from reliable and consistent pulse sequences. A widely used approach in NMR relies on ^{15}N relaxation rates yielding to stunning dynamic insights in protein function^{151,158,159,160,161,162,163,164,165,166,167,168,169}. Advances in magnet technology are leading to higher available magnetic fields (up to 1200 MHz). Established pulse sequences need to be tested and tailored to these higher field strengths. The good relaxation properties of IDPs leads to long T_2 times and slow magnetization decay, which opens a discussion about the readout scheme of the pulse program for achieving the best signal-to-noise ratio (SNR). The deuteration of side chain protons of the protein backbone is often used to reduce the relaxation rate constants^{170,171,172}, but it is cost-intensive. As IDPs tend to have small relaxation rates, the benefit of deuteration is questionable if no effects on relaxation rates are visible. In chapter 8, I present NMR ^{15}N R_1 and $R_{1\rho}$ relaxation experiments with a sensitivity-enhanced readout scheme and improved water suppression. They offer two-times higher SNR compared to the previously published TROSY readout scheme. These sequences work well for field frequencies between 600 MHz and 1200 MHz and fully protonated protein.

The work on NMR relaxation experiments motivated projects including the setup of relaxation rate experiments for other protein systems, data evaluation, and interpretation of the relaxation rates. When encountering NMR relaxation methods, the learning curve is challenging for beginners, but the information content of relaxation rates is worth the effort regarding protein backbone dynamics. NMR relaxation measurements should be easily accessible to all members of the community in order to advance science. This motivated us to publish paper number three on ‘NMR ^{15}N Relaxation Experiments for the Investigation of Picosecond to Nanoseconds Structural Dynamics of Proteins’ (chapter 9).

Setting up of a ^{15}N - ^1H hetNOE experiment with a sensitivity-enhanced readout scheme¹⁷³ at field strengths above 600 MHz, completed the set of ^{15}N NMR relaxation experiments. This allowed the determination of the relaxation rate constants ^{15}N R_1 , ^{15}N R_2 and hetNOE values at several field strengths. In the history of biomolecular NMR, multiple methods were developed for a quantitative analysis of these relaxation parameters: model-free¹¹, extended model-free¹³⁶, Spectral density mapping¹⁷⁴, IMPACT¹³⁷, Detectors¹³⁹. For SNAP25a, an IDP, we were interested in the performance of these methods, especially using relaxation data from a 1200 MHz spectrometer. This project led to the manuscript ‘Internal Backbone Dynamics of SNAP25a’ (chapter 10).

The structural dynamics of proteins happen on a broad range of timescales from femtoseconds to several seconds^{175,176,177}. The relaxation rates R_1 and R_2 , as well as the hetNOE values only cover picosecond- and nanosecond-scale dynamics. Since several protein dynamics occur on the millisecond- and microsecond-scale^{177,145,144,12,178,179}, we needed an experimental approach to detect these dynamics: The pure Hahn echo sequence, which has been less tested at high magnetic fields (1200 MHz). To complete the set of relaxation rate experiments, I prepared the manuscript ‘R2 Hahn echo Experiment for Detection of ms Dynamics’ (chapter 11).

6 Summary

For the project ‘Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its Pre-fusion Conformation’ (chapter 7) we started with the expression and purification of an 150 μM $^1\text{H}^{15}\text{N}^{13}\text{C}$ SNAP25a sample in 20 mM sodium phosphate buffer (pH 6.5), 150 mM NaCl and 0.1 mM TCEP. By CD-spectroscopy, we detected a prominent minimum at 203 nm, indicating that a substantial part of the protein is intrinsically disordered. The second minimum at 221 nm was interpreted as a less-dominant α -helical portion. Computational analysis using the software BeStSel¹⁸⁰ fitted 16.9% helical conformation and at least 50.6% random coil conformation.

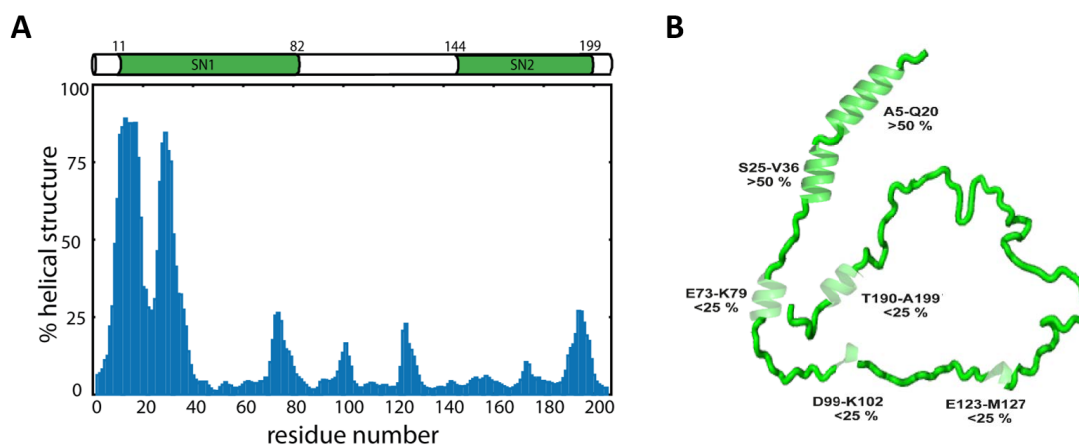


Figure 6.1: Population of α -helical structure in % along the protein backbone of SNAP25a. A: Bar plot of the population of local helical structure (in percent) of SNAP25a (calculated by the software CheSPI). B: Model of monomeric SNAP25a. The model was constructed manually (modified from PDB ID:3HD7), considering secondary structure information derived from secondary chemical shifts and using the program PyMOL (Schrödinger, Cambridge, MA). Figure 6.1 B was created with BioRender.com.²⁹

NMR spectroscopy provides residue-specific insights into the secondary structure of proteins based on the secondary chemical shifts. The secondary structure of proteins is sensitive to the $\Delta C^{\alpha 140}$, which is defined as the difference between the C^{α} and the random coil shift of the amino acid predicted by POTENCI¹⁸¹.

The C α chemical shifts were extracted from the 3D backbone assignment of SNAP25a that the author successfully completed as part of this project based on a set of three-dimensional experiments (hnco, hnca, hncacb, hn(co)ca, hn(co)cacb). The SNAP25a assignment was published in the BMRB (entry 51598). Using the software CheSPI¹⁸² the proportion of α -helical structure along the protein backbone was determined. Figure 6.1 A indicates that the N-terminal region A5 to V36, which partially overlaps with the SNARE motif SN1, has a strong α -helical propensity. Furthermore, the regions E73 to K79 (SN1), D99 to K102 (loop region), E123 to M127 (loop region), and T190 to A199 (SN2) show weak α -helical propensities (Figure 6.1 B). These findings are consistent with the total amount of secondary structure observed by CD-spectroscopy.

Using several disorder predictors and the AlphaFold 2 model, we were unable to predict a protein structure whose secondary structure matched NMR spectroscopy results.

Small angle X-ray scattering (SAXS) revealed an IDP typical Rg/Rh of 0.96 (disordered myelin basic protein e.g. shows Rg/Rh=1.06, for comparison¹⁸³), leading to the conclusion that the SNAP25 configuration is more compact than a Gaussian coil, but still possesses dominant characteristics of the random chain, as expected for intrinsically disordered proteins. SAXS also excluded a dimer formation of SNAP25a.

We further measured ¹⁵N relaxation rate constants at 600 MHz (14.1 T). The low hetNOE data of V36 to G206 revealed fast internal dynamics on the picosecond timescale, which are characteristic for IDPs and agree well with the secondary chemical shifts. The ¹⁵N R₁ relaxation rates further supported these findings. Apart from the N-terminus, low ¹⁵N R₂ values showed high internal dynamics, consistent with the ¹⁵N R₁ rates and hetNOE values. The region E10 to V36 showed increased ¹⁵N R₂ relaxation rates, in line with the secondary structure, as indicated by the secondary chemical shifts, which attribute an α -helical content to the N-terminal region.

In view of an N-terminal to C-terminal zippering of SNARE proteins during SNARE complex formation, we suggest that the N-terminus of SNAP25a acts as the nucleation site for the initiation of the SNARE zippering.

To gain further dynamic insights into SNAP25a, including high-field NMR relaxation data at 1200 MHz, we first tailored and optimized the underlying NMR relaxation experiments for high-field applications and improved signal-to-noise (which also reduced the experimental measurement time). Therefore, the aim of the following project was to optimize NMR ^{15}N R_1 and $R_{1\rho}$ relaxation experiments in terms of their accuracy and water suppression for high magnetic fields. Two samples of Synaptobrevin-2 were expressed and purified for this project (in collaboration with Katharina Vormann): 171 μM $^1\text{H}^{15}\text{N}$ Syb-2 (1-96) and $^2\text{H}^{15}\text{N}$ Syb-2 (1-96), both in 50 mM MES (pH 6.0), 150 mM NaCl, 0.1 mM TCEP and 1 mM EDTA.

The starting point for the pulse sequence optimization was the work of Lakomek et al. 2012¹⁷³. In these sequences, the sensitivity-enhanced readout is combined with an Echo-/Antiecho encoding for quadrature detection. Compared to a TROSY readout scheme, sensitivity-enhanced readout schemes increase the SNR at a similar number of scans (for proteins ≤ 25 kDa). The difficult and tedious task of improving water suppression in the pulse sequences described by Lakomek et al. was the starting point. By integrating new selective pulses and changing the gradient setup to shorter gradients (Figure 6.2), we were able to substantially improve water suppression, enabling robust application across different magnetic fields and spectrometers, using the same experimental parameter setup. The 1200 MHz spectrometer enabled us to test the sequences at magnetic fields above the GHz barrier, which are becoming increasingly available to the NMR community. At high field strengths, it is important to optimize pulse lengths, as they directly influence the excitation range, which is larger at higher field strengths. Technical limitations prevent ever shorter pulse lengths, as they are associated with stronger pulses and higher power deposition.

Within the scope of this paper, we also demonstrated the resolution enhancement of the 1200 MHz spectrometer relative to the 600 MHz and 900 MHz spectrometers by comparing HSQC experiments. The SNR enhancement between the 600 MHz and 1200 MHz spectrometers was approximately two-fold. The HSQC detection scheme also showed approximately twice the SNR compared to the TROSY readout. We had expected such results for small proteins (≤ 20 kDa) like Syb-2, as TROSY only shows its advantages for large and heavy proteins with short relaxation times. The water suppression enhancement compared to Lakomek et al 2012 was demonstrated by comparison of the first FID file. This proved enhanced water suppression with new, shorter gradients and an additional IBURP-2 pulse in the R_1 experiment. Better water suppression is associated with lower saturation of the water protons during the pulse program, which would exchange with the amide protons.

The comparison of relaxation rates between the HSQC readout scheme and the TROSY readout scheme showed high agreement for both R_1 rates and R_2 rates at 600 MHz and 1200 MHz. High Pearson correlation coefficients R of approximately 0.99 and low root mean square deviations confirmed this strong correlation. Similar results were obtained with the deuterated sample. Comparison of the protonated sample with the deuterated sample at 1200 MHz revealed a high correlation for the R_1 rates and slightly 3% faster R_2 relaxation for the protonated sample.

We concluded that deuteration is not absolutely necessary for NMR ^{15}N relaxation measurements of small proteins or IDPs, thereby reducing the cost of NMR sample preparation. The sensitivity-enhanced HSQC-detected pulse scheme works well for IDPs and for small- and medium-sized proteins. For making the correct readout scheme decision, we introduced a brief guideline: TROSY has the better spatial resolution and better water suppression, whereas HSQC has the higher SNR for small and medium-sized molecules, especially for IDPs. For measurements with high fields (≥ 800 MHz), the break-even point for better signal-to-noise (TROSY compared to HSQC) shifts to lower molecular weights, also for IDPs, which generally have longer relaxation times. We recommend using the HSQC readout scheme when SNR is the main criterion.

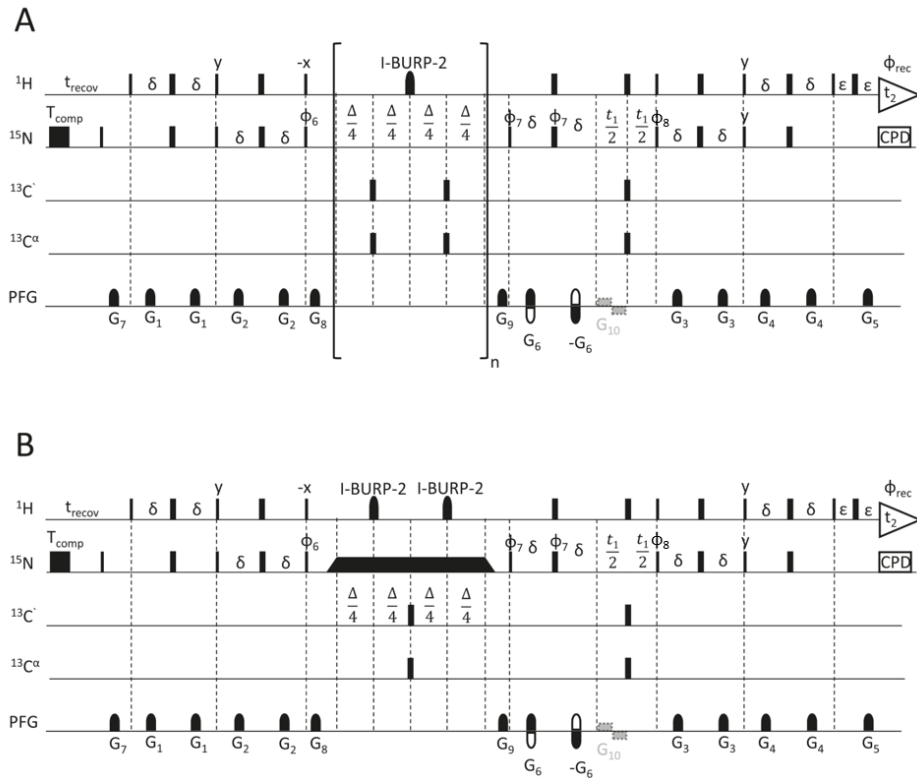


Figure 6.2: Pulse sequence schemes of NMR relaxation experiments using a sensitivity-enhanced HSQC readout scheme. The following phase cycling is applied: $\varphi_6 = y, y, -y, -y$, $\varphi_7 = y, -y$, $\varphi_{rec} = y, -y, -y, y$. The quadrature detection is implemented with the polarity inversion of the encoding gradient G_6 and simultaneous inversion of the phase $\varphi_8 = x$ (Echo/ Anti-Echo detection)¹⁸⁴. A: Pulse sequence scheme of the used ^{15}N R_1 relaxation experiment. Narrow rectangles correspond to 90° hard pulses with phase x, if not marked otherwise. 180° hard pulses are displayed as broad rectangles. B: Pulse sequence scheme of the used ^{15}N $R_{1\rho}$ experiment. Apart from the bracketed relaxation period, the $R_{1\rho}$ experiment is similar to the R_1 experiment.

NMR spectroscopy provides experiments for detecting dynamics over a broad range of timescales (Figure 6.3). As NMR relaxation data provide very valuable insights into protein dynamics, but are sometimes not easy to grasp for non-NMR experts, we decided to publish a hand-on protocol on NMR relaxation experiments in the Journal of Visualized Experiments (JoVE). NMR beginners are frequently not very familiar with NMR theory, nor familiar with the spectrometer software TopSpin or with spectra processing software, such as nmrPipe.

Briefly, the chemical shift anisotropy (CSA) and the dipolar coupling (D) are the main interactions driving ^{15}N relaxation in the range of picoseconds and nanoseconds, faster than the rotational correlation time. The picosecond and nanosecond dynamics of the protein backbone are therefore contained in the ^{15}N R_1 , ^{15}N R_2 relaxation rates and hetNOE values. These dynamics describe the local flexibility of the protein backbone (Figure 6.3) and can be assigned to the fast timescale of protein dynamics.

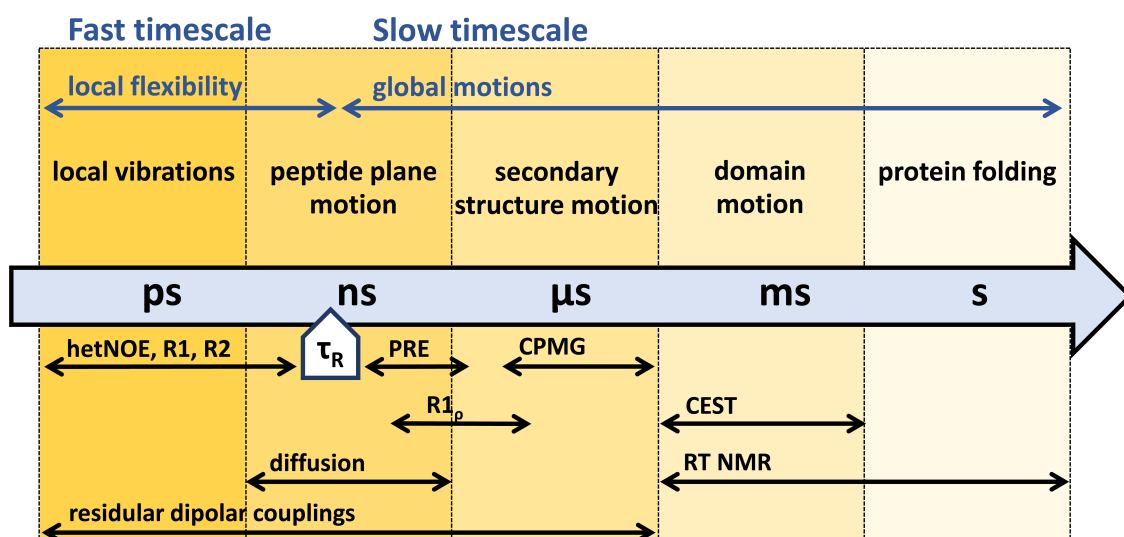


Figure 6.3: Time scales of protein backbone dynamics and sensitive time window of different NMR dynamics experiments. NMR offers a variety of methods to characterize protein backbone dynamics across a wide range of timescales. Different motions experienced by the protein backbone are indicated at their respective timescales. The protein's rotational correlation time, τ_R , is the time the protein needs for an overall rotation (by one radian). Motions faster than the protein's rotational correlation time, τ_R , can be associated with the protein's internal flexibility. Various NMR experiments and their sensitivity to the respective timescales are indicated below the arrow.

The published protocol includes the set-up of the relaxation pulse schemes presented in Stief et al. 2024 and the hetNOE pulse scheme introduced in Lakomek et al 2012. At the spectrometer, the protocol includes the most important steps: importing pulse schemes, calibrating the spectrometer (oscillating circuit and shim), calibrating the 90° pulse lengths, calibrating the carrier frequency, determining spectral widths and acquisition times, and setting up relaxation experiments. In the last step, the calibration of the amide-selective IBURP pulses and the calibration of power levels of the decoupling and spin-lock are described. The instructions for evaluating relaxation experiments contain a step-by-step protocol for using the nmrPipe scripts provided online. In addition, screenshots and a movie were produced for the support on the spectrometer and with nmrPipe.

The example relaxation data of Syb-2 presented in the paper shows a highly dynamic N-terminus (low R_2 rates and low hetNOE values), an internal increasing rigidity (increasing R_2 rates) from the N-terminus to the C-terminus (linker region), where low hetNOE values and low R_2 values indicate a highly flexible C-terminus dominated by fast nanosecond and picosecond dynamics. These findings agree well with the conclusions from Lakomek et al 2019¹⁸⁵. The discussion about the interscan delay, spin-lock power, spin-lock duration, saturation length, and TROSY readout schemes was provided for advanced users, who wish to deepen their knowledge of the application of NMR relaxation experiments.

The aim of the project ‘Internal Backbone Dynamics of SNAP25a’ (chapter 10) is to gain a deeper understanding of the internal backbone dynamics of SNAP25a, whose investigation was started in Stief et al. 2023. The optimization of NMR ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments, as well as the implementation of the hetNOE experiment, enabled the time-efficient detection of three relaxation rates at three different field strengths, which can be used for further investigation. This extensive quantitative analysis of ^{15}N relaxation data was done by using three different established approaches: model-free^{11,136}, IMPACT¹³⁷, Detectors^{139,186}. These approaches led to the determination of the timescales of motions in the protein backbone, which are not evident from the first look at the ^{15}N relaxation rates. The model-free analysis was performed using the software package relax^{187,188}, which provides a script-based model-free analysis, as a graphical user interface (gui)¹⁸⁹ for performing various analysis of NMR data, including a model-free analysis. In proteins with IDRs, structured regions can be described by a diffusion tensor^{169,9}, disordered regions have to be described by a local correlation time. These local correlation times describe the segmental motion of the polypeptide chain and influence the slow dynamics of each specific residue^{14,190,191}. Blackledge and coworkers identified three dynamic modes occurring in the backbone of IDPs: segmental motions in the range of several nanoseconds, dihedral angle fluctuation in the range of hundreds of picoseconds up to nanoseconds, and bond librations faster than 50 picoseconds^{191,192}.

We applied the model-free approach to the IDP SNAP25a, whose secondary structure was solved in Stief et al. 2023 (chapter 7). The relaxation data revealed slow segmental motions in the range of 8 nanoseconds in the helical N-terminal region. The disordered region of SNAP25a showed segmental motions in the four nanoseconds regime. Faster motions between 0.5 ns and 1.5 ns were detected by the slower internal correlation time τ_s . Local fluctuations in the backbone dihedral angles are causing these fast nanosecond dynamics, which Blackledge and coworkers also observed. Fast internal dynamics were detected by the internal correlation time τ_f , which indicated motions in the 20 ns to 80 ns time range. These amplitudes of motion are caused by the librations of the amide bonds, which can be described by a wobble in a cone model associated to the model-free analysis¹¹. The model-free analysis was able to detect the three most important dynamic modes characterizing the backbone dynamics in IDPs, whose existence in other proteins had been verified previously by MD simulations^{193,194} and NMR spectroscopy^{137,191}.

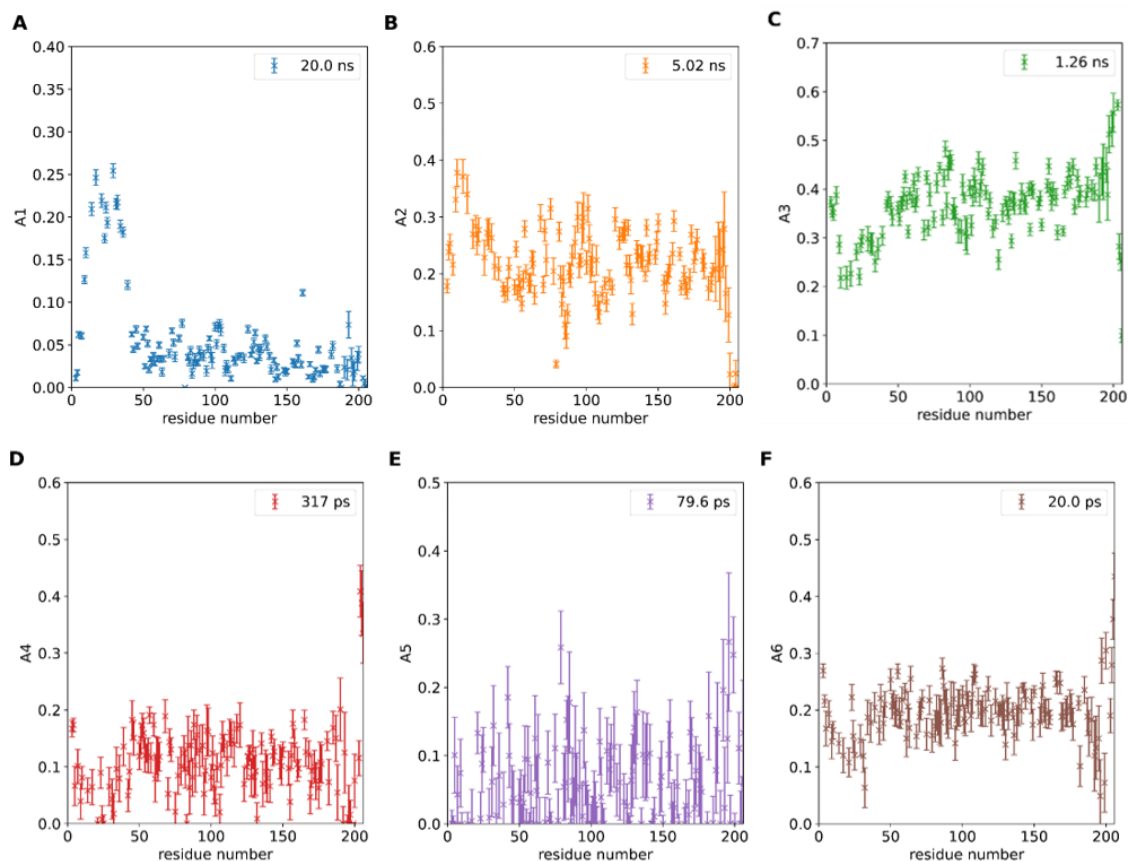


Figure 6.4: IMPACT analysis of the dynamic motions along the protein backbone of SNAP25a. For the input, we used R_1 and R_2 relaxation rate constants, as the ^{15}N - ^1H NOE values at three different magnetic fields (600 MHz, 900 MHz, 1200 MHz). The IMPACT analysis was performed with six correlation times: A: 20.0 ns, B: 5.02 ns, C: 1.26 ns, D: 317 ps, E: 79.6 ps, F: 20.0 ps each representing a specific time scale of motion. The amplitudes A: A1, B: A2, C: A3, D: A4, E: A5 and F: A6 define the response of the individual backbone motions at different time scales to these specific correlation times.

The IMPACT analysis was performed to receive a more detailed picture of the different dynamic modes occurring in the backbone of SNAP25a, as IMPACT allows the study of more than three correlation times that contribute to the spectral density function. The IMPACT analysis (Figure 6.4) showed segmental motions in the range of 20 ns for the helical N-terminus and motions in the range of 5 ns for all residues. The calculated tumbling time from the IMPACT analysis ($\tau_m = (A_1 * \tau_1 + A_2 * \tau_2) / (A_1 + A_2)$) correlates well with the local correlation time of the model-free analysis, showing high consistency between both approaches. For the disordered backbone part of SNAP25a, the most present dynamics are dihedral angle fluctuations in the nanosecond regime. In the picoseconds dynamic range, IMPACT revealed a dynamic mode around 300 ps, mostly visible for the disordered

part and a strong mode for dynamics around 20 ps and faster picosecond dynamics, which are rather present in the disordered regions of SNAP25a. Here, the model-free approach and the IMPACT approach showed differences in the timescale of fast internal motions, resulting from the sampling of two different correlations times $\tau_5 = 80$ ps and $\tau_6 = 20$ ps in the IMPACT analysis. Therefore, the IMPACT analysis is able to extract faster picosecond dynamic modes from the relaxation data at a more accurate level. Especially the relaxation data measured at 1200 MHz, which are sensitive to faster dynamics compared to relaxation data at lower fields, in combination with the IMPACT analysis, enables these fast dynamic insights.

The Detectors approach uses a total distribution of correlation times and based on that the detector response is an indicator for motion in a specific detector window. Segmental motions of several nanoseconds were detected for most residues and are dominant in the structured N-terminus. These motional restricted residues (because of the α -helix) showed less response to detectors of faster correlation times, especially in the picosecond regime. The intrinsically disordered residues, however, showed strong responses to the detector of 1.7 ns. In contrast to the IMPACT analysis, the Detectors approach showed a stronger response to dynamics in the hundreds of ps range. Dynamics slower than 100 ps, including bond librations, mainly had a response in the disordered region. Therefore, the three most important dynamic modes were resolved using this approach.

All three approaches showed high consistency in detecting dynamic modes that occur in the backbone dynamics of SNAP25a. In general, the Detectors analysis appears more sensitive to differences of regions with distinguishable dynamic modes. IMPACT showed the highest resolution, enabling the discrimination of overlapping dynamic modes, especially in the ns regime. The model-free approach could not show that resolution, but has the benefit of extracting easy to grasp amplitudes of motions, which include the information on dynamic restriction regarding the dynamics of the amide bond vectors.

The final manuscript (chapter 10) will be submitted soon.

The ^{15}N Hahn echo experiment complements the set of relaxation experiments. The R_2 relaxation rate from a Hahn echo experiment consists of the relaxation rate R_{20} and an exchange contribution R_{ex} , which is sensitive to chemical exchange in the millisecond and microsecond regime. We built the Hahn echo pulse sequence from the sensitivity-enhanced readout pulse schemes of Stief et al. 2024 by replacing the relaxation element with a ^{15}N Hahn echo element. The water suppression of this scheme was enhanced using a Hahn echo delay that matches the super cycle of the protein decoupling scheme, which is active during the Hahn echo relaxation period. ^{13}C decoupling was implemented by an adiabatic CHIRP pulse every half of the super cycle. The pulse sequence was tested on a ^{15}N labeled and on a ^{15}N - ^{13}C labeled human Ubiquitin sample, both supplemented with only 2% D_2O to minimize the impact of the deuterium isotope effect on R_2 relaxation rates¹⁹⁵.

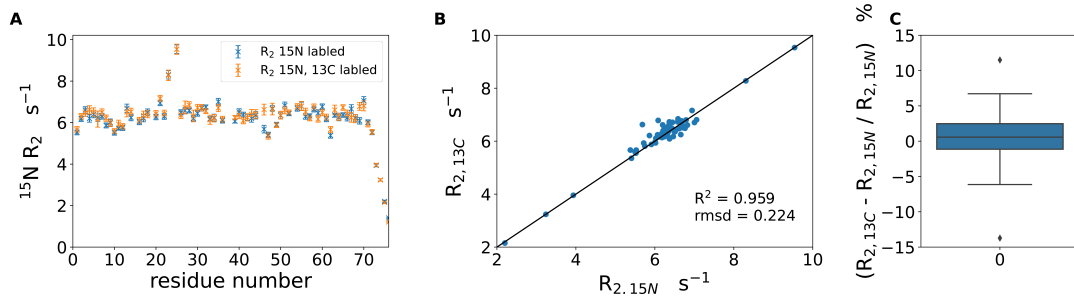


Figure 6.5: Comparison of R_2 relaxation rates of ^1H - ^{15}N and ^1H - ^{15}N - ^{13}C human ubiquitin acquired with the Hahn echo pulse scheme (see chapter 11) at 600 MHz and 25 °C. A: Residue-specific comparison of relaxation rate R_2 of the ^1H - ^{15}N sample (blue) and the ^1H - ^{15}N - ^{13}C (Orange). B: Correlation plot of the compared R_2 relaxation rate constants. C: Box plot of the residue-specific percentual differences of R_2 relaxation rate constants.

For the validation of the pulse schemes, we compared the ^{15}N R_2 Hahn relaxation rates with the R_2 relaxation rates, calculated from the R_1 and $R_{1\rho}$ rates, because we were expecting small exchange contribution along the proton backbone of human ubiquitin¹². We found high correlation between the Hahn echo R_2 data and the R_2 from $R_{1\rho}$ data with a Pearson correlation coefficient of $R^2 = 0.96$ at 600 MHz. We also tested the Hahn echo at 1200 MHz, for which the Pearson correlation coefficient of 0.95 also validated the Hahn echo sequence for high magnetic fields. In further experiments, we compared the R_2 rates from the ^{15}N labeled sample and the ^{15}N - ^{13}C labeled sample using the Hahn echo sequence. Again, we found a high Pearson correlation factor of 0.96 (see Figure 6.5), confirming the efficiency of the implemented ^{13}C decoupling scheme at 600 MHz. Beside the validation of the Hahn echo pulse scheme, we could not detect any R_2 exchange contribution for human ubiquitin at a pH of 6.0 and a sample temperature of 25 °C. After further experiments for pulse sequence validation, the final manuscript (chapter 11) will be submitted soon.

7 Intrinsic Disorder of SNAP25a

The neuronal exocytosis on the molecular level is not well understood, but scientific approaches were developed to reach the understanding of this mechanism in detail. We contributed to this, by using NMR spectroscopy to solve the structure and structural dynamics of SNAP25a in its prefusion conformation.

The author expressed and purified the ^{15}N ^{13}C labeled SNAP25a sample in the biochemical laboratory. The expression and purification protocols were optimized in a previous master thesis. After the sample preparation, the author started his work at the NMR spectrometers. 2D heteronuclear single quantum coherence (HSQC) experiments and 3D backbone resonance experiments were performed by the author. Afterwards the author processed the NMR data for the following evaluation and interpretation. The 3D backbone assignment of SNAP25a was completed successfully by the author. The relaxation experiments, the processing of the spectra and the evaluation of the relaxation rates were also done by the author. In cooperation with Lothar Gremer the author acquired circular dichroism (CD) spectroscopy data of SNAP25a. As a final step, the author deposited the 3D data and the final backbone assignment in the Biological Magnetic Resonance Data Bank (BMRB) data bank. The author contributed text and figures for the final publication.

Journal: jmb

Volume: Volume 435, Issue 10, 15 May 2023, 168069

Authors: Tobias Stief
Lothar Gremer
Sonja Pribicevic
Delane F. Espinueva
Katharina Vormann
Ralf Biehl
Reinhard Jahn
Angel Perez-Lara
Nils-Alexander Lakomek

DOI: 10.1016/j.jmb.2023.168069

© 2023. This manuscript version is made available under the CC-BY-NC-ND 4.0

license <https://creativecommons.org/licenses/by-nc-nd/4.0/>

Published article: J Mol Biol. 2023 May 15;435(10):168069. doi: 10.1016/j.jmb.2023.168069

Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its Pre-fusion Conformation

Tobias Stief,^{a,b} Lothar Gremer,^{a,b} Sonja Pribicevic,^c Delane F. Espinueva,^c Katharina Vormann,^{a,b} Ralf Biehl,^d Reinhard Jahn,^c Ángel Pérez-Lara,^e and Nils-Alexander Lakomek^{a,b,*}

^a Forschungszentrum Jülich, Structural Biochemistry (IBI-7) and Jülich Center for Structural Biology (JuStruct), Wilhelm-Johnen-Straße, 52425 Jülich, Germany

^b Heinrich Heine University of Düsseldorf, Physical Biology, Universitätsstraße 1, 40225 Düsseldorf, Germany

^c Max-Planck Institute for Multidisciplinary Sciences, Dept. of Neurobiology, Am Faßberg 11, 37077 Göttingen, Germany

^d Jülich Centre for Neutron Science (JCNS-1) and Institute for Biological Information Processing (IBI-8), Forschungszentrum Jülich GmbH, 52428 Jülich, Germany.

^e Dept. of Physical Chemistry, University of Granada, Campus Universitario de Cartuja, 18071 Granada, Spain

Correspondence: Nils-Alexander.Lakomek@hhu.de, n.lakomek@fz-juelich.de

Abstract:

The neuronal SNARE protein SNAP25a (isoform 2) forms part of the SNARE complex eliciting synaptic vesicle fusion during neuronal exocytosis. While the post-fusion cis-SNARE complex has been studied extensively, little is known about the pre-fusion conformation of SNAP25a. Here we analyze monomeric SNAP25a by NMR spectroscopy, further supported by small-angle X-ray scattering (SAXS) experiments. SAXS data indicate that monomeric SNAP25 is more compact than a Gaussian chain but still a random coil. We show that for monomeric SNAP25a, before SNAP25a interacts with its SNARE partners to drive membrane fusion, only the N-terminal part (region A5 to V36) of the first SNARE motif, SN1 (L11 - L81), is helical, comprising two α -helices (ranging from A5 to Q20 and S25 to V36). From E37 onwards, SNAP25a is mostly disordered and displays high internal flexibility, including the C-terminal part of SN1, almost the entire second SNARE motif (SN2, N144-A199), and the connecting loop region. Apart from the N-terminal helices, only the C-termini of both SN1 (E73 - K79) and SN2 (region T190 - A199), as well as two short regions in the connecting loop (D99 - K102 and E123 - M127) show a weak α -helical propensity (α -helical population < 25%). We speculate that the N-terminal helices (A5 to Q20 and S25 to V36) which constitute the N-terminus of SN1 act as a nucleation site for initiating SNARE zippering.

Keywords: SNARE proteins, SNAP25a, NMR spectroscopy, intrinsically disordered proteins, protein dynamics

Introduction

SNARE proteins and their assembly into helical SNARE complexes drive membrane fusion during exocytosis and vesicle trafficking [1, 2]. Because of their fundamental importance for brain function, neuronal SNARE complexes belong to the best-studied SNARE complexes [3, 4]. They are the key engine eliciting synaptic vesicle membrane fusion with the plasma membrane, which propagates into fusion pore formation and subsequent neurotransmitter release into the synaptic cleft for signal transmission [1, 2].

The SNARE proteins SNAP25 (Synaptosomal associated protein of 25 kilodaltons) and syntaxin-1a reside at the plasma membrane, waiting for the vesicular SNARE protein, synaptobrevin-2, to initiate SNARE complex zippering. Formation of the SNARE complex will lead to the juxtaposition of the synaptic vesicle membrane and the plasma membrane (Figure 1), with synaptobrevin-2 anchored in the synaptic vesicle membrane and syntaxin-1a anchored in the plasma membrane, both via their C-terminal transmembrane domains [1, 2]. The SNARE coiled-coil is formed by four heptad repeat motifs, called SNARE motifs, contributed by the individual SNARE proteins [4]. Here, synaptobrevin-2 and syntaxin-1a contribute one SNARE motif each and SNAP25 two (Figure 1). The central layer of the helix bundle, also termed the “zero layer” [1, 5] contains three Gln (Q) residues contributed by SNAP25 and syntaxin-1a, therefore dubbed Q-SNAREs, while synaptobrevin-2 provides an Arg (R) and is therefore named R-SNARE. It is widely accepted that the initial stages of the SNARE complex formation are templated by two accessory proteins, Munc13 and Munc18, interacting with syntaxin-1a [2]. However, the exact order of assembly is still under debate [6].

SNAP25 exists in two isoforms, SNAP25b (isoform 1) and SNAP25a (isoform 2), with SNAP25a expressed in the embryonic brain while SNAP25b becomes the dominant form in the adult brain [7, 8]. However, SNAP25a remains the predominant isoform in endocrine and neuroendocrine cells throughout life [8]. Mutations in the gene encoding SNAP25 are associated with epileptic encephalopathies [9] and neurodevelopmental and neurological disorders [7, 10].

Previous NMR studies on the monomeric forms of the SNAREs synaptobrevin-2 and syntaxin-1a provided structural insights into their pre-fusion conformations [11-15]. However, the structure of SNAP25 in its monomeric pre-fusion conformation is still unclear. Early circular dichroism measurements detected intrinsic disorder and some helical content of SNAP25 [16]. The intrinsic disorder of SNAP25 was later confirmed by EPR spectroscopy using several site-specific spin-labels [17]. However, detailed residue-specific insights into its secondary structure propensity have remained elusive.

Here, we investigate the secondary structure and structural dynamics of the monomeric pre-fusion conformation of SNAP25a (isoform 2 of SNAP25, from *Rattus norvegicus*, Uniprot accession number: P60881-2, see Figure S1 for sequence information) in a residue-specific manner by solution NMR spectroscopy, further supported by far-UV circular dichroism (CD) spectroscopy and small angle X-ray scattering (SAXS). Importantly, our construct includes the SNAP25 loop region connecting the N-terminal SNARE motif, SN1 (region L11-L81), with the C-terminal SNARE motif, SN2 (region N144-A199). Wild-type SNAP25a comprises four cysteine residues, located in this loop region between SN1 and SN2 and adjacent to SN1. These four cysteines become posttranslationally palmitoylated and act as membrane anchor [16, 18]. Although a structural study on fully palmitoylated SNAP25 would be highly desirable, it is hard to achieve yields compatible with structural investigations, particularly in the context of NMR isotope labeling. We, therefore, decided to use a variant in which the four cysteines are replaced by serines (C84S, C85S, C90S, C92S), thus lacking the palmitoyl residues [17]. (For detailed sequence information on SNAP25a, see Figure S1.)

We find that SNAP25a is intrinsically disordered and highly dynamic, except of two adjacent N-terminal α -helices, which we interpret as a potential nucleation site for the initiation of SNARE zippering.

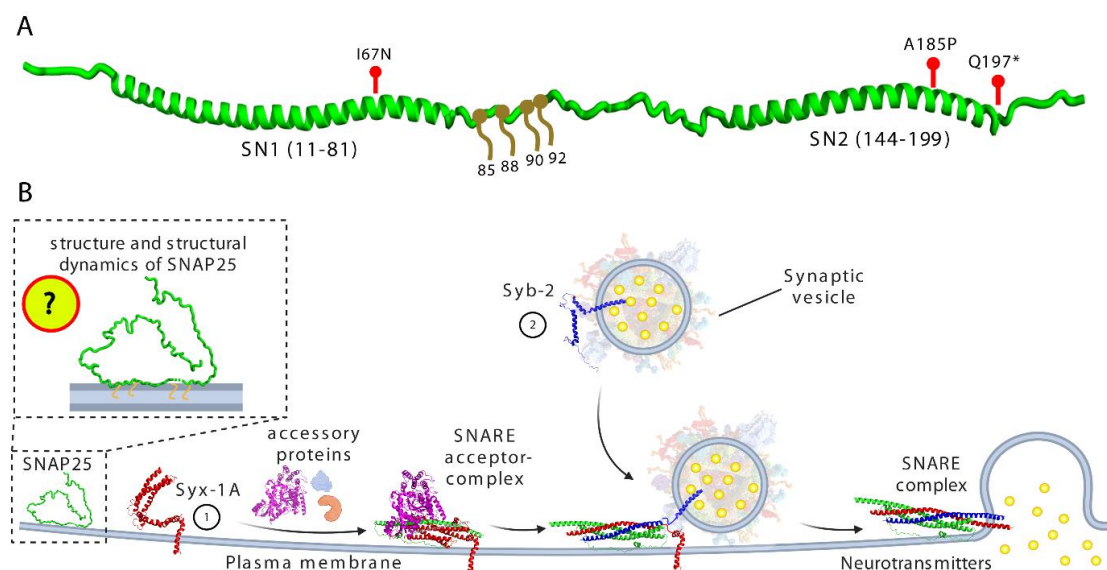


Figure 1. (A) SNAP25 domain organization showing the palmitoylation sites (in brown) and main disease mutations (in red) in humans. Its helical state is revealed in the SNARE complex. Mutations I67N and A185P lead to congenital myasthenic syndrome 18 (CMS18)[19], while Q197* leads to early infantile epileptic encephalopathy in humans (from UniProt database, <https://www.uniprot.org/>).

(B) Scheme of SNARE assembly driving neuronal exocytosis. Note that the pre-fusion conformations of the SNARE proteins are not well understood. (Syx-1A, syntaxin-1A, modified from PDB ID: 1BR0 and 2M8R, and syb-2, synaptobrevin-2, modified from PDB ID: 2KOG. The accessory protein Munc18 appears in magenta, modified from PDB ID: 3C98, the SNARE acceptor complex is modified from the AlphaFold model AF-P60881-F1-model_v4 and PDB ID: 3C98, and the SNARE complex is modified from PDB ID: 3HD7).

Results

NMR chemical shift data reveal intrinsic disorder of SNAP25a at a residue-specific resolution

As a first inspection of the overall fold, we recorded far-UV-CD spectra of SNAP25a (Figure 2). CD spectra exhibit evident characteristics of a predominantly intrinsically disordered state through a prominent minimum at 203 nm. A second minimum is detected at 221 nm and can be interpreted as the presence of an α -helical portion to a lesser extent. Although the buffer conditions at close to

physiological salt concentrations (20 mM sodium phosphate buffer (pH 6.5), 150 mM NaCl, 0.1 mM TCEP) precluded CD data recording below 198 nm, and, therefore, did not allow unambiguous computational secondary structure calculations, analysis of the derived CD data (in the data range from 250 to 200 nm) fits 16.9% helical and at least 50.6% random-coil conformation, using the program BeStSel [20]. Those data agree with early CD data recorded on SNAP25 [16], suggesting a large random-coil contribution and some helical content. Residue-specific insights into which parts are more structured and which are less have remained absent. We, therefore, turned to solution NMR spectroscopy which is well-suited to provide residue-specific insights into intrinsically disordered proteins [21-25].

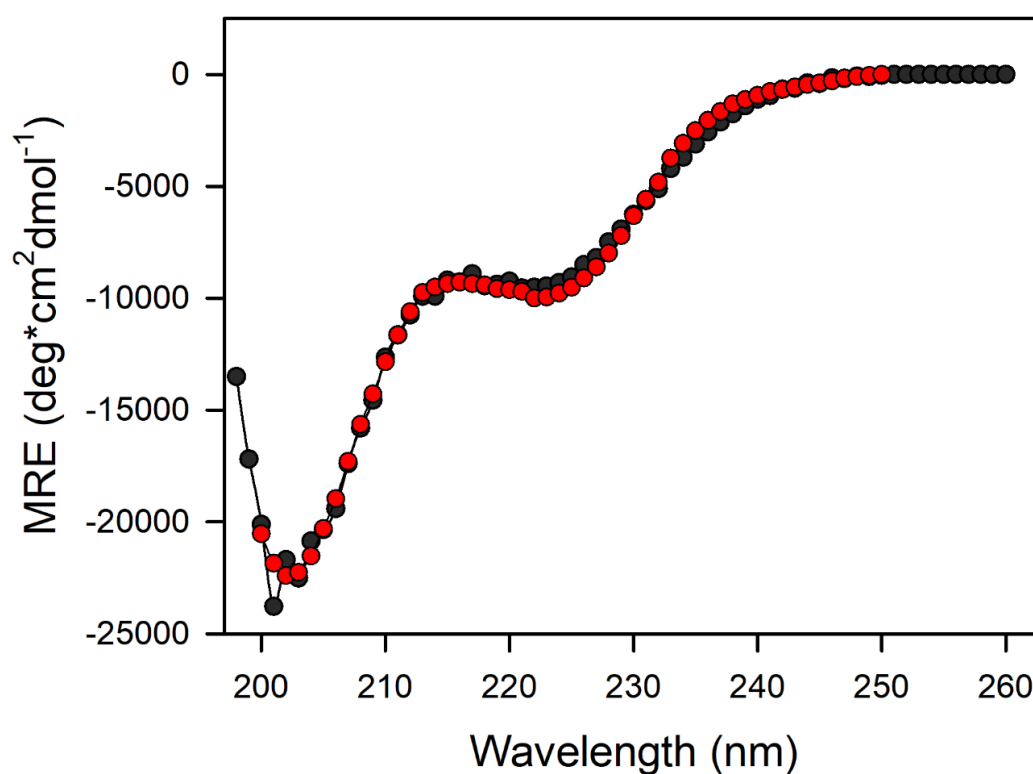


Figure 2. Far-UV circular dichroism (CD) spectrum of SNAP25a. Analysis of the data (black dots, experimental data; red dots, fitted data between 200 and 250 nm using BeStSel[20]) indicates the presence of a random-coil dominated secondary structure of SNAP25a, as evident from a strong minimum at 203 nm and the absence of any positive CD signal portions in the recorded data range down

to 198 nm. MRE stands for mean residue ellipticity. The less pronounced minimum at 221 nm indicates the additional presence of a folded portion to a lesser extent.

For the NMR analysis, first, we performed NMR spectral backbone assignments of SNAP25a, employing three-dimensional NMR experiments described in the Materials and Methods section. Except for two proline residues (P91 and P117), complete backbone resonance assignments could be obtained (Table S4), which have been deposited to the Biological Magnetic Resonance Bank (BMRB ID 51598). A two-dimensional ^1H - ^{15}N HSQC spectrum of SNAP25a, including assignments, is shown in Figures 3A and B. The narrow spectral dispersion in the proton dimension, with ^1H resonances ranging between 7.6 and 8.5 ppm, suggests an intrinsic disorder of SNAP25a. For a fully α -helical conformation, a slightly wider spectral dispersion (between about 7 and 9 ppm) would be expected. However, just from the ^1H dimension, it is difficult to distinguish between an α -helical or intrinsically disordered secondary structure. Therefore, we turned to $\text{C}\alpha$ secondary chemical shifts, allowing for a much better distinction of secondary structure elements.

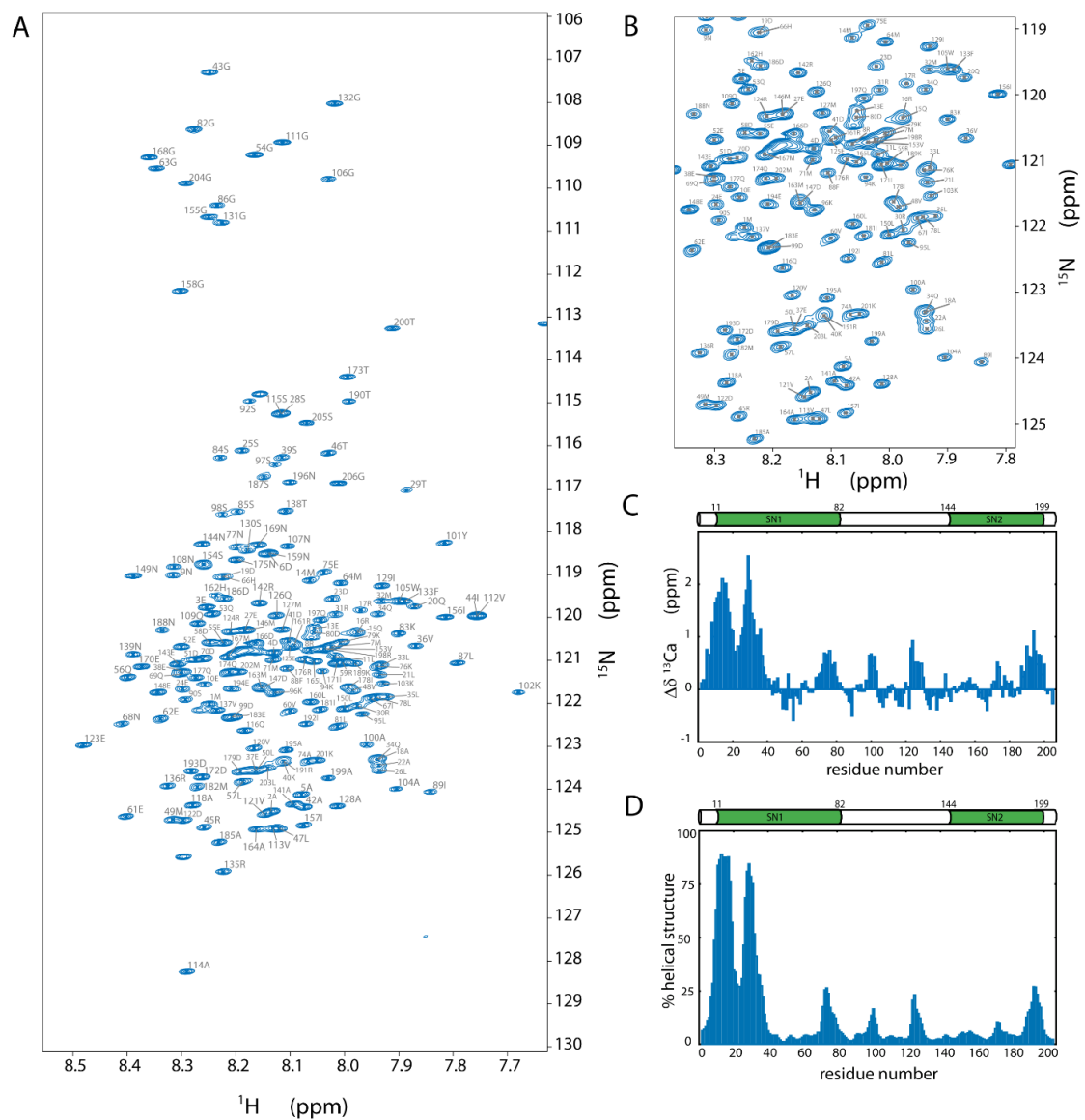


Figure 3. (A) ^1H - ^{15}N HSQC spectrum of 150 μM ^{15}N ^{13}C SNAP25a in 20mM sodium phosphate buffer (pH 6.5), 150mM NaCl, 0.1mM TCEP, recorded at 900 MHz and 15°C. (B) Zoom into the most crowded region of the ^1H - ^{15}N HSQC spectrum of SNAP25a shown in panel a. (C) NMR C α secondary chemical shift of SNAP25a plotted along the sequence. Positive values (> 0.5 ppm) indicate α -helical propensity (about 3-4 ppm stating full α -helical content). Negative values (< -0.5 ppm) show β -sheet propensity. Values between -0.5 ppm and 0.5 ppm report random-coil characters, respectively. (D) Bar plot of population of local helical structure (in percent) of the SNAP25a (calculated by the Software

CheSPI)[26]. Our data indicate that SNAP25a is primarily intrinsically disordered, apart from the N-terminal region A5 to V36, which shows a strong α -helical propensity and partially overlaps with the N-terminal SNARE motif SN1.

From the $C\alpha$ chemical shifts, we calculated residue-specific secondary chemical shifts to obtain information on the secondary structure propensity of SNAP25a (see Materials and Methods section). Figure 3C shows the $C\alpha$ secondary chemical shifts of the individual SNAP25a residues along the sequence (Table S5). The regions A5 to Q20 and S25 to V36 show strong α -helical propensity with positive secondary chemical shifts up to 2.5 ppm. They are connected by a short, slightly less structured, linker. From E37 to Q69, secondary chemical shifts indicate an intrinsic disorder. From E73 to K79, weak α -helical propensity marks the C-terminus of the N-terminal SNARE motif, SN1, according to the post-fusion structure of SNAP25 in the assembled SNARE complex [3, 4]. L11 marks the N-terminus of SN1 in the post-fusion structure. Therefore, the region A5 to V36 overlaps with the N-terminus of SN1. The α -helical propensity declines, however, well before the zero-layer positioned at Q53. Within the loop region (G82 to E143) that connects SN1 to the second SNARE motif, SN2, residues D99 to K102 and E123 to M127 show weak α -helical propensity. The adjacent second SNARE motif SN2 (N144 to A199) displays secondary chemical shifts close to zero ppm indicating intrinsic disorder. Only the C-terminus of SN2 (T190 to A199) shows weak α -helical propensity. We also predicted the population of α -helical structure from the chemical shift values using the CheSPI software [26]. The α -helical population (in percent), shown in Figure 3D, follows the pattern of the $C\alpha$ chemical shift values (see also Figure S2). We have tested different computational disorder predictors (fIDPnn [27], Metapredict [28], IDP-Seq2Seq [29], IUPred3 [30]) (Figure S3). While most of them correctly predict intrinsic disorder of SNAP25a, they fail to predict the N-terminal helices. One of them, Metapredict [28], predicts helical structure for both SN1 and SN2 and disorder for the loop region, therefore more similar to what is expected for SNAP25 in its post-fusion conformation. A similar model is predicted by AlphaFold2 [31, 32] (www.alphafold.ebi.ac.uk/entry/P60881). It has been discussed previously that caution is required when assessing AlphaFold2 predictions of IDP secondary structure [33, 34].

SAXS analysis

Small angle X-ray scattering (SAXS) data were measured for several concentrations of SNAP25a and corrected for background. Figure S4A shows SAXS data scaled by concentrations. Samples were checked for aggregation by dynamic light scattering (Zetasizer Nano, Malvern, U.S.A.), showing no significant aggregate formation. The hydrodynamic radius R_h extrapolated to zero concentration was $R_h=4.46$ nm. SAXS shows a typical pattern for Gaussian chains. The highest concentration was fitted by a wormlike chain model [35] showing a radius of gyration $R_g=4.7$ nm with a persistence length of 1.4 nm leading to a weaker power law decay at large Q ($\sim Q^{-d}$ with $d<2$). The compactness of the configuration can be examined looking at $R_g/R_h=0.96$. For an ideal Gaussian chain, we expect 0.66 [36] while a sphere results in 1.29, respectively a globular protein like myoglobin in 1.26 [37]. The pair distance distribution, $P(r)$, shows an end-to-end distance of ~ 12.5 nm (Figure S4B). Consequently, the SNAP25 configuration is more compact than a Gaussian coil, but still a random chain as expected for intrinsically disordered proteins. The disordered myelin basic protein e.g., shows $R_g/R_h=1.06$ [38].

Structural dynamics by NMR relaxation measurements

To characterize the structural dynamics of SNAP25, we turned to NMR relaxation measurements. Low heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE data, ranging between 0.47 for M14 and -1.54 for G206, reveal high internal dynamics on the picosecond timescale (Figure 4A shows the $\{^1\text{H}\}$ - ^{15}N NOE values between -0.1 to 1, which are observed for the region M1-T200; for the full range including the highly dynamic C-terminus, see Figure S5A). These high internal dynamics are characteristic of an intrinsically disordered protein (IDP) [39] and are in line with the NMR secondary chemical shift data. Consistent with the $\{^1\text{H}\}$ - ^{15}N NOE values, ^{15}N R_1 relaxation rate constants further support the finding of high internal dynamics (Figure 4B). ^{15}N R_2 rate constants, derived from $R_{1\rho}$ measurements, show elevated rates between 8.0 s^{-1} and 10.5 s^{-1} for the region E10 to V36 (Figure 4C). In comparison, most remaining residues show an R_2 ($R_{1\rho}$) rate constant between 3 s^{-1} and 6 s^{-1} , apart from the C-terminus with values down to 0.8 s^{-1} . Low R_2 ($R_{1\rho}$) rates show high internal dynamics, consistent with the $\{^1\text{H}\}$ - ^{15}N NOE and

^{15}N R_1 relaxation rate constants, further supporting the finding of intrinsic disorder. The higher R_2 ($R_{1\rho}$) rates observed for the N-terminus of SN1 indicate the presence of a secondary structure, in line with the secondary chemical shifts indicating α -helical content. R_2 ($R_{1\rho}$) rate constants are sensitive to ps-ns dynamics, while NOE values are dominated by very fast ps dynamics (< 100 ps). To investigate the presence of conformational exchange, we recorded Hahn-echo-based ^{15}N R_2 rate constants at two magnetic fields, 14.1 T (corresponding to 600 MHz ^1H Larmor frequency) and 21.15 T (corresponding to 900 MHz ^1H Larmor frequency). While in the $R_{1\rho}$ experiments, with an applied spin-lock frequency of 2 kHz, all exchange processes slower than 80 μs have been refocused, and $R_2(R_{1\rho})$ rate constants primarily report on fast ps-ns dynamics (faster than the rotational correlation time τ_c), these Hahn-echo-based R_2 measurements are sensitive to all conformational exchange processes slower than about 10 ms. The exchange processes scale with the square of the magnetic field and therefore have a more substantial impact on relaxation at higher fields. Figure 4D shows the difference between spin-echo-based R_2 rate constants measured at 900 MHz minus those measured at 600 MHz (for the individual rate constants, see Figure S5B and Table S6). For regions N9 to Q34, we observe differences higher than two s^{-1} for most resonances, while most residues for the remaining protein show differences lower than one s^{-1} . This suggests the presence of a conformational exchange process for the N-terminus of SN1, like, e.g., α -helix formation, which would be consistent with the high α -helical propensity revealed by the secondary chemical shifts data (Figure 3).

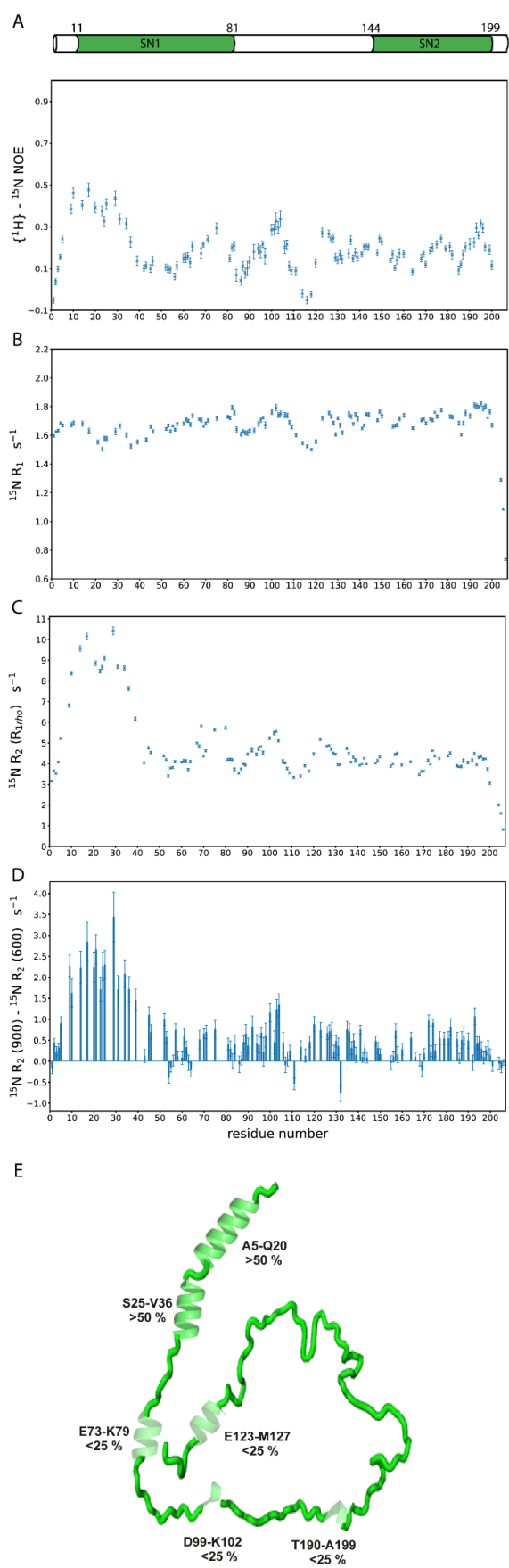


Figure 4. NMR relaxation data reveal high intrinsic dynamics of SNAP25a. (A) Low $\{^1\text{H}\}$ - ^{15}N NOE values indicate a high degree of internal dynamics on the fast ps timescale (for full range of data, including the negative values of the C-terminus, see Figure S5A), which is also in line with the (B) ^{15}N R_1 rate constants. (C) Low ^{15}N R_2 ($R_{1\rho}$) rate constants underline the finding of a high degree of intrinsic dynamics; elevated ^{15}N R_2 rate constants for the N-terminal region A5 to V36 point to secondary structure. (D) Hahn-echo-based ^{15}N R_2 rate constants are sensitive to conformational exchange effects. Higher ^{15}N R_2 (900 MHz) minus R_2 (600 MHz) rate constants support a conformational exchange process for regions A5 to V36, such as, e.g., transient α -helix formation (for individual rate constants see Figure S5B). (E) Model of monomeric SNAP25a. The model was constructed manually (modified from PDB ID:3HD7), taking into account secondary structure information derived from secondary chemical shifts (see also Figures 3C and D) and using the program PyMOL (Schrödinger, Cambridge, MA). Figure 4E was created with BioRender.com.

SAXS data (see above) do not show indications of dimer formation of SNAP25a. We have further validated the monomeric character of SNAP25a by repeating the ^{15}N R_2 ($R_{1\rho}$) measurements on a sample of lower concentration (70 μM vs. 150 μM). The ^{15}N R_2 ($R_{1\rho}$) rate constants are sensitive to changes of the rotational correlation time τ_c , because of their $J(\omega_1) \approx J(0)$ dependence, with the spin-lock frequency ω_1 (which is 2 kHz in our case and small compared to the Larmor frequencies). ^{15}N R_2 ($R_{1\rho}$) rate constants of the 70 μM sample overlay well with the 150 μM sample and do not show a concentration dependence (Figure S6). We conclude that SNAP25 is monomeric at both concentrations.

Our $C\alpha$ secondary chemical shift and NMR relaxation data reveal that SNAP25 is intrinsically disordered in its monomeric conformation, apart from two N-terminal helices A5-Q20 and S25-V36, with α -helical propensity $> 75\%$. This region coincides with the N-terminus of SN1. We assign the N-terminal SNARE motif SN1 to residues L11 to L81. In the different X-ray structures of the post-fusion SNARE complex (PDB codes 1SFC, 1N7S, 3HD7) [3, 4, 40], the SN1 has fully α -helical conformation, with the N-terminus showing some variation regarding the beginning of the helix. The second SNARE motif SN2, which we assign to region N144-A199, is also fully helical in the post-fusion conformation of SNAP25 in the assembled cis-SNARE complex, but intrinsically disordered in the monomeric form; here only the C-terminus shows a weak α -helical propensity ($< 25\%$). All X-ray structures (PDB codes 1SFC, 1N7S, 3HD7) were solved using two isolated constructs of SN1 and SN2, with the connecting loop region lacking. For a better visualization of the post-fusion conformation of SNAP25 its structure is highlighted in the context of the assembled (truncated) SNARE complex (Figure S7A, PDB code 1N7S). B-factors (or temperature factors, respectively) of the structure are color-coded onto the structure and plotted versus the residue number in Figure S7A. Towards the N- and C-termini of SN1 and SN2 B-factors increase, maybe not unexpectedly. For the first structure of the SNARE complex, 1SFC, which has been solved at lower resolution (2.4 Å compared to 1.45 Å for 1N7S), B-factors are overall higher, and also show an increase towards the N- and C-termini (Figure S7B). A similar effect is observed for 3HD7, the only structure of the SNARE complex that includes the syx-1a and syb-2 transmembrane regions and the only one which has been solved in a lipid-mimicking detergent environment on a non-soluble construct (Figure S7B).

Discussion

To summarize, we have studied the secondary structure and structural dynamics of SNAP25a using NMR chemical shifts and NMR relaxation data. Our data reveal that SNAP25a is intrinsically disordered in its monomeric pre-fusion form. Comparing NMR relaxation data to those obtained for synaptobrevin-2 [15], SNAP25a exhibits less intrinsic dynamics (and disorder) than synaptobrevin-2.

NMR data on monomeric SNAP25a indicate that the second SNARE motif of SNAP25a, SN2, is intrinsically disordered, while the N-terminus of SN1 (up to residue V36) shows a strong α -helical propensity, with two α -helices A5-Q20 and S25-V36 connected by a short, less structured, linker. SAXS data indicate that SNAP25a is more compact than a Gaussian chain but still behaves like an intrinsically disordered protein. We speculate that the helical SNAP25a N-terminus may act as a nucleation site for N- to C-terminal zippering of the SNARE complex or upstream for the formation of an intermediate SNARE acceptor complex, presumably composed of Munc18:syntaxin-1a and SNAP25 (1:1:1) [41]. Fasshauer and Margittai found previously, that N-terminal but not C-terminal truncations of SNAP25 prevented SNARE complex assembly [42]. Interestingly, while purified SNARE complexes containing a synaptobrevin-2 N-terminal (1-59) fragment are structured only N-terminally, purified SNARE complexes containing a synaptobrevin-2 C-terminal (60-96) fragment appear well-structured both for their N- and C-termini [43], suggesting higher order of the syntaxin-1a and SNAP25 N-termini. This is in line with solution NMR ^1H - ^{15}N HSQC spectra recorded on the homologous binary complex from yeast, composed of Sso1 (equivalent to Syntaxin-1a) and Sec9 (equivalent to SNAP25). Those spectra indicated that the C-terminus of Sso1 is less structured in the binary complex [44]. (As Sec9, similar to SNAP25, was not labeled with NMR-active isotopes, Sec9 was not directly observable in the NMR HSQC spectra.) Further, a SNARE complex containing a synaptobrevin-2 C-terminal fragment shows a more substantial decrease of the activation barrier for the assembly reaction than a SNARE complex containing a synaptobrevin-2 N-terminal fragment [45]. These results suggest that the N-termini of the SNAP25 and the syntaxin-1a SNARE motif exhibit a higher propensity to adopt an α -helical structure than the SNAP25 C-termini, which would favor SNARE complex assembly via an N- to C-terminal

zippering. This has been confirmed previously by NMR spectroscopy for the SNARE motif of syntaxin-1a in its pre-fusion conformation [13], and we find this now for the N-terminus of SNAP25. Therefore, our data close the gap for SNAP25 and support a model of N-terminal nucleation and N- to C-terminal zippering for SNARE complex assembly.

Materials and Methods

NMR sample preparation

We studied the non-palmitoylated form of SNAP25a (isoform 2 of SNAP25 from *Rattus norvegicus*, Uniprot accession number: P60881-2, 206 amino acids, 23.3 kDa) with the four cysteine sites mutated to serines (C84S, C85S, C90S, C92S). The gene encoding SNAP25a from *Rattus norvegicus*, subcloned in a pET28a(+) vector with an N-terminal His₆-tag and Thrombin cleavage site, was heterologously expressed in BL21 (DE3) cells. For NMR sample preparation, cells were grown in M9 minimal media supplemented with ¹⁵N enriched ammonium chloride (99%, Cambridge Isotope Laboratories, USA) and ¹³C enriched Glucose (U-¹³C6, 99 %, Cambridge Isotope Laboratories, Tewksbury, MA, USA) as single sources for protein synthesis. Following an overnight preculture in 2-YT broth media (ThermoFisher, Waltham, MA, USA), cells were grown in M9 media for 4-5 h until an OD₆₀₀ of 0.7 was reached, and protein expression was induced by the addition of 0.5 mM IPTG, and cells incubated over-night at 20°C. SNAP25a was purified by Ni²⁺-NTA affinity chromatography, followed by ion exchange chromatography (using a Resource Q column, Cytiva, Marlborough, MA, USA) and size exclusion chromatography (using a HiLoad 16/600 Superdex 75 pg column, Cytiva, USA), following the protocol described by Pobatti et al. [43]. The final NMR sample contained 150 μM ¹⁵N¹³C SNAP25a in 20 mM sodium phosphate buffer at pH 6.5, 150 mM NaCl and 0.1mM TCEP.

NMR backbone resonance assignment

Spectral backbone resonance assignment was performed using a suite of three-dimensional HNCO, HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, and HN(CA)CO experiments. Spectra were referenced to DSS internally (0.2 μ M), which was added to the NMR sample buffer. Measurements were conducted on a Bruker 750 MHz Avance III spectrometer equipped with a ^1H , ^{13}C , ^{15}N triple resonance Bruker TCI cryoprobe (Bruker, Billerica, MA, USA). The experimental temperature was 15°C. Details on the experimental setup are summarized in Table S1. Spectra were processed using the Bruker Topspin 3.5 and analyzed using the CCPN Analysis 3.0 software [46]. Secondary chemical shift data were derived from the backbone chemical shift assignments and random-coil chemical shifts predicted by the POTENCI software [47], using the SNAP25 amino acid sequence and experimental buffer conditions as inputs. The secondary structure populations were predicted from chemical shift assignments using the CheSPI software [26].

NMR relaxation data

^{15}N R_1 , ^{15}N $R_{1\rho}$ relaxation rate constants and heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE data were recorded at 600 MHz using a Bruker Avance III HD spectrometer equipped with a ^1H , ^{13}C , ^{15}N triple resonance Bruker TCI cryoprobe, using the pulse programs described in [48]. Here, the pulse programs employing a sensitivity-enhanced HSQC read-out scheme were used. The experimental temperature was 15°C.

Rate constants and heteronuclear NOE data were evaluated using the NMRPipe software [49] and dedicated scripts as detailed in [48]; R_2 rate constants were derived from the $R_{1\rho}$ by correcting for R_1 contributions. Details on the experimental setup are summarized in Table S3.

^{15}N R_2 data, including potential R_{ex} contributions due to chemical or conformational exchange, were recorded using a Hahn-echo-based R_2 experiment; the pulse sequence scheme is shown in Figure S8. Conformational exchange processes result in a R_{ex} contribution to $R_{2,0}$ auto-relaxation, such that the Hahn-echo measured R_2 rate constants become $R_{2,\text{app}} = R_{2,0} + R_{\text{ex}}$. Data were recorded at 600 MHz and 900 MHz (using a Bruker 900 MHz Avance Neo Spectrometer equipped with a cryogenic ^1H , ^{13}C , ^{15}N

TCI probe). As the R_{ex} contribution scales quadratically with the magnetic field, $R_{ex} \sim B^2$, the difference R_2 (900 MHz) minus R_2 (600 MHz) will indicate R_{ex} contributions due to conformational exchange.

Circular dichroism (CD) spectroscopy

Far-UV-CD spectroscopy of SNAP25a protein samples was performed on a JASCO J-815 CD spectropolarimeter (Jasco, Gross-Umstadt, Germany) using 1 mm path length quartz cuvettes (Helma, Müllheim, Germany). Typically, spectra were recorded at protein concentrations of ~6 to 12 μ M SNAP25 in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 6.5 at 20 °C with instrument settings as follows: 0.1 to 1 nm step size, 50 nm min⁻¹ scan speed, 1 nm band width. The signal-to-noise ratio was improved by the accumulation of 10 scans per sample. The mean residue ellipticity $[\theta]_{mrw}$ in deg·cm²·dmol⁻¹ was calculated from the equation $[\theta]_{mrw} = (\theta_{obs} \times MRW) / (c \times d \times 10)$, with θ_{obs} , observed ellipticity (in degrees); c , concentration (in g/ml); d , cell path length (in cm); MRW (mean residue weight), molecular weight divided by number of peptide bonds.

Secondary structure analysis by BeStSel [20] (<https://BeStSel.elte.hu/>) was applied on CD data recorded at 11.55 μ M SNAP25 in the 200 to 250 nm range with a scale factor of 1.0.

Small angle X-ray Scattering (SAXS) analysis

Small angle X-ray Scattering (SAXS). SAXS experiments were performed at the Jülich Center for Neutron Science (JCNS) at Forschungszentrum Jülich, Germany. The instrument “Ganesha-Air” from SAXSLAB/XENOCs was used. The X-ray source of the laboratory-based “Ganesha-Air” system is a D2-MetalJet (Excillum) with a liquid metal anode operating at 70 kV and 3.57 mA with Ga- $K\alpha$ radiation (wavelength $\lambda = 0.13414$ nm). The data were acquired with a position-sensitive detector (PILATUS 300 K, Dectris). After calibration with silver behenate, the distance from the sample to the detector was set to cover a Q-range 0.1 – 6 nm⁻¹. Data analysis was done using the Python-based project Jscatter [50]. The pair distance distribution function, $P(r)$ was calculated from the form factor with concentration 8 mg/ml, using the DATGNOM4 tool of the ATSAS software package [51].

Accession numbers

NMR backbone chemical shift assignments (^1H , ^{15}N , $^{13}\text{C}'$, and $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$) for SNAP25a have been deposited to the BMRB with accession code 51598 and provided as supplementary Table S4.

CRedit authorship contribution statement

Tobias Stief: Investigation, Methodology, Formal analysis, Resources, Writing. **Lothar Gremer:** Investigation, Formal analysis, Writing. **Sonja Pribicevic:** Resources, Writing. **Delane F. Espinueva:** Resources. **Katharina Vormann:** Resources. **Ralf Biehl:** Investigation, Formal Analysis, Writing. **Reinhard Jahn:** Conceptualization, Writing. **Angel Perez-Lara:** Conceptualization, Writing. **Nils-Alexander Lakomek:** Conceptualization, Methodology, Writing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

A.P.-L. acknowledges funding by the Ramon y Cajal grant (RYC2018-023837-I) and R.J. financial support by the National Institutes of Health (NIH grant number P01 GM072694). N.L. acknowledges the German Science Foundation for funding through the Heisenberg Program (DFG grant number 433700474) as well as access to the Jülich-Düsseldorf Biomolecular NMR Center, jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU).

References

- [1] Jahn R, Fasshauer D. Molecular machines governing exocytosis of synaptic vesicles. *Nature*. 2012;490:201-7.
- [2] Rizo J, Sudhof TC. The Membrane Fusion Enigma: SNAREs, Sec1/Munc18 Proteins, and Their Accomplices-Guilty as Charged? *Annu Rev Cell Dev Biol*. 2012;28:279-308.
- [3] Stein A, Weber G, Wahl MC, Jahn R. Helical extension of the neuronal SNARE complex into the membrane. *Nature*. 2009;460:525-8.
- [4] Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature*. 1998;395:347-53.
- [5] Hernandez JM, Stein A, Behrmann E, Riedel D, Cypionka A, Farsi Z, et al. Membrane fusion intermediates via directional and full assembly of the SNARE complex. *Science*. 2012;336:1581-4.
- [6] Zhang Y, Hughson FM. Chaperoning SNARE Folding and Assembly. *Annu Rev Biochem*. 2021;90:581-603.
- [7] Irfan M, Daraio T, Bark C. SNAP-25 Puts SNAREs at Center Stage in Metabolic Disease. *Neuroscience*. 2019;420:86-96.
- [8] Bark IC, Hahn KM, Ryabinin AE, Wilson MC. Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. *Proc Natl Acad Sci U S A*. 1995;92:1510-4.
- [9] Alten B, Zhou Q, Shin OH, Esquivies L, Lin PY, White KI, et al. Role of Aberrant Spontaneous Neurotransmission in SNAP25-Associated Encephalopathies. *Neuron*. 2021;109:59-72 e5.
- [10] Antonucci F, Corradini I, Fossati G, Tomasoni R, Menna E, Matteoli M. SNAP-25, a Known Presynaptic Protein with Emerging Postsynaptic Functions. *Front Synaptic Neurosci*. 2016;8:7.
- [11] Ellena JF, Liang B, Wiktor M, Stein A, Cafiso DS, Jahn R, et al. Dynamic structure of lipid-bound synaptobrevin suggests a nucleation-propagation mechanism for trans-SNARE complex formation. *Proc Natl Acad Sci U S A*. 2009;106:20306-11.
- [12] Liang B, Dawidowski D, Ellena JF, Tamm LK, Cafiso DS. The SNARE motif of synaptobrevin exhibits an aqueous-interfacial partitioning that is modulated by membrane curvature. *Biochemistry-US*. 2014;53:1485-94.
- [13] Liang BY, Kiessling V, Tamm LK. Prefusion structure of syntaxin-1A suggests pathway for folding into neuronal trans-SNARE complex fusion intermediate. *Proc Natl Acad Sci USA* 2013;110:19384-9.
- [14] Brewer KD, Li W, Horne BE, Rizo J. Reluctance to membrane binding enables accessibility of the synaptobrevin SNARE motif for SNARE complex formation. *Proc Natl Acad Sci U S A*. 2011;108:12723-8.
- [15] Lakomek NA, Yavuz H, Jahn R, Perez-Lara A. Structural dynamics and transient lipid binding of synaptobrevin-2 tune SNARE assembly and membrane fusion. *Proc Natl Acad Sci U S A*. 2019;116:8699-708.
- [16] Fasshauer D, Bruns D, Shen B, Jahn R, Brunger AT. A structural change occurs upon binding of syntaxin to SNAP-25. *J Biol Chem*. 1997;272:4582-90.
- [17] Margittai M, Fasshauer D, Pabst S, Jahn R, Langen R. Homo- and heterooligomeric SNARE complexes studied by site-directed spin labeling. *J Biol Chem*. 2001;276:13169-77.
- [18] Veit M, Sollner TH, Rothman JE. Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25. *Febs Lett*. 1996;385:119-23.
- [19] Shen XM, Selcen D, Brengman J, Engel AG. Mutant SNAP25B causes myasthenia, cortical hyperexcitability, ataxia, and intellectual disability. *Neurology*. 2014;83:2247-55.
- [20] Micsonai A, Moussong E, Wien F, Boros E, Vadaszi H, Murvai N, et al. BeStSel: webserver for secondary structure and fold prediction for protein CD spectroscopy. *Nucleic Acids Res*. 2022.
- [21] Arai M, Sugase K, Dyson HJ, Wright PE. Conformational propensities of intrinsically disordered proteins influence the mechanism of binding and folding. *Proc Natl Acad Sci U S A*. 2015;112:9614-9.
- [22] Bah A, Vernon RM, Siddiqui Z, Krzeminski M, Muhandiram R, Zhao C, et al. Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch. *Nature*. 2015;519:106-9.

- [23] Borgia A, Borgia MB, Bugge K, Kissling VM, Heidarsson PO, Fernandes CB, et al. Extreme disorder in an ultrahigh-affinity protein complex. *Nature*. 2018;555:61-6.
- [24] Schneider R, Blackledge M, Jensen MR. Elucidating binding mechanisms and dynamics of intrinsically disordered protein complexes using NMR spectroscopy. *Curr Opin Struct Biol*. 2019;54:10-8.
- [25] Dyson HJ, Wright PE. NMR illuminates intrinsic disorder. *Curr Opin Struct Biol*. 2021;70:44-52.
- [26] Nielsen JT, Mulder FAA. CheSPI: chemical shift secondary structure population inference. *J Biomol Nmr*. 2021;75:273-91.
- [27] Hu G, Katuwawala A, Wang K, Wu Z, Ghadermarzi S, Gao J, et al. fIDPnn: Accurate intrinsic disorder prediction with putative propensities of disorder functions. *Nat Commun*. 2021;12:4438.
- [28] Emenecker RJ, Griffith D, Holehouse AS. Metapredict: a fast, accurate, and easy-to-use predictor of consensus disorder and structure. *Biophys J*. 2021;120:4312-9.
- [29] Tang YJ, Pang YH, Liu B. IDP-Seq2Seq: identification of intrinsically disordered regions based on sequence to sequence learning. *Bioinformatics*. 2021;36:5177-86.
- [30] Erdos G, Pajkos M, Dosztanyi Z. IUPred3: prediction of protein disorder enhanced with unambiguous experimental annotation and visualization of evolutionary conservation. *Nucleic Acids Res*. 2021;49:W297-W303.
- [31] Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596:583-9.
- [32] Varadi M, Anyango S, Deshpande M, Nair S, Natassia C, Yordanova G, et al. AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res*. 2022;50:D439-D44.
- [33] Ruff KM, Pappu RV. AlphaFold and Implications for Intrinsically Disordered Proteins. *J Mol Biol*. 2021;433:167208.
- [34] Wilson CJ, Choy WY, Karttunen M. AlphaFold2: A Role for Disordered Protein/Region Prediction? *Int J Mol Sci*. 2022;23.
- [35] Kholodenko AL. Analytical calculation of the scattering function for polymers of arbitrary flexibility using the Dirac propagator. *Macromolecules*. 1993;26:4179-83.
- [36] Doi M, Edwards SF. *The Theory of Polymer Dynamics* Oxford University Press. 1988.
- [37] Stadler AM, Pellegrini E, Johnson M, Fitter J, Zaccai G. Dynamics-stability relationships in apo- and holomyoglobin: a combined neutron scattering and molecular dynamics simulations study. *Biophys J*. 2012;102:351-9.
- [38] Stadler AM, Stingaciu L, Radulescu A, Holderer O, Monkenbusch M, Biehl R, et al. Internal nanosecond dynamics in the intrinsically disordered myelin basic protein. *J Am Chem Soc*. 2014;136:6987-94.
- [39] Wright PE, Dyson HJ. Intrinsically disordered proteins in cellular signalling and regulation. *Nat Rev Mol Cell Biol*. 2015;16:18-29.
- [40] Ernst JA, Brunger AT. High resolution structure, stability, and synaptotagmin binding of a truncated neuronal SNARE complex. *J Biol Chem*. 2003;278:8630-6.
- [41] Jakhanwal S, Lee CT, Urlaub H, Jahn R. An activated Q-SNARE/SM protein complex as a possible intermediate in SNARE assembly. *Embo Journal*. 2017;36:1788-802.
- [42] Fasshauer D, Margittai M. A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. *J Biol Chem*. 2004;279:7613-21.
- [43] Pobbati AV, Stein A, Fasshauer D. N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science*. 2006;313:673-6.
- [44] Fiebig KM, Rice LM, Pollock E, Brunger AT. Folding intermediates of SNARE complex assembly. *Nat Struct Biol*. 1999;6:117-23.
- [45] Li F, Tiwari N, Rothman JE, Pincet F. Kinetic barriers to SNAREpin assembly in the regulation of membrane docking/priming and fusion. *Proc Natl Acad Sci U S A*. 2016;113:10536-41.
- [46] Skinner SP, Fogh RH, Boucher W, Ragan TJ, Mureddu LG, Vuister GW. CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. *J Biomol Nmr*. 2016;66:111-24.
- [47] Nielsen JT, Mulder FAA. POTENCI: prediction of temperature, neighbor and pH-corrected chemical shifts for intrinsically disordered proteins. *J Biomol Nmr*. 2018;70:141-65.

-
- [48] Lakomek NA, Ying JF, Bax A. Measurement of N-15 relaxation rates in perdeuterated proteins by TROSY-based methods. *J Biomol Nmr*. 2012;53:209-21.
- [49] Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol Nmr*. 1995;6:277-93.
- [50] Biehl R. Jscatter, a program for evaluation and analysis of experimental data. *PLoS One*. 2019;14:e0218789.
- [51] Petoukhov MV, Konarev PV, Kikhney AG, Svergun DI. ATSAS 2.1 - towards automated and web-supported small-angle scattering data analysis. *Journal of Applied Crystallography*. 2007;40:s223-s8.

8 Improved R_1 and $R_{1\rho}$ Relaxation Experiments

Protein dynamics are highly important, as they are closely connected to protein functions and protein interactions⁹. An NMR based approach to detect the dynamics of a protein backbone are ^{15}N relaxation rates, which are sensitive to backbone dynamics at different time scales in the picosecond and nanosecond dynamic regime. The basic requirement for such a dynamic analysis is reproducible and error-free relaxation rates, derived from reliable and consistent pulse sequences.

In this work the author established the presented pulse sequences in the IBI-7 NMR facility of Forschungszentrum Jülich. Following this task, the author optimized the pulse sequences and tested them at different magnetic fields. The final published data was acquired and analyzed by the author (2D HSQCs, ^{15}N relaxation experiments). During the writing process, the author contributed text and figures.

Journal: Methods

Volume: Volume 223, March 2024, Pages 1-15

Authors: Tobias Stief
Katharina Vormann
Nils-Alexander Lakomek

DOI: 10.1016/j.ymeth.2024.01.008

Sensitivity-enhanced NMR ^{15}N R_1 and $R_{1\rho}$ relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields

Tobias Stief^{1,2}, Katharina Vormann^{1,2}, and Nils-Alexander Lakomek^{1,2,*}

¹ Institute of Biological Information Processing (IBI-7), Forschungszentrum Jülich, Jülich, Germany

² Institute of Physical Biology, Faculty of Mathematics and Natural Sciences, Heinrich Heine

University Düsseldorf, Düsseldorf, Germany

*Correspondence:

Dr. Nils-Alexander Lakomek
Heinrich-Heine-Universität Düsseldorf
Institut für Physikalische Biologie (IPB)
Gebäude 26.44, Ebene U1
Universitätsstraße 1
40225 Düsseldorf
Germany
Phone: +49 211 811 5702
E-Mail: Nils-Alexander.Lakomek@hhu.de

Forschungszentrum Jülich
IBI-7: Strukturbiochemie
Gebäude 05.8v
Wilhelm-Johnen-Strasse
52425 Jülich
Germany
Phone: +49 2461 61 85345
E-Mail: n.lakomek@fz-juelich.de

Abstract

NMR relaxation experiments provide residue-specific insights into the structural dynamics of proteins. Here, we present an optimized set of sensitivity-enhanced ^{15}N R_1 and $R_{1\rho}$ relaxation experiments applicable to fully protonated proteins. The NMR pulse sequences are conceptually similar to the set of TROSY-based sequences and their HSQC counterpart (Lakomek et al., J. Biomol. NMR 2012). Instead of the TROSY read-out scheme, a sensitivity-enhanced HSQC read-out scheme is used, with improved and easier optimized water suppression. The presented pulse sequences are applied on the cytoplasmic domain of the SNARE protein Synpatobrevin-2 (Syb-2), which is intrinsically disordered in its monomeric pre-fusion state. A two-fold increase in the obtained signal-to-noise ratio is observed for this intrinsically disordered protein, therefore offering a four-fold reduction of measurement time compared to the TROSY-detected version. The inter-scan recovery delay can be shortened to two seconds. Pulse sequences were tested at 600 MHz and 1200 MHz ^1H Larmor frequency, thus applicable over a wide magnetic field range. A comparison between protonated and deuterated protein samples reveals high agreement, indicating that reliable ^{15}N R_1 and $R_{1\rho}$ rate constants can be extracted for fully protonated and deuterated samples. The presented pulse sequences will benefit not only for IDPs but also for an entire range of low and medium-sized proteins.

Keywords: NMR, relaxation measurements, protein dynamics, intrinsically disordered proteins, sensitivity-enhanced HSQC, protein backbone

1. Introduction

Intrinsically disordered proteins (IDPs), or proteins with intrinsically disordered regions (IDRs), compose about 30% of the human proteome and fulfill essential functions in cellular regulation and signaling [1-4]. They are characterized by high disorder and increased structural dynamics, resulting in high local mobility and flexibility, allowing them to fold and adapt to various binding partners [5-7]. Recently, IDPs have received increased attention as they are involved in organizing membrane-less organelles [8-13]. Misfolding of IDPs can lead to several diseases, including neurodegenerative diseases [14-17]. Characterizing the structural dynamics of intrinsically disordered proteins is critical to a deeper understanding of their function, conformational space, and binding interactions [18, 19]. The high internal dynamics of IDPs pose challenges to state-of-the-art structural biology techniques such as X-ray crystallography or cryo-EM due to diffuse electron density. NMR spectroscopy is, however, well suited to studying IDPs and their conformational dynamics [5, 7, 20-22].

^{15}N relaxation experiments are the “working horse” for a protein dynamics investigation by NMR spectroscopy. The original “standard” NMR ^{15}N relaxation methods, developed in the early 1990s, cover the ps-ns time range faster than the overall tumbling rotational correlation time of the molecule [23-30]. Several sophisticated extensions were developed, e.g., *relaxation dispersion* experiments, which are sensitive to modulations of the isotropic part of the chemical shift tensor and can provide insights into the μs -ms time range [31-37]. Applications of NMR relaxation and relaxation dispersion experiments have led to numerous insights into protein structural dynamics, shaping our current understanding of the protein energy landscape [5, 38-42], but also for RNA [43-45].

This manuscript focuses on the “standard” ^{15}N NMR relaxation methods addressing dynamics on the ps-ns time range, which can be considered as the flexibility of the protein. For these standard methods, several improvements have been added [25, 27, 46-53]. Systematic errors of used pulse sequences must be minimized to guarantee a reliable characterization of protein dynamics. This is particularly acute for IDPs as they are characterized by small relaxation rate constants and a high dynamic range.

Due to the long history of NMR relaxation experiments, most pulse sequences have been developed at magnetic field strengths of 14.1 T, corresponding to a ^1H Larmor frequency of 600 MHz and lower.

Frequently, these experiments have been designed for and tested on small, well-folded globular proteins. Advances in magnet technology have led to the development of increasing magnetic field strengths of NMR spectrometers, with a 28 T magnet (1200 MHz ^1H Larmor frequency) recently becoming commercially available [54]. These high field strengths offer unprecedented resolution and new opportunities for investigating protein dynamics by NMR. They are particularly beneficial for studying IDPs (because of their narrow signal dispersion in the ^1H dimension). At the same time, high field strengths put new demands on underlying spectrometer electronics and probe design, as well as NMR pulse sequence design. Existing methods have to be tested and may have to be adapted, and novel NMR methods will be developed.

In the following, we introduce two optimized ^{15}N R_1 and $R_{1\rho}$ pulse schemes with sensitivity-enhanced HSQC detection. The presented pulse schemes have been tested at 14.1 T and 28 T magnets, corresponding to ^1H Larmor frequencies of 600 MHz and 1200 MHz, respectively.

Because research on IDPs represents a highly active and growing field within biomolecular solution NMR [5, 7, 20-22], we have tested the presented pulse sequences on an IDP, the vesicular SNARE protein Synaptobrevin-2 (Syb-2), which in its monomeric pre-fusion state behaves as an IDP [55-57]. Due to the small relaxation rate constants of IDPs, even small systematic errors of less than 1 s^{-1} will lead to substantial percentage-wise errors in rate constants. Therefore, IDPs present an ideal test case for NMR relaxation experiments and will identify even small systematic errors in the used pulse sequence. We have tested the optimized sensitivity-enhanced ^{15}N R_1 and $R_{1\rho}$ pulse sequences both at 600 MHz and 1200 MHz, using both a fully protonated as well as a deuterated sample, and compared the results to the original TROSY-based sequences, which had been tested on deuterated GB3 and 600 MHz [51]. For both fully protonated and deuterated samples, we find a high reproducibility and robustness at both field's strengths, suggesting that the presented sensitivity-enhanced sequences, as well as the original TROSY-based sequences [51], will also be applicable at any magnetic field strength between 14.1 T and 28 T. High agreement of extracted relaxation rate constants, measured either in the sensitivity-enhanced HSQC-detected or the TROSY-detected sequence, is observed. Independent of HSQC- or TROSY-detection, we also find high agreement between the protonated and the deuterated samples.

2. Materials and Methods

2.1. NMR sample preparation

The gene base sequence encoding Synaptobrevin-2 (1-96), Syb-2 (1-96), the soluble part of Syb-2 (wt) without the transmembrane region, from *Rattus norvegicus* (UniProt accession number: P63045, 96 amino acids, 10.52 kDa) was codon-optimized for expression in *E. coli* and subcloned in a pET28a(+) vector. To facilitate protein isolation, a hexa-histidine tag was included at the N-terminus. Additionally, a Thrombin-cleavage site (amino acid sequence: LVPR'GS) was inserted for tag removal.

The pET28a(+)-His-Syb-2(1-96) plasmid was transformed into chemically competent *E. coli* BL21 (DE3) cells. Protein expression and purification followed protocols by Pobbati et al. [57, 58]. Briefly, for precultures, cells were grown at 37°C in 2xYT medium (Thermo Fisher, Waltham, MA, USA) overnight. For isotope labeling of ^{15}N Syb-2 (1-96), cells were grown in M9 minimal medium supplemented with ^{15}N - $\text{NH}_4\text{-Cl}$ (99%, Cambridge Isotope Laboratories, USA) as a single source for protein synthesis. The main culture was incubated at 37°C until an OD_{600} of 0.8 was reached; at this point, protein expression was induced with 0.5 mM IPTG. Cultures were incubated at 20°C after induction of expression for 18 hours. Bacterial cells were harvested for 10 min at 6,000 x g (4 °C) using centrifugation. The cell pellet was resuspended in 20 mM HEPES (pH 7.4), 500 mM NaCl, 8 mM Imidazole, and 0.1 mM TCEP supplemented with protease inhibitors (Complete, Roche) and DNase I for nucleic acid digestion. Cells were lysed for 20 min by sonication, followed by an additional centrifugation step for 30 min at 45,000 rpm (4 °C). The lysate was purified by Ni^{2+} -NTA affinity chromatography. His-tagged Syb-2 (1-96) was eluted with 20 mM HEPES (pH 7.4), 500 mM NaCl, 400 mM Imidazole, and 0.1 mM TCEP. Thrombin cleavage was combined with dialysis overnight at 4 °C in 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.1 mM TCEP, and 1 mM EDTA. Thrombin was separated from digested protein by ion exchange chromatography using 20 mM HEPES (pH 7.4), 0.1 mM TCEP, 1 mM EDTA, and a Resource Q column (Cytiva, Marlborough, MA, USA) for isolation. For elution, the same buffer composition was used with 1000 mM NaCl added. To increase the purity of the sample, a size exclusion chromatography was performed afterward using a HighLoad 16/600 Superdex 75 pg

column (Cytiva, USA) equilibrated in 50 mM MES (pH 6.0), 150 mM NaCl, 0.1 mM TCEP and 1 mM EDTA.

For the expression of deuterated $^2\text{H}^{15}\text{N}^{13}\text{C}$ Syb-2 (1-96) and $^2\text{H}^{15}\text{N}$ Syb-2 (1-96), commonly used filtered and deionized water (Milli-Q, Merck, USA) was replaced by D_2O . To reduce stressing conditions for cell growth, the transition from 2xYT medium into minimal medium with D_2O was performed slowly. First, cells were precultured in 2xYT medium and ddH_2O at 37°C overnight. Then, cells were precultured in M9 minimal medium supplemented with $^{15}\text{N}\text{-NH}_4\text{-Cl}$ in ddH_2O at 37°C for 8 hours. In the third overnight preculture, ddH_2O was replaced by D_2O . The main culture of deuterated $^2\text{H}^{15}\text{N}^{13}\text{C}$ Syb-2 (1-96) was supplemented with $^{15}\text{N}\text{-NH}_4\text{-Cl}$ and $^{13}\text{C}\text{-D-glucose}$ (99% Cambridge Isotope Laboratories, USA) in D_2O . The main culture of deuterated $^2\text{H}^{15}\text{N}$ Syb-2 (1-96) was supplemented with $^{15}\text{N}\text{-NH}_4\text{-Cl}$ in D_2O . Induction of expression with IPTG and protein isolation was performed as described for ^{15}N Syb-2 (1-96); see above.

2.2. NMR spectroscopy

2.2.1. Potential sources of artifacts in standard NMR relaxation experiments

Well-known systematic errors are generated by cross-correlated relaxation (CCR) of the $^1\text{H}\text{-}^{15}\text{N}$ dipolar coupling (DD) and the ^{15}N chemical shift anisotropy (CSA) [27, 46, 48], which increases at higher magnetic fields [52]. Refocusing of the respective CCR during the NMR relaxation is usually achieved by 180° ^1H pulses [27]. For several ^{15}N R_1 experiments, frequently, a train of hard 180° ^1H pulses, spaced by a 5 ms delay, was used to suppress CCR. As discussed in the earlier work on ^{15}N NMR relaxation experiments with TROSY-detection [51], this pulse train leads, however, to partial saturation of the water magnetization. The degree of water saturation will vary depending on the length of the relaxation period. In combination with the long T_1 times of the water in the order of 3-4 s, this can substantially attenuate the available amide proton magnetization via direct exchange with the water magnetization or through $^1\text{H}\text{-}^1\text{H}$ NOEs to nearby exchangeable protons [51], as also pointed out by Chen and Tjandra

independently [50]. The impact of progressive water saturation during the ^{15}N T_1 relaxation period increased the measured ^{15}N R_1 rate constant and was discussed in detail in [51]. This apparent (artificial) increase could be correlated to the solvent exposure of the respective amide group, with the most solvent-exposed amide groups displaying the strongest increase [51]. That systematic error can be circumvented by choosing very long interscan recovery delays (up to 10 s), leading to very long experimental times that are frequently not affordable. Another possibility is to saturate the water resonance entirely before the relaxation period starts, resulting in intensity losses for amide groups subject to solvent exchange. As discussed in the context of the TROSY-based sequences [51], shaped I-BURP-2 180° ^1H pulses, being selective on the amide protons and spaced by a 40 ms delay, provide an alternative [51]. The 40 ms inter-pulse delay was sufficient to refocus CCR but, simultaneously (in combination with amide-selective shaped pulses), disturbs the water magnetization only minimally. We use the same strategy for the sensitivity-enhanced HSQC-detection scheme (Figure 1). As pointed out, the key is keeping or returning the water magnetization to the z-axis. This is also achieved by the additional ^1H $90(-x)$ pulse at the end of the refocused INEPT transfer, which fulfills two purposes: Returning the water to the z-axis and removing any residual antiphase coherence.

Radiation damping of the water magnetization [59] is an additional but related concern, particularly acute for cryoprobes and high-field magnetics [60-62]. Very weak gradients can dephase the water magnetization and prevent radiation damping [63]. An alternative is to return the water magnetization to the z-axis using water-flip back pulses [49, 62, 64]. The TROSY-based- and HSQC-based experiments (Figure 1, see below) have been designed to return the water magnetization to the z-axis in the refocused ^1H - ^{15}N INEPT transfer before the relaxation period. Therefore, the water magnetization is aligned along the z-axis at the start of the relaxation period. The R_1 relaxation delay element is designed to disturb the water magnetization minimally (see above).

For the $R_{1\rho}$ relaxation period, we introduce amide-selective IBURP-2 pulses, which will be discussed further below. Therefore, systematic errors due to varying degrees of water saturation will be alleviated

in the examined sequences. Any disturbance of the water magnetization after the relaxation period will reduce the achievable signal-to-noise ratio of the NMR experiment but will not introduce additional systematic errors.

The TROSY detection element is advantageous regarding water suppression, as decoupling on the ^1H channel during t_1 evolution is avoided; for the sensitivity-enhanced HSQC detected relaxation experiments, a 180° (^1H) is used to refocus J-coupling evolution. A second 180° (^1H) pulse at the beginning of the t_1 evolution is used within the gradient-based Echo-/ Anti-Echo encoding element. The water magnetization will be returned to the z-axis after the second 180° (^1H) pulse. Radiation damping can occur in principle for longer t_1 times and the associated spacing of the two 180° (^1H) pulses. Although at the two spectrometers tested, we did not observe strong radiation damping, an optional weak rectangular gradient ($< 2\%$) can be included in the pulse sequence (Figure 1, gradient G10, grey) to prevent radiation damping [63], similarly as used in [51]. We further minimized the impact of the water signal caused by using hard 90° and 180° ^1H pulses by carefully choosing gradients (Figure 1, see below). Using amide-selective detection schemes, applied in SOFAST-HMQC or Band-selective Excitation Short-Transient (BEST) detection schemes, presents an alternative [65, 66]. Very recently, during the revision of this manuscript, broad-band shaped pulses designed by optimum control theory were introduced to cover the large spectral width at very high magnetic fields [67]. Using those pulses, a significant SNR increase in a ^1H , ^{15}N TROSY-HSQC experiment could be obtained while requiring only moderate RF power levels.

2.2.2. General setup of NMR relaxation experiments

We have recorded NMR relaxation experiments using a $171.1\ \mu\text{M}$ ^{15}N Syb-2 (1-96) sample and, for comparison, a $171.7\ \mu\text{M}$ $^2\text{H}^{15}\text{N}$ Syb-2 (1-96) sample in 50 mM MES (pH 6.0) buffer containing 150 mM NaCl, 0.1 mM TCEP and 1 mM EDTA. (The ^{15}N R_1 experiment, investigating the influence of the recovery delay, had been recorded on a $210\ \mu\text{M}$ $^2\text{H}^{15}\text{N}^{13}\text{C}$ Syb-2 (1-96) sample.)

All NMR experiments were performed at 278.15 K using a 250 μl volume filled in a 3 mm NMR sample tube. Experiments at 600 MHz ^1H Larmor frequency were conducted on a Bruker 600 MHz AVANCE

III HD spectrometer equipped with a Bruker 5 mm QCI ^1H , ^{15}N , ^{13}C , ^{31}P quadruple resonance cryoprobe. Experiments at 1200 MHz ^1H Larmor frequency were recorded on a Bruker 1200 MHz AVANCE NEO spectrometer (Bruker, Billerica, MA, USA) equipped with a 3 mm TCI ^1H , ^{15}N , ^{13}C triple resonance cryoprobe.

The optimized ^{15}N R_1 and $R_{1\rho}$ pulse sequences with sensitivity-enhanced HSQC detection presented here (Figure 1) employ a sensitivity-enhanced HSQC detection scheme [27, 68] and can be applied to fully protonated proteins. The basic building blocks of those two sequences are conceptually similar to the previous TROSY-based sequences and their HSQC counterparts, initially designed in the Bax laboratory [51]. At that time, those pulse sequences had been tested on deuterated GB3 at 600 MHz. While the TROSY-detection [69, 70] offers the best water suppression by using fewer ^1H pulses and avoiding ^1H decoupling, the sensitivity-enhanced HSQC scheme provides a higher signal-to-noise ratio (SNR) in principle (up to two-fold theoretically). In the sensitivity-enhanced read-out scheme, more ^1H pulses are used by design. While care was taken to return the water magnetization to the z-axis by the end of the pulse sequence, radiation damping of the water magnetization during the pulse sequence and its associated water trajectory is a potential pitfall. Previously, radiating damping was (partially) avoided by the use of soft rectangular gradients (of fixed duration) placed in the delay periods, requiring, however (in our hands), a very careful and frequently tedious gradient optimization. In turn, the potential gain in SNR of the HSQC-based sequence was often limited by a still strong water signal, limiting the affordable receiver gain setting [51].

The ^{15}N R_1 and $R_{1\rho}$ pulse sequences with sensitivity-enhanced HSQC detection presented here offer an improved water suppression (see Results section, Figure 3), and an easier to optimize water suppression (in our hands), by replacing those previous weak rectangular gradients by soft shaped gradients that can be adjusted in their gradient strengths and duration. Further, the ^{15}N $R_{1\rho}$ experiment uses two shaped ^1H pulses rather than two hard 180 (^1H) rectangular pulses spaced by a weak rectangular gradient in the delay period. To obtain the best water suppression in the sensitivity-enhanced HSQC experiments, we recommend optimization of the ^1H carrier frequency using a pre-saturation experiment (Bruker: zgpr sequence) and adjusting the ^1H carrier frequency (Bruker: o1) such that the water signal is minimized. Because of the easier-optimized water suppression combined with the high SNRs that can be achieved

(see below), we anticipate these sequences to be of practical importance and valuable for various fully protonated small to medium-sized proteins.

In the following, we briefly discuss the magnetization transfer pathway of the ^{15}N R_1 and $R_{1\rho}$ pulse sequences shown in Figure 1. After the recovery delay, a hard 90° (^{15}N) pulse destroys any residual non-Boltzmann magnetization that could be present on ^{15}N . A refocused INEPT element (two consecutive INEPT transfers [70]) transfers the initial ^1H Boltzmann magnetization to in-phase magnetization on the ^{15}N nucleus. During the following relaxation period, magnetization on the ^{15}N nucleus decays - depending on the length of the relaxation delay and resulting in decreased intensities as a function of the relaxation delay. The observed decay of intensities will be fitted by a mono-exponential decay function later to obtain the relaxation rate constants. Residue-specific intensities and rate constants will be acquired by the following t_1 evolution period of the transverse ^{15}N magnetization. For quadrature detection (phase sensitivity in the ^{15}N dimension), ^{15}N coherences are encoded by an Echo/ Anti-Echo encoding scheme with alternating gradients [27, 68]. The ^{15}N evolution period is followed by a refocused INEPT element, which transfers the magnetization to the amide protons. During a final Hahn-Echo element, the decoding gradient of the Echo/ Anti-Echo scheme follows. The complete pulse scheme is illustrated in Figure 1. Gradients during the INEPT elements are implemented both for coherence selection and to prevent radiation damping of the water magnetization (here, only weak gradient strengths are used) [63, 71].

2.2.3. ^{15}N R_1 experiment

The central part of pulse schemes is the relaxation period, which will be discussed in more detail in the following: In the ^{15}N R_1 experiment, the relaxation period is elongated by increasing a loop counter and the associated number of repetitions (n) of the bracketed part of the pulse scheme (Figure 1A). The entire relaxation period is omitted in the first experiment, resulting in the $\tau_1 = 0$ reference point. Cross-correlated relaxation of the ^{15}N chemical shift anisotropy (CSA) and the ^1H - ^{15}N dipolar coupling interactions during the relaxation period is refocused by a central I-BURP-2 180° pulse [72], selective

to the amide proton resonances at $\Delta/2$, the center of the respective loop element. The relaxation loop is repeated an even number of times ($n = 0, 2, \dots$) to balance the evolution of longitudinal cross-correlated relaxation during the relaxation period Δ . These I-BURP-2 pulses invert the amide proton magnetization during the relaxation period. Here, it is essential to use selective pulses to avoid exciting the water resonance, as partial saturation of the water magnetization for more extended relaxation periods would impact the return to ^1H Boltzmann equilibrium adversely [49], resulting in erroneous and too high ^{15}N R_1 rate constants for solvent-exposed residues (see section 2.2.1.) [50, 51]. Any amide proton will spend half of the relaxation period in the α -state and half the time in the β -state (assuming an ideal case, without any random ^1H - ^1H spin-flips during the relaxation period). As a result, the longitudinal cross-correlated relaxation between the ^1H - ^{15}N dipolar coupling and the ^{15}N chemical shift anisotropy will be averaged out.

In the following, we briefly discuss the experimental parameters of the ^{15}N R_1 experiment: The duration of the delay in the INEPT transfer is $\delta = 2.65$ ms; the delay ϵ corresponds to the decoding gradient G4 (201 μs). This delay makes inserting the decoding gradient without adding a linear phase error to the proton dimension possible. The variable delay corresponds to the delay between two shaped 180° I-BURP-2 pulses, selective on the amide proton region. For the duration of the individual I-BURP-2 pulses, we choose a length of 1000 μs at 1200 MHz to cover the amide ^1H chemical shift region of the IDP Syb-2 (1-96). We used a delay of 40 ms; the choice of the delay depends on the relaxation properties of the protein, which must be faster than the cross-correlated relaxation rate of the protein. The relaxation period is elongated by increasing the loop counter n and the associated repetition of the bracketed element of the pulse scheme. In the first experiment, the entire relaxation period will be jumped over. The relaxation loop is repeated an even number of times ($n = 0, 2, \dots$) to match the chosen relaxation delay. For the elimination of any cross-correlated relaxation between the ^{13}C and ^{15}N nuclei (applicable to ^{13}C labeled samples) on the ^{13}C channel, four 180° pulses, two selective rectangular 180° ^{13}C pulses are applied on $^{13}\text{C}'$ as well as on $^{13}\text{C}^\alpha$, at $\Delta*1/4$ and $\Delta*3/4$ of the relaxation period. The duration of the selective rectangular $^{13}\text{C}'$ pulse is chosen such that the $^{13}\text{C}^\alpha$ resonances are not excited and vice versa (see below) [64]. The length of these selective rectangular 180° pulses is defined by $\frac{\sqrt{3}}{2\Omega}$ [64]. Ω

corresponds to the chemical shift difference between $^{13}\text{C}^\alpha$ and $^{13}\text{C}'$; therefore, the pulse length of the 180° pulses was set to $23.7\ \mu\text{s}$ at 1200 MHz. Gradients are sine-bell shaped, identical to the sine.20 gradient shape of the Bruker gradient library, apart from G7, which is a sine.50 shaped. The gradient strengths are G_1 (200 μs , 1% (at our spectrometer setting), corresponding to 0.58 G/cm), and G_2 (200 μs , 2%, 1.16 G/cm), G_3 (800 μs , 31%, 17.98 G/cm), G_4 (200 μs , 11%, 6.38 G/cm), G_5 (201 μs , 50%, 29.00 G/cm), G_6 (1000 μs , 50%, 29.00 G/cm), G_7 (200 μs , 50%, 29.00 G/cm), G_8 (200 μs , 5%, 2.90 G/cm), and G_9 (200 μs , 14%, 8.12 G/cm). Further, we include an optional Gradient G_{10} (t1/4, 0.5%, 0.29 G/cm) during the first half of the t1 evolution period that can be switched on in case radiation damping is observed. In the ^{15}N dimension, a garp decoupling with an RF amplitude of 1.25 kHz, corresponding to a 90° pulse length of 200 μs , is applied.

For the measurements conducted at 600 MHz we used the identical gradient setup. Therefore, this gradient setup should be widely applicable to various spectrometer frequencies between 600 MHz and 1200 MHz. At least the suggested gradient setup provides a good starting point for any further (and spectrometer dependent) optimization of the water suppression.

2.2.4. ^{15}N $R_{1\rho}$ experiment

For the ^{15}N $R_{1\rho}$ experiment (Figure 1B), providing rate constants of the transverse relaxation, R_2 (see below), an adiabatic half-passage pulse turns the ^{15}N magnetization from the z-axis to the transverse plane, or the effective field direction for off-resonance nuclei, respectively [73, 74]. The adiabatic half tanh/tan pulse [74] has the same strength as the applied spin-lock RF amplitude (2 kHz). During the relaxation period, the magnetization is spin-locked in the rotating frame, and the intensity decay of the respective resonances is measured as a function of the lengths of the relaxation delay period Δ .

In principle, the RF amplitude should be as high as affordable to reduce exchange contributions and off-resonance effects at the edges of the spectrum. The higher the spectrometer frequency, the more significant potential off-resonance effects will be. At 1200 MHz, for example, a 2 kHz spin-lock RF amplitude will be equivalent to 1 kHz at 600 MHz in terms of off-resonance effects. Particular care must be taken to properly align the magnetization along the effective field axis using an adiabatic pulse with

the same RF amplitude as the following spin-lock. Confirming that the adiabatic pulse fulfills its adiabaticity condition for the given RF amplitude is important. Otherwise, magnetization components orthogonal to the effective field axis will be generated that will get dephased during the initial spin-lock period, leading to a sudden intensity drop in the decay curve and, consequently, a bi- or multi-exponential decay.

During the relaxation period, two 180° I-BURP-2 pulses, selective to the amide proton resonances, are applied at $\Delta^*1/4$ and $\Delta^*3/4$ of the relaxation period to average out contributions by transverse ^{15}N chemical shift anisotropy (CSA)/ ^1H - ^{15}N dipolar coupling cross-correlated relaxation. In the case of ^{13}C labeled samples, a selective soft rectangular 180° pulse is applied, one at the $^{13}\text{C}'$ and the other at the $^{13}\text{C}^\alpha$ carrier, to prevent any errors potentially introduced by cross-correlated relaxation between ^{15}N and ^{13}C . In both $R_{1\rho}$ and R_1 experiments, a temperature-compensation element [51] is applied off-resonance on the ^{15}N channel. On modern NMR probes, that temperature compensation element will not necessarily be required and could be switched off to reduce the power deposition in the probe, at least in our experience.

In the following, we briefly describe the setup of the ^{15}N $R_{1\rho}$ experiment: Apart from the bracketed relaxation period, the $R_{1\rho}$ experiment is similar to the R_1 experiment. The gradient setup is identical. The additional gradient G9 is a sine-bell-shaped (sine.20 Bruker gradient library), with a duration of 200 μs and a strength of 10%, corresponding to 5.8 G/cm. (This gradient will dephase residual orthogonal magnetization. A stronger gradient is advisable but has to be adjusted as a trade-off between dephasing efficiency and good overall water suppression.) The variable delay is equal to the length of the relaxation period. A spinlock with an RF amplitude of 2 kHz is applied during the relaxation period. Before the spinlock, an adiabatic half passage pulse (AHP) with a duration of 3000 μs aligns the magnetization on the effective field axis [63], and a second AHP returns the magnetization to the z-axis after the spinlock period. Those AHPs are displayed as triangle pulses before and after the spinlock. They correspond to the first and second half of a tangent-hyperbolic tangent (tanh/tan) adiabatic inversion pulse, as defined in the Bruker pulse library (100 kHz total sweep width, $\zeta = 10$, $\tan(\kappa) = 20$, $\omega_{\text{max}} = 2$ kHz). As for the R_1 experiment, 180° pulses on the $^{13}\text{C}'$ and the $^{13}\text{C}^\alpha$ are used to eliminate the cross-correlated effects resulting from the ^{13}C - ^{15}N dipolar interactions in ^{13}C labeled samples. The two I-BURP-2 pulses stop

the transverse cross-correlated relaxation contributions from the ^{15}N chemical shift anisotropy and the ^1H - ^{15}N dipolar coupling interactions.

The optimized NMR pulse sequences can be downloaded at www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences.

2.2.5. Application to intrinsically disordered proteins

To test the applicability of the pulse sequences on fully-protonated intrinsically disordered proteins and at high magnetic field strengths, we recorded ^{15}N R_1 and $R_{1\rho}$ relaxation experiments on monomeric Syb-2 (1-96), see above, which is intrinsically disordered in its monomeric state [55, 57, 75]. NMR spectra were acquired at 1200 MHz and 5°C, with 16.03 ppm spectral width in the ^1H direct dimension and 30.02 ppm spectral width in the ^{15}N indirect dimension. Altogether, eight different relaxation delays were recorded for the ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments. Each spectrum (= plane of the pseudo-3D) was acquired with 1024 complex data points in the ^1H dimension, corresponding to an acquisition time of 53.25 ms in the ^1H dimension. In the ^{15}N dimension, experiments were performed with an acquisition time of 70.14 ms and 256 complex data points per spectrum. Frequency carriers were placed for protons at 4.690 ppm, nitrogen at 117 ppm, and carbon at 176 ppm. For the R_1 experiment, the following relaxation delays were randomly shuffled: 0 ms, 960 ms, 240 ms, 800 ms, 160 ms, 640 ms, 320 ms, and 480 ms. In the $R_{1\rho}$ experiment, the following delays were used: 5 ms, 65 ms, 25 ms, 45 ms, 15 ms, 55 ms, 35 ms, and 10 ms. The first delay serves as the reference point. For the spinlock on the ^{15}N channel, a radio frequency (RF) amplitude of 2 kHz was applied. A four-step phase cycle was employed, limiting the applicable number of scans to multiples of four. As an interscan delay, 2.0 s were used unless specified otherwise. The experiments at 1200 MHz had a duration of 12 h 12 min total for the R_1 experiment and a duration of 10 h 19 min for the $R_{1\rho}$ experiment. A detailed overview of the chosen experimental parameters is shown in Table S1. Experimental NMR parameters for the relaxation experiments recorded at 600 MHz are shown in Table S2.

For comparison, TROSY experiments were acquired as described in [51].

2.2.6. Evaluation of NMR relaxation data

To evaluate the experimental data, the dedicated NMR pipe [76] scripts originally published in the context of the TROSY-based sequences [51] were modified slightly. Scripts can be downloaded under the following link:

www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences.

The R_2 rate constants were calculated by the following formula [77, 78]:

$$R_2 = \frac{R_{1\rho}}{\sin^2 \theta} - \frac{R_1}{\tan^2 \theta} \quad (1).$$

The angle $\theta = \arctan\left(\frac{\omega_1}{\Omega}\right)$ represents the angle between the axis of the effective magnetic field B_{eff} and the external magnetic field B_0 , ω_1 is the RF amplitude of the spin-lock and Ω the chemical shift offset (the difference between the ^{15}N chemical shift of the respective residue and the ^{15}N carrier frequency) [77, 78].

The experimental error was estimated using the NMR pipe-integrated Monte-Carlo-based error analysis [76]. The signal-to-noise ratio obtained for the various experiments (cf. Table 1) was calculated by taking the mean of the intensities of the three most intense resonances in the reference spectrum (first entry in the relaxation delay list). Those signals had to be non-overlapping signals. Their average intensity was divided by the noise estimated using nmrDraw / nmrPipe scripts [75]. Further, a “weak” SNR takes the average intensity of the three least intense signals and is divided by the noise calculated with nmrDraw. All SNR values were calculated for the reference spectrum acquired with the first entry of the vplist in the $R_{1\rho}$ experiment or with the first entry of the vclist in the R_1 experiment.

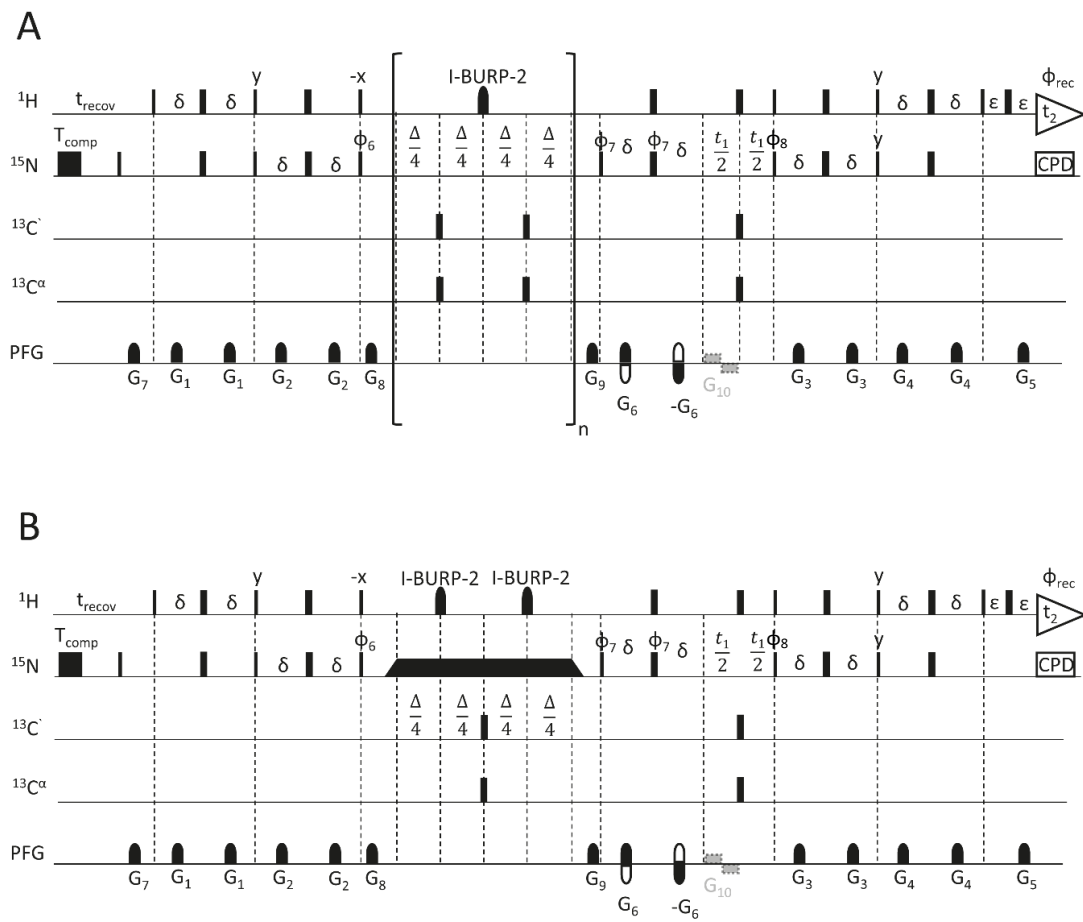


Figure 1: Pulse sequence schemes of NMR relaxation experiments using a sensitivity-enhanced HSQC read-out scheme. The following phase cycling is applied: $\phi_6 = y, y, -y, -y$; $\phi_7 = y, -y$, $\phi_{\text{rec}} = y, -y, -y, y$. The quadrature detection is implemented with the polarity inversion of the encoding gradient G_6 and the simultaneous inversion of $\phi_8=x$ (Echo/ Anti-Echo detection [27]). **A:** Pulse sequence scheme of the used ^{15}N R_1 relaxation experiment. Narrow rectangles correspond to 90° hard pulses with phase x, if not marked otherwise. 180° hard pulses are displayed as broad rectangles. **B:** Pulse sequence scheme of the used ^{15}N $R_{1\rho}$ experiment. Apart from the bracketed relaxation period, the $R_{1\rho}$ experiment is similar to the R_1 experiment.

3. Results and Discussion

We have tested the optimized NMR relaxation experiments for their applicability to intrinsically disordered proteins. As a model system, we used the soluble cytoplasmic part of the vesicular SNARE protein Synaptobrevin-2 (1-96), frequently referred to as VAMP-2 (vesicle-associated membrane protein 2). Synaptobrevin-2 (1-96), dubbed Syb-2 (1-96), is intrinsically disordered in its monomeric pre-fusion state. As a first assessment of the IDP character of Syb-2 (1-96), we recorded two-dimensional ^1H - ^{15}N HSQC spectra on ^{15}N Syb-2 (1-96), both at 600 MHz and 1200 MHz.

3.1. Comparison of ^1H - ^{15}N HSQC spectra of ^{15}N Syb-2 (1-96) recorded at 600 MHz and 1200 MHz

Figure 2 shows spectra of fully-protonated Syb-2 (1-96) measured at 600 MHz (Figure 2A) and 1200 MHz (Figure 2B). Both spectra have been recorded with an acquisition time of 106.5 ms in the direct ^1H dimension, corresponding to 1024 complex points in the direct ^1H dimension at 600 MHz and 2048 complex points at 1200 MHz. In the indirect ^{15}N dimension, 256 complex points were recorded. The timewise increment is defined by $1/\text{SWH}$ (or $1/(2*\text{SWH})$, respectively, depending on the employed quadrature detection and implementation of the pulse sequence), with the spectral width in Hz dubbed SWH. Therefore, at 1200 MHz, the length of the timewise increment, $1/\text{SWH}$, will be only half as long as the corresponding one at 600 MHz. Thus, the double number of increments must be recorded at 1200 MHz relative to 600 MHz to achieve the same total acquisition time and resulting spectral resolution in Hz (which correlates inversely to the total acquisition time). While a longer acquisition time in the direct ^1H dimension will minimally increase the overall experimental time, the twice-as-long acquisition time in the indirect dimension due to the doubling of the recorded complex points will double the experimental time. However, when doing so, the resolution can be further increased (Figure 2C). As evident from Figure 2, higher magnetic field strengths will be beneficial for recording highly resolved NMR spectra of IDPs, even when fully protonated.

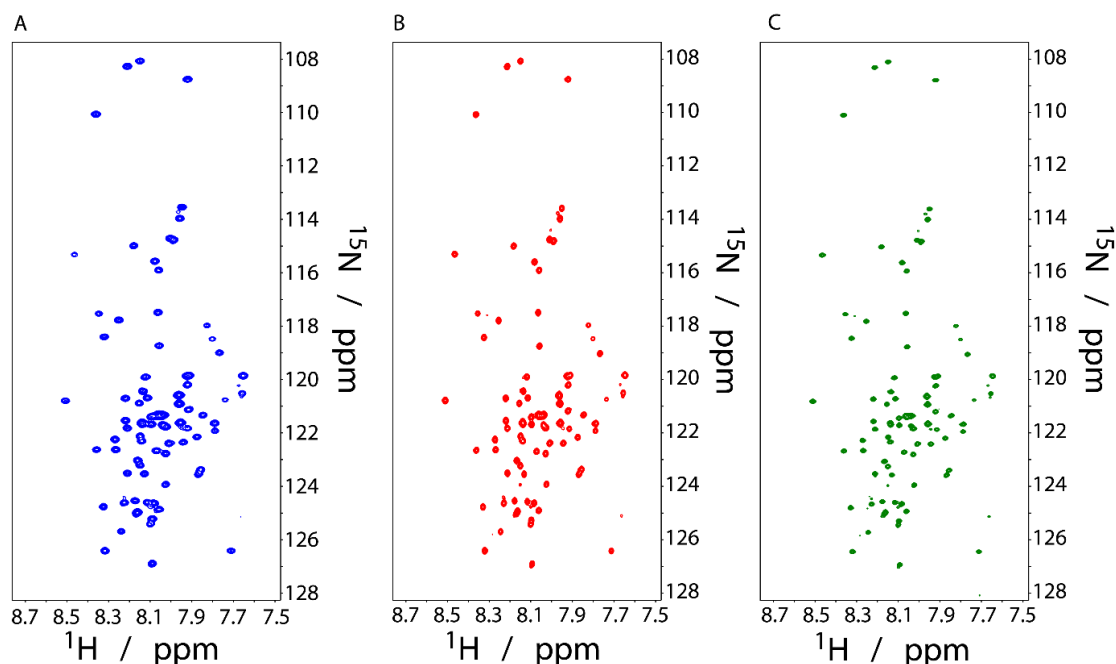


Figure 2: Two-dimensional ^1H - ^{15}N HSQC spectra of fully protonated Syb-2 (1-96). All spectra were acquired at 5°C with 8 scans, an interscan delay of 1.2 s, and a spectral width of 16 ppm in ^1H dimension and 30 ppm in ^{15}N dimension. **A:** HSQC at 600 MHz with 1024 complex points in the ^1H dimension and 256 complex points recorded in the ^{15}N dimension. **B:** HSQC at 1200 MHz with 2048 complex points in the ^1H dimension and 256 in the ^{15}N dimension. **C:** HSQC at 1200 MHz with 2048 complex points in the ^1H dimension and 512 complex points in the ^{15}N dimension, resulting in the same acquisition time as 600 MHz (but doubling the experimental time).

3.2. ^{15}N R_1 , $R_{1\rho}$ with sensitivity-enhanced HSQC-detection and improved water suppression

3.2.1. Modifications in the ^{15}N R_1 and $R_{1\rho}$ pulse sequences

We briefly reiterate the main modifications for the ^{15}N R_1 and ^{15}N $R_{1\rho}$ relaxation experiments using a sensitivity-enhanced HSQC read-out scheme: starting from the original TROSY-based NMR relaxation experiments and their HSQC counterpart [51], we have implemented ^{15}N R_1 and $R_{1\rho}$ pulse sequences using a sensitivity-enhanced (Rance-Kay) HSQC read-out scheme with improved water suppression (Figure 1). We emphasize that for both read-out schemes, the TROSY and the HSQC-detected ones, the “standard” ^{15}N R_1 and $R_{1\rho}$ (auto-relaxation) rate constants are measured. Relative to the original implementation (compare SI Figure S1 in [51]), instead of weak rectangular gradients of fixed duration,

we have used shorter shaped gradients that can be adjusted both in duration and time, allowing an easier to optimize water suppression. For the $R_{1\rho}$ experiment, we replaced the two hard 180° pulses (plus the soft gradient in between, preventing radiation damping) with two shaped I-BURP-2 pulses, which are selective on the amide protons. Figure 1 shows the improved set of NMR R_1 and $R_{1\rho}$ relaxation pulse sequences with sensitivity-enhanced (Rance-Kay) HSQC detection and improved water suppression, applicable at high-field magnets. We refer to the Materials and Methods section for details on the experimental setup.

3.2.2. Improved water suppression

Figure 3 displays the improved water suppression obtained using the presented ^{15}N R_1 and $R_{1\rho}$ NMR relaxation experiments with sensitivity-enhanced HSQC detection, tested both at 1200 MHz and 600 MHz. For comparison, the water-suppression using the previous implementation (SI Figure S1 in [51]) is shown [51]. The water-suppression obtained for the deuterated Syb-2 sample (see below) is shown in Figure S2. Apart from the ^{15}N R_1 experiment at 600 MHz, the level of water suppression of the new implementation is improved compared to the previous implementation. At 1200 MHz, using a 3 mm Bruker TCI cryoprobe, the level of improvement is higher than at 600 MHz using a 5 mm Bruker QCI probe. The same sample in a 3 mm NMR tube was used for both NMR spectrometers.

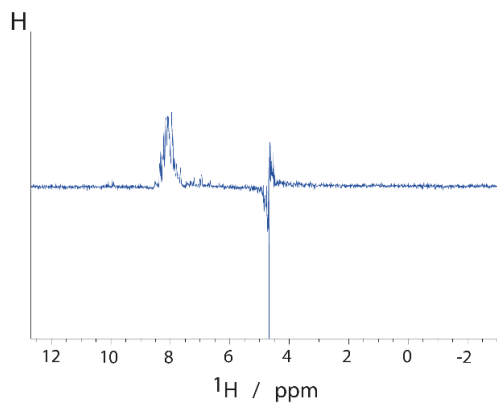
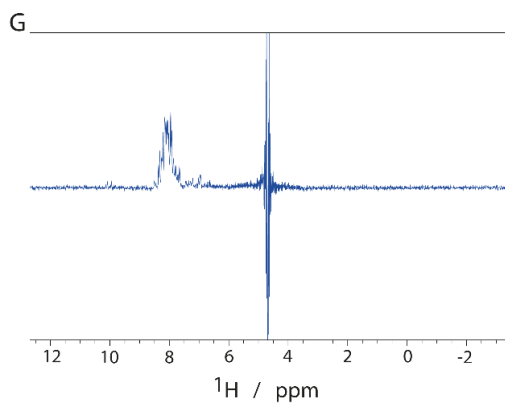
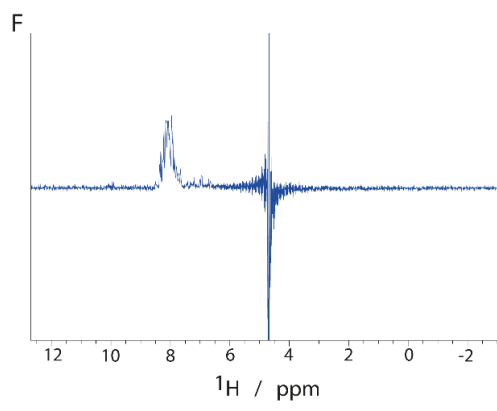
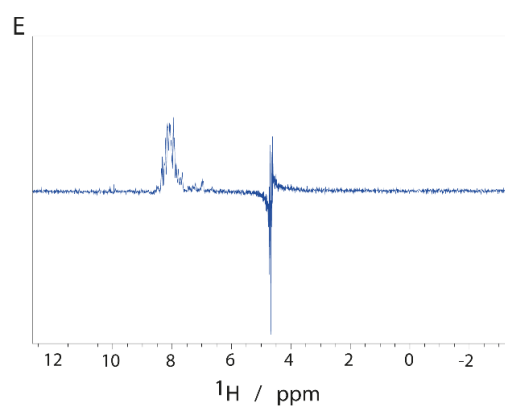
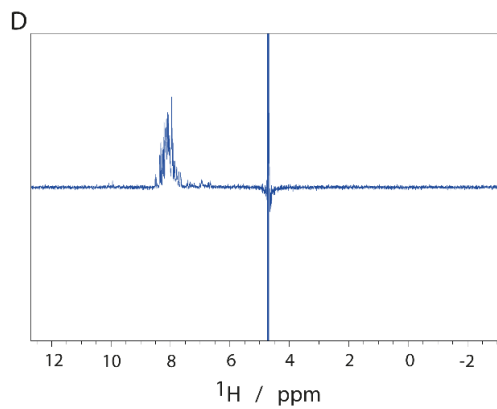
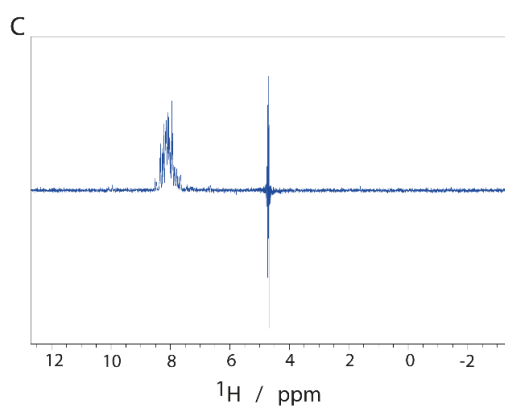
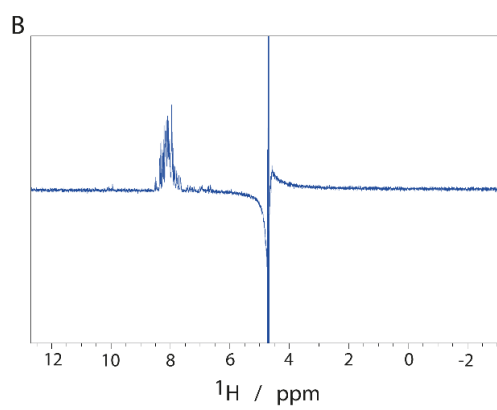
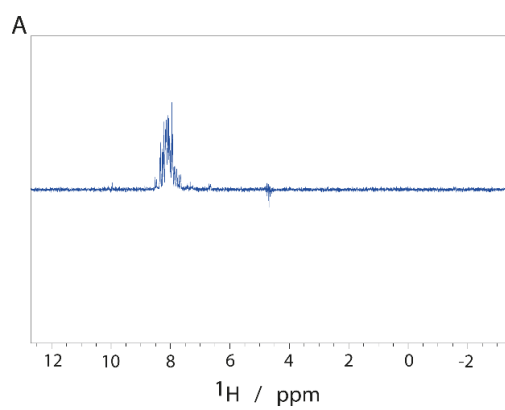


Figure 3: Improvement of water suppression in ^{15}N $R_{1\rho}$ and R_1 NMR relaxation experiments with sensitivity-enhanced HSQC-detection using the new implementation (left, see also Figure 1) and compared to the old implementation (right, SI Figure S1 in [51]). Shown is the 1D spectrum obtained from the first FID recorded on the protonated ^{15}N Syb-2 sample. (A) Optimized ^{15}N $R_{1\rho}$ experiment at 1200 MHz compared to (B) previous implementation. ^{15}N R_1 experiment at 1200 MHz: (C) New and (D) old. ^{15}N $R_{1\rho}$ experiment at 600 MHz: (E) new and (F) old. ^{15}N R_1 experiment at 600 MHz: (G) new and (H) old.

3.3. Protonated sample: Comparison between TROSY- and HSQC-detection scheme

3.3.1. Application at 1200 MHz

The ^{15}N R_1 and $R_{1\rho}$ relaxation rate constants of the fully protonated ^{15}N Syb-2 (1-96) sample measured at 1200 MHz using the sensitivity-enhanced HSQC detection scheme displayed in Figure 1 are shown in Figures 4A and 4D (blue) and compared to the data obtained using TROSY-detected pulse sequences (orange) [51]. The ^{15}N R_2 rate constants are derived from the measured ^{15}N R_1 and $R_{1\rho}$ rate constants using the formula (1). Low ^{15}N R_2 rate constants indicate high internal dynamics at the N-terminus of the Syb-2 (1-96), consistent with the intrinsically disordered state of monomeric Syb-2. Increasing relaxation rate constants along the protein backbone to the C-terminus point to decreasing internal dynamics. In the region of residue S80 up to W90 the R_2 relaxation rate constants reach a maximum before the rates drop due to the internal highly dynamic amino acids at the C-terminus. These observations correspond well to our earlier finding on $^2\text{H}^{15}\text{N}^{13}\text{C}$ Syb-2 (1-96) [57]. The R_1 data only show small variations along the sequence and do not follow a clear trend.

We re-emphasize that the HSQC-detected and the TROSY-detected schemes measure the “standard” R_1 and $R_{1\rho}$ auto-correlation rate constants.

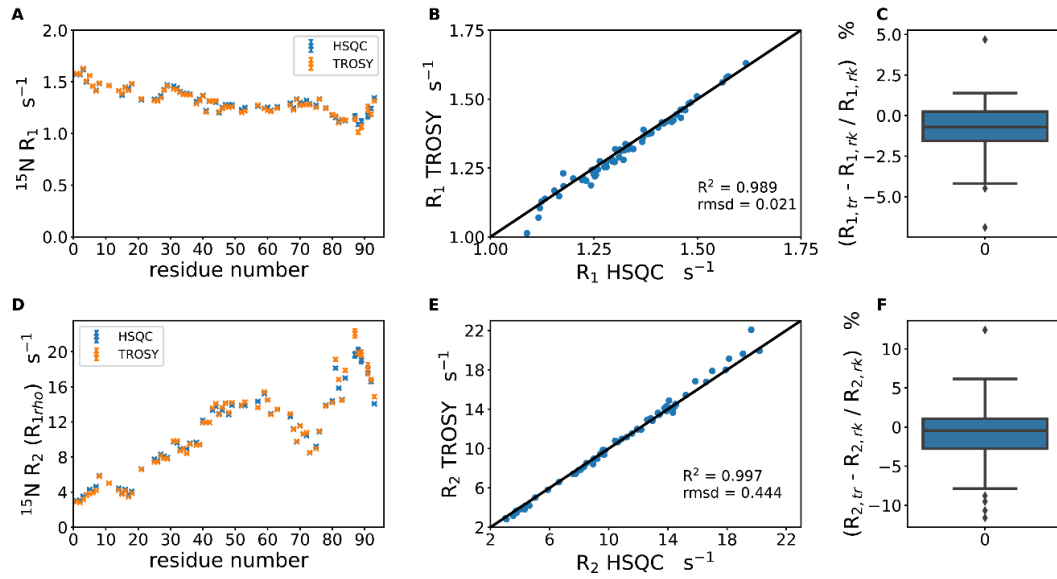


Figure 4: Comparison of relaxation rates of fully protonated ^1H - ^{15}N Syb-2 (1-96) acquired with the pulse schemes in Figure 1 A, B at 1200 MHz and compared to data recorded on the same sample using the TROSY read-out pulse schemes. **A:** Residue-specific R_1 relaxation rate comparison. **B:** Correlation plot of the compared R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rate constants. **D-F:** Same plots as in A-C, illustrating the comparison of R_2 (R_{1p}) rate constants.

The ^{15}N R_1 rate constants, measured using either the HSQC or the TROSY-detection scheme, follow a very similar pattern and show a high correlation, with a Pearson correlation coefficient of $R = 0.989$ and a root mean square deviation (rmsd) of 0.021 (Figure 4B). A box plot analysis was performed to assess the agreement quality better. In Figure 4C, the difference of rate constants is measured using the HSQC or TROSY detection scheme. The agreement is best if the average deviation is zero and the width of the distribution, shown by the box plot bar (interquartile range), is small. We find that the average of the R_1 rate constants measured using the TROSY-detection scheme minus those measured using the HSQC-detection scheme is about -0.76%. This means that rate constants measured using the TROSY-detection scheme are underestimated compared to those obtained using the HSQC-detection scheme by about 0.76%. We observe an experimental error between 0.2% (most intense resonances) and 3% (least low resonances) for the HSQC-detected and between 0.3% and 4% for the TROSY experiment.

The ^{15}N R_2 rate constants (obtained from the ^{15}N $R_{1\rho}$ rate constants by formula 1) are, on average, about -0.84% lower for the TROSY-detection scheme than for the HSQC-detection scheme (Figure 4F) but agree with a Pearson correlation coefficient $R=0.997$ and an $\text{rmsd}=0.444$. The experimental error is 0.3% and 3% for the HSQC-detected experiment and between 0.4% and 4% for the TROSY-detected experiments.

Compared to the TROSY-based $R_{1\rho}$ experiment [51], in the new pulse scheme presented in Figure 1B, we modified the way the evolution of cross-correlated relaxation is refocused. In the new experiment, two 180° I-BURP-2, selective to the amide protons and spaced at $\Delta^*1/4$ and $\Delta^*3/4$ duration of the relaxation period, refocus the cross-correlated relaxation. Previously, this was achieved by two hard 180° ^1H composite pulses. To avoid radiation damping of the water magnetization, a very weak gradient (that alternated its phase halfway) was introduced between those two 180° ^1H composite pulses [51]. As evident from Figure S3, we find excellent agreement between relaxation rate constants measured using either of both implementations. In our hand, the new implementation using amide selective I-BURP-2 pulses shows slightly better overall water suppression.

The 2 kHz spinlock RF amplitude used at 1200 MHz seems sufficient to avoid off-resonance effects. At least we do not observe any unexpected deviations of the ^{15}N R_2 rate constants of residues with the most ^{15}N upfield or downfield resonances (Figure S4).

3.3.2. Application at 600 MHz

To test the applicability of the pulse sequences with sensitivity-enhanced HSQC detection on a 600 MHz spectrometer, we recorded ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments on the protonated sample, using either the HSQC or the original TROSY-detection scheme. Also, at 600 MHz, the ^{15}N R_1 rate constants show good agreement (Figure 5A-C), with a Pearson correlation coefficient of $R = 0.949$ and a rmsd of 0.032 (Figure 5B). For the box plot analysis, the average deviation between both data sets is -0.45% and a relatively even distribution around that is observed (Figure 5C), pointing to a stochastic experimental error.

The ^{15}N R_2 rate constants, derived from the ^{15}N $R_{1\rho}$ rate constants by formula 1, show good agreement with a Pearson correlation coefficient of $R = 0.995$ and a rmsd of 0.216 (Figure 5E). ^{15}N R_2 data obtained using the TROSY-detection scheme are, on average -0.43% lower than the ones obtained using the sensitivity-enhanced HSQC-detection scheme. For both experiments the average deviation lies within the experimental error margin (between 0.5% and 2% for HSQC and 0.5% and 3% for TROSY).

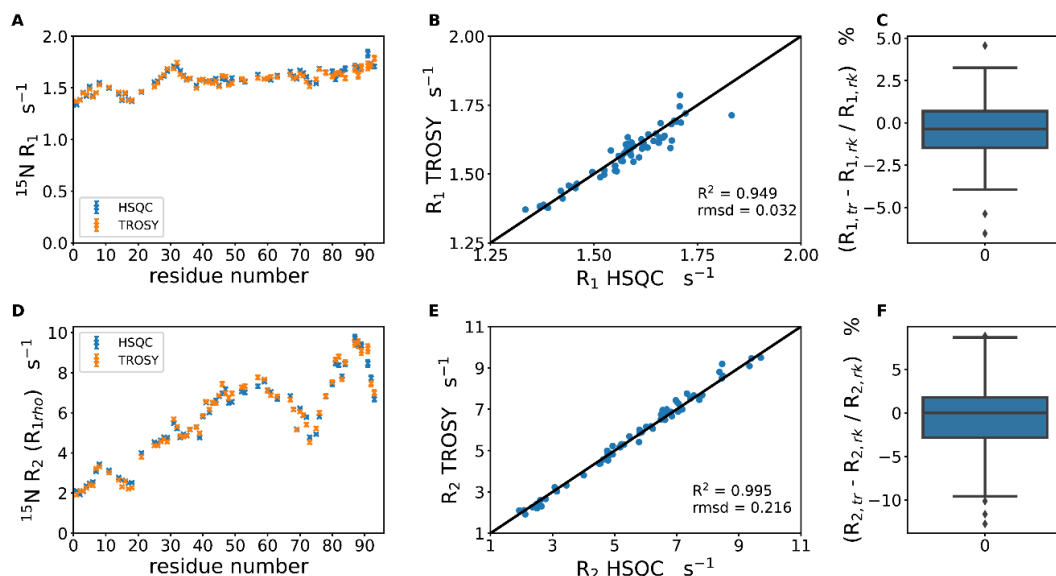


Figure 5: Comparison of relaxation rate constants of protonated ^{15}N Syb-2 (1-96) at 600 MHz, acquired with the pulse schemes in Figures 1 A, B, using sensitivity-enhanced HSQC detection and, for comparison, with the TROSY-detection. **A:** Residue-specific R_1 relaxation rate constants comparison. **B:** correlation plot of the R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rates. **D-F:** Same plots as in A-C, illustrating the comparison of R_2 ($R_{1\rho}$) rate constants.

3.3.3. Signal-to-noise comparison

TROSY-detection schemes offer several advantages, such as excellent water suppression (see above) due to fewer pulses and the absence of proton decoupling. Also, in terms of line widths, TROSY-detection will yield smaller line widths and, therefore, better resolution compared to HSQC-detection because of the partial cancellation of the transverse auto-relaxation and cross-correlated relaxation, resulting in slower transverse relaxation and in turn reduced line widths. In addition, in principle, longer acquisition times in the direct dimension can be afforded due to the absence of composite pulse

decoupling during the acquisition period, so resolution is not limited by the acquisition time. For higher molecular weight proteins (> 20 kDa), TROSY will also win over HSQC regarding signal-to-noise.

However, for small and medium-sized proteins, in particular, at low concentrations, the sensitivity-enhanced detection scheme offers a higher signal-to-noise ratio (SNR). Table 1 shows a signal-to-noise comparison between the HSQC and TROSY-detection schemes. For fully protonated Syb-2, which is an IDP, we find an approximately two-fold increase in the SNR when using the sensitivity-enhanced HSQC scheme compared to the TROSY-detection scheme, which agrees with the maximum theoretically expected improvement of the HSQC vs TROSY-detection (which is two-fold), based on detection of the H^- operator of the sensitivity-enhanced HSQC detection compared to detection of the H^-N^β operator for (sensitivity-enhanced) TROSY detection. However, we also find slower 1H T_1 relaxation of the H^-N^β TROSY line used for detection than the H^- operator in the HSQC scheme (see Figure S5). We estimated 1H T_1 (TROSY) = 1.24 s, compared to 1H T_1 (HSQC) = 0.86 s at 1200 MHz, which further enhances the SNR in the HSQC-detection scheme, given the inter-scan recovery delay of 2 s, used in both experiments.

Table 1. Signal-to-noise comparison for the ^{15}N R_1 and R_2 ($R_{1\rho}$) NMR relaxation experiments recorded on 1H ^{15}N Syb-2, using the sensitivity-enhanced HSQC-detection or for comparison using the TROSY-detection scheme. **(A)** The SNR is calculated by taking the average of the three most intense resonances. For comparison, the “weak” SNR is also calculated by taking the average of the three least intense (“weakest”) resonances **(B)** to get a better impression of the dynamic range.

A

Experiment	NS	read out	field / MHz	SNR (strong)	extrapolated SNR (at 8 NS) ¹
$R_{1\rho}$	4	HSQC	1200	688	972
$R_{1\rho}$	8	TROSY	1200	388	388
R_1	4	HSQC	1200	717	1014
R_1	8	TROSY	1200	404	404
$R_{1\rho}$	4	HSQC	600	332	469
$R_{1\rho}$	8	TROSY	600	237	237
R_1	4	HSQC	600	337	477
R_1	8	TROSY	600	228	228

B

Experiment	NS	read out	field MHz	/	SNR (weak)	extrapolated SNR (at 8 NS) ¹
R _{1ρ}	4	HSQC	1200		47	66
R _{1ρ}	8	TROSY	1200		35	35
R ₁	4	HSQC	1200		55	78
R ₁	8	TROSY	1200		38	38
R _{1ρ}	4	HSQC	600		65	91
R _{1ρ}	8	TROSY	600		57	57
R ₁	4	HSQC	600		71	100
R ₁	8	TROSY	600		57	57

1 Extrapolated SNR: As the HSQC-detected schemes were recorded with four scans only, their measured SNR was multiplied by a factor $\sqrt{2}$, to be comparable to the TROSY-detection schemes, which were recorded with eight scans.

3.4. Relaxation rate constants of deuterated vs. protonated ¹⁵N Syb-2 (1-96)

3.4.1. ¹⁵N R₁ experiment

Figures 4 and 5 show the rate constants measured on a fully protonated ¹⁵N Syb-2 (1-96) sample. In principle, the N-H amide group can be considered an isolated two-spin system in good approximation. However, we cannot exclude a residual interaction with the surrounding proton network. Therefore, measuring relaxation rate constants on a deuterated sample, with aliphatic side chains deuterated, and amide protons and exchangeable side-chain protons fully back-exchanged to protons (in a protonated buffer) is preferable in principle [79-81]. However, deuteration is cost-extensive, and frequently, deuteration puts an additional burden on the biochemical sample preparation. We, therefore, tested how well the rate constants agree for a fully protonated versus a deuterated sample, with amide protons fully back-exchanged (Figure 6).

Figure 6A shows a residue-wise comparison of the measured ¹⁵N R₁ rate constants of the fully protonated ¹⁵N Syb-2 (1-96) sample (compare Figure 4A) with those measured on the deuterated ²H¹⁵N Syb-2 (1-96) sample (of equal concentration). Both samples were measured using the sensitivity-enhanced HSQC-detection scheme. On both samples, the ¹⁵N R₁ rate constants follow a very similar pattern and show a high correlation, with a Pearson correlation coefficient of $R = 0.976$ and a root mean square

deviation (rmsd) of 0.026 (Figure 6B). In Figure 6C, the difference in rate constants is measured on the protonated and deuterated samples. We find that the average deviation of the R_1 rate constants measured on the protonated sample minus those on the deuterated sample is close to zero on average.

For deuterated samples, ^1H T_1 times increase because of the more dilute proton network. We, therefore, compared different interscan recovery delays (2s vs. 4s). Figure S6 compares the ^{15}N R_1 experiment (Figure 1A), recorded with a 2 s vs 4 s interscan recovery delay, both for the fully protonated (Figure S6 A-C) and the deuterated sample (Figure S6 D-F). The 2 s and the 4 s experiment correlate with $R^2 = 0.992$ (protonated) and $R^2 = 0.985$ (deuterated), and a rmsd of 0.016 and 0.02, respectively, indicating high correspondence between both experiments (2 s vs 4 s interscan recovery delay). Also, data points scatter stochastically, and the average difference is close to zero. Thus, we concluded that a 2 s interscan delay is sufficient.

A modified ^{15}N R_1 relaxation experiment has been suggested [82], which maintains the transverse magnetization of aliphatic protons in a dephased state (saturated state) during the variable ^{15}N relaxation period T and uses a cosine modulated I-BURP-2 scheme that inverts both the amide and the aliphatic protons simultaneously. In our hands, ^{15}N R_1 data recorded on the fully protonated sample agree well with those on the deuterated sample, arguing against a strong impact of the magnetization state of the aliphatic protons.

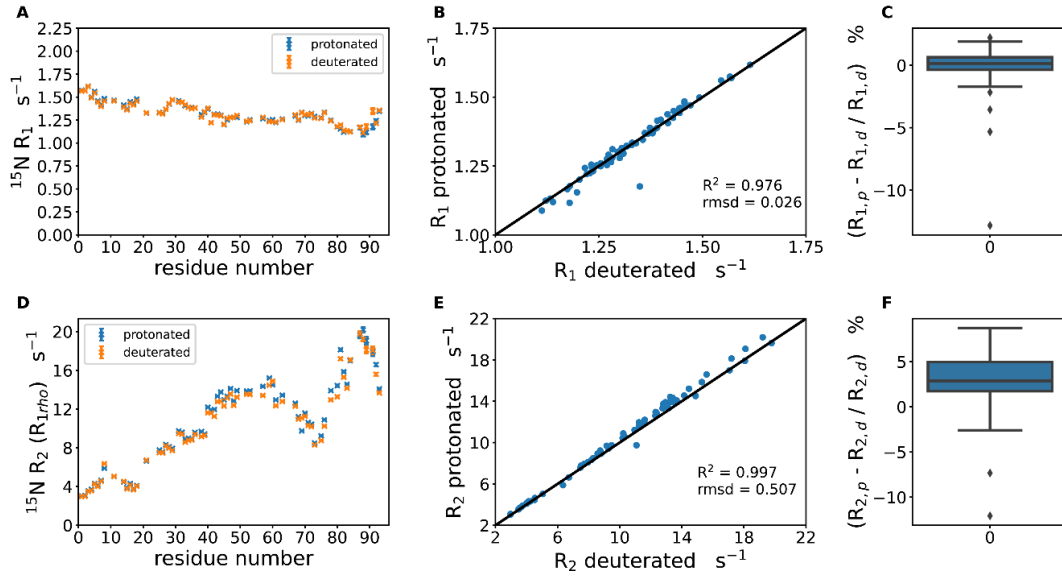


Figure 6: Comparison of relaxation rate constants of deuterated ^2H - ^{15}N Syb-2 (1-96) and protonated ^1H - ^{15}N Syb-2 (1-96) acquired with the pulse schemes in Figure 1 A, B at 1200 MHz. **A:** Residue-specific comparison of ^{15}N R_1 relaxation rate constants. **B:** Correlation plot of the compared R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rate constants. **D-F:** Same comparing plots as in A-C, but illustrating the comparison of R_2 ($R_{1\rho}$) rate constants.

3.4.2. ^{15}N $R_{1\rho}$ experiment

Figure 6D compares the ^{15}N R_2 rate constants measured using the HSQC vs the TROSY detection scheme. While we find a Pearson correlation coefficient of $R = 0.997$ and rmsd of 0.507 (Figure 6E), indicating a close correlation, we also observe an offset of, on average, 2.79% higher values observed for the protonated sample (Figure 6F). More structure/rigid residues show a more substantial deviation.

Deuteration of protein side chains may have a minimal impact on protein dynamics [83], e.g., the C-D bond length is marginally shorter than the C-H bond lengths (approx. 0.005 Å) [84]. However, this impact is subtle and less clear than dissolving the protein in a buffer of heavy water (D_2O) [83, 85]. Here, we work with a deuterated protein dissolved in H_2O buffer (with only 5% D_2O added for reference). The slightly reduced C-D bond lengths may lead to an overall marginally smaller hydrodynamic radius, affecting the overall tumbling correlation time. This may explain the slightly lower ^{15}N R_2 rate constants observed for the deuterated sample.

3.5. Deuterated sample: Comparison between TROSY- and HSQC-detection scheme

To test whether, for the deuterated Syb-2 sample, the TROSY-detection scheme yields the same rate constants as when using a sensitivity-enhanced HSQC read-out scheme, we repeated the analysis described in section 3.3. (protonated sample) also for the deuterated sample.

3.5.1. Application at 1200 MHz

Figure 7 compares the ^{15}N R_1 and $R_{1\rho}$ experiments recorded with the TROSY-detection scheme and the sensitivity-enhanced HSQC-detection scheme. Data were recorded at 1200 MHz. In Figure 7A, both R_1 data sets show only minor, non-systematic differences and follow a similar pattern. The correlation plot also shows a strong correlation between both data sets (Figure 7B). As demonstrated in the box plot in Figure 7C, ^{15}N R_1 and $R_{1\rho}$ rate constants are, on average, 0.59% and 0.80% higher for the HSQC-detected experiments vs. the TROSY-detected ones. An even stronger correlation between both pulse schemes is observed for the R_2 datasets (Figure 7 D-F), underlined by a correlation coefficient $R = 0.997$ and rmsd of 0.359.

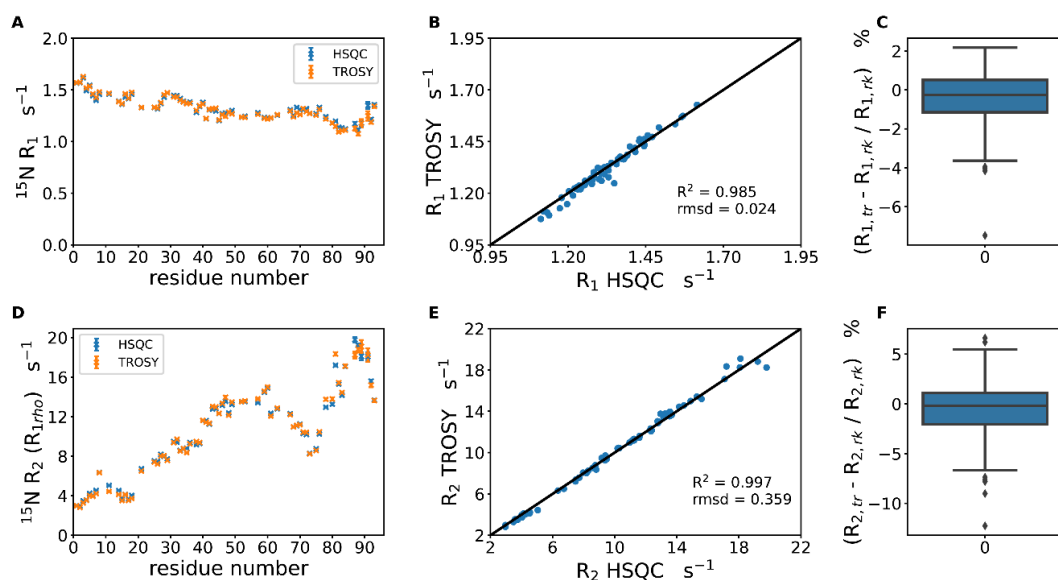


Figure 7: Comparison of relaxation rates of deuterated ^2H - ^{15}N Syb-2 (1-96) acquired with the pulse schemes in Figure 1 A, B at 1200 MHz and of deuterated ^2H - ^{15}N Syb-2 (1-96) obtained with the TROSY read-out schemes [51] at 1200 MHz. **A:** Residue-specific R_1 relaxation rate comparison. **B:** Correlation plot of the compared R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rate constants. **D-F:** Same plots as in A-C, but illustrating the R_2 ($R_{1\rho}$) rates comparison.

3.5.2. Application at 600 MHz

We also recorded the ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments with sensitivity-enhanced HSQC detection and, for comparison with TROSY-detection at 600 MHz spectrometer, using the deuterated sample. Also, at 600 MHz, the ^{15}N R_1 rate constants show good agreement (Figure 8A-C), with a Pearson correlation coefficient of $R = 0.955$ and a rmsd of 0.031(Figure 8B). For the box plot analysis, a close to zero average deviation between both data sets and a relatively even distribution around that is observed (Figure 8C), pointing to a stochastic experimental error.

The ^{15}N R_2 rate constants, derived from the ^{15}N $R_{1\rho}$ rate constants by formula 1, show good agreement with a Pearson correlation coefficient of $R = 0.994$ and a rmsd of 0.214 (Figure 8E) and close to zero average deviation.

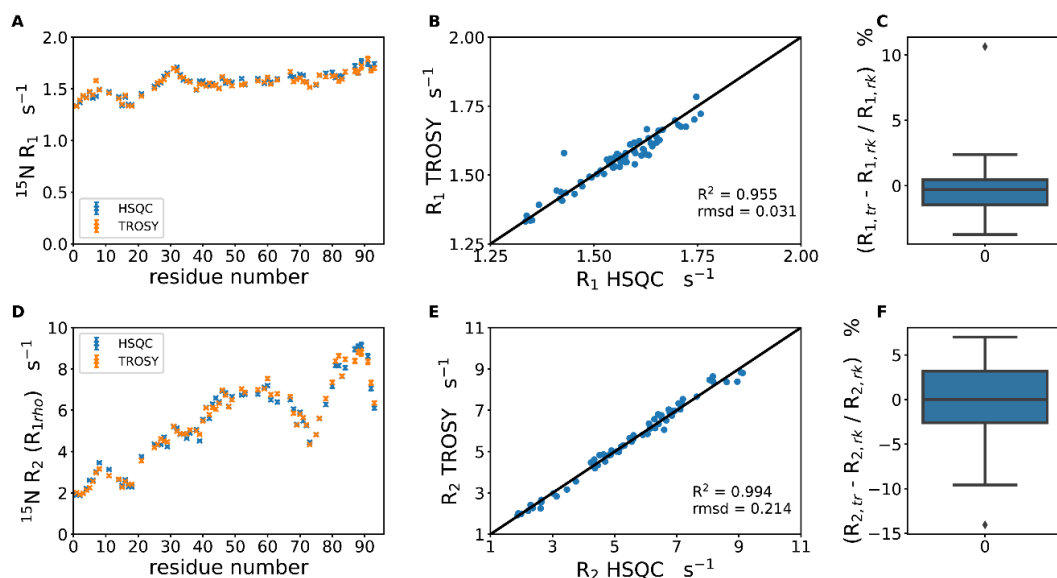


Figure 8. Comparison of relaxation rates of deuterated ^2H - ^{15}N Syb-2 (1-96) acquired with the pulse schemes in Figure 1 A, B at 600 MHz and of deuterated ^2H - ^{15}N Syb-2 (1-96) obtained with the TROSY read-out schemes [51] at 600 MHz. **A:** Residue-specific R_1 relaxation rate comparison. **B:** Correlation plot of the compared R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rate constants. **D-F:** Same plots as in A-C, but illustrating the R_2 ($R_{1\rho}$) rates comparison.

4. Conclusions

Advances in magnet technology have led to higher available field strengths, which have improved spectral resolution. Hence, new opportunities are offered, especially regarding the investigations of IDPs.

Recently, NMR relaxation measurements could provide novel insights into fast internal protein dynamics of intrinsically disordered proteins [19-21, 86-99]. Temperature-dependent NMR relaxation experiments have allowed for identifying different dynamics modes in an intrinsically disordered protein [87, 89] or examining the effect of crowding on IDP dynamics [13, 86, 98]. Detailed atomic-resolution insights on IDP dynamics can be obtained when NMR relaxation measurements are combined with molecular dynamics simulations [88, 100] or further experimental probes, such as e.g. fluorescence or SAXS measurements, are included [101-103].

We established sensitivity-enhanced HSQC-based R_1 and $R_{1\rho}$ relaxation pulse sequences with improved water suppression and tested them at 14.1 T (600 MHz ^1H Larmor frequency) and 28 T (1200 MHz ^1H Larmor frequency). We showed that relaxation rate constants obtained using these pulse sequences correspond well with those obtained using the TROSY-based pulse schemes introduced previously [51]. These pulse schemes reduce systematic errors from partial water saturation and cross-correlated relaxation. As we observe high robustness and reproducibility of the ^{15}N R_1 and $R_{1\rho}$ experiments both at 600 MHz and 1200 MHz, we conclude that both the sensitivity-enhanced HSQC-detected experiments as well as the TROSY-detected experiments can be applied for any magnetic field in between, ranging between 600 MHz and 1200 MHz.

Further, we showed a high correlation and high agreement of relaxation rate constants between a deuterated and non-deuterated protein sample, using both sets of pulse sequences (sensitivity-enhanced HSQC and TROSY-based). Our data demonstrate that reliable relaxation rate constants can be obtained on fully protonated samples, and deuteration (for getting isolated ^1H - ^{15}N spin pairs) is not required per se. Consequently, the subset of proteins accessible to a backbone structural dynamic investigation is enlarged, simultaneously reducing the costs of NMR sample preparation. The presented NMR relaxation sequences sensitivity-enhanced HSQC-detected are not limited to IDPs but will work equally well for small and medium-sized proteins.

Which detection scheme to use? TROSY or HSQC?

The $\text{H}\cdot\text{N}^\beta$ TROSY-line detected in the TROSY-based read-out scheme shows slower relaxation (and sharper linewidth) than the H^α line detected in the sensitivity-enhanced HSQC experiments. (The H^α operator results from applying the H^+ detection operator on the H_x or H_y operator, respectively.). The slower relaxation of the TROSY line originates from the partial cancellation of the R_2 auto-relaxation and the transverse cross-correlated relaxation of N-H dipolar coupling and the ^{15}N chemical shift anisotropy [69]. This partial cancellation becomes effective at ^1H Larmor frequencies above 500 MHz and reaches a maximum of around 1000 MHz [104, 105]. The corresponding line widths will also be smaller because of the TROSY line's slower relaxation. Therefore, TROSY detection is always preferable in terms of line width. However, as only one doublet component is detected, the signal intensity will only be half if relaxation is neglected. Therefore, for a small and highly dynamic protein (which is characterized by small transverse relaxation rate constants), such as the IDP investigated here, the HSQC signal intensity will be twice, as both the $\text{H}\cdot\text{N}^\beta$ and $\text{H}\cdot\text{N}^\alpha$ line will add up and be detected in the decoupled HSQC spectra.

The resulting signal intensity, HSQC vs TROSY, will now be a trade-off between the double signal intensity of the HSQC spectra (in the absence of relaxation) and the TROSY line ($\text{H}\cdot\text{N}^\beta$ in the direct ^1H dimension and $\text{N}\cdot\text{H}^\beta$ in the indirect ^{15}N dimension, respectively) becoming gradually stronger for increased transverse relaxation. At some point, the intensity of the TROSY line will become more

intense than the HSQC line (which is the average of the TROSY and anti-TROSY line because of $H\cdot N^\beta + H\cdot N^\alpha = 2 H\cdot$). This is the breakeven that will be reached when TROSY outperforms HSQC, not only in terms of line width but also in terms of SNR. For a fully protonated rigid globular folded protein, the breakeven will be reached at a molecular weight (MW) of about 20-30 kDa. For even higher MWs, deuteration of the protein is recommended to dilute the proton network and reduce transverse relaxation due to 1H - 1H dipolar couplings with the surrounding proton network. This breakeven for proteins with high internal dynamics will be shifted towards higher MWs as fast internal dynamics will lead to lower transverse relaxation rates.

In the following, we will give a brief guideline on when to choose the TROSY and when to select the sensitivity-enhanced HSQC detected ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments:

1. When the main priority is to obtain the highest resolution (smallest line widths), the TROSY-detected experiments will always be the best option.
2. TROSY-detected experiments will also always give the best water suppression.
3. When a high signal-to-noise ratio (SNR) is the main criterion, it will be a judgment call, depending on the following points: (A) The molecular weight of the investigated biomolecule, (B) the (expected) internal dynamics of the studied biomolecule, (C) the magnetic field the sample is measured at, and (C) whether a fully protonated or a deuterated sample is used.

(A) Below 20 kDa, we recommend using the sensitivity-enhanced HSQC detection when SNR is the main criterion.

(B) Proteins with high internal dynamics (like IDPs) will give more intense resonances due to smaller transverse relaxation. Therefore, for IDPs, the HSQC detection may be beneficial up to 30 kDa.

(C) Below 800 MHz, the TROSY effect will be less effective. Therefore, the breakeven point for TROSY may be shifted towards higher MW.

(D) the TROSY effect will be more effective for deuterated proteins, shifting the breakeven points to lower MW.

This brief guideline can, however, only be over-simplified because all factors A to D contribute and will influence each other. Therefore, we strongly recommend to follow the following empirical approach:

TROSY will always win in terms of line width. If SNR is the main criterion, we suggest the following approach:

1. Set up both the TROSY-detected and the HSQC-detected NMR relaxation experiments. (With the easier-to-optimize HSQC-detected NMR experiments, this should be easy.)
2. Record the first FID of each sequence. The corresponding 1D spectrum (after the Fourier-Transformation of the first FID) will yield a good impression of the expected SNR. Choose the detection scheme that offers higher SNR.
3. To estimate the maximum duration that should be sampled, record, e.g., the second FID with the most extended relaxation delay period. If possible, the entire exponential decay curve down to 30% of the initial signal intensity should be sampled.
4. For the ^{15}N $R_{1\rho}$ experiment, be careful not to exceed the (probe and spectrometer-specific) maximum power and duration of the CW irradiation during the spinlock period of the relaxation delay.

Author contributions

Tobias Stief: Investigation, Methodology, Formal Analysis, Writing. **Katharina Vormann:** Resources (Sample preparation), Writing. **Nils-Alexander Lakomek:** Conceptualization, Methodology, Writing, Supervision, Funding acquisition.

Acknowledgments

We thank Robin Backer, Lothar Gremer, Filip Hasecke, Nina Kirchgässler, Anne Pfitzer, Celina M. Schulz, Nasrollah Rezaei-Ghaleh, and Marie Schützmann for helpful discussions. N.L. thanks the German Science Foundation for funding through the Heisenberg Program (DFG grant number

433700474). This work is further supported by the project “Virological and immunological determinants of COVID-19 pathogenesis – lessons to get prepared for future pandemics (KA1-Co-02 “COVIPA”), a grant from the Helmholtz Association's Initiative and Networking Fund. We acknowledge generous access to the Jülich-Düsseldorf Biomolecular NMR Center, jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU).

Supplementary material

Experimental details on the NMR experiments, supplementary NMR data, and ^{15}N R_1 and $R_{1\rho}$ rate constants can be found in the Supplementary Material.

References

- [1] P. Tompa, Intrinsically unstructured proteins, *Trends Biochem Sci* 27(10) (2002) 527-33.
- [2] V.N. Uversky, Natively unfolded proteins: a point where biology waits for physics, *Protein Sci* 11(4) (2002) 739-56.
- [3] H.J. Dyson, P.E. Wright, Intrinsically unstructured proteins and their functions, *Nat Rev Mol Cell Biol* 6(3) (2005) 197-208.
- [4] P.E. Wright, H.J. Dyson, Intrinsically disordered proteins in cellular signalling and regulation, *Nat Rev Mol Cell Biol* 16(1) (2015) 18-29.
- [5] K. Sugase, H.J. Dyson, P.E. Wright, Mechanism of coupled folding and binding of an intrinsically disordered protein, *Nature* 447(7147) (2007) 1021-5.
- [6] T. Mittag, J.D. Forman-Kay, Atomic-level characterization of disordered protein ensembles, *Curr Opin Struct Biol* 17(1) (2007) 3-14.
- [7] A. Bah, R.M. Vernon, Z. Siddiqui, M. Krzeminski, R. Muhandiram, C. Zhao, N. Sonenberg, L.E. Kay, J.D. Forman-Kay, Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch, *Nature* 519(7541) (2015) 106-9.
- [8] K.A. Burke, A.M. Janke, C.L. Rhine, N.L. Fawzi, Residue-by-Residue View of In Vitro FUS Granules that Bind the C-Terminal Domain of RNA Polymerase II, *Mol Cell* 60(2) (2015) 231-41.
- [9] T.J. Nott, E. Petsalaki, P. Farber, D. Jervis, E. Fussner, A. Plochowitz, T.D. Craggs, D.P. Bazett-Jones, T. Pawson, J.D. Forman-Kay, A.J. Baldwin, Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles, *Mol Cell* 57(5) (2015) 936-947.

-
- [10] C.W. Pak, M. Kosno, A.S. Holehouse, S.B. Padrick, A. Mittal, R. Ali, A.A. Yunus, D.R. Liu, R.V. Pappu, M.K. Rosen, Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein, *Mol. Cell* 63(1) (2016) 72-85.
- [11] S. Boeynaems, S. Alberti, N.L. Fawzi, T. Mittag, M. Polymenidou, F. Rousseau, J. Schymkowitz, J. Shorter, B. Wolozin, L. Van den Bosch, P. Tompa, M. Fuxreiter, Protein Phase Separation: A New Phase in Cell Biology, *Trends Cell Biol.* 28(6) (2018) 420-435.
- [12] M. Boehning, C. Dugast-Darzacq, M. Rankovic, A.S. Hansen, T. Yu, H. Marie-Nelly, D.T. McSwiggen, G. Kokic, G.M. Dailey, P. Cramer, X. Darzacq, M. Zweckstetter, RNA polymerase II clustering through carboxy-terminal domain phase separation, *Nat Struct Mol Biol* 25(9) (2018) 833-840.
- [13] S. Guseva, V. Schnapka, W. Adamski, D. Maurin, R.W.H. Ruigrok, N. Salvi, M. Blackledge, Liquid-Liquid Phase Separation Modifies the Dynamic Properties of Intrinsically Disordered Proteins, *J Am Chem Soc* 145(19) (2023) 10548-10563.
- [14] C.M. Dobson, Protein folding and misfolding, *Nature* 426(6968) (2003) 884-90.
- [15] C.W. Bertoncini, Y.S. Jung, C.O. Fernandez, W. Hoyer, C. Griesinger, T.M. Jovin, M. Zweckstetter, Release of long-range tertiary interactions potentiates aggregation of natively unstructured alpha-synuclein, *Proc Natl Acad Sci U S A* 102(5) (2005) 1430-5.
- [16] A.K. Buell, C. Galvagnion, R. Gaspar, E. Sparr, M. Vendruscolo, T.P. Knowles, S. Linse, C.M. Dobson, Solution conditions determine the relative importance of nucleation and growth processes in alpha-synuclein aggregation, *Proc Natl Acad Sci U S A* 111(21) (2014) 7671-6.
- [17] A. Patel, H.O. Lee, L. Jawerth, S. Maharana, M. Jahnel, M.Y. Hein, S. Stoyanov, J. Mahamid, S. Saha, T.M. Franzmann, A. Pozniakovski, I. Poser, N. Maghelli, L.A. Royer, M. Weigert, E.W. Myers, S. Grill, D. Drechsel, A.A. Hyman, S. Alberti, A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation, *Cell* 162(5) (2015) 1066-77.
- [18] M.R. Jensen, L. Salmon, G. Nodet, M. Blackledge, Defining conformational ensembles of intrinsically disordered and partially folded proteins directly from chemical shifts, *J Am Chem Soc* 132(4) (2010) 1270-2.
- [19] A.R. Camacho-Zarco, V. Schnapka, S. Guseva, A. Abyzov, W. Adamski, S. Milles, M.R. Jensen, L. Zidek, N. Salvi, M. Blackledge, NMR Provides Unique Insight into the Functional Dynamics and Interactions of Intrinsically Disordered Proteins, *Chem Rev* 122(10) (2022) 9331-9356.
- [20] N. Rezaei-Ghaleh, G. Parigi, A. Soranno, A. Holla, S. Becker, B. Schuler, C. Luchinat, M. Zweckstetter, Local and Global Dynamics in Intrinsically Disordered Synuclein, *Angew Chem Int Ed Engl* 57(46) (2018) 15262-15266.
- [21] R. Schneider, M. Blackledge, M.R. Jensen, Elucidating binding mechanisms and dynamics of intrinsically disordered protein complexes using NMR spectroscopy, *Curr Opin Struct Biol* 54 (2019) 10-18.
- [22] T.R. Alderson, L.E. Kay, NMR spectroscopy captures the essential role of dynamics in regulating biomolecular function, *Cell* 184(3) (2021) 577-595.
- [23] L.E. Kay, D.A. Torchia, A. Bax, Backbone dynamics of proteins as studied by ¹⁵N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease, *Biochemistry* 28(23) (1989) 8972-9.
- [24] J.W. Peng, G. Wagner, Mapping of the spectral densities of N-H bond motions in eglin c using heteronuclear relaxation experiments, *Biochemistry* 31(36) (1992) 8571-86.
- [25] N.A. Farrow, R. Muhandiram, A.U. Singer, S.M. Pascal, C.M. Kay, G. Gish, S.E. Shoelson, T. Pawson, J.D. Formankay, L.E. Kay, BACKBONE DYNAMICS OF A FREE AND A PHOSHOPEPTIDE-COMPLEXED SRC HOMOLOG-2 DOMAIN STUDIED BY N-15 NMR RELAXATION, *Biochemistry* 33(19) (1994) 5984-6003.
- [26] G. Barbato, M. Ikura, L.E. Kay, R.W. Pastor, A. Bax, Backbone dynamics of Calmodulin studied by N-15 relaxation using inverse detected 2-dimensional NMR-spectroscopy - The central helix is flexible, *Biochemistry* 31(23) (1992) 5269-5278.
- [27] L.E. Kay, P. Keifer, T. Saarinen, Pure Absorption Gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, *J. Am. Chem. Soc.* 114(26) (1992) 10663-10665.

- [28] L.E. Kay, Protein dynamics from NMR, *Nat Struct Biol* 5 (1998) 513-517.
- [29] R. Ishima, D.A. Torchia, Protein dynamics from NMR, *Nat. Struct. Biol.* 7(9) (2000) 740-743.
- [30] S.F. Lienin, T. Bremi, B. Brutscher, R. Bruschweiler, R.R. Ernst, Anisotropic intramolecular backbone dynamics of ubiquitin characterized by NMR relaxation and MD computer simulation, *J. Am. Chem. Soc.* 120(38) (1998) 9870-9879.
- [31] M. Akke, A.G. Palmer, Monitoring macromolecular motions on microsecond to millisecond time scales by R(1)rho-R(1) constant relaxation time NMR spectroscopy, *J. Am. Chem. Soc.* 118(4) (1996) 911-912.
- [32] J.P. Loria, M. Rance, A.G. Palmer, A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy, *J. Am. Chem. Soc.* 121(10) (1999) 2331-2332.
- [33] M. Tollinger, N.R. Skrynnikov, F.A.A. Mulder, J.D. Forman-Kay, L.E. Kay, Slow dynamics in folded and unfolded states of an SH3 domain, *J. Am. Chem. Soc.* 123(46) (2001) 11341-11352.
- [34] F.A.A. Mulder, N.R. Skrynnikov, B. Hon, F.W. Dahlquist, L.E. Kay, Measurement of slow (μ s-ms) time scale dynamics in protein side chains by N-15 relaxation dispersion NMR spectroscopy: Application to Asn and Gln residues in a cavity mutant of T4 lysozyme, *J. Am. Chem. Soc.* 123(5) (2001) 967-975.
- [35] D.F. Hansen, P. Vallurupalli, L.E. Kay, An improved (15)N relaxation dispersion experiment for the measurement of millisecond time-scale dynamics in proteins, *J Phys Chem B* 112(19) (2008) 5898-5904.
- [36] A.G. Palmer, NMR characterization of the dynamics of biomacromolecules, *Chem. Rev.* 104(8) (2004) 3623-3640.
- [37] A. Mittermaier, L.E. Kay, Review - New tools provide new insights in NMR studies of protein dynamics, *Science* 312(5771) (2006) 224-228.
- [38] D.D. Boehr, D. McElheny, H.J. Dyson, P.E. Wright, The dynamic energy landscape of dihydrofolate reductase catalysis, *Science* 313(5793) (2006) 1638-1642.
- [39] K. Henzler-Wildman, D. Kern, Dynamic personalities of proteins, *Nature* 450(7172) (2007) 964-972.
- [40] P. Neudecker, P. Robustelli, A. Cavalli, P. Walsh, P. Lundstrom, A. Zarrine-Afsar, S. Sharpe, M. Vendruscolo, L.E. Kay, Structure of an Intermediate State in Protein Folding and Aggregation, *Science* 336(6079) (2012) 362-366.
- [41] E.A. Morrison, G.T. DeKoster, S. Dutta, R. Vafabakhsh, M.W. Clarkson, A. Bahl, D. Kern, T. Ha, K.A. Henzler-Wildman, Antiparallel EmrE exports drugs by exchanging between asymmetric structures, *Nature* 481(7379) (2012) 45-U50.
- [42] J.B. Stiller, R. Otten, D. Haussinger, P.S. Rieder, D.L. Theobald, D. Kern, Structure determination of high-energy states in a dynamic protein ensemble, *Nature* 603(7901) (2022) 528-+.
- [43] Q. Zhang, X.Y. Sun, E.D. Watt, H.M. Al-Hashimi, Resolving the motional modes that code for RNA adaptation, *Science* 311(5761) (2006) 653-656.
- [44] I.J. Kimsey, K. Petzold, B. Sathyamoorthy, Z.W. Stein, H.M. Al-Hashimi, Visualizing transient Watson-Crick-like mispairs in DNA and RNA duplexes, *Nature* 519(7543) (2015) 315-+.
- [45] J. Rinnenthal, J. Buck, J. Ferner, A. Wacker, B. Furtig, H. Schwalbe, Mapping the Landscape of RNA Dynamics with NMR Spectroscopy, *Accounts Chem Res* 44(12) (2011) 1292-1301.
- [46] J. Boyd, U. Hommel, I.D. Campbell, Influence of cross-correlation between dipolar and anisotropic chemical-shift relaxation mechanisms upon longitudinal relaxation rates of N-15 in macromolecules, *Chem. Phys. Lett.* 175(5) (1990) 477-482.
- [47] L.E. Kay, L.K. Nicholson, F. Delaglio, A. Bax, D.A. Torchia, Pulse sequences for removal of the effects of cross-correlation between dipolar and chemical-shift anisotropy relaxation mechanism on the measurement of heteronuclear T1 and T2 values in proteins, *J. Magn. Reson.* 97(2) (1992) 359-375.
- [48] A.G. Palmer, N.J. Skelton, W.J. Chazin, P.E. Wright, M. Rance, SUPPRESSION OF THE EFFECTS OF CROSS-CORRELATION BETWEEN DIPOLAR AND ANISOTROPIC CHEMICAL-SHIFT RELAXATION MECHANISMS IN THE MEASUREMENT OF SPIN SPIN RELAXATION RATES, *Mol. Phys.* 75(3) (1992) 699-711.

-
- [49] S. Grzesiek, A. Bax, The importance of not saturating H₂O in protein NMR - Application to sensitivity enhancement and NOE measurements, *J. Am. Chem. Soc.* 115(26) (1993) 12593-12594.
- [50] K. Chen, N. Tjandra, Water proton spin saturation affects measured protein backbone ¹⁵N spin relaxation rates, *J Magn Reson* 213(1) (2011) 151-7.
- [51] N.A. Lakomek, J. Ying, A. Bax, Measurement of (1)(5)N relaxation rates in perdeuterated proteins by TROSY-based methods, *J Biomol NMR* 53(3) (2012) 209-21.
- [52] R. Ishima, A probe to monitor performance of (1)(5)N longitudinal relaxation experiments for proteins in solution, *J Biomol NMR* 58(2) (2014) 113-22.
- [53] T. Yuwen, N.R. Skrynnikov, Proton-decoupled CPMG: a better experiment for measuring (15)N R₂ relaxation in disordered proteins, *J Magn Reson* 241 (2014) 155-69.
- [54] P. Wikus, W. Frantz, R. Kummerle, P. Vonlanthen, Commercial gigahertz-class NMR magnets, *Supercond Sci Tech* 35(3) (2022).
- [55] J.F. Ellena, B. Liang, M. Wiktor, A. Stein, D.S. Cafiso, R. Jahn, L.K. Tamm, Dynamic structure of lipid-bound synaptobrevin suggests a nucleation-propagation mechanism for trans-SNARE complex formation, *Proc Natl Acad Sci U S A* 106(48) (2009) 20306-11.
- [56] J. Hazzard, T.C. Sudhof, J. Rizo, NMR analysis of the structure of synaptobrevin and of its interaction with syntaxin, *J Biomol NMR* 14(3) (1999) 203-7.
- [57] N.A. Lakomek, H. Yavuz, R. Jahn, A. Perez-Lara, Structural dynamics and transient lipid binding of synaptobrevin-2 tune SNARE assembly and membrane fusion, *Proc Natl Acad Sci U S A* 116(18) (2019) 8699-8708.
- [58] A.V. Pobbati, A. Stein, D. Fasshauer, N- to C-terminal SNARE complex assembly promotes rapid membrane fusion, *Science* 313(5787) (2006) 673-676.
- [59] N. Bloembergen, R.V. Pound, Radiation Damping in Magnetic Resonance Experiments, *Phys Rev* 95(1) (1954) 8-12.
- [60] R. Ishima, Effects of Radiation Damping for Biomolecular NMR Experiments in Solution: A Hemisphere Concept for Water Suppression, *Concept Magn Reson A* 44a(5) (2015) 252-262.
- [61] D. Shishmarev, G. Otting, Radiation damping on cryoprobes, *J. Magn. Reson.* 213(1) (2011) 76-81.
- [62] J.P. Loria, M. Rance, A.G. Palmer, Transverse-relaxation-optimized (TROSY) gradient-enhanced triple-resonance NMR spectroscopy, *J. Magn. Reson.* 141(1) (1999) 180-184.
- [63] V. Sklenar, Suppression of Radiation Damping in Multidimensional Nmr Experiments Using Magnetic-Field Gradients, *J Magn Reson Ser A* 114(1) (1995) 132-135.
- [64] M. Sattler, J. Schleucher, C. Griesinger, Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients, *Prog Nucl Mag Res Sp* 34(2) (1999) 93-158.
- [65] P. Schanda, H. Van Melckebeke, B. Brutscher, Speeding up three-dimensional protein NMR experiments to a few minutes, *J. Am. Chem. Soc.* 128(28) (2006) 9042-9043.
- [66] P. Schanda, B. Brutscher, Very fast two-dimensional NMR spectroscopy for real-time investigation of dynamic events in proteins on the time scale of seconds, *J. Am. Chem. Soc.* 127(22) (2005) 8014-8015.
- [67] D. Joseph, C. Griesinger, Optimal control pulses for the 1.2-GHz (28.2-T) NMR spectrometers, *Sci Adv* 9(45) (2023) eadj1133.
- [68] A.G. Palmer, J. Cavanagh, P.E. Wright, M. Rance, Sensitivity Improvement in Proton-Detected 2-Dimensional Heteronuclear Correlation Nmr-Spectroscopy, *J. Magn. Reson.* 93(1) (1991) 151-170.
- [69] K. Pervushin, R. Riek, G. Wider, K. Wuthrich, Attenuated T₂ relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, *Proceedings of the National Academy of Sciences of the United States of America* 94(23) (1997) 12366-12371.
- [70] M. Salzmann, G. Wider, K. Pervushin, K. Wuthrich, Improved sensitivity and coherence selection for [N-15,H-1]-TROSY elements in triple resonance experiments, *Journal of Biomolecular Nmr* 15(2) (1999) 181-184.
- [71] G. Wider, Technical aspects of NMR spectroscopy with biological macromolecules and studies of hydration in solution, *Prog Nucl Mag Res Sp* 32 (1998) 193-275.

- [72] H. Geen, R. Freeman, Band-Selective Radiofrequency Pulses, *J. Magn. Reson.* 93(1) (1991) 93-141.
- [73] F.A.A. Mulder, R.A. de Graaf, R. Kaptein, R. Boelens, An Off-resonance Rotating Frame Relaxation Experiment for the Investigation of Macromolecular Dynamics Using Adiabatic Rotations, *J Magn Reson* 131(2) (1998) 351-7.
- [74] M. Garwood, Y. Ke, Symmetrical Pulses to Induce Arbitrary Flip Angles with Compensation for Rf Inhomogeneity and Resonance Offsets, *J. Magn. Reson.* 94(3) (1991) 511-525.
- [75] X. Shao, T.C. Sudhof, J. Rizo, Assignment of the ^1H , ^{15}N and ^{13}C resonances of the calcium-free and calcium-bound forms of the first C2-domain of synaptotagmin I, *J Biomol NMR* 10(3) (1997) 307-8.
- [76] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes, *Journal of Biomolecular Nmr* 6(3) (1995) 277-293.
- [77] H. Desvaux, P. Berthault, Study of dynamic processes in liquids using off-resonance rf irradiation, *Prog Nucl Mag Res Sp* 35(4) (1999) 295-340.
- [78] A.G. Palmer, F. Massi, Characterization of the dynamics of biomacromolecules using rotating-frame spin relaxation NMR spectroscopy, *Chem. Rev.* 106(5) (2006) 1700-1719.
- [79] D.M. Lemaster, Isotope Labeling in Solution Protein Assignment and Structural-Analysis, *Prog Nucl Mag Res Sp* 26 (1994) 371-419.
- [80] S. Grzesiek, P. Wingfield, S. Stahl, J.D. Kaufman, A. Bax, 4-Dimensional N-15-Separated Noesy of Slowly Tumbling Perdeuterated N-15-Enriched Proteins - Application to Hiv-1 Nef, *J. Am. Chem. Soc.* 117(37) (1995) 9594-9595.
- [81] A. Eletsky, A. Kienhofer, K. Pervushin, TROSY NMR with partially deuterated proteins, *Journal of Biomolecular Nmr* 20(2) (2001) 177-180.
- [82] M. Gairi, A. Dyachenko, M.T. Gonzalez, M. Feliz, M. Pons, E. Giralt, An optimized method for $(^{15}\text{N})\text{R}(1)$ relaxation rate measurements in non-deuterated proteins, *J Biomol NMR* 62(2) (2015) 209-20.
- [83] P.J. Nichols, I. Falconer, A. Griffin, C. Mant, R. Hodges, C.J. McKnight, B. Vogeli, L. Vugmeyster, Deuteration of nonexchangeable protons on proteins affects their thermal stability, side-chain dynamics, and hydrophobicity, *Protein Science* 29(7) (2020) 1641-1654.
- [84] J.S. Mugridge, R.G. Bergman, K.N. Raymond, Does Size Really Matter? The Steric Isotope Effect in a Supramolecular Host-Guest Exchange Reaction, *Angew Chem Int Edit* 49(21) (2010) 3635-3637.
- [85] P. Cioni, G.B. Strambini, Effect of heavy water on protein flexibility, *Biophys J* 82(6) (2002) 3246-3253.
- [86] W. Adamski, N. Salvi, D. Maurin, J. Magnat, S. Milles, M.R. Jensen, A. Abyzov, C.J. Moreau, M. Blackledge, A Unified Description of Intrinsically Disordered Protein Dynamics under Physiological Conditions Using NMR Spectroscopy, *J. Am. Chem. Soc.* 141(44) (2019) 17817-17829.
- [87] N. Salvi, A. Abyzov, M. Blackledge, Multi-Timescale Dynamics in Intrinsically Disordered Proteins from NMR Relaxation and Molecular Simulation, *J Phys Chem Lett* 7(13) (2016) 2483-2489.
- [88] N. Salvi, A. Abyzov, M. Blackledge, Solvent-dependent segmental dynamics in intrinsically disordered proteins, *Sci Adv* 5(6) (2019).
- [89] A. Abyzov, E. Mandelkow, M. Zweckstetter, N. Rezaei-Ghaleh, Fast Motions Dominate Dynamics of Intrinsically Disordered Tau Protein at High Temperatures, *Chem-Eur J* 29(17) (2023).
- [90] N. Rezaei-Ghaleh, G. Parigi, M. Zweckstetter, Reorientational Dynamics of Amyloid-beta from NMR Spin Relaxation and Molecular Simulation, *J Phys Chem Lett* 10(12) (2019) 3369-3375.
- [91] D. Kurzbach, G. Kontaxis, N. Coudeville, R. Konrat, NMR Spectroscopic Studies of the Conformational Ensembles of Intrinsically Disordered Proteins, *Adv Exp Med Biol* 870 (2015) 149-185.
- [92] S. Kim, K.P. Wu, J. Baum, Fast hydrogen exchange affects N-15 relaxation measurements in intrinsically disordered proteins, *Journal of Biomolecular Nmr* 55(3) (2013) 249-256.
- [93] M.L. Gill, R.A. Byrd, A.G. Palmer, Dynamics of GCN4 facilitate DNA interaction: a model-free analysis of an intrinsically disordered region, *Phys Chem Chem Phys* 18(8) (2016) 5839-5849.
- [94] J.P. Brady, P.J. Farber, A. Sekhar, Y.H. Lin, R. Huang, A. Bah, T.J. Nott, H.S. Chan, A.J. Baldwin, J.D. Forman-Kay, L.E. Kay, Structural and hydrodynamic properties of an intrinsically disordered region of

-
- a germ cell-specific protein on phase separation, *Proceedings of the National Academy of Sciences of the United States of America* 114(39) (2017) E8194-E8203.
- [95] D.S. Libich, V. Tugarinov, G.M. Clore, Intrinsic unfoldase/foldase activity of the chaperonin GroEL directly demonstrated using multinuclear relaxation-based NMR, *Proceedings of the National Academy of Sciences of the United States of America* 112(29) (2015) 8817-8823.
- [96] Z. Solyom, P.X. Ma, M. Schwarten, M. Bosco, A. Polidori, G. Durand, D. Willbold, B. Brutscher, The Disordered Region of the HCV Protein NS5A: Conformational Dynamics, SH3 Binding, and Phosphorylation, *Biophys J* 109(7) (2015) 1483-1496.
- [97] S.C. Chiliveri, Y. Shen, J.L. Baber, J.F. Ying, V. Sagar, G. Wistow, P. Anfinrud, A. Bax, Experimental NOE, Chemical Shift, and Proline Isomerization Data Provide Detailed Insights into Amelotin Oligomerization, *J. Am. Chem. Soc.* 145(32) (2023) 18063-18074.
- [98] T. Gruber, M. Lewitzky, L. Machner, U. Weininger, S.M. Feller, J. Balbach, Macromolecular Crowding Induces a Binding Competent Transient Structure in Intrinsically Disordered Gab1, *Journal of Molecular Biology* 434(5) (2022).
- [99] K. Kämpf, S.A. Izmailov, S.O. Rabdano, A.T. Groves, I.S. Podkorytov, N.R. Skrynnikov, What Drives N Spin Relaxation in Disordered Proteins? Combined NMR/MD Study of the H4 Histone Tail, *Biophys J* 115(12) (2018) 2348-2367.
- [100] N. Salvi, V. Zapletal, Z. Jasenakova, M. Zachrdla, P. Padrta, S. Narasimhan, T. Marquardsen, J.M. Tyburn, L. Zidek, M. Blackledge, F. Ferrage, P. Kaderavek, Convergent views on disordered protein dynamics from NMR and computational approaches, *Biophys J* 121(20) (2022) 3785-3794.
- [101] S. Milles, D. Mercadante, I.V. Aramburu, M.R. Jensen, N. Banterle, C. Koehler, S. Tyagi, J. Clarke, S.L. Shammass, M. Blackledge, F. Grater, E.A. Lemke, Plasticity of an Ultrafast Interaction between Nucleoporins and Nuclear Transport Receptors, *Cell* 163(3) (2015) 734-745.
- [102] S. Naudi-Fabra, M. Tengo, M.R. Jensen, M. Blackledge, S. Milles, Quantitative Description of Intrinsically Disordered Proteins Using Single-Molecule FRET, NMR, and SAXS, *J. Am. Chem. Soc.* 143(48) (2021) 20109-20121.
- [103] A. Borgia, M.B. Borgia, K. Bugge, V.M. Kissling, P.O. Heidarsson, C.B. Fernandes, A. Sottini, A. Soranno, K.J. Buholzer, D. Nettels, B.B. Kragelund, R.B. Best, B. Schuler, Extreme disorder in an ultrahigh-affinity protein complex, *Nature* 555(7694) (2018) 61-66.
- [104] C. Fernandez, G. Wider, TROSY in NMR studies of the structure and function of large biological macromolecules, *Curr Opin Struct Biol* 13(5) (2003) 570-80.
- [105] A.G. Tzakos, C.R. Grace, P.J. Lukavsky, R. Riek, NMR techniques for very large proteins and rnas in solution, *Annu Rev Biophys Biomol Struct* 35 (2006) 319-42.

9 Userfriendly Relaxation Experiments

^{15}N NMR relaxation is a complicated chapter in the theory of NMR with a flat learning curve, especially for beginners. But ^{15}N relaxation rates are a strong tool to catch protein backbone dynamics and therefore NMR relaxation measurements should be easily accessible to everyone.

For this publication the author had to develop a step-by-step protocol for the acquisition of ^{15}N relaxation experiments. This protocol has been tested by several people, which were not familiar with ^{15}N relaxation experiments, under supervision of the author. The author acquired the spectra, processed the spectra, evaluated and analyzed the data. The published video clips were recorded by the author. In the publishing process the author contributed text and figures.

Journal: JoVE

Volume: -

Authors: Tobias Stief
Katharina Vormann
Nils-Alexander Lakomek

DOI: 10.3791/67088-v

TITLE:

NMR ^{15}N Relaxation Experiments for the Investigation of Picosecond to Nanoseconds Structural Dynamics of Proteins

AUTHORS AND AFFILIATIONS:

Tobias Stief^{1,2}, Katharina Vormann^{1,2}, Nils-Alexander Lakomek^{1,2,*}

¹ Institute of Biological Information Processing (IBI-7), Forschungszentrum Jülich, Jülich, Germany

² Institute of Physical Biology, Faculty of Mathematics and Natural Sciences, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

*Correspondence:

Dr. Nils-Alexander Lakomek

Heinrich-Heine-Universität Düsseldorf

Institut für Physikalische Biologie (IPB)

Gebäude 26.44, Ebene U1

Universitätsstraße 1

40225 Düsseldorf

Germany

Phone: +49 211 811 5702

E-Mail: Nils-Alexander.Lakomek@hhu.de

Forschungszentrum Jülich

IBI-7: Strukturbiochemie

Gebäude 05.8v

Wilhelm-Johnen-Strasse

52425 Jülich

Germany

Phone: +49 2461 61 85345

E-Mail: n.lakomek@fz-juelich.de

SUMMARY:

Nuclear magnetic resonance (NMR) spectroscopy can characterize structural protein dynamics in a residue-specific manner. We provide a hands-on protocol for recording NMR ^{15}N R_1 and R_2 relaxation and $\{^1\text{H}\}$ - ^{15}N heteronuclear Overhauser effect (hetNOE) experiments, sensitive to the picoseconds to nanoseconds timescale.

ABSTRACT:

Nuclear magnetic resonance (NMR) spectroscopy allows studying proteins in solution and under physiological temperatures. Frequently, either the amide groups of the protein backbone or the methyl groups in side chains are used as reporters of structural dynamics in proteins. A structural dynamics study of the protein backbone of globular proteins on ^{15}N labeled and fully protonated samples usually works well for proteins with a molecular weight of up to 50 kDa. When side chain deuteration in combination with Transverse Relaxation Optimized Spectroscopy (TROSY) is applied, this limit can be extended up to 200 kDa for globular proteins and up to 1 MDa when the focus is on the side chains. When intrinsically disordered proteins (IDPs) or proteins with intrinsically disordered regions (IDRs) are investigated, these weight limitations do not apply but can go well beyond. The reason is that IDPs or IDRs, characterized by high internal flexibility, are frequently dynamically decoupled. Various NMR methods offer atomic-resolution insights into structural protein dynamics across a wide range of time scales, from picoseconds up to hours. Standard ^{15}N relaxation measurements overview a protein's internal flexibility and characterize the protein backbone dynamics experienced on the fast pico- to nanosecond timescale. This article presents a hands-on protocol for setting up and recording NMR ^{15}N R_1 , R_2 , and heteronuclear Overhauser effect (hetNOE) experiments. We show exemplary data and explain how to interpret them simply qualitatively before any more sophisticated analysis.

INTRODUCTION:

The function of a protein is determined not only by its three-dimensional structure but also by its structural dynamics, encompassing its internal flexibility and structural transitions between different conformations the protein will adopt. NMR spectroscopy can investigate the structural dynamics of proteins in solution¹⁻³. Recent developments in proton-detected solid-state NMR also allow for the characterization of protein dynamics in a less soluble state, such as, e.g., a lipid bilayer membrane⁴⁻⁶. In solution NMR, the structural dynamics of the protein backbone and protein side chains can be studied. For a globular protein, a structural dynamics study of the protein backbone can be achieved up to 50 kDa once the protein is ¹⁵N isotopically labeled. When side chain deuteration and TROSY are employed, this limit can be extended up to 200 kDa^{7,8}. When the focus lies on side-chain dynamics, the range of accessible proteins and complexes can be extended up to 1 MDa^{2,9}.

The named weight limitations do not apply to IDPs, which frequently show high intrinsic dynamics. Over 30% of the eukaryotic proteome comprises IDPs or IDRs¹⁰⁻¹³. They play central roles in many cellular processes, such as, e.g., signal transduction and transcription [1], and are frequently involved in intracellular phase separation¹⁴⁻¹⁷. IDPs lack a well-defined three-dimensional native structure under physiological conditions and have a weakly funneled or rugged energy landscape^{17,18}. Due to a low hydrophobicity and strong electrostatic repulsion distributed over the backbone of IDPs or IDRs, a driving force for folding to a rigid structure is missing¹⁹. IDPs frequently adopt a folded conformation when complex with other binding partners^{10,20,21}. Also, post-translational modifications (PTMs) expand the folding possibilities of IDPs or IDRs^{22,23}. Misfolding of IDPs has been identified as a cause of various diseases, including neurodegenerative diseases^{15,24-26}.

IDPs and IDRs show high internal flexibility^{21,27,28}. Conformational ensembles displaying the variation of atomic positions and dihedral angles were derived from molecular dynamics simulations and restraints obtained from experimental data²⁹⁻³². Due to dynamics and resulting disorder in the frozen state, the diffuse electron density makes it difficult to structurally characterize them using state-of-the-

art methods in structural biology, such as cryo-EM or X-ray crystallography. Also, crystallization conditions or sample preparation techniques for experiments at cryogenic temperatures may impact the conformational space experienced by IDPs. However, solution NMR works well for highly dynamic proteins and is therefore well-suited for investigating IDPs ^{16,20,22,28-38}.

As introduced above, solution NMR offers various techniques to study internal protein dynamics across a wide range of time scales (Figure 1), primarily based on spin relaxation ^{31,33,38-42}.

Spin relaxation of the ¹⁵N nuclei in the amide groups of the protein backbone is induced by ¹H-¹⁵N bond angle orientation changes due to internal protein dynamics and collective motions (including, when relevant, rotational diffusion) ^{27,43-51}. At timescales shorter than the rotational correlation time τ_R (the time the molecule needs to rotate one radian, also dubbed overall tumbling correlation time), the chemical shift anisotropy (CSA) and the dipolar coupling (D) are active and not averaged by the rotational diffusion of the protein. Internal dynamics of the protein backbone, comprising variations in bond angles, reorientations of bonds, and rotational tumbling, induce stochastic fluctuations of the CSA and dipolar coupling tensor, resulting in a variation of the local magnetic field, ultimately leading to NMR spin relaxation ^{47,48,52,53}. These fluctuations can be described by an overall correlation function. The Fourier transform of the overall correlation function is called the spectral density function. In the semi-classical Redfield relaxation theory, the NMR relaxation rate constants can be described by linear combinations of these spectral density functions ⁵⁴.

Backbone ¹⁵N NMR relaxation experiments developed in the early 1990s comprise ¹⁵N R_1 , $R_{1\rho}$, and $\{^1\text{H}\}$ -¹⁵N nuclear Overhauser effect experiments, sensitive to the fast picosecond (ps) nanosecond (ns) timescale, faster than the rotational correlation time τ_R of the protein ^{45,55-57}. To characterize backbone dynamics slower than the rotational correlation time τ_R , so-called relaxation dispersion experiments, $R_{1\rho}$, and CPMG (Carr-Purcell-Meiboom-Gill) experiments sensitive to microsecond (μ s) – millisecond (ms) dynamics ^{44,46,58-61} are used. Dynamics slower than microseconds can be captured by ¹⁵N CEST (Chemical exchange saturation transfer) NMR ⁶², exchange spectroscopy (EXSY, milliseconds to

seconds), or RT (Real-time) NMR (seconds to hours) ^{63,64}. The PRE (paramagnetic relaxation enhancement) effect of paramagnetic probes, as well as residual dipolar couplings (RDCs), can be used to assess the entire range of ps to ms dynamics ⁶⁵⁻⁶⁸.

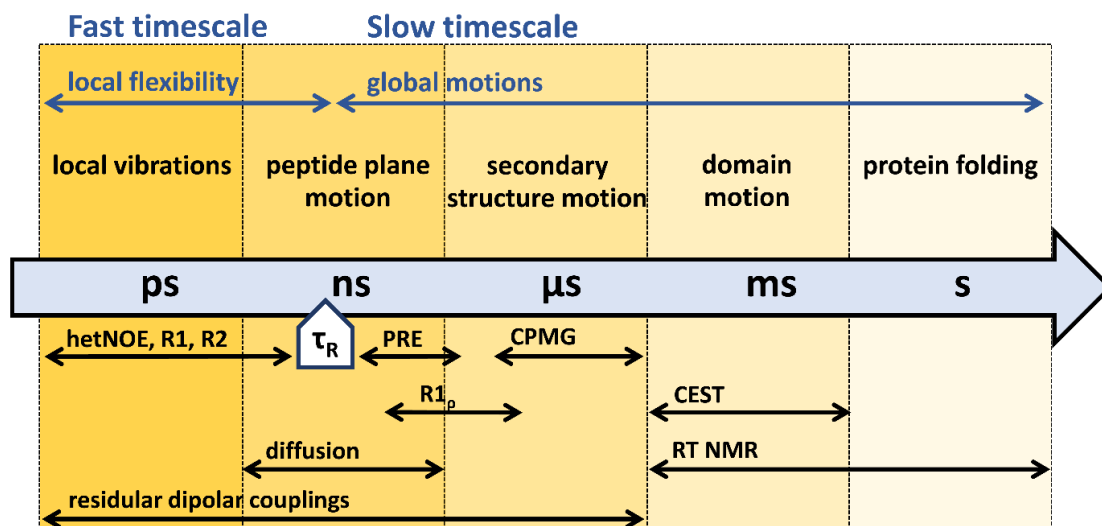


Figure 1: Time scales of protein backbone dynamics and sensitive time window of different NMR dynamics experiments. NMR offers a variety of methods to characterize protein backbone dynamics across a wide range of timescales. Different motions experienced by the protein backbone are indicated at their respective timescales. The protein's rotational correlation time, τ_R , is the time the protein needs for an overall rotation (by one radian). Motions faster than the protein's rotational correlation time, τ_R , can be associated with the protein's internal flexibility. Various NMR experiments and their sensitivity to the respective timescales are indicated below the arrow.

The protocol below describes the setup of NMR relaxation experiments by Lakomek et al. ⁶⁹ and Stief et al. ⁷⁰, using a sensitivity-enhanced HSQC detection scheme.

Before proceeding to the experimental implementation, a very brief overview of NMR spin relaxation and NMR relaxation experiments is given. Due to size restraints and to keep this protocol comprehensible, this overview must remain simplistic (and therefore incomplete).

The longitudinal or spin-lattice relaxation, characterized by the T_1 time or the $R_1 = 1/T_1$ rate constant, describes the return of magnetization to Boltzmann equilibrium. In equilibrium, the magnetization is aligned along the axis of the outer magnetic field, which defines the z-axis of the laboratory frame. Spectral densities at high (^1H) and small (^{15}N) Larmor frequencies (the NMR resonance frequencies, e.g., 600 MHz for ^1H for a 14.1 Tesla magnet) and linear combinations of these Larmor frequencies contribute to the ^{15}N R_1 relaxation, which is characterized by the ^{15}N R_1 rate constants measured in rad s^{-1} . Motions on timescales inverse to these Larmor frequencies, thus, motions on the picosecond to nanosecond timescale contribute to the relaxation rate constant R_1 . For molecules that show overall tumbling and where a rotational correlation time can be defined, the $R_1(T_1)$ curve shows a maximum (minimum) for $\omega\tau_R = 1$, with the rotational correlation τ_R and the Larmor frequency ω of the spin under consideration. If multiple Larmor frequencies contribute, the one with the lowest frequency is the dominant one, e.g., ω_N in the case of ^{15}N R_1 . The fast motion regime ($\omega\tau_R \ll 1$) applies to small molecules that tumble very fast and for low magnetic fields and low viscosity. The slow motion regime ($\omega\tau_R \gg 1$) is valid for larger molecules that tumble slower and for high magnetic fields and high viscosity.

Globular folded proteins show overall tumbling in solution, and a rotational correlation time can be assigned. However, the concept of overall tumbling is no longer valid for intrinsically disordered proteins and frequently differs from assigning a single rotational correlation time. Here, the residue-specific internal correlation time becomes more critical.

The described pulse sequence measuring ^{15}N R_1 relaxation rates (Figure 2B) is based on a sensitivity-enhanced HSQC read-out experiment with an Echo/ Anti-Echo detection for quadrature detection⁶⁹⁻⁷¹. Short gradients with variable strength and length are used for coherence selection and improved water suppression⁷⁰. During that time, ^{15}N longitudinal polarization will relax. Longer decay times lead to

reduced intensities in the associated 2D planes of this pseudo-3D spectrum (delay data points are recorded in the third dimension). A loop element, described below, is executed an increasing number of times for longer relaxation times. As cross-correlated relaxation between the ^{15}N chemical shift anisotropy (CSA) and the ^1H and ^{15}N dipolar coupling (D) is also active during the relaxation delay, a central I-BURP-2 180° pulse ⁷², selective on the amide protons, is necessary to refocus the contribution by cross-correlated relaxation (which if not refocused would lead to skewed and erroneous ^{15}N R_1 rate constants).

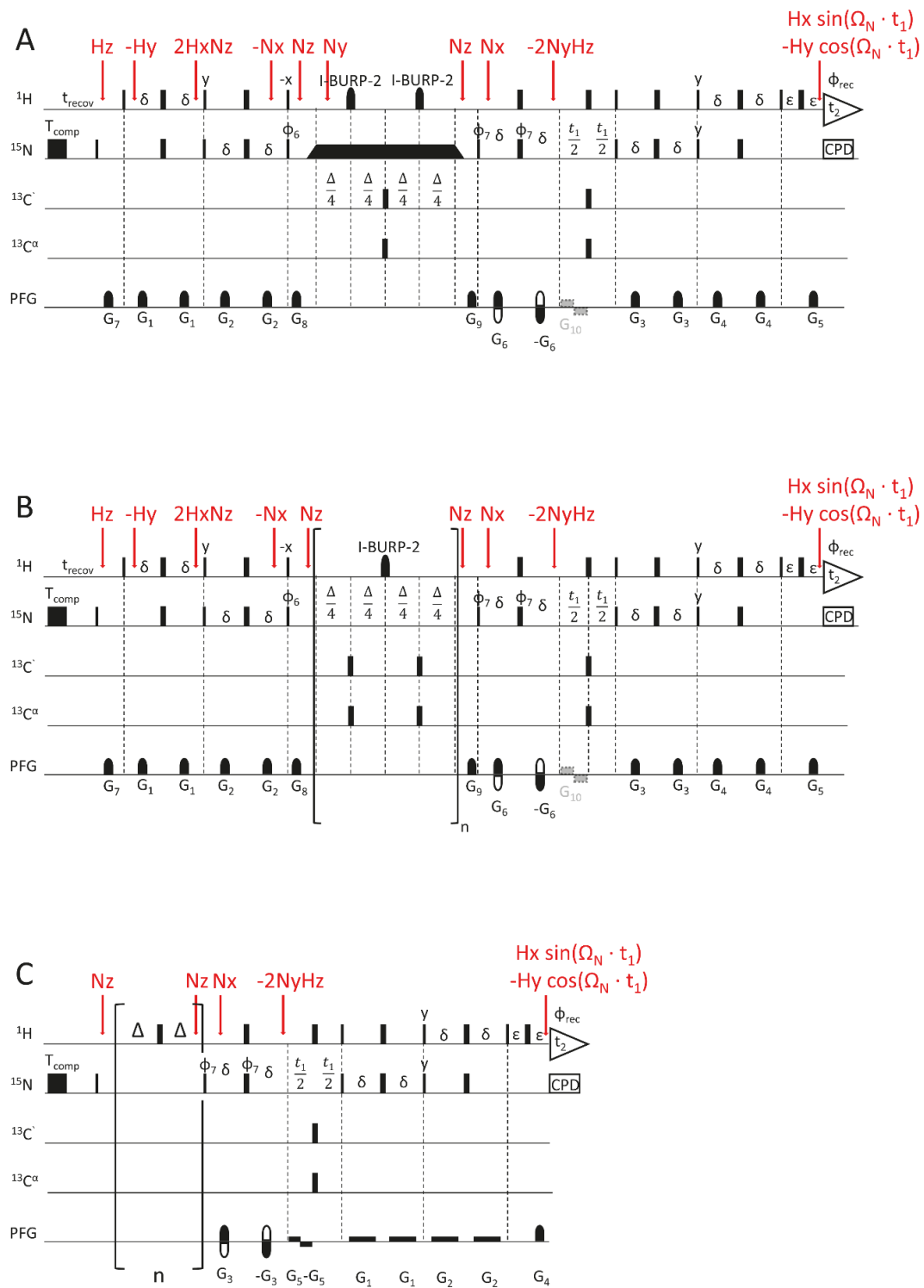


Figure 2: NMR pulse sequence schemes for determining the NMR relaxation rate constants. (A)

^{15}N $R_{1\rho}$, (B) ^{15}N R_1 , and (C) hetNOE experiment, using a sensitivity-enhanced HSQC read-out scheme^{69,70}. $90^\circ(x)$ pulses are visualized by narrow rectangles and $180^\circ(x)$ pulses by wide rectangles, unless indicated otherwise. The following phase cycle is applied: $\phi_6 = y, y, -y, -y$; $\phi_7 = y, -y$, $\phi_{\text{rec}} = y, -y, -y, y$.

Quadrature detection is achieved by inverting the polarity of gradient G_5 and the phase cycle of ϕ_7 (Echo/ Anti-Echo detection). (A) ^{15}N $R_{1\rho}$ experiment: The black rectangular represents the spin-lock, for which the duration varies to acquire different relaxation delays. The triangles before and after the spin-lock indicate the adiabatic shape pulses that align the magnetization along the effective magnetic field axis B_{eff} . G_{10} is an optional gradient to prevent radiation damping of the water magnetization during the evolution phase. (B) ^{15}N R_1 experiment: The bracketed part shows the sequence's loop element, repeated an n number of times to match the desired relaxation delay. (C) The hetNOE pulse scheme is similar to the second half of R_1 and $R_{1\rho}$ pulse schemes, namely the t_1 evolution time and HSQC-detection element. However, ^{15}N magnetization is excited directly without any INEPT. Saturation of the proton magnetization (to achieve cross-relaxation between ^1H and ^{15}N) is achieved by a train of $180(^1\text{H})$ pulses applied for at least 5 s. An idle delay of the same lengths (here, 5 s) without any pulse train is applied for the reference experiment. G_5 is an optional gradient to prevent radiation damping, and the inversion in polarity of gradient G_4 , in combination with phase $\phi_7 = y, -y, -y, y$, achieves quadrature detection. The magnetization transfer steps, represented by product operators, are marked in red.

The relaxation rate constant R_2 describes the relaxation of transverse polarization (in the xy -plane orthogonal to the external magnetic field) due to loss of phase coherence between the spin, leading to decay of detectable magnetization^{53,54}. The spectral density function at high and small frequencies contributes to R_2 , similar to R_1 . However, the biggest contribution to R_2 originates from the spectral density at zero frequency. For this reason, R_2 is very sensitive to the rotational tumbling, described by the rotational correlation time τ_R , which is in the order of a few ns for a small globular protein at room temperature. Thus, slower backbone motions in the hundreds of ps to low ns regime contribute the most. Exchange dynamics of the backbone that cause a modulation of the isotropic part of the chemical shift tensor of the ^{15}N nuclei, in principle, add an exchange contribution $R_{2\text{ex}}$ to the R_2 rate constants^{43,44,49,60,61}. In the described experiments, the $R_{2\text{ex}}$ contribution is suppressed by a spin-lock that refocuses dynamics slower than the inverse circular frequency of the spin-lock. The spin-lock is a long continuous wave radiofrequency pulse that keeps the magnetization aligned along the effective magnetic field axis B_{eff} .

(the vector sum of the spin-lock ω_1 field and the chemical shift offset from the ^{15}N carrier frequency (see below)). The relaxation of the magnetization aligned along the $B_{1,\text{eff}}$ axis is called $R_{1\rho}$ relaxation, which has a R_1 component and a R_2 component. Using formula (1), R_2 can be calculated from $R_{1\rho}$ and R_1 ^{44,73}:

$$R_2 = \frac{R_{1\rho}}{\sin^2 \theta} - \frac{R_1}{\tan^2 \theta} \quad (1).$$

The angle between the axis of the effective magnetic field B_{eff} and the external magnetic field B_0 is $\theta = \arctan\left(\frac{\omega_1}{\Omega}\right)$. ω_1 is the RF amplitude of the spin-lock and Ω the chemical shift offset between the ^{15}N chemical shift of the corresponding residue and the ^{15}N carrier frequency ^{44,73}.

The $R_{1\rho}$ pulse scheme (Figure 2A, ⁷⁰) is very similar to the ^{15}N R_1 scheme except for the relaxation delay. To measure the ^{15}N $R_{1\rho}$ relaxation rates, the spin-lock must be active after the magnetization has been aligned along the effective field axis B_{eff} by an adiabatic pulse with the same radio-frequency (RF) amplitude as the spin-lock. The length of the spin-lock will be varied to obtain the different relaxation delays.

The steady-state $\{^1\text{H}\}$ - ^{15}N nuclear Overhauser effect (^1H - ^{15}N NOE), dubbed hetNOE in the following, is the ratio of the cross-relaxation rate and the ^{15}N longitudinal relaxation rate. It leads to a reduction of the steady-state polarization on ^{15}N due to cross-relaxation with the proton upon saturation of the proton polarization ^{45,53,54,74,75}. The cross-relaxation depends on spectral density functions of the sum and difference of the ^1H and ^{15}N Larmor frequencies. Therefore, the hetNOE is sensitive both to fast picosecond dynamics (< 100 ps) and also to ps-ns dynamics (due to its R_1 dependence). The sequence (Figure 2C, ⁶⁹) is based on a sensitivity-enhanced HSQC read-out with Echo/ Anti-Echo gradients for quadrature detection. For saturation of the proton magnetization and the resulting hetNOE, the equilibrium proton magnetization is inverted and subsequently saturated by rapidly pulsing 180° pulses for about 5 times the ^{15}N T_1 . For the reference experiment, the recovery delay is equal to the saturation delay but without the ^1H 180° pulse train. An extra delay of $D1 = 2$ s is added for the reference experiment and the one with ^1H saturation. Both experiments are recorded back-to-back and differ only in applying ^1H 180° pulses (saturation) or not (reference). The ratio of spectral intensities recorded in the experiment with ^1H saturation divided by the intensities of the reference experiment (without the 180° proton pulse train) gives the $\{^1\text{H}\}$ - ^{15}N NOE (hetNOE) value.

The following protocol describes the setup of NMR relaxation experiments by Lakomek et al. ⁶⁹ and Stief et al. ⁷⁰. We focus on NMR pulse sequences using a sensitivity-enhanced HSQC detection scheme. The ^{15}N R_1 and $R_{1\rho}$ experiments are implemented as described in detail by Stief et al. ⁷⁰, and the hetNOE experiment is described by Lakomek et al. ⁶⁹.

1. NMR sample preparation

NOTE: Isotope labeling of the proteins is performed for higher-dimensional NMR and advanced NMR experiments. When protein expression in *E. coli* and protein purification had been established using rich media (e.g., LB or 2YT) with a yield of several milligrams per liter, preparing an isotopically labeled NMR sample is usually relatively straightforward.

1.1. For isotope labeling, use M9 minimal media for gene expression, where ^{15}N ammonium chloride is the only nitrogen source and ^{13}C -enriched glucose is the only carbon source.

1.2. For protein purification, follow the standard protocol established in the laboratory for the respective protein.

NOTE 1: The results section shows representative results for an intrinsically disordered protein (IDP), the monomeric SNARE protein Synaptobrevin-2 (1-96). NMR isotope labeling of Synaptobrevin-2 has been performed as described in ⁷⁰.

NOTE 2: For very high-precision measurements, using low D_2O concentrations, less than 5%, is recommended. This is because of the deuterium isotope effect, which can be observed in Hahn-Echo ^{15}N T_2 and at low-frequency ^{15}N CPMG relaxation dispersion experiments (not discussed here) but has only a minimal impact in ^{15}N $R_{1\rho}$ experiments (where it is primarily refocused) ⁷⁶.

[Place **Figure 2** here]

2. Preparations for running the NMR relaxation experiments on the spectrometer

NOTE: The described NMR relaxation experiments are specific for Bruker spectrometers. They have been tested on cryogenic and room temperature ^1H , ^{15}N , and ^{13}C triple resonance probes and Avance III and Avance Neo consoles operated by the Bruker software Topspin 3.6 or higher.

2.1. Precautions

2.1.1. Do a safety briefing before working at the NMR spectrometer.

2.1.2. Check the probe's power specifications of the used spectrometer and choose an appropriate pulse length and pulse power for every pulse, e.g., by comparing consistency with the Topspin “prosol” table entries.

2.2. Importing the pulse sequences

2.2.1.- Download the NMR pulse programs either from the website indicated below or from the extended Biological Magnetic Resonance Bank (BMRB) repository (bmrbig102) to the directory */opt/topspin4.1.4/exp/stan/nmr/lists/pp/user* of the computer operating the NMR spectrometer. Please adjust the topspin version (here: topspin4.1.4 to the respective version installed on the spectrometer).

2.2.2. Download the NMR shaped pulses either from the website indicated below or from the extended Biological Magnetic Resonance Bank (BMRB) repository (bmrbig102) to the directory */opt/topspin4.1.4/exp/stan/nmr/lists/pp/user* of the computer operating the NMR spectrometer. Please adjust the topspin version (here: topspin 4.1.4 to the respective version installed on the spectrometer).

2.3. Calibration of the spectrometer and sample

2.3.1. Set the temperature of interest. For temperature calibration, apply your facility's protocols. For example, a Methanol-d₄ sample can be used, and the observed chemical shift difference can be interpreted using the web application “NMR thermometer”

(<https://chem.ch.huji.ac.il/nmr/software/thermometer.html>); see also⁷⁷.

2.3.2. Insert the sample (plus an additional 5% D₂O for the lock) and wait approximately 10 min to allow the sample to equilibrate its temperature. Lock the deuterium (²H) signal (Bruker command: **lock**, select H₂O+D₂O).

2.3.2. Match and tune the ¹H, ¹⁵N, and ¹³C channels, e.g., using the Bruker **atma** or **atmm** routine. If no automatic matching and tuning unit is installed, manually match and tune the probe using the **wobb** command.

2.3.3. Perform shimming for a homogenous B₀ field, e.g., using the Bruker topshim routine **tg**.

2.4. Calibration of the 90° (¹H) pulse length

2.4.1. Use the Bruker zg experiment. Use **getprosol** to import the probe parameters. Choose a short duration of 5 μs for P1. Check and correct the specified power level of the probe for PLW1 if needed.

2.4.2. Start the experiment with **zg**. Process the data with **efp**. Adjust the phase with **.ph** so that the baseline is even and the NMR signal of the water resonance is in phase.

2.4.3. Estimate a 360° (¹H) pulse by taking a common or approximate 90° (¹H) pulse (e.g., from the listed spectrometer specifications) and multiplying it by a factor of four. Run the experiment and process it.

NOTE: The chosen duration is too long if the signal is positive and too short if it is negative. The 360° (¹H) pulse will be the zero crossing.

2.4.4. Adjust the pulse length accordingly. Do this iteratively to find the zero crossing. Divide the obtained value by four. That will be your 90° (¹H) pulse.

NOTE: An alternative to the described 360° method is the Bruker TOPSPIN *pulscal* routine, which can be started from the Bruker command line and allows an automated ¹H pulse calibration

2.5. Calibration of the carrier frequency O1 to the water resonance

2.5.1. Copy the zg experiment by typing *edc* in the command line to a new experiment number. Type *rpar* to open the parameter sets. (Figure S1A)

2.5.2. Search for the parameter set ZGPR. Click on the parameter set ZGPR (Figure S1A), click on *Read...*, choose *execute "getprosol"* and click *OK* (Figure S1B).

2.5.3. Change P1 to the calibrated 90° (¹H) pulse length. Choose a saturation length of D1 = 2 s and a pulse power of PLW9 of 50 dB. Set the receiver gain (**rg**) to 16. Alternatively, execute the receiver gain adjustment by typing **rga** and setting the receiver gain to the specified value. (But avoid “receiver gain overflow”).

2.5.4. Type **gs** in the command line to start for a repeated, continuous acquisition. Go to the acquisition window and change the O1 to reduce the FID area dominated by the water signal (Figure S2).

2.5.5. Click below the marker to decrease the O1, and click above the marker to increase it. The sensitivity button can be used to change the step size. *Save* the current O1; *Close* stops the repeated acquisition (Figure S2).

2.6. Calibration of the 90° (¹⁵N) pulse length

2.6.1. Copy the zg experiment to a new experiment number (type *edc*). Type *rpar* to read the parameter set HSQCFFPF3GPPHWG to change the pulse program to a ¹H-¹⁵N HSQC experiment. Use the option *Keep parameters P1, O1, and PLW1* (Figure S1).

2.6.2. Type **getprosol 1H <calibrated 90° (¹H) pulse length in μs> <90° (¹H) pulse strength in db>** in the command line to import the probe parameters. Set the number of scans (**NS**) to 8 and the dummy scans (**DS**) to 2.

NOTE: <> specifies here the values to be set. For example: getprosol 1H 8.5 -11.79.

2.6.3. Run the first increment of the experiment by typing **zg** in the command line. Copy the first increment to a new experiment number with *rser 1 <new experiment number>*. Process the data with *efp* and phase the spectrum with *.ph*. Use the first increment (1D experiment) of the experiment for the following steps.

NOTE: The <> specify the chosen number for the new experiment, e.g., rser 1 101.

2.6.4. Type **ased** in the command line to open the pulse sequence parameters of the 1D experiment. Click on the **E** in the PULPROG line (see Figure S3A) to open the pulse program editor.

2.6.5. Use the following workflow to save the pulse sequence under a new name: Click *File* (left upper corner), *Save as...*, enter the new name, and click OK. Then click Set PULPROG to the dataset (see Figure S3B) and click *OK*.

2.6.6. Modify the original ^1H - ^{15}N HSQC sequence (saved under a new name, see 2.6.4.) by multiplying the 90° (^{15}N) after the t_1 evolution period (before the back transfer) by a factor of 2 (the 90° (^{15}N) pulse is now 180° (^{15}N)). Press *Ctrl S* to save the pulse sequence. Set the pulse length to 90° (^{15}N) (P21) so that the NMR signal of the first increment will vanish (at the zero crossing) (Figure S3B).

2.6.7. Open the ^1H - ^{15}N HSQC experiment (2D experiment) and enter the calibrated 90° (^{15}N) pulse length (P21). Type **getprosol 1H <calibrated 90° (^1H) pulse length in μs > < 90° (^1H) pulse strength in db> 15N <calibrated 90° (^{15}N) pulse length in μs > < 90° (^{15}N) pulse strength in db>** in the command line.

2.7. Determination of the spectral widths and appropriation of acquisition times

2.7.1. In the acquisition parameter window for the ^1H - ^{15}N HSQC experiment (type *eda* in the command line), adjust the spectral width (Bruker acquisition parameter SW) and the number of dimension increments in direct and indirect dimensions (Bruker acquisition parameter TD).

NOTE: Choose for the SW 16 ppm in the direct (^1H) and 30 ppm in the indirect (^{15}N) dimension.

Choose for the TD 2048 in the direct (^1H) and 512 in the indirect (^{15}N) dimension.

2.7.2. Acquire the ^1H - ^{15}N HSQC experiment. Check that all signals of interest are detected and no aliasing artifacts are present in the spectrum. Change the O3P if the center of the spectrum doesn't fit with the center of the protein signal distribution along the ^{15}N axis. Increase the SW if the spectral window is too small. Increase the TD to gain a higher FID resolution (Check the probe specifications for the maximum duration of decoupling!).

NOTE: This is the general setup and the starting point for the setup of NMR relaxation experiments.

We recommend recording the ^1H - ^{15}N HSQC experiment at the beginning and end of the entire set of experiments and, for more extended experimental times, also in between to monitor sample integrity.

3. Implementing the NMR relaxation experiments.

NOTE: The NMR relaxation pulse sequences (Figure 2) are available at

<https://www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences>, or at the extended Biological Magnetic Resonance Bank (BMRB) repository (bmrbig102).

3.1. Setting up the ^{15}N $\text{R}_{1\rho}$ experiment

3.1.1. Copy the HSQC experiment (type *edc*) to a new directory. Type *ased* and change the pulse program to the ^{15}N $\text{R}_{1\rho}$ experiment by clicking on the three dots of the parameter line PULPROG in the pulse sequence parameters (*ased*, see Figure S3A). Double-click on the pulse program, click on Set PULPROG to dataset (see Figure S3B), and click *OK*.

NOTE: Some error messages may appear. Close these messages.

3.1.2. Type **gpnam** in the command line to insert all missing **gradient shapes** (gpnam1 - gpnam9) as described in the pulse sequence (Figure S4).

3.1.3. Type **vpulist** in the command line. Click on the three dots (Figure S5A), *File* (upper left corner), and *New....* (Figure S5B).

3.1.4. Type a name for a new dummy vplist, and press *OK*. Create the dummy vplist (5m, 65m, 25m, 45m, 15m, 55m, 35m, 10m; a new row for each vplist entry). Press *Ctrl S* to save the list and close the window of the vplist (Figure S5C).

3.1.5. Double-click on the name of the newly created vplist (Figure S5B).

NOTE: This is necessary to avoid compilation errors in the pulse sequence. The appropriate entries of the vplist will be determined further below (point 3.1.29.).

3.1.6. Type *ased* to open the pulse sequence parameters. Enter all missing **gradient strengths** and **gradient lengths** as given in the pulse sequence (Figure S6D).

3.1.7. Enter for the loop counter L_3 (number of complex points in the indirect dimension) 1/2 of the ^{15}N TD entry in the Bruker acquisition parameter window (**eda**). Enter for the loop counter L_6 the number of recorded relaxation data points used for fitting the relaxation curve.

NOTE: This number L_6 corresponds to the number of vplist entries, here: 8 (Figure S6A).

3.1.8. Set the adiabatic TanhTan_half.nl pulse, downloaded from the website specified above, as the shape of the ^{15}N SP8 pulse. Set the adiabatic TanhTan_2nd.nl pulse from the website as the shape of the ^{15}N SP9 pulse (Figure S6C).

3.1.9. Set the P8 entry to the length of the adiabatic TanhTan pulse - sufficiently long to guarantee the adiabaticity of the pulse; here $P8 = 3000\ \mu\text{s}$ is chosen (Figure S6C).

3.1.10. Set the ^{15}N decoupling (CPDPRG 3) to a garp decoupling scheme. Use for the length of decoupling PCPD3 $200\ \mu\text{s}$ (Figure S6C).

3.1.11. Set the interscan recovery delay to at least $D1 = 2\ \text{s}$ or longer.

NOTE: For the choice of a proper recovery delay, see the Discussions section.

3.1.12. Set the dummy scans (DS) to at least 64 for the final experiment (during the setup, DS = 4 is sufficient for time reasons). As a starting point, use a number of scans (NS) of 4; use multiples of 4 if the signal-to-noise (SNR) is too low (Figure S6A).

3.1.13. Set the O1 to the calibrated carrier frequency of step 2.5. Set the O2P to 176 ppm and copy the O3P from the ^1H - ^{15}N HSQC experiment (Figure S6A-C).

3.1.14. Set the pulse length P7 to the 90° (^{15}N) pulse length calibrated in step 2.6. Also, copy the pulse power level of the 90° (^{15}N) pulse from step 2.6 to PLW3 and PLW7 (Figure S6C).

3.1.15. Set the pulse length P1 and P19 to the 90° (^1H) pulse length calculated in step 2.4. (Figure S6B).

3.1.16. Set the number of increments in the indirect dimension $\text{TD} = \text{I}_3 * 2 * \text{I}_6$ (**eda** or type **td** in the command line).

NOTE: All pulse sequence parameters described here are additionally annotated in the pulse sequence.

3.1.17. Set the shape pulse SP5 to an I-BURP2 shape (Iburp2.1000). Set the pulse length P15 to 2000 μs . Open the shape tool display by clicking on the *E* next to the I-BURP2 shape pulse in the Topspin pulse sequence parameter window (**ased**) (Figure S6B).

3.1.18. Simulate the shape pulse with the correct length by clicking the *Start NMR Simulation* button. Check the Shaped pulse length [μs] (= 2000 μs) and the Rotation angle [$^\circ$] (= 180°) in the Simulation window. Click on *Start NMR-SIM* (Figure S7A).

3.1.19. Check the excitation range and choose the appropriate I-BURP2 pulse length to cover the protein's ^1H spectral dispersion but avoid excitation of the water at the same time. Set P15 to the Shaped pulse length of the Simulation window with which you simulated your best IBURP-2 pulse (Figure S7B).

NOTE: The proper length is chosen if the I-BURP2 excites ($> 95\%$ effectiveness) the entire amide region. To simulate an I-BURP2 pulse with a different length, close the Excitation profile window and edit the Shaped pulse length in the Simulation window. Click on *Start NMR-SIM*. Pulses with a shorter length have a broader excitation range in Hz.

3.1.20. Set SPOFFS5 to change the carrier frequency of the I-BURP2 pulse and shift the excitation range to the left or right to avoid the I-BURP2 pulse touching and disturbing the water magnetization (Figure S6B).

3.1.21. To determine the appropriate power level of the shaped pulse, open the Bruker shape tool, click the *Start NMR Simulation* button, and set your I-BURP2 pulse length to Shaped pulse length [μs]. Remember the corresponding length of a soft rectangular 90° (^1H) pulse, visible in the Simulation window at Corresp. 90° square pulse [μs] (Figure S7A).

3.1.22. Type **calcpowlev**. Use the hard 90° (^1H) pulse (P1) in μs as the reference length (Figure S8B) and the 90° (^1H) soft rectangular pulse corresponding to the I-BURP2 pulse in μs as the new length (Figure S8A). Remember the power difference Δ in dB between the hard 90° (^1H) pulse and the soft rectangular pulse (Figure S8C). Copy the pulse strength of the hard 90° (^1H) pulse in dB to SPW5 and add the memorized difference Δ in dB (Figure S6B).

3.1.23. To determine the power level of the spin-lock, calculate the corresponding 90° (^{15}N) pulse length of the spin-lock. Use the formula: **90° (^{15}N) pulse length in s = $0.25 / (\text{spin-lock power in Hz})$** .

3.1.24. Use **calcpowlev** to calculate the power difference Δ in dB between the power of the spin-lock and the 90° (^{15}N) hard pulse (P7). New: 90° (^{15}N) pulse length of the spin-lock in μs (Figure S8A). Ref: hard 90° (^{15}N) pulse length in μs (Figure S8B). Copy the power level PLW7 of the 90° (^{15}N) hard pulse in dB to the spin-lock power level PLW8 (Figure S6C), and add the calculated power difference Δ in dB (Figure S8C).

3.1.25. Copy the power level of the spin-lock PLW8 to the power levels SPW8 and SPW9. (Figure S6C).

3.1.26. To determine the power level of the ^{15}N decoupling, use **calcpowlev** to calculate the power difference Δ between the power of the 90° (^{15}N) decoupling pulse (PCPD3) and the 90° (^{15}N) hard pulse (P7). New: PCPD3 in μs (200) (Figure S8A). Ref: hard 90° (^{15}N) pulse length in μs (Figure S8B). Copy the power level PLW7 of the 90° (^{15}N) hard pulse in dB to the decoupling level PLW31 (Figure S6C) and add Δ in dB (Figure S8C).

3.1.27. To use temperature compensation, include `#define TEMP_COMPENSATION` (by deleting the semicolon in the pulse sequence). Set P18 to the maximum duration of the spin-lock used in the ^{15}N $\text{R}_{1\rho}$ experiment.

NOTE: We recommend switching off the temperature compensation for the first trial of the experiment.

3.1.28. In the case of $^{15}\text{N}^{13}\text{C}$ labeled samples, include `#` to *define LABEL_CN* in the pulse program (by deleting the semicolon in the pulse sequence). Set P4 to the pulse length calculated with the formula in ⁷⁸. For example, it corresponds to 23.70 μs at 600 MHz and 11.85 μs at 1200 MHz. Type **calcpowlev** to calculate the power difference in dB between P4 and the (^{13}C) hard pulse. Copy the (^{13}C) hard pulse power in dB to PLW4 and add the Δ in dB. Copy PLW4 to PLW2 (Figure S6B).

3.1.29. Determine the relaxation delays for appropriate sampling. Run the first eight FIDs (in case $L_6 = 8$) and process these (*rser <number of FID> <new experiment number>*). Choose the entries in the

vplist that the peak intensity of the experiment with the longest delay decreases to at least 1/e (approx. 37%), but not less than 25% compared to the experiment with the shortest delay (which serves as the reference). Adjust the remaining entries of the vplist to achieve an even sampling of the intensity decreases between the FID with the longest delay and the FID with the shortest delay. Read the NOTE and the power specifications of the probe!

NOTE: The slowly relaxing residues will dominate the first FID. Adjust the remaining entries of the vplist to achieve an even sampling of the intensity decreases between the first and second FID. **Do not exceed the probe's maximum allowed spin-lock power and length x (consult probe manual and probe specifications).** In case of temperature compensation, check P18 (maximum spin-lock duration), as the overall spin-lock duration of P18 will be applied, split between the relaxation delay and temperature compensation. It holds $\text{time (relaxation delay)} + \text{time (temperature compensation)} = \text{const} = \text{P18}$. For a 2 kHz spin-lock, we prefer not to exceed a spin-lock duration of more than 65 ms, using a recovery delay (D1) of 2 s. In that case, a compromise between the maximum spin-lock duration and the longest relaxation delay must be taken in favor of the maximum allowed spin-lock duration. (In case of longer D1 recovery delays, longer spin-lock powers may be applicable. What counts is the overall power deposition in the probe over time. If longer spin-lock durations are required, only increase the spin-lock lengths in small steps and watch the lock signal. (As a rule of thumb, adjust the lock level in the upper third of your lock display. If the lock level reduces by more than two squares during the execution of the pulse sequence, the spin-lock power deposited in the probe is too much.)

3.1.30. Type *rga* in the Topspin command line to determine the receiver gain. For a good signal-to-noise ratio, aim for a receiver gain (*rg*) value between 128 and 256, which proves good water suppression.

NOTE: If a receiver gain between 128 and 256 cannot be reached, this indicates a too strong water signal. In that case, gently adjust the gradient power and durations until the receiver gain is reached. See also the Discussions section.

3.1.31. Start a test run of the ^{15}N R_{1ρ} experiment by typing *zg* in the Topspin command line. Check that the water signal is well suppressed for all delays (1 to 8, in case of 8 delays in the vplist). Check also increment 9, which is the second increment of the quadrature detection scheme (Echo/ Anti-Echo).

3.2. Setting up the ^{15}N R_1 experiment

3.2.1. Copy the ^{15}N $\text{R}_{1\rho}$ experiment and change the pulse program to the ^{15}N R_1 experiment.

3.2.2. Type ***vc*list**, generate a vclist, and enter the dummy vclist described in ⁷⁰: 0, 24, 6, 20, 4, 16, 8, 12; a new row for each vclist entry).

3.2.3. Open ***ased***. Check the gradient lengths and strengths. Change GPZ9 to 14%.

3.2.4. Check pulse lengths, pulse shapes, and pulse strengths (the correct values should have been copied from the ^{15}N $\text{R}_{1\rho}$ experiment).

3.2.5. In case of $^{15}\text{N}^{13}\text{C}$ **labeled samples** use ***#define LABEL_CN*** in the pulse program.

3.2.6. For the use of **temperature compensation**, include ***#define TEMP_COMPENSATION***.

3.2.7. Run the first eight FIDs (in case $L_6 = 8$) and process these (***rser <number of FID> <new experiment number>***). Choose the entries in the vclist that the peak intensity of the experiment with the longest delay decreases to at least $1/e$ (approx. 37%), but not less than 25% compared to the experiment with the shortest delay. Adjust the remaining entries of the vplist to achieve an even sampling of the intensity decreases between the FID with the longest delay and the FID with the shortest delay.

NOTE: Only even numbers are allowed for vclist entries!

3.3. Setting up the $\{^1\text{H}\}$ - ^{15}N NOE experiment

3.3.1 Copy the ^{15}N R_1 relaxation experiment and change the pulse sequence to the $\{^1\text{H}\}$ - ^{15}N NOE pulse scheme.

3.3.2. Set the length of the saturation period with the loop counter L_8 . The spacing between 180° (^1H) pulses is 22 ms ($2 * 1 / J_{\text{NH}}$, ⁷⁹). To achieve the most accurate results, use a saturation period of about 5 times the protein's ^{15}N $T_1(^{15}\text{N})$. Here: 5 s. That would correspond to $L_8 = 230$.

NOTE: The Discussion section provides additional advice on choosing the saturation period/recovery delay.

3.3.3. Change the length and strength of the gradients as described in the pulse scheme. Use sine.20 gradient shapes.

3.3.4. Set the number of increments in the indirect dimension equal to $\text{TD} = \text{I}_3 * 2 * 2$

3.3.5. In the case of $^{15}\text{N}^{13}\text{C}$ labeled samples, use `#define LABEL_CN` and `-DLABEL_CN` as a ZGPTNS.

3.3.6. Run the pulse schemes by typing **zg** in the command line.

4. Processing and analysis of the recorded NMR experiments

NOTE: Spectra have been recorded using a Bruker system.

Processing is performed using a Unix or Linux operating system. Spectra processing and data analysis were performed using NMRPipe ⁸⁰ and python3.

The NMRPipe software can be downloaded at <https://www.ibbr.umd.edu/nmrpipe/index.html>.

NMRPipe-based processing scripts from the website:

<https://www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences>.

or from the extended Biological Magnetic Resonance Bank (BMRB) repository (bmrbig102).

NOTE: We recommend using NMRPipe. If NMRPipe is not available or desired, alternatives such as CCPN⁸¹ or SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, or its successors NMRFAM-SPARKY⁸² or POKY⁸³) can be used.

4.1. Processing and analysis of ¹⁵N R₁ experiment.

NOTE: The following commands are executed in a Unix Terminal.

4.1.1. Copy the files in the downloaded directory R1_processing to the directory containing the experimental R1 data (working directory).

4.1.2. Make the files executable by the terminal command: > **chmod u+x *.com**

4.1.3. > **bruker** to open the NMRPipe Conversion Utility and generate the fid.com file. Click “Read Parameters”. Change the Dimension Count to two if three dimensions are counted, and press “Read Parameters” again. Then press “Clear Script,” “Update Script,” and “Save Script” (Figure S9).

NOTE: If the carrier frequency is not correct, change the Center Position PPM after clicking “Read Parameters” the second time.

4.1.4. > **./fid.com**

4.1.5. Convert the vclist in a vplist with ms entries: delay in ms = 40 ms * entry of vclist. Use > **cp vclist vplist** and edit the vplist.

4.1.6. > **./split.com** to split the pseudo 3D data into eight 2D files.

4.1.7. **Edit the tauValues** (vplist values in order of the vplist) and the idxExpmt values (order of tauValues regarding the time) in nmrproc.com (Figure S10).

4.1.8. > **./nmrproc.com** to process the raw data of all eight 2D spectra.

4.1.9. > **nmrDraw** to draw the spectra. Use the reference spectrum (first entry in vplist) for these steps. Switch to that spectrum by changing the z dimension to one (upper left corner). Adjust the contour level using the + and - next to the Factor button. Redraw the spectrum with the button “Draw.” Use a contour level where you can recognize all signals of interest with only a few signals coming from the noise (the contour level is at the edge of the noise level) (Figure S11).

4.1.10. Correct the spectrum phase, type “v” for vertical 1D-signal projection and “h” for horizontal 1D-signal projection. Adjust the phase with P0 and P1 (Figure S11), remember the phase difference, and correct the phase in the nmrproc.com file (Figure S10).

NOTE: If the spectrum is flipped in the ¹⁵N dimension, remove the *-neg* in the line: | *nmrPipe -fn FT - neg * (Figure S10).

4.1.11. Close nmrDraw and process again with > **nmrproc.com**. For Peak picking (after the spectrum is phased correctly and the reference spectrum is displayed with a contour level where all peaks are present), type “K” (shift + “k”), press “Detect”, and click on “Save”. (Figure S11).

4.1.12. Copy a peak list to your working directory and change *-assName* in **ass.com** to the name of your peak list (Figure S12).

NOTE: An example of a peaklist in the correct format is available at

<https://www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences>

and at the extended Biological Magnetic Resonance Bank (BMRB) repository (bmrbig102).

4.1.13. > **./ass.com** to assign the spectra. Verify the assignment that the script suggests for the various peaks. Use the left mouse button to assign and the right mouse button to deassign peaks. Use the “Next” button to go to the following amino acid and “Prev” to go to the previous amino acid (Figure S13).

NOTE: You can change the contour level with + and – at the top right corner. Use the button “Draw” to redraw the spectrum with the new contour level (Figure S13).

4.1.14. After completing the assignment, click “Save” and then “Save” again. Afterward, click on “Quit”, “Cancel”, and “Exit” (Figure S13).

4.1.15. > **python3 NoneDeleter.py** to create *relax.tab*, which contains only information on assigned peaks.

4.1.16. > **./relax.com** to generate the autofit.com script.

4.1.17. > **./autoFit.com** to perform a Fourier Interpolation and generate pseudo ratios of the different delay times for each amino acid (*axt.tab* and *nlin.tab* are generated).

4.1.18. > **./model.com** to generate the modelExp.com script based on the data in *nlin.tab*.

4.1.19.> **./modelExp.com** to fit the evolution curve of each residue with a Non-Linear Least-Squares Optimization model.

4.1.20. > **bash summary.tcl -in relax.tab > t1.tab**. Ignore a potential error message: *GDB Notice: 21 missing values in the table file can occur*.

4.1.21. Copy *t1.tab* into a newly created directory *R1R2*.

4.2. Processing and analysis of the ^{15}N $\text{R}_{1\rho}$ experiment.

4.2.1. Copy the files in the downloaded directory *R1rho_processing* to the directory containing the experimental $\text{R}_{1\rho}$ data (working directory).

4.2.2. > **chmod u+x *.com**

4.2.3. > **bruker** to open the NMRPipe Conversion Utility and generate the *fid.com* file. See also the processing of the ^{15}N R_1 spectra.

4.2.4. > **./fid.com**

4.2.5. > **./split.com** to split the pseudo 3D data into eight 2D files.

4.2.6. > **./nmrproc.com** (Edit the *tauValues* [*vplist* values] and the *idxExpmt* beforehand) to process the raw data of all eight 2D spectra (Figure S10).

4.2.7. > **nmrDraw** to draw the spectra and pick the peaks. Use the reference spectrum (first entry in *vplist*) for these steps. Follow the same procedure as for the ^{15}N R_1 experiment.

4.2.8. Copy a peak list to your working directory and change *-assName* in **ass.com** to the name of your peak list (Figure S12).

4.2.9. > **./ass.com** Follow the same procedure as for the ^{15}N R_1 experiment.

4.2.10. > **python3 NoneDeleter.py** to create *relax.tab*, which contains only information on assigned peaks.

4.2.11. > **./relax.com**

4.2.12. > **./autoFit.com**

4.2.13. > **./model.com**

4.2.14. > **./modelExp.com**

4.2.15. > **bash summary.tcl -in relax.tab > t1rho. tab**. Ignore potential error message: *GDB Notice: 21 missing values in the table file* may occur.

4.2.16. > Copy the following header in the *t1rho.tab* file (you get the values from the *fid.com* file) (Figure S14):

REMARK 600MHz for T1rho

REMARK -yOBS = 60.818 (put here the value found in *fid.com*)

REMARK -yCAR = 119.06 (put here the value found in *fid.com*)

4.2.17. > **./extract_PPM.com** will generate a table called *fusionOffset.tab* that contains the offset of the peak ^{15}N frequency relative to the carrier frequency (which will be needed to calculate the angle θ for the extraction of R_2 data from the R_1 and $\text{R}_{1\rho}$ data).

4.3. Summary of the relaxation data

4.3.1. Copy the *t1rho.tab* and the *fusionOffset.tab* into the directory *R1R2*.

4.3.2. Copy the script *crtR1p.tcl* in the directory *R1R2*, adjust the spectrometer frequency and spin-lock power, and execute **bash crtR1p.tcl | sort -n > R1R2.tab**. (Figure S15)

4.3.3. *R1R2.tab* will contain the fitted $R_{1\rho}$ rate constants, theta angle, R_2 rate constants, and R_1 rate constants.

4.4. Processing and analysis of the hetNOE experiment.

4.4.1. Copy the files in the downloaded directory *NOE_processing* to the experimental NOE data directory (working directory).

4.4.2. > **chmod u+x *.com**

4.4.3. > **bruker** to open the NMRPipe Conversion Utility and generate the *fid.com* file. Click Read Parameters. Change the Dimension Count to two if three dimensions were counted and press “Read Parameters” again. Then press “Clear Script”, “Update Script”, and “Save Script”.

4.4.4. > **./fid.com**

4.4.5. > **./split.com** (A *vplist* with two entries (0, 1) has to be created before)

4.4.6. > **./ft2.com**

4.4.7. > **nmrDraw** to draw the spectrum and pick the peaks in the reference spectrum (z-dimension = 1). Extract and remember the noise (press T = shift + “t”). Phase correction and peak picking follow the same procedure as the ^{15}N R_1 experiment.

4.4.8. Copy a peak list to your working directory and change *-assName* in **ass.com** to the name of your peak list (Figure S12)

4.4.9.> **/ass.com** to assign the spectra. Follow the same procedure as detailed for the ^{15}N R_1 experiment.

4.4.10. > **python3 NoneDeleter.py** to create *relax.tab*.

4.4.11. > **./script.com**

4.4.12. > **./autoFit.com**

4.4.13. > Change the noise in *error_hetnoe.tcl*.

4.4.14. > **bash error_hetnoe.tcl |sort -n > hetNOE.tab**

REPRESENTATIVE RESULTS:

The following shows some exemplary NMR relaxation data recorded on the vesicular SNARE protein Synaptobrevin-2 (1-96), frequently called VAMP2 (vesicle-associated protein 2). For recording the NMR data, we used a 171 μM ^{15}N Synaptobrevin-2 (1-96) sample (dubbed Syb-2 in the following) in 50 mM MES (pH 6.0) buffer containing 150 mM NaCl, 0.1 mM TCEP and 1 mM EDTA. All experimental data was recorded at 278.15 K using a 250 μl volume filled in a 3 mm NMR sample tube. Experiments were performed at a Bruker 600 MHz AVANCE III HD spectrometer equipped with a Bruker 5 mm QCI ^1H , ^{15}N , ^{13}C , ^{31}P quadruple resonance cryoprobe.

Figure 3 displays the residue-specific ^{15}N R_1 and R_2 relaxation rate constants fitted to the NMR relaxation curves obtained from the NMR relaxation experiments. On the y-axis, the respective relaxation rate constants are plotted for the individual residues along the protein sequence (on the x-axis). Also, ^1H - ^{15}N heteronuclear NOE values (hetNOE) extracted from the intensity ratios of the saturated and non-saturation (reference) experiments of the ^1H - ^{15}N heteronuclear NMR measurement are shown.

Heteronuclear NOE data and ^{15}N R_2 rate constants are more easily interpreted than ^{15}N R_1 data.

Therefore, we will start with them.

Heteronuclear NOE:

Heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE are sensitive to internal dynamics on the fast picosecond timescale (< 100 ps, because of its cross-relaxation dependence) but also to ps - ns dynamics (due to its R_1 dependence) and can take values usually between 0 and (a bit less than) 1 for folded proteins. At higher fields they will be systematically higher (but always lower than 1).

They describe the internal flexibility of the protein. When hetNOE values at the same field strengths are compared, it holds: The smaller the $\{^1\text{H}\}$ - ^{15}N NOE, the more dynamic the respective residue is.

For IDPs, typically small residue-specific $\{^1\text{H}\}$ - ^{15}N NOE are observed, e.g. in the order of 0.5 and smaller at 600 MHz. However, negative $\{^1\text{H}\}$ - ^{15}N NOE are also found for IDPs, even down to -2 at 500 MHz. For monomeric Syb-2, $\{^1\text{H}\}$ - ^{15}N NOE measured at 600 MHz range between -0.5 for the N-terminus and 0.5 for the C-terminal linker domain. Values increase gradually from the N- to the C-terminus, indicating gradually decreasing flexibility / increasing rigidity (Figure 3C).

^{15}N R_2 rate constants

^{15}N R_2 rate constants are sensitive to ps - ns dynamics that modulate the N-H bond vector orientation by the overall tumbling of the protein, the motion of the peptide planes, and the torsion of backbone dihedral angles. Different from the $\{^1\text{H}\}$ - ^{15}N NOE values and R_1 , the ^{15}N R_2 rate constants are dominated by the $J(0)$ spectral density associated with the overall (rotational) tumbling of the protein. Therefore, for globular folded proteins, the ^{15}N R_2 rate constants are scaled in size according to the overall tumbling and size of the protein.

For true IDPs, no global rotational correlation time can be assigned. Still, a chain-length dependent slow correlation time can be fitted ⁸⁴, as expected for polymers, but residue-specific internal dynamics have a larger impact on the ^{15}N R_2 rate constants. Within the same protein, ^{15}N R_2 rate constants allow for the discrimination of structures vs non-structured regions with different internal residue-specific mobility. The smaller the observed ^{15}N R_2 rate constant, the more mobile the respective residue is. High ^{15}N R_2 rate constants are characteristic of secondary structure elements, such as α -helices or β -strands. Low R_2 relaxation rate constants are characteristic of loop and intrinsically disordered regions. For monomeric Syb-2 (1-96), we observe overall low ^{15}N R_2 rate constants consistent with its intrinsically disordered character. The internal rigidity increases (mobility decreases) from the N-terminus up to the C-terminal linker domain before trailing off to the flexible C-terminus (Figure 3B).

¹⁵N R₁ rate constants

The R₁ relaxation rates are sensitive to picosecond (ps) and faster nanosecond (ns) dynamics. However, their direct interpretation of internal dynamics is more complex than previous ones. There is a field T₁ (R₁) minimum (maximum) when T₁ is plotted as a function of the rotational correlation τ_R : T_{1min} at $\tau_{R, \min} = 1/\omega$. For fast tumbling molecules with a $\tau_R < \tau_{R, \min}$, e.g., small molecules or small globular proteins, lower R₁ rate constants indicate increased protein backbone dynamics (same as for R₂). In the case of larger proteins, with $\tau_R > \tau_{R, \min}$, it is the opposite, and higher R₁ relaxation rate constants represent increased dynamics (opposite to R₂).

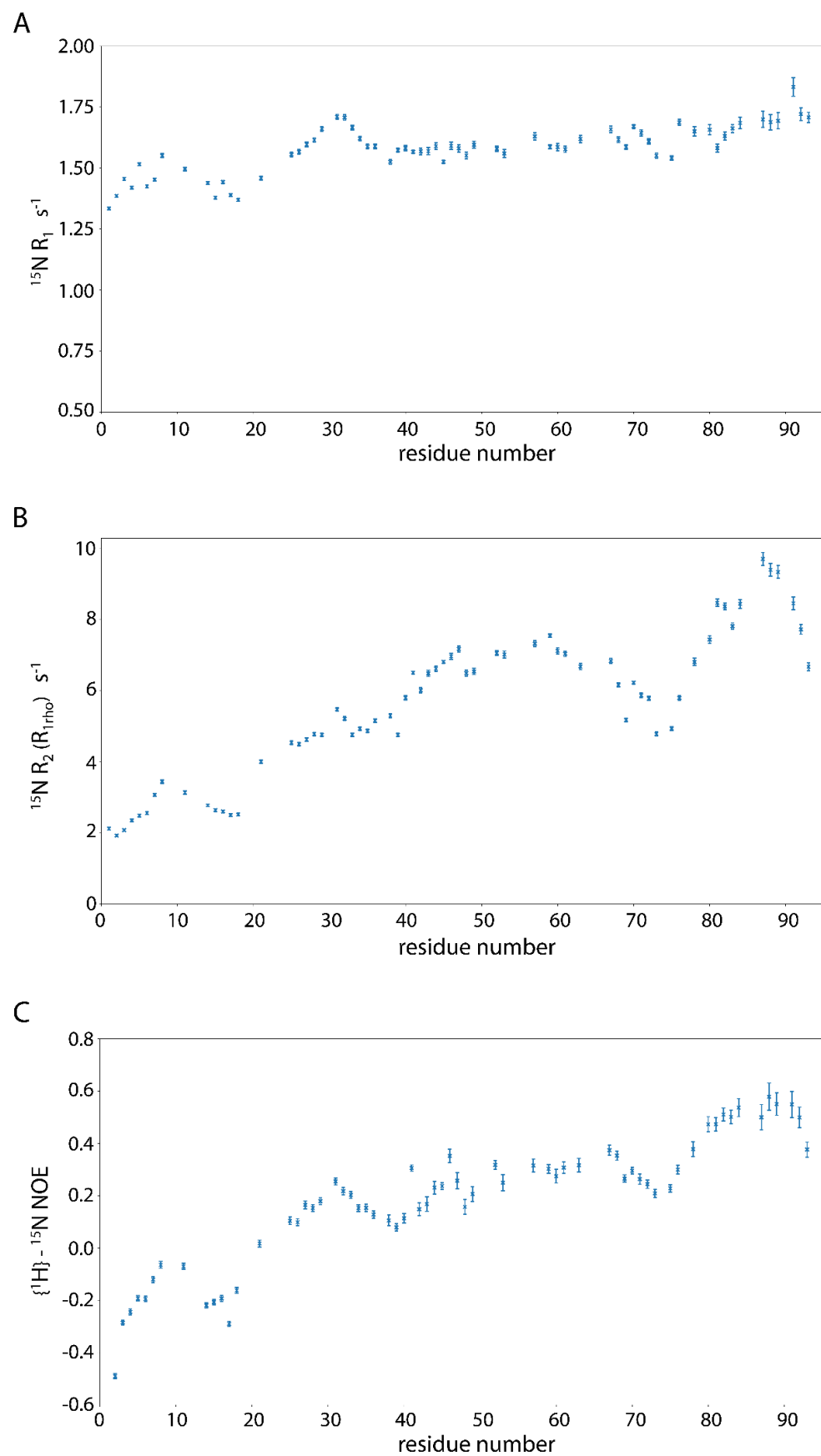


Figure 3: NMR relaxation data. Data were recorded and evaluated following the protocol and workflow described above. A: Residue-specific ^{15}N R_1 relaxation rate constants along the protein backbone of Syb2 (1-96). B: ^{15}N R_2 relaxation rate constants, calculated from $R_{1\rho}$ and R_1 data using formula (1). C: hetNOE transfer rates along the backbone of Syb2 (1-96).

FIGURE AND TABLE LEGENDS:

Figure S1: Reading in of NMR parameters. The interface of the Bruker Topspin macro *rpar* is displayed. A: Overview of all parameter sets. (The red box marks the search bar. The red numbers (here 5.2. and 6.1.) refer to the respective point in the protocol.) DoubleClick to choose a parameter set. B: Overview of parameters that can be imported to the current experiment. Options to execute *getprosol* or to keep specific parameters of the current experiment are marked with red boxes.

Figure S2: Positioning the ^1H carrier frequency for optimal water suppression. The interface of the Bruker macro *gs* is displayed. While the experiment is looped it is possible to change different parameters of the experiment. The marker can be used here to change the chosen parameter during the acquisition. Clicking above the marker raises the parameter by the value specified under “sensitivity”. The sensitivity switch can be used to adjust the step size of the marker. On the right side, the live FID is visible. For calibration of the O1, the aim is to minimize the FID area marked with a red box, as the FID is usually dominated by the water signal. The reduction of the FID area goes in hand with decreased water signal and improved water suppression.

Figure S3: A closer look at the ^1H - ^{15}N HSQC pulse sequence and its acquisition parameters.

A: Pulse sequence parameters of the ^1H - ^{15}N HSQC experiment HSQCFPF3GPPHWG. Click on “E” to open the pulse program editor window in B. B: Pulse program editor of the ^1H - ^{15}N HSQC experiment HSQCFPF3GPPHWG. The commented line (line 91) with a ^{15}N 90° pulse shows the original line for a ^1H - ^{15}N HSQC experiment. In line 92, the ^{15}N 90° pulse is changed to a ^{15}N 180° pulse. This change makes the ^1H - ^{15}N HSQC experiment a calibration pulse program for the 90° ^{15}N hard pulse calibration.

To edit bruker pulse sequences, save the sequence under a new name first. Use the following workflow: Click *File, Save as...*, enter the new name, click *OK*, and click *Set PULPROG to the dataset*.

Figure S4: Setting up the Gradients. The interface of the *gpnam* macro to change gradient shapes, lengths, and strengths is shown. To open the pulse sequence parameter interface used, all gradient shapes GPNAM must be entered.

Figure S5: Setting up the relaxation delays. Interface of the *vpulist* macro (identical to the *vclist* macro). A: Interface to choose a *vpulist*. By clicking on the three dots, interface B opens. B: Interface to choose a *vpulist* by DoubleClick, or create a new list by clicking on *File*, and *New....* C: Opens by creating a new list or editing an existing list. The list can be saved by clicking *File* and *Save*.

Figure S6: Pulse sequence parameters of the ^{15}N R1 ρ experiment. All essential parameters that must be changed in the protocol are marked with red boxes and the associated step.

Figure S7: Interface of the *shapetool* macro. An I-BURP2 (used in the experiments) is opened as a shape pulse. A: Parameters of the selected shape pulse. By clicking the *Start NMR Simulation* button marked with a red box, the Simulation parameter windows open with the simulation parameters Shaped pulse length [μs] (= 2000 μs) and the Rotation angle [$^{\circ}$] (= 180 $^{\circ}$). Clicking the *Start NMR-SIM* button opens the simulation window in B. B, which shows the inversion bandwidth of the I-BURP2 pulse.

Figure S8: Interface of the *calcpowlev* macro. A: Input window for the pulse length of the pulse whose power is to be calculated. B: Input window of the reference pulse (mostly the 90 $^{\circ}$ hard pulse). C: The result window shows the pulse strength difference in dB between a pulse with the new pulse length and a pulse with the reference pulse length. Attention: This difference is only valid if both pulse lengths correspond to the identical pulse regarding shape and rotation angle!

Figure S9: NMR spectra processing: Interface of the NMRPipe conversion tool. Clicking on *Read Parameters* automatically reads the experiment's parameters. The conversion script is shown on the right. All parameters can be set individually. After changing parameters using *Clear Script*, Updating the *Script* is recommended. Click on *Save Script* to save the script under fid.com, as shown on the right.

Figure S10: The nmrproc.com file for spectral processing. The code used in the nmrproc.com file to process the raw data in NMRPipe format is shown. The tauValues entry and idxExpmt entry have to be set before processing. The tauValues correspond to the vplist entries in ms for the ^{15}N R_{1ρ} experiment (vclist entries recalculated in ms for the ^{15}N R₁ experiment). Regarding their relaxation delay length, the idxExpmt entries correspond to the order of the vplist entries (vclist entries for the ^{15}N R₁ experiment). In the script, the positions to change the phase of the spectrum are marked with red boxes.

Figure S11: NMR spectra display and peak picking. An overview of nmrDraw with an open spectrum is shown. Use the “+” and “-” to change the Factor, which goes in hand with a change in the contour level. Changing the “Z” dimension allows for the switch between the HSQC experiment with different relaxation delays. Using *Draw* to redraw the spectrum with the changed parameters. The “P0” slider and “P1” slider allow changes in the phase of the spectrum (These phase changes will not be saved in the processed data; the phase change has to be entered in the nmrproc.com file manually, and the nmrproc.com file has to be executed again). The Peak Detection macro allows picking the spectrum peaks at the contour level set.

Figure S12: Semi-automated NMR peak assignment with NMRDraw. The code of the ass.com file is shown, and it must be executed to start the NMR signal assignment. Change -assName to the peak list of interest you generated yourself. This peak list has to be in the same format as the attached peak list ass.syb2.hn_1200.tab in the folder peaklist (In the zip folder downloaded at <https://www.ipb.hhu.de/en/teams/team-lakomek/pulsesequences>).

Figure S13: Visual NMR peak and assignment inspection. The Interface of the assignment macro that is opened by executing `ass.com`. Use the left mouse button to assign peaks and the right mouse button to deassign peaks. Use “Next” to go to the following amino acid and “Prev” to go to the previous amino acid. With “+” and “-,” the contour level can be changed using “Draw,” which redraws the spectrum. Using the workflow: “Quit”, “Save”, and “Exit” saves the assignment and ends the program.

Figure S14: Extracting the ^{15}N R_2 rate constants from $R_{1\rho}$ and R_1 data. Step 1. The content of the `tlrho.tab` file is shown. A code marked with a red box has to be added before calculating the R_2 rates from the $R_{1\rho}$ rates.

Figure S15: Extracting the ^{15}N R_2 rate constants from $R_{1\rho}$ and R_1 data. Step 2. The content of the `crtR1p.tcl` file is shown. Before executing, the entries in the red box must be changed as described in the protocol.

DISCUSSION:

This protocol described the setup of NMR ^{15}N relaxation experiments by Lakomek et al.⁶⁹ and Stief et al.⁷⁰. We focused on NMR pulse sequences using a sensitivity-enhanced HSQC detection scheme. The ^{15}N R_1 and $R_{1\rho}$ experiments are implemented as described in detail by Stief et al.⁷⁰, and the hetNOE experiment is described by Lakomek et al.⁶⁹.

When setting up the NMR relaxation experiments, the ^{15}N $R_{1\rho}$ is the most critical one, as choosing a too-long maximum relaxation period or a too-high RF amplitude of the spin-lock can damage the probe. For the relaxation period, sampling the full decay curve equally from 100% intensity to 25% intensity (compared to the reference experiment) is desirable. For slowly relaxing proteins, such as IDPs, with

long ^{15}N T_2 times, it will frequently not be affordable to sample the decay curve to the full extent because the power deposition into the probe needs to be limited.

For the ^{15}N radio frequency (RF) amplitude, theoretically, the higher the RF amplitude, $\nu_{\text{spin-lock}}$, the better, as more exchange processes can be refocused. As a rule of thumb, exchange processes slower than $1 / (2\pi \nu_{\text{spin-lock}})$ can be refocused. However, RF power limitations apply. For example, in our hand, we would NOT(!) exceed a maximum spin-lock period of 65 ms when an RF amplitude of 2 kHz is applied, to stay on the safe side. At the same time, the spin-lock RF amplitude at a 600 MHz spectrometer should be higher than 1 kHz, e.g., 1.4 kHz at 600 MHz, which has proven sufficient for the small globular protein gb3⁶⁹. To cover the spectral widths and avoid off-resonance effects at higher field strengths, the corresponding RF amplitude should scale with the magnetic field, e.g., 1.5 times higher at 900 MHz ($\nu_{\text{spin-lock}} \approx 2$ kHz). But must stay within the limitations of the probe. (Refer to the local list of spectrometer power limitations and contact your NMR facility manager for advice.)

For the choice of the spacing of relaxation delays between the reference experiment (shorted delay) and the longest delay period (determined by the factors discussed above), we prefer equal spacing of the relaxation delay data points and choose a number of 8 (if allocated measurement time and signal-to-noise allows). The first increment (and subsequent ones) will be dominated by the most intense (and frequently slowest relaxing) residues. Therefore, depending on the dynamic range of the protein, the NMR signals of several residues in the protein sequence may have already decayed after the chosen maximum relaxation period. By equally spacing delay points, we ensure that the relaxation curves of the fast relaxing residues will also be covered by a sufficient number of data points sampling the relaxation curve.

Also, a good choice of recovery delay is essential to get accurate relaxation data and avoid long-term damage to the probe. We choose a recovery delay of at least $D1 = 2$ s to prevent any damage to the probe. For accurate results, the protein ^1H and ^{15}N longitudinal polarization must also be given sufficient time to return to equilibrium or at least a stable, steady state. The ^1H T_1 usually varies between 1 s and 4 s at 600 MHz for a small or medium-sized protein (up to 20 kDa); for larger proteins and higher fields, the ^1H T_1 will increase further. For the small globular protein gb3, we found $D1 = 3.5$ s sufficient for

the ^{15}N R_1 and $R_{1\rho}$ experiments. For the small IDP Syb-2 presented in this protocol, we recently showed that $D1 = 2$ s was adequate ⁷⁰.

The ^{15}N hetNOE has the lowest sensitivity, as excitation starts on ^{15}N , and no ^1H - ^{15}N INEPT at the beginning of the pulse sequence is applied. Further, the ^1H saturation period and ^{15}N recovery delay should be 5 times ^{15}N T_1 to obtain accurate data. While for the IDP Syb-2 presented here, a 5 s saturation/recovery delay plus an extra 2 s $D1$ recovery delay (total 7 s) was sufficient, for larger proteins and/ or deuterated proteins, this time can be substantially longer, e.g, 10 s or longer. However, if qualitative data are good enough, a slight systematic offset can be accepted. In that case, reasonable results can be obtained already at 3 times ^{15}N T_1 , as 95% of the saturation will be achieved. In that case, resulting hetNOE values will show a systematic offset to higher values.

In this protocol, we worked with fully protonated ^{15}N Syb-2, which is most cost-efficient. Side-chain deuteration is recommended to reduce the number of relaxation pathways and work with the most isolated ^1H - ^{15}N backbone amide spin pairs. However, comparing $^{15}\text{N}^2\text{H}$ Syb-2 and ^{15}N syb-2, we notice only very few differences between deuterated and fully protonated samples ⁷⁰. For practical purposes, NMR relaxation data are frequently recorded on the double or triple-labeled samples ($^{15}\text{N}^{13}\text{C}$ or $^2\text{H}^{15}\text{N}^{13}\text{C}$). The discussed NMR pulse sequences are capable of refocusing J coupling evolution between ^{13}C with ^1H or ^{15}N nuclei. Obviously, also relaxation pathways will increase when ^{13}C labeling is introduced. However, in our hands, for practical purposes, the introduced error, if at all, is less than 5% and might be acceptable when only qualitative data are needed.

When working with proteins of different sizes, for detection, there is frequently the question of whether to choose sensitivity-enhanced HSQC-detected experiments ⁷⁰ or TROSY-detected experiments ⁶⁹. For small proteins, the sensitivity-enhanced HSQC-detected NMR experiments offer a two-fold gain in signal-to-noise (provided that good water suppression can be obtained). For larger proteins, the TROSY-detected experiments will win over. The break-even is roughly 20 kDa for folded proteins. For IDPs, for which low R_2 rate constants can be obtained that increase roughly linearly with increasing chain

lengths, the break-even may be shifted to higher molecular weights. For practical purposes, we recommend implementing both the sensitivity-enhanced HSQC-detected or TROSY-detected experiments when in doubt. Then, choose the one that gives the best signal-to-noise in the first increment.

The discussion of whether to choose the sensitivity-enhanced HSQC-detected or TROSY-detected experiments only applies when signal-to-noise is the main criterion. When the smallest line widths and highest resolution are required, TROSY will always win. TROSY also always offers the best water suppression, as fewer proton pulses and no proton decoupling are required. We refer to ⁶⁹ for a more extended discussion.

One more remark on the issue of water suppression. While setting the receiver gain (rg) to 16 may be sufficient on Bruker Avance NEO consoles, a receiver gain in the range of 128-256 is usually desirable for (older) Bruker Avance consoles. The achievable rg (determined by the Bruker command rga) is frequently obtained from the intensity of the residual water signal to the noise level. A bad water suppression will always introduce additional noise in the spectra. Thus, even if an rg = 16 can be achieved on a Bruker Avance NEO console, the remaining water signal can substantially contribute to the noise level. Therefore, the signal-to-noise ratio would be reduced. Consequently, we recommend aiming at an rg level of 128-256. The presented pulse sequences ⁷⁰ have been tested at 600 MHz and 1200 MHz. Both field strengths work with the same gradient setup, as written in the pulse sequence. We recommend them at least as a good starting point for further optimization.

As mentioned in the introduction, the discussed NMR relaxation experiments cover only the ps - ns time range. For insights into the μ s - ms dynamics, we refer to relaxation dispersion experiments, CPMG (Carr-Purcell-Meiboom-Gill) experiments, and an extension of the $R_{1\rho}$ experiments with changing spin-lock amplitudes (or carrier frequencies) are used ^{44,46,58-61,85,86}. Dynamics slower than microseconds can be captured ¹⁵N CEST (Chemical exchange saturation transfer) NMR ⁶², exchange spectroscopy (EXSY, ms to s), or RT (Real-time) NMR (seconds to hours) ^{63,64}. The PRE (paramagnetic relaxation

enhancement) effect of paramagnetic probes, as well as residual dipolar couplings (RDCs), can be used to assess the entire range of ps to ms dynamics ⁶⁵⁻⁶⁸.

All these experiments cover only backbone dynamics. To investigate side-chain dynamics, Kay and co-workers introduced another plethora of NMR experiments, providing information on side-chain dynamics for proteins up to 1 MDa ^{2,87}.

Further analysis and interpretation of NMR relaxation data

To obtain more quantitative insights into protein backbone dynamics, a combined analysis of all three NMR parameters, ¹⁵N R₁, R₂, and hetNOE, is necessary - sometimes involving even further parameters, such as cross-correlated relaxation rate constants. Different sophisticated analysis methods have been developed. The so-called model-free approach has been widely applied in biomolecular NMR ^{43,88-91}. Also, spectral density mapping can provide valuable insights ^{55,92,93}. While these are powerful analysis methods for globular proteins with well-defined rotational correlation time, their applicability is limited for IDPs. A folded domain is necessary to define a rotational correlation time ³⁹. This can be partially circumvented when correlation function C(t) can be modeled by the sum of exponentially decaying functions in case the different timescales involved can be well-separated ^{23,31,84}. In the absence of a well-defined rotational correlation time, alternative approaches involving a distribution of correlation times, e.g., the IMPACT analysis ⁹⁴ or the DETECTOR analysis ^{95,96}, are an alternative. Furthermore, a combined analysis of NMR relaxation data and MD simulations is promising also for IDPs ⁹⁷; currently, however, still facing sampling and force-field limitations for full-atom simulations of larger IDPs ^{31,98}.

ACKNOWLEDGMENTS:

We thank Melinda Jaspert and Kevin Bochinsky for the helpful discussions. N.L. thanks the German Science Foundation for funding through the Heisenberg Program (DFG grant number 433700474). This

work is further supported by the project “Virological and immunological determinants of COVID-19 pathogenesis – lessons to get prepared for future pandemics (KA1-Co-02 “COVIPA”), a grant from the Helmholtz Association's Initiative and Networking Fund. We acknowledge generous access to the Jülich-Düsseldorf Biomolecular NMR Center, jointly run by Forschungszentrum Jülich and Heinrich Heine University Düsseldorf (HHU).

DISCLOSURES:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES:

- 1 Sekhar, A. & Kay, L. E. An NMR View of Protein Dynamics in Health and Disease. *Annu Rev Biophys.* **48** 297-319, doi:10.1146/annurev-biophys-052118-115647, (2019).
- 2 Rosenzweig, R. & Kay, L. E. Bringing dynamic molecular machines into focus by methyl-TROSY NMR. *Annu Rev Biochem.* **83** 291-315, doi:10.1146/annurev-biochem-060713-035829, (2014).
- 3 Palmer, A. G., 3rd. Enzyme dynamics from NMR spectroscopy. *Acc Chem Res.* **48** (2), 457-465, doi:10.1021/ar500340a, (2015).
- 4 Reif, B. Ultra-high resolution in MAS solid-state NMR of perdeuterated proteins: implications for structure and dynamics. *J Magn Reson.* **216** 1-12, doi:10.1016/j.jmr.2011.12.017, (2012).
- 5 Schanda, P. & Ernst, M. Studying Dynamics by Magic-Angle Spinning Solid-State NMR Spectroscopy: Principles and Applications to Biomolecules. *Prog Nucl Magn Reson Spectrosc.* **96** 1-46, doi:10.1016/j.pnmrs.2016.02.001, (2016).
- 6 Le Marchand, T. *et al.* (1)H-Detected Biomolecular NMR under Fast Magic-Angle Spinning. *Chem Rev.* **122** (10), 9943-10018, doi:10.1021/acs.chemrev.1c00918, (2022).
- 7 Pervushin, K., Riek, R., Wider, G. & Wuthrich, K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci U S A.* **94** (23), 12366-12371, doi:10.1073/pnas.94.23.12366, (1997).
- 8 Tzakos, A. G., Grace, C. R., Lukavsky, P. J. & Riek, R. NMR techniques for very large proteins and rnas in solution. *Annu Rev Biophys Biomol Struct.* **35** 319-342, doi:10.1146/annurev.biophys.35.040405.102034, (2006).
- 9 Sprangers, R. & Kay, L. E. Quantitative dynamics and binding studies of the 20S proteasome by NMR. *Nature.* **445** (7128), 618-622, doi:10.1038/nature05512, (2007).
- 10 Wright, P. E. & Dyson, H. J. Intrinsically disordered proteins in cellular signalling and regulation. *Nat Rev Mol Cell Biol.* **16** (1), 18-29, doi:10.1038/nrm3920, (2015).
- 11 van der Lee, R. *et al.* Classification of intrinsically disordered regions and proteins. *Chem Rev.* **114** (13), 6589-6631, doi:10.1021/cr400525m, (2014).
- 12 Tompa, P. Intrinsically unstructured proteins. *Trends Biochem Sci.* **27** (10), 527-533, doi:10.1016/s0968-0004(02)02169-2, (2002).
- 13 Dyson, H. J. & Wright, P. E. Coupling of folding and binding for unstructured proteins. *Curr Opin Struct Biol.* **12** (1), 54-60, doi:10.1016/s0959-440x(02)00289-0, (2002).

- 14 Uversky, V. N. Intrinsically disordered proteins in overcrowded milieu: Membrane-less organelles, phase separation, and intrinsic disorder. *Current Opinion in Structural Biology*. **44** 18-30, doi:10.1016/j.sbi.2016.10.015, (2017).
- 15 Patel, A. *et al.* A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell*. **162** (5), 1066-1077, doi:10.1016/j.cell.2015.07.047, (2015).
- 16 Abyzov, A., Blackledge, M. & Zweckstetter, M. Conformational Dynamics of Intrinsically Disordered Proteins Regulate Biomolecular Condensate Chemistry. *Chemical Reviews*. **122** (6), 6719-6748, doi:10.1021/acs.chemrev.1c00774, (2022).
- 17 Uversky, V. N. Unusual biophysics of intrinsically disordered proteins. *Biochim Biophys Acta*. **1834** (5), 932-951, doi:10.1016/j.bbapap.2012.12.008, (2013).
- 18 Papoian, G. A. Proteins with weakly funneled energy landscapes challenge the classical structure-function paradigm. *Proc Natl Acad Sci U S A*. **105** (38), 14237-14238, doi:10.1073/pnas.0807977105, (2008).
- 19 Uversky, V. N., Gillespie, J. R. & Fink, A. L. Why are "natively unfolded" proteins unstructured under physiologic conditions? *Proteins*. **41** (3), 415-427, doi:10.1002/1097-0134(20001115)41:3<415::aid-prot130>3.0.co;2-7, (2000).
- 20 Bah, A. *et al.* Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch. *Nature*. **519** (7541), 106-109, doi:10.1038/nature13999, (2015).
- 21 Tompa, P., Schäd, E., Tantos, A. & Kalmar, L. Intrinsically disordered proteins: emerging interaction specialists. *Curr Opin Struct Biol*. **35** 49-59, doi:10.1016/j.sbi.2015.08.009, (2015).
- 22 Dyson, H. J. & Wright, P. E. Perspective: the essential role of NMR in the discovery and characterization of intrinsically disordered proteins. *J Biomol NMR*. **73** (12), 651-659, doi:10.1007/s10858-019-00280-2, (2019).
- 23 Adamski, W. *et al.* A Unified Description of Intrinsically Disordered Protein Dynamics under Physiological Conditions Using NMR Spectroscopy. *J Am Chem Soc*. **141** (44), 17817-17829, doi:10.1021/jacs.9b09002, (2019).
- 24 Dobson, C. M. Protein folding and misfolding. *Nature*. **426** (6968), 884-890, doi:10.1038/nature02261, (2003).
- 25 Bertonecini, C. W. *et al.* Release of long-range tertiary interactions potentiates aggregation of natively unstructured alpha-synuclein. *Proc Natl Acad Sci U S A*. **102** (5), 1430-1435, doi:10.1073/pnas.0407146102, (2005).
- 26 Buell, A. K. *et al.* Solution conditions determine the relative importance of nucleation and growth processes in alpha-synuclein aggregation. *Proc Natl Acad Sci U S A*. **111** (21), 7671-7676, doi:10.1073/pnas.1315346111, (2014).
- 27 Salvi, N., Abyzov, A. & Blackledge, M. Atomic resolution conformational dynamics of intrinsically disordered proteins from NMR spin relaxation. *Progress in Nuclear Magnetic Resonance Spectroscopy*. **102** 43-60, doi:10.1016/j.pnmrs.2017.06.001, (2017).
- 28 Schneider, R., Blackledge, M. & Jensen, M. R. Elucidating binding mechanisms and dynamics of intrinsically disordered protein complexes using NMR spectroscopy. *Curr Opin Struct Biol*. **54** 10-18, doi:10.1016/j.sbi.2018.09.007, (2019).
- 29 Rezaei-Ghaleh, N., Blackledge, M. & Zweckstetter, M. Intrinsically disordered proteins: from sequence and conformational properties toward drug discovery. *Chembiochem*. **13** (7), 930-950, doi:10.1002/cbic.201200093, (2012).
- 30 Jensen, M. R., Salmon, L., Nodet, G. & Blackledge, M. Defining conformational ensembles of intrinsically disordered and partially folded proteins directly from chemical shifts. *J Am Chem Soc*. **132** (4), 1270-1272, doi:10.1021/ja909973n, (2010).
- 31 Camacho-Zarco, A. R. *et al.* NMR Provides Unique Insight into the Functional Dynamics and Interactions of Intrinsically Disordered Proteins. *Chem Rev*. **122** (10), 9331-9356, doi:10.1021/acs.chemrev.1c01023, (2022).
- 32 Mittag, T. & Forman-Kay, J. D. Atomic-level characterization of disordered protein ensembles. *Curr Opin Struct Biol*. **17** (1), 3-14, doi:10.1016/j.sbi.2007.01.009, (2007).
- 33 Dyson, H. J. & Wright, P. E. Unfolded proteins and protein folding studied by NMR. *Chem Rev*. **104** (8), 3607-3622, doi:10.1021/cr030403s, (2004).

-
- 34 Dyson, H. J. & Wright, P. E. NMR illuminates intrinsic disorder. *Current Opinion in Structural Biology*. **70** 44-52, doi:10.1016/j.sbi.2021.03.015, (2021).
- 35 Sugase, K., Dyson, H. J. & Wright, P. E. Mechanism of coupled folding and binding of an intrinsically disordered protein. *Nature*. **447** (7147), 1021-1025, doi:10.1038/nature05858, (2007).
- 36 Bessa, L. M. *et al.* The intrinsically disordered SARS-CoV-2 nucleoprotein in dynamic complex with its viral partner nsp3a. *Science Advances*. **8** (3), doi:ARTN eabm403410.1126/sciadv.abm4034, (2022).
- 37 Milles, S., Salvi, N., Blackledge, M. & Jensen, M. R. Characterization of intrinsically disordered proteins and their dynamic complexes: From in vitro to cell-like environments. *Progress in Nuclear Magnetic Resonance Spectroscopy*. **109** 79-100, doi:10.1016/j.pnmrs.2018.07.001, (2018).
- 38 Salvi, N., Abyzov, A. & Blackledge, M. Atomic resolution conformational dynamics of intrinsically disordered proteins from NMR spin relaxation. *Prog Nucl Magn Reson Spectrosc*. **102-103** 43-60, doi:10.1016/j.pnmrs.2017.06.001, (2017).
- 39 Gill, M. L., Byrd, R. A. & Palmer, A. G., III. Dynamics of GCN4 facilitate DNA interaction: a model-free analysis of an intrinsically disordered region. *Phys Chem Chem Phys*. **18** (8), 5839-5849, doi:10.1039/c5cp06197k, (2016).
- 40 Bertocini, C. W. *et al.* Structural characterization of the intrinsically unfolded protein beta-synuclein, a natural negative regulator of alpha-synuclein aggregation. *J Mol Biol*. **372** (3), 708-722, doi:10.1016/j.jmb.2007.07.009, (2007).
- 41 Rezaei-Ghaleh, N. *et al.* Local and Global Dynamics in Intrinsically Disordered Synuclein. *Angew Chem Int Ed Engl*. **57** (46), 15262-15266, doi:10.1002/anie.201808172, (2018).
- 42 Rezaei-Ghaleh, N., Parigi, G. & Zweckstetter, M. Reorientational Dynamics of Amyloid-beta from NMR Spin Relaxation and Molecular Simulation. *Journal of Physical Chemistry Letters*. **10** (12), 3369-3375, doi:10.1021/acs.jpclett.9b01050, (2019).
- 43 Palmer, A. G. NMR characterization of the dynamics of biomacromolecules. *Chemical Reviews*. **104** (8), 3623-3640, doi:10.1021/cr030413t, (2004).
- 44 Palmer, A. G. & Massi, F. Characterization of the dynamics of biomacromolecules using rotating-frame spin relaxation NMR spectroscopy. *Chemical Reviews*. **106** (5), 1700-1719, doi:10.1021/cr0404287, (2006).
- 45 Kay, L. E., Torchia, D. A. & Bax, A. Backbone dynamics of proteins as studied by ¹⁵N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. *Biochemistry*. **28** (23), 8972-8979, doi:10.1021/bi00449a003, (1989).
- 46 Mittermaier, A. & Kay, L. E. Review - New tools provide new insights in NMR studies of protein dynamics. *Science*. **312** (5771), 224-228, doi:10.1126/science.1124964, (2006).
- 47 Abragam, A. Principles of Nuclear Magnetism. *Oxford University Press*. (1983).
- 48 Ernst, R. R., Bodenhausen, G., Wokaun, A. Principles of Nuclear Magnetic Resonance in One and Two Dimensions. *Oxford University Press*. (1990).
- 49 Cavanagh, J., Fairbrother, W.J., Palmer, A.G. III, Skelton, N.J. Protein NMR Spectroscopy: Principles and Practice. *Academic Press*. (1995).
- 50 Levitt, M. H. Spin Dynamics: Basics of Nuclear Magnetic Resonance *Wiley*. (2008).
- 51 Keeler, J. Understanding NMR Spectroscopy. *Wiley*. (2010).
- 52 Bloembergen, N., Purcell, E. M. & Pound, R. V. Relaxation Effects in Nuclear Magnetic Resonance Absorption. *Physical Review*. **73** (7), 679-712, doi:DOI 10.1103/PhysRev.73.679, (1948).
- 53 Wangsness, R. K. & Bloch, F. The Dynamical Theory of Nuclear Induction. *Physical Review*. **89** (4), 728-739, doi:DOI 10.1103/PhysRev.89.728, (1953).
- 54 Redfield, A. G. On the Theory of Relaxation Processes. *Ibm Journal of Research and Development*. **1** (1), 19-31, doi:DOI 10.1147/rd.11.0019, (1957).

- 55 Peng, J. W. & Wagner, G. Mapping of the spectral densities of N-H bond motions in eglin c using heteronuclear relaxation experiments. *Biochemistry*. **31** (36), 8571-8586, doi:10.1021/bi00151a027, (1992).
- 56 Farrow, N. A. *et al.* BACKBONE DYNAMICS OF A FREE AND A PHOSPHOPEPTIDE-COMPLEXED SRC HOMOLOG-2 DOMAIN STUDIED BY N-15 NMR RELAXATION. *Biochemistry*. **33** (19), 5984-6003, doi:10.1021/bi00185a040, (1994).
- 57 Tolman, J. R. & Ruan, K. NMR residual dipolar couplings as probes of biomolecular dynamics. *Chemical Reviews*. **106** (5), 1720-1736, doi:10.1021/cr040429z, (2006).
- 58 Mulder, F. A. A., Skrynnikov, N. R., Hon, B., Dahlquist, F. W. & Kay, L. E. Measurement of slow (μ s-ms) time scale dynamics in protein side chains by N-15 relaxation dispersion NMR spectroscopy: Application to Asn and Gln residues in a cavity mutant of T4 lysozyme. *Journal of the American Chemical Society*. **123** (5), 967-975, doi:10.1021/ja003447g, (2001).
- 59 Tollinger, M., Skrynnikov, N. R., Mulder, F. A. A., Forman-Kay, J. D. & Kay, L. E. Slow dynamics in folded and unfolded states of an SH3 domain. *Journal of the American Chemical Society*. **123** (46), 11341-11352, doi:10.1021/ja011300z, (2001).
- 60 Akke, M. & Palmer, A. G. Monitoring macromolecular motions on microsecond to millisecond time scales by R(1) ρ -R(1) constant relaxation time NMR spectroscopy. *J. Am. Chem. Soc.* **118** (4), 911-912, doi:10.1021/ja953503r, (1996).
- 61 Loria, J. P., Rance, M. & Palmer, A. G. A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *Journal of the American Chemical Society*. **121** (10), 2331-2332, doi:DOI 10.1021/ja983961a, (1999).
- 62 Vallurupalli, P., Bouvignies, G. & Kay, L. E. Studying "Invisible" Excited Protein States in Slow Exchange with a Major State Conformation. *Journal of the American Chemical Society*. **134** (19), 8148-8161, doi:10.1021/ja3001419, (2012).
- 63 Jeener, J., Meier, B. H., Bachmann, P. & Ernst, R. R. Investigation of Exchange Processes by 2-Dimensional Nmr-Spectroscopy. *Journal of Chemical Physics*. **71** (11), 4546-4553, doi:Doi 10.1063/1.438208, (1979).
- 64 Palmer, A. G., Kroenke, C. D. & Loria, J. P. Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules. *Nuclear Magnetic Resonance of Biological Macromolecules, Pt B*. **339** 204-238, doi:Doi 10.1016/S0076-6879(01)39315-1, (2001).
- 65 Clore, G. M. & Iwahara, J. Theory, Practice, and Applications of Paramagnetic Relaxation Enhancement for the Characterization of Transient Low-Population States of Biological Macromolecules and Their Complexes. *Chemical Reviews*. **109** (9), 4108-4139, doi:10.1021/cr900033p, (2009).
- 66 Iwahara, J. & Clore, G. M. Detecting transient intermediates in macromolecular binding by paramagnetic NMR. *Nature*. **440** (7088), 1227-1230, doi:10.1038/nature04673, (2006).
- 67 Lakomek, N. A. *et al.* Residual dipolar couplings as a tool to study molecular recognition of ubiquitin. *Biochem Soc Trans*. **36** (Pt 6), 1433-1437, doi:10.1042/BST0361433, (2008).
- 68 Johnson, C. N. & Libich, D. S. Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins. *Jove-Journal of Visualized Experiments*. (175), doi:10.3791/63057, (2021).
- 69 Lakomek, N. A., Ying, J. & Bax, A. Measurement of (1)(5)N relaxation rates in perdeuterated proteins by TROSY-based methods. *J Biomol NMR*. **53** (3), 209-221, doi:10.1007/s10858-012-9626-5, (2012).
- 70 Stief, T., Vormann, K. & Lakomek, N. A. Sensitivity-enhanced NMR (15)N R(1) and R(1 ρ) relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields. *Methods*. **223** 1-15, doi:10.1016/j.ymeth.2024.01.008, (2024).
- 71 Kay, L. E., Keifer, P. & Saarinen, T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *Journal of the American Chemical Society*. **114** (26), 10663-10665, doi:10.1021/ja00052a088, (1992).

-
- 72 Geen, H. & Freeman, R. Band-selective radiofrequency pulses. *Journal of Magnetic Resonance* (1969). **93** (1), 93-141, doi:[https://doi.org/10.1016/0022-2364\(91\)90034-Q](https://doi.org/10.1016/0022-2364(91)90034-Q), (1991).
- 73 Desvaux, H. & Berthault, P. Study of dynamic processes in liquids using off-resonance rf irradiation. *Progress in Nuclear Magnetic Resonance Spectroscopy*. **35** (4), 295-340, doi:Doi 10.1016/S0079-6565(99)00011-4, (1999).
- 74 Overhauser, A. W. Polarization of Nuclei in Metals. *Physical Review*. **91** (2), 476-476 (1953).
- 75 Overhauser, A. W. Polarization of Nuclei in Metals. *Physical Review*. **92** (2), 411-415, doi:DOI 10.1103/PhysRev.92.411, (1953).
- 76 Kumari, P., Frey, L., Sobol, A., Lakomek, N. A. & Riek, R. (15)N transverse relaxation measurements for the characterization of micro-ms dynamics are deteriorated by the deuterium isotope effect on (15)N resulting from solvent exchange. *J Biomol NMR*. **72** (3-4), 125-137, doi:10.1007/s10858-018-0211-4, (2018).
- 77 Karschin, N., Krennek, S., Heyer, D. & Griesinger, C. Extension and improvement of the methanol-d(4) NMR thermometer calibration. *Magn Reson Chem*. **60** (2), 203-209, doi:10.1002/mrc.5216, (2022).
- 78 Sattler, M., Schleucher, J. & Griesinger, C. Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Progress in Nuclear Magnetic Resonance Spectroscopy*. **34** (2), 93-158, doi:Doi 10.1016/S0079-6565(98)00025-9, (1999).
- 79 Ferrage, F., Cowburn, D. & Ghose, R. Accurate Sampling of High-Frequency Motions in Proteins by Steady-State N-{H} Nuclear Overhauser Effect Measurements in the Presence of Cross-Correlated Relaxation. *Journal of the American Chemical Society*. **131** (17), 6048-6049, doi:10.1021/ja809526q, (2009).
- 80 Delaglio, F. *et al.* Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *Journal of Biomolecular Nmr*. **6** (3), 277-293, doi:Doi 10.1007/Bf00197809, (1995).
- 81 Skinner, S. P. *et al.* CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. *J Biomol NMR*. **66** (2), 111-124, doi:10.1007/s10858-016-0060-y, (2016).
- 82 Lee, W., Tonelli, M. & Markley, J. L. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics*. **31** (8), 1325-1327, doi:10.1093/bioinformatics/btu830, (2015).
- 83 Lee, W., Rahimi, M., Lee, Y. & Chiu, A. POKY: a software suite for multidimensional NMR and 3D structure calculation of biomolecules. *Bioinformatics*. **37** (18), 3041-3042, doi:10.1093/bioinformatics/btab180, (2021).
- 84 Abyzov, A. *et al.* Identification of Dynamic Modes in an Intrinsically Disordered Protein Using Temperature-Dependent NMR Relaxation. *Journal of the American Chemical Society*. **138** (19), 6240-6251, doi:10.1021/jacs.6b02424, (2016).
- 85 Singh, A., Purslow, J. A. & Venditti, V. 15N CPMG Relaxation Dispersion for the Investigation of Protein Conformational Dynamics on the micro-ms Timescale. *J Vis Exp*. (170), doi:10.3791/62395, (2021).
- 86 Gopalan, A. B., Hansen, D. F. & Vallurupalli, P. CPMG Experiments for Protein Minor Conformer Structure Determination. *Methods Mol Biol*. **1688** 223-242, doi:10.1007/978-1-4939-7386-6_11, (2018).
- 87 Tugarinov, V. & Kay, L. E. Methyl groups as probes of structure and dynamics in NMR studies of high-molecular-weight proteins. *Chembiochem*. **6** (9), 1567-1577, doi:10.1002/cbic.200500110, (2005).
- 88 Lipari, G. & Szabo, A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity. *Journal of the American Chemical Society*. **104** (17), 4546-4559, doi:10.1021/ja00381a009, (1982).
- 89 Lipari, G. & Szabo, A. Model-Free Approach to the Interpretation of Nuclear Magnetic-Resonance Relaxation in Macromolecules .2. Analysis of Experimental Results. *Journal of the American Chemical Society*. **104** (17), 4559-4570, doi:DOI 10.1021/ja00381a010, (1982).

- 90 Clore, G. M. *et al.* Deviations from the simple two-parameter model-free approach to the interpretation of nitrogen-15 nuclear magnetic relaxation of proteins. *Journal of the American Chemical Society*. **112** (12), 4989-4991, doi:10.1021/ja00168a070, (1990).
- 91 Lemaster, D. M. Larmor frequency selective model free analysis of protein NMR relaxation. *J Biomol NMR*. **6** (4), 366-374, doi:10.1007/BF00197636, (1995).
- 92 Peng, J. W. & Wagner, G. Mapping of spectral density functions using heteronuclear NMR relaxation measurements. *Journal of Magnetic Resonance (1969)*. **98** (2), 308-332, doi:[https://doi.org/10.1016/0022-2364\(92\)90135-T](https://doi.org/10.1016/0022-2364(92)90135-T), (1992).
- 93 Farrow, N. A., Zhang, O. W., FormanKay, J. D. & Kay, L. E. Characterization of the backbone dynamics of folded and denatured states of an SH3 domain. *Biochemistry*. **36** (9), 2390-2402, doi:DOI 10.1021/bi962548h, (1997).
- 94 Khan, S. N. *et al.* Distribution of Pico- and Nanosecond Motions in Disordered Proteins from Nuclear Spin Relaxation. *Biophys J*. **109** (5), 988-999, doi:10.1016/j.bpj.2015.06.069, (2015).
- 95 Smith, A. A., Ernst, M., Meier, B. H. & Ferrage, F. Reducing bias in the analysis of solution-state NMR data with dynamics detectors. *J Chem Phys*. **151** (3), 034102, doi:10.1063/1.5111081, (2019).
- 96 Zumpfe, K. & Smith, A. A. Model-Free or Not? *Front Mol Biosci*. **8** 727553, doi:10.3389/fmolb.2021.727553, (2021).
- 97 Robustelli, P., Trbovic, N., Friesner, R. A. & Palmer, A. G. Conformational Dynamics of the Partially Disordered Yeast Transcription Factor GCN4. *Journal of Chemical Theory and Computation*. **9** (11), 5190-5200, doi:10.1021/ct400654r, (2013).
- 98 Salvi, N., Abyzov, A. & Blackledge, M. Multi-Timescale Dynamics in Intrinsically Disordered Proteins from NMR Relaxation and Molecular Simulation. *Journal of Physical Chemistry Letters*. **7** (13), 2483-2489, doi:10.1021/acs.jpclett.6b00885, (2016).

10 Internal Backbone Dynamics of SNAP25a

IDPs and IDRs fulfill a variety of many different functions, whereby the dynamics of the protein backbone are close related to the protein function and interactivity⁹. NMR spectroscopy methods, especially ¹⁵N NMR relaxation experiments are sensitive to protein backbone dynamics in the picosecond and nanosecond regime, so that several quantitative methods were developed to derive the dynamic timescales, as well as dynamic restriction of individual backbone dynamics from the ¹⁵N relaxation rates^{11,136,135,137,139}.

The author performed ¹⁵N relaxation experiments at three different magnetic fields for this work. Also, the postprocessing and evaluation of the relaxation rates was done by the author. For the model-free analysis the author used the software package relax. The author implemented the IMPACT analysis by writing a python script. The Detectors analysis was performed by the author using the software package pyDIFRATE. For the final publication the author contributed text and figures. The according manuscript will be submitted for publication within the next weeks.

Journal: -

Volume: -

Authors: Tobias Stief
Katharina Vormann
Nils-Alexander Lakomek

DOI: -

Abstract

Protein function is strongly related to protein structure and internal backbone dynamics. A particularly dynamic class of proteins are intrinsically disordered proteins (IDPs) or proteins with large intrinsically disordered regions (IDRs), which together compose about 40% of the human proteome. Because of their high internal flexibility and conformational variability, they are extremely versatile and can fulfill a wide range of functions. We studied the fast internal dynamics in the picoseconds to nanoseconds regime of the SNARE protein SNAP25a, which assembles together with two other SNARE proteins to the SNARE complex, driving membrane fusion at the neuronal synapse. In its monomeric pre-fusion form, SNAP25a is intrinsically disordered. NMR ^{15}N relaxation R_1 and R_2 rate constants, as well as $^{15}\text{N}\{-^1\text{H}\}$ NOE values, were measured at three different magnetic field strengths (600 MHz, 900 MHz, 1200 MHz ^1H Larmor frequencies). For the quantitative dynamics analysis, an extended model-free approach, an IMPACT analysis, and a Detector analysis were performed. All evaluation methods yielded consistently dynamic modes at hundreds of picoseconds, roughly 1 ns, and 5 ns to 20 ns, depending on whether a structured or an intrinsically disordered region was involved. For residues with increased alpha-helical propensities, the several nanoseconds timescale was dominant, whereas intrinsically disordered residues showed the highest amplitudes of motion around one nanosecond and the faster picosecond timescale. Due to the participation of SNAP25a in the SNARE zippering, we speculate that the regions along the backbone with increased alpha-helical propensities (A5-Q20, S25-V36, E73-K79, D99-K102, E123-M127, T190-A199), now characterized by restricted segmental motions in the several ns regime, act as anchor points for the zippering process during the SNARE complex assembly.

Introduction

For information exchange between neurons, neurotransmitters must be released from the presynaptic neuron, cross the synaptic cleft, and activate receptors on the postsynaptic neuron. The neurotransmitters are located in vesicles in the axon terminal of the presynaptic neuron. For neurotransmitter release, the vesicle membrane has to fuse with the plasma membrane of the presynaptic neuron, which requires three SNARE proteins (Synaptobrevin-2, Syntaxin-1, and SNAP25a) and further accessory proteins (Munc13,

Munc18, Synaptotagmin, complexin). This highly complex and, on a molecular level, not fully understood fusion process is called neuronal exocytosis [1, 2].

In its monomeric, non-assembled pre-fusion form, SNAP25a shows large regions of intrinsic disorder [3]. Intrinsic disorder means a lack of tertiary structure associated with residues resembling a random-coil conformation where all backbone dihedral angles are randomly distributed within the allowed Ramachandran regions [4-11]. This observation led to a dynamic picture in which IDPs switch rapidly between various conformations [4]. Studying intrinsically disordered regions (IDRs) and intrinsically disordered proteins (IDPs) proves to be challenging by state-of-the-art techniques in structural biology, such as X-ray crystallography or electron microscopy, due to low and diffuse electron density [4], being a consequence of the high flexibility of IDRs and IDPs [12-14]. Solution NMR spectroscopy, on the other hand, provides multiple insights into secondary structure propensities, structural dynamics, and binding interactions of IDPs and IDRs with residue-specific resolution [15-21].

¹⁵N NMR relaxation measurements are widely used to investigate the ps-ns dynamics of the backbone amide groups of proteins, which report on the residue-specific local dynamics [22-24]. The Palmer and Blackledge groups pioneered the study of IDP structural dynamics using ¹⁵N relaxation measurements [25, 26]. Both groups used an extended model-free approach or a conceptually similar derivative to analyze the data. Blackledge and co-workers describe IDPs' backbone dynamics in terms of three dynamic modes, each defined by a correlation time τ [26-28]. By comparing them to molecular dynamics simulations, Salvi et al. connected those modes to the following dynamics observed in an molecular dynamics (MD) trajectory: The well-known local bond librations on a time scale $\tau \leq 50$ ps, but in addition to that also backbone dihedral angle sampling on the low ns timescale and segmental motions of several ns due to the chain-like characteristics of proteins [26-28].

The underlying model-free (MF) analysis by Lipari and Szabo [29] is the most widespread approach for the quantitative analysis of ¹⁵N relaxation data. This was further developed into the extended model-free (EMF) approach by Clore et al. [30]. Both approaches were developed in the framework of globular proteins. In solution, proteins, like any other globular molecule, show an overall tumbling described by a rotational correlation time. Depending on how much the protein shape deviates from a sphere, the rotational correlation time is associated with an anisotropic diffusion tensor, with different correlation

times along the principal axes. Further, a separation of time scales of at least an order of magnitude (10-fold) is assumed between the internal residue-specific motions and the overall tumbling correlation time. Methods developed for the study of globular protein dynamics do not in per-se work for describing IDP dynamics. One requirement for the applicability of the (extended) model-free analysis is the separation of timescales for local residue-specific motions and the global rotational tumbling of the protein (see above). This is fulfilled for globular proteins but not necessarily for IDPs, as they do not show a clearly defined global rotational tumbling due to their lack of rigidity and frequently extended non-globular shape [16, 25, 26]. To account for that dilemma, Ferrage and co-workers developed the so-called IMPACT approach. Here, the relaxation rate constants are fitted by a correlation function, described by a sum of exponentials [31]. Using this approach, several dynamic modes in the backbone can be extracted from the ^{15}N relaxation rates, whereby a model-free approach is limited to a maximum of three modes. Another method to detect dynamic regimes, the so-called Detectors approach, was recently developed by Smith and co-workers, originally in the context of assuming a distribution of correlation times rather than the fixed correlation times used in IMPACT to reconstruct the relaxation rates [32]. Catching all specific dynamic interactions separately is extremely difficult, as ^{15}N relaxation rates are sensitive to the reorientation of the NH bond vector, which is influenced by a wide variety of dynamics that differ for each residue, resulting in a complex multidimensional description [33].

In this work, we investigate the residue-specific internal backbone dynamics of the SNARE protein SNAP25a in the range of picoseconds and nanoseconds. We used NMR relaxation experiments to measure the ^{15}N relaxation rates R_1 and R_2 and the ^{15}N - $\{^1\text{H}\}$ NOE data. These rate constants contain information on backbone dynamics in the ps and ns time ranges and are a widely used approach for analyzing the structural dynamics of proteins [13, 34-36]. Using three different evaluation methods, model-free[29, 30], IMPACT[31], and Detectors[32], we extract motional amplitudes and timescales, characterizing the residue-specific backbone dynamics of SNAP25a.

Material and Methods

NMR sample preparation

The gene base sequence encoding the synaptosomal-associated protein of 25 kDa (SNAP25), referred as isoform 2 of SNAP25a, from *Rattus norvegicus* (UniProt accession number: P60881-2; 206 residues, 23.3 kDa; with the four cysteine sites C84S, C85S, C90S, C92S mutated to serines) was codon-optimized for expression in *E. coli* and subcloned into a pET28a(+) vector including a N-terminal *hexa*-histidine tag and a Thrombin-cleavage site. Protein expression and purification followed the protocols by Pobatti *et al.*[37]. Pure protein samples of ^1H - ^{15}N SNAP25a were concentrated in 20 mM sodium phosphate (pH 6.5), 150 mM NaCl and 0.1 mM TCEP. For later NMR measurements, protein samples with a final concentration of 100 μM were supplemented with 1% (v/v) NaN_3 .

NMR relaxation experiments

NMR experiments were acquired at 15°C on three Bruker NMR spectrometers with different magnetic field strengths: 14.1 T (600 MHz Avance III), 21.15 T (900 MHz Avance Neo), and 28.2 T (1200 MHz Avance Neo). The relaxation rate constants R_1 , $R_{1\rho}$, and ^{15}N - $\{^1\text{H}\}$ NOE values were recorded using the pulse sequences described by Stief *et al.* 2024 [38] (Validation of R_1 and $R_{1\rho}$ sequence) and Stief *et al.* 2024 JoVE[39] (detailed description of the setting up process), conceptually similar to pulse sequences developed by Lakomek *et al.* in the Bax group[40], but improved for water-suppression of the sensitivity-enhanced HSQC experiments and tested for application at high-field magnets and fully protonated protein samples. The R_2 relaxation rate constants were calculated from the R_1 and $R_{1\rho}$ rate constants. In the $R_{1\rho}$ experiment, we applied a 2-kHz spin-lock field. Detailed experimental parameters are listed in Table S1, Table S2, and Table S3 in the Supporting Information.

For processing and evaluating the NMR experiments, NMRPipe was used [41]. The model-free analysis was performed with relax[42, 43] (<https://www.nmr-relax.com/>). The implementation of the IMPACT analysis is based on Khan *et al.* 2016[31]. For the detectors analysis[32] we made use of the python implementation pyDIFRATE, available under: <http://nmr4dynamics.de/page2.html>.

Model-Free Analysis (MF)

The model-free analysis was performed using the software package relax 5.1.0[42, 43]. The model-free approach by Lipari and Szabo [S^2 , τ_e] [29] and the extended model-free model [S^2 , τ_f , S_f^2 , τ_s] [30] were used to fit the three field relaxation data, each with a residue-specific local correlation time τ_M and without a chemical exchange contribution. The local correlation time τ_M describes segmental motions of the polypeptide chain [25, 28], as IDPs cannot be described by a single global tumbling time due to their high internal dynamics, frequently combined a non-globular shape. The software Relax chooses the best-fitting model for each residue individually based on the Akaike information criterion (AIC), which also weights model complexity in addition to minimizing chi-square. The anisotropic chemical shift was set to -172 ppm, and the average amide bond length was set to 1.02 Å. 11 grid search increments and 500 Monte-Carlo simulations were performed.

Impact Analysis

The interpretation of motions by a projection onto an array of correlation times (IMPACT) analysis was implemented similarly to that of Khan et al. 2015 [31]. However, different from the original implementation, we used $R_{1\rho}$ data rather than cross-correlated relaxation rate constants to calculate the spectral density at $\omega = 0$. $J(0)$ was calculated using equation (1) to reduce the number of required NMR relaxation experiments and ensure better comparability with model-free and detector methods. The IMPACT analysis fits the spectral density points using n amplitudes A_i , corresponding to n correlation times τ_i . A variable number n of τ_i were tested ($n = 3, 4, 5, 6, 7, 8$). The exact value of τ_i was calculated as a logarithmic time point on a defined interval; 100 intervals [1 ps, 1 ns] up to [100 ps, 100 ns] were tested. The best parameter setting was chosen based on the AIC criterion. The amplitudes A_i were selected using a global minimization method called Basin-hopping that uses a SLSQP (Sequential Least Squares Programming) algorithm for local minimization[44, 45]. This global minimization method was implemented by using SciPy[46]. The anisotropic chemical shift was set to -172 ppm, and the average amide bond length was set to 1.02 Å, similar to the model-free analysis. For the error analysis, we used a Monte-Carlo approach with 100 simulation steps.

$$J(0) = \frac{R_2 - \frac{R_1}{2} - \frac{3d^2}{4} J(\omega_H)}{\frac{d^2}{2} + \frac{2}{3}c^2} \quad (1)[47, 48]$$

Detectors analysis

The protein dynamic analysis ‘Detectors’ was performed using the pyDIFRATE software package [32]. The dipole-dipole cross-relaxation rate constant σ_{NH} was calculated using equation (2). Additionally, the ^{15}N R_1 and ^{15}N $R_{1\rho}$ relaxation rate constants were used as input. The number of detectors was set to 6, to guarantee a high comparability with the IMPACT analysis, which calculated the best fit using six correlation times.

$$\sigma_{NH} = (NOE - 1) \frac{\gamma_N}{\gamma_H} R_1 \quad (2)[48]$$

Results and Discussion

For the analysis of the backbone dynamics of SNAP25a in the picoseconds (ps) to nanoseconds (ns) time range, ^{15}N relaxation rate constants were acquired at three different magnetic field strengths: 14.1 T (600 MHz), 21.15 T (900 MHz), and 28.2 T (1200 MHz) (Figure 1). All relaxation rate constants are presented in Table S4 attached in the SI. While both ^{15}N R_1 and R_2 relaxation rate constants are sensitive to tens to hundreds of ps dynamics, the R_2 rates are additionally influenced by the globular motion of the protein, which would be the overall tumbling of a globular protein being described by one or up to five rotational correlation times (depending on the degree of anisotropy of the diffusion tensor). The ^{15}N - $\{^1\text{H}\}$ NOE, dubbed hetNOE in the following, is dominated by fast motions on the tens of picoseconds timescale.

Figure 1 displays the ^{15}N relaxation rate constants measured at three different magnetic fields. The ^{15}N R_1 rates at 600 MHz (Figure 1A) show a flat curve along the protein backbone of SNAP25a with rates of around $1.6 \text{ rad} / \text{s}^{-1}$. The ^{15}N R_1 rate constants at 900 MHz and 1200 MHz show lower values of around $1.4 \text{ rad} / \text{s}^{-1}$ and, particularly, for the N-terminus rates at about $1.2 \text{ rad} / \text{s}^{-1}$, indicating slower dynamics for the N-terminus than for the rest of the protein backbone. The ^{15}N R_1 rate constants at 900 MHz and 1200 MHz reveal two dynamic regimes along the protein backbone of SNAP25a, which are not visible in the 600 MHz data. The ^{15}N R_2 relaxation rate constants of SNAP25a (Figure 1B) exhibit

very similar curves across all magnetic fields, with a systematic increase at higher magnetic fields, as expected. For the N-terminus, the ^{15}N R_2 rate constants are up to 10 rad/s^{-1} at 600 MHz, up to 15 rad/s^{-1} at 900 MHz, and 19 rad/s^{-1} at 1200 MHz. Following residues along the protein backbone relax with rate constants of around 5 rad/s^{-1} at 600 MHz, 6 rad/s^{-1} at 900 MHz, and 7 rad/s^{-1} at 1200 MHz, consistent with the intrinsically disordered character of SNAP25a (apart from the helical N-terminus). The increase in R_2 rate constants for the N-terminus of SNAP25a, indicate the presence of secondary structure, consistent with the increased α -helical propensity extracted from the secondary chemical shifts[3, 49]. The hetNOE values of SNAP25a range from -1.6 (C-terminus) to 0.4 (N-terminus), with most residues showing 0.1-0.2 at 600 MHz, which is typical for intrinsically disordered proteins with fast internal dynamics, for which hetNOE values are sensitive. The hetNOE data at 900 MHz and 1200 MHz show increased values, for most residues higher than 0.3 at 900 MHz and higher than 0.5 for 1200 MHz. Additionally, the hetNOE data curve at 900 MHz and 1200 MHz seem to flatten compared to the 600 MHz data, that show increased hetNOE values for the N-terminus. This is explained by the fact that the different time-scale windows probed at higher magnetic fields shift to faster ps dynamics, which are less prevalent in the N-terminal region of SNAP25a.

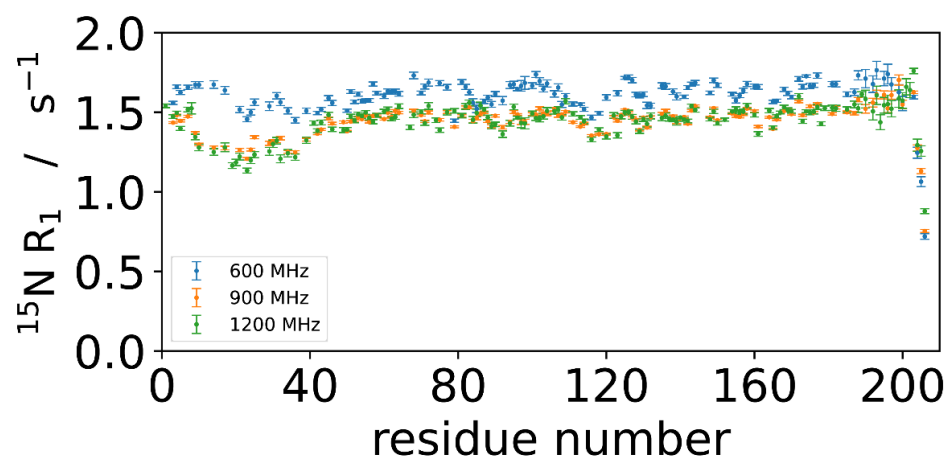
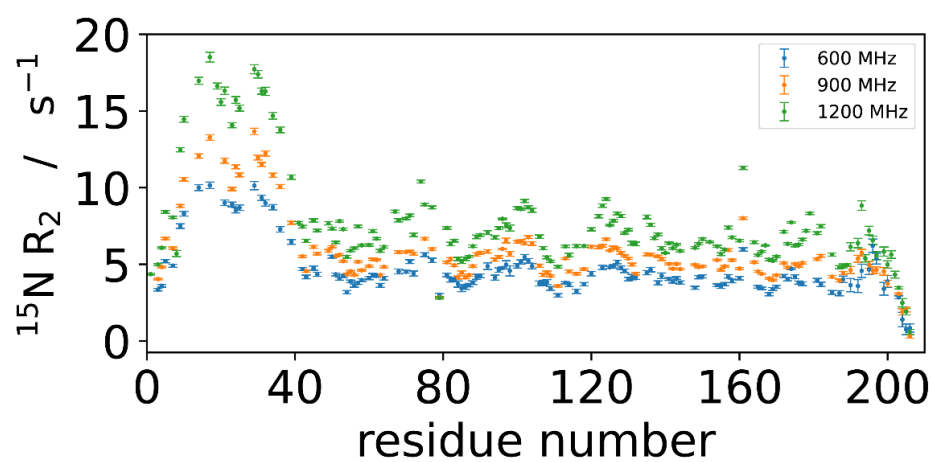
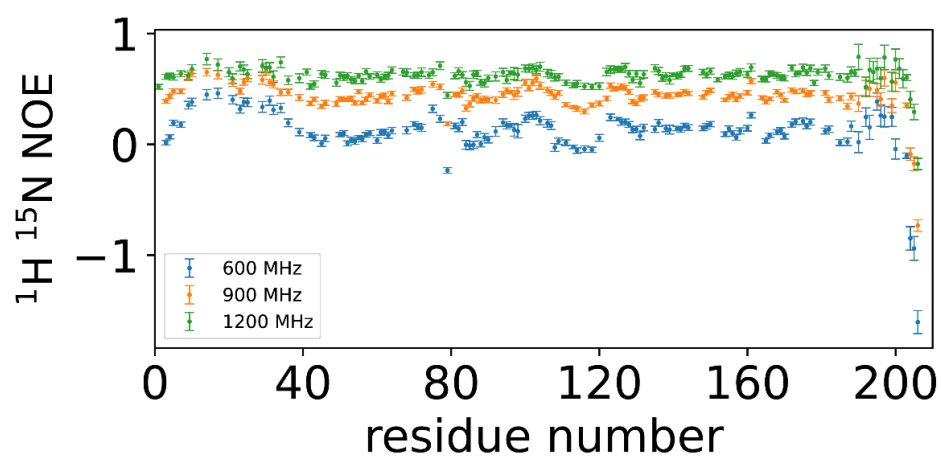
A**B****C**

Figure 1: ^{15}N NMR relaxation data of SNAP25a at three different magnetic fields at 15 °C. 14.1 T (600 MHz) in Blue, 21.15 T (900 MHz) in orange, and 28.2 T (1200 MHz) in Green. A: ^{15}N R_1 relaxation rates, sensitive to backbone dynamics in the range of picoseconds and nanoseconds. B: ^{15}N R_2 relaxation rates, calculated from R_1 and $R_{1\rho}$ relaxation rates, are mostly sensitive to ns dynamics. C: ^{15}N - $\{^1\text{H}\}$ NOE values along the backbone of SNAP25a, sensitive to backbone dynamics in the range of picoseconds.

Model Free Analysis

A model-free analysis of the SNAP25a ^{15}N relaxation rate constants and hetNOE values was performed using the RELAX software (data in Table S5). Because of the missing secondary structure of SNAP25a and accounting for the absence of a globular overall shape of an intrinsically disordered protein, which is better described by a polymer chain [50-52], a local, residue-specific correlation time, τ_m , for each residue, instead of a global tumbling time for the whole molecule (Figure 2 A) was used. This local correlation time is in the range between one nanosecond for the highly flexible C-terminus and ten nanoseconds for the more rigid N-terminus (see Figure 2A), as discussed in more detail in the following paragraph. For the N-terminus, the internal dynamics of the α -helix structure occur on a timescale of approximately eight to ten nanoseconds. Residues A42 up to G206 of the backbone of SNAP25a, which are mostly intrinsically disordered, as revealed by secondary chemical shifts previously [3], and do not possess a regular secondary structure, show local correlation times in the range of five to six nanoseconds. These dynamics over several nanoseconds define a dynamic mode which has been related to segmental motions of the polypeptide chain [26].

For each residue, the standard MF results (Lipari and Szabo) as well as the extended MF results (Clore et al.) were calculated, and the best fitting model was selected based on the AIC criteria. For most residues along the backbone, the extended model-free model with parameters S^2 , τ_f , S_f^2 , τ_s was the most successful model selected by the RELAX software (Figure S1). As shown in SI Figure S2, the back-calculated relaxation rates fit well with the experimental data, underscoring the robustness of the approach. The fast internal correlation time τ_f represents the timescale of the faster of two independent internal motions; the timescale of the slower internal motions is modelled with the slow internal correlation time τ_s . For τ_f and τ_s the following constraint is implemented: $\tau_f, \tau_s \leq 2\tau_m$. The fast correlation

times τ_f (Figure 2B) occur for most backbone residues between 20 ps and 80 ps. This relatively narrow margin of the fast correlation time τ_f of around 50 ± 30 ps for most residues indicates the presence of a fast dynamic mode in the ps time range. These dynamics are present along the whole protein backbone, and differences in the timescale between the structured N-terminus and the disordered part of SNAP25 are not clearly visible. The slow correlation time τ_s (slower than τ_f but faster than τ_m , Figure 2C), shows a relatively flat curve along the protein backbone. Most τ_s , correlation times lie between 0.5 ns and 1.5 ns. This indicated the presence of a second dynamic mode around 1 ± 0.5 ns.

In the model-free analysis, amplitudes of motion are described by a residue-specific order parameter S^2 . Despite its drawbacks, such an order parameter allows immediate physical visualization of the amplitudes of motion of the N-H bond vector at the inspected time scale. In the extended model-free analysis, three order parameters are fitted to the relaxation rate constant data: S^2 , S_f^2 , and S_s^2 . The generalized order parameter S^2 quantifies the amplitude of backbone motions, S_f^2 defines the amplitude of the fast internal motions. S_s^2 represents the amplitude of the slow internal motion at the time scale τ_s . All order parameters are linked by the following equation $S^2 = S_f^2 * S_s^2$.

The residue-specific order parameter S_f^2 (Figure 2E) shows values up to 0.95 for the helical structure at the N-terminus (A5-Q20 and S25-V36), the dynamics of the remaining residues along the backbone have S_f^2 values between 0.6 and 0.85. The fast internal dynamics on the picosecond timescale are severely restricted at the N-terminus of SNAP25, consistent with the present secondary structure. Less restricted are the fast internal motions of the other residues along the backbone of SNAP25a, which is predominantly disordered.

The order parameter of slow internal dynamics S_s^2 (Figure 2 F) shows a similar pattern as the order parameter of fast internal dynamics S_f^2 (Figure 2 E). For the N-terminus, the order parameters S_s^2 are between 0.4 and 0.7, for all other residues S_s^2 lies between 0.2 and 0.6. For the C-terminus, very low values around 0.1 were fitted by the extended model-free analysis. Therefore, slow internal motions at the timescale of one nanosecond show a higher degree of freedom in movement than the fast internal motions. Especially the C-terminus has nearly no restrictions on freedom of motion in the nanosecond regime. Nevertheless, the slow internal dynamics of the N-terminus are more restricted than the

dynamics of the other residues along the backbone of SNAP25, resulting from the α -helical structure of the N-terminus.

The residue-specific order parameter $S^2 = S_f^2 * S_s^2$ (Figure 2D) shows values up to 0.7 for the helical structure at the N-terminus (A5-Q20 and S25-V36). The dynamics of the remaining residues along the backbone have S^2 values between 0.2 and 0.4. Therefore, the slower dynamics of the α -helical structure at the N-terminus (5 ns – 10 ns) are more restricted than the dynamics of the intrinsically disordered residues on a faster timescale (5 ns - 6 ns), which show a higher degree of freedom of movement as indicated by lower S^2 values.

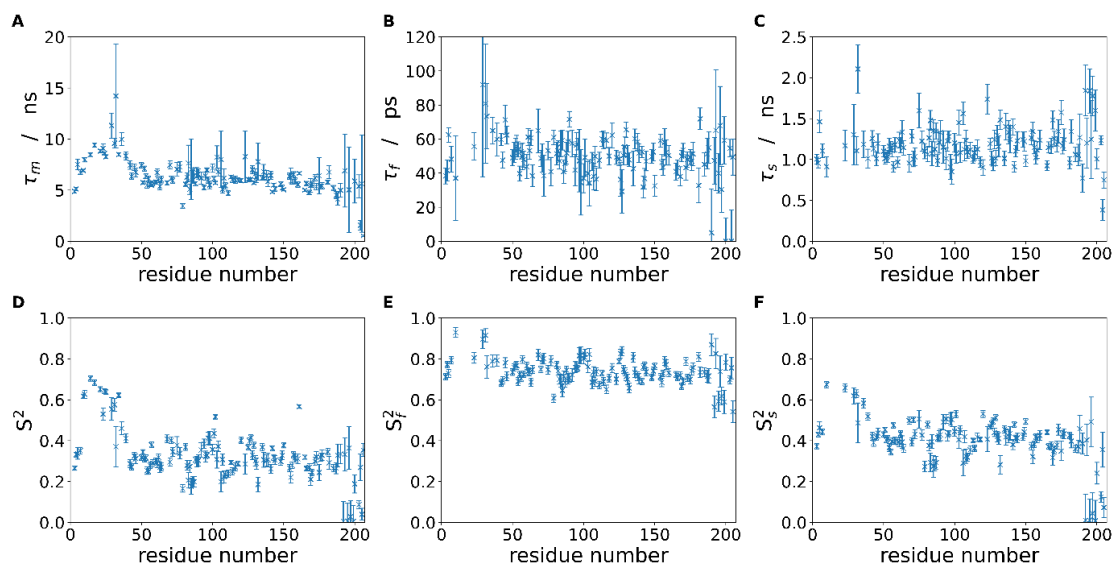


Figure 2: Model Free Analysis of the backbone dynamics of SNAP25a. As Input, the relaxation rates R_1 , R_2 , and the $^{15}\text{N}\{-^1\text{H}\}$ NOE values were used. Due to the absence of secondary structure for most of SNAP25a (apart from the N-terminus), a model-free analysis using a local correlation time τ_m (rather than a global rotational correlation time) was performed. A: Local correlation time τ_m for each residue. B: Fast internal correlation time τ_f represents the timescale of the faster of two independent internal motions. C: The timescale of the slower internal motions is modelled with the slow internal correlation time τ_s . D: Order parameter $S^2 = S_f^2 * S_s^2$. E: Order parameter S_f^2 of fast internal motions. F: Order parameter S_s^2 of slow internal motions.

IMPACT

As it is, per se, not evident that an extended model-free analysis (despite all its drawbacks) would still be sufficient to describe the ps-ns dynamics of the IDP SNAP25a, a more detailed analysis of the specific backbone motions in SNAP25a was performed using the IMPACT approach. For the input, we used the R_1 and R_2 relaxation rate constants, along with hetNOE values at three magnetic fields (600 MHz, 900 MHz, 1200 MHz). The IMPACT analysis was performed with six correlation times: 20.0 ns, 5.02 ns, 1.26 ns, 317 ps, 79.6 ps, 20.0 ps, each representing a specific time scale of motion. The amplitudes A_1 , A_2 , A_3 , A_4 , A_5 , and A_6 define the extent of the individual backbone motions at different time scales to these specific correlation times. The number of correlation times and the specific values used to fit the spectral density function, were selected using an AIC criterion that weights the number of model parameters alongside the model's prediction error (Figure S3).

Figure 3 shows the Amplitudes A_1 – A_6 as responses to the backbone dynamics of SNAP25; all amplitudes are also provided in the SI (Table S6). A_1 is sensitive to dynamics of around 20 ns and dynamics slower than 20 ns. For the N-terminal α -helices (A5-Q20 and S25-V36), A_1 is close to 0.25, whereas A_1 is smaller than 0.1 for the intrinsically disordered part of SNAP25a. Compared to the model-free analysis, IMPACT reveals dynamics in the range of 20 ns and slower for the α -helical region, while the model-free analysis only detects motions of around 10 ns. Additionally, the IMPACT analysis shows distinct dynamic regimes in some regions, with slightly increased α -helical propensity (D99-K102, E123-M127) in the IDP region of SNAP25a, accompanied by a slightly increased A_1 response. Dynamics in the range of 5 ns contribute more to backbone dynamics than those in the range of 20 ns, as observed from the higher amplitude A_2 compared to A_1 . For the IDP region of SNAP25a, A_2 is mostly between 0.15 and 0.3, the α -helical region shows responses between 0.2 and 0.4. These observations indicate a higher presence of slow ns motions (> 5 ns) in the α -helical region compared to the IDP region of SNAP25a. Specifically, in the IDP region of SNAP25a, the findings of low A_1 and higher A_2 underscore a prevalence of high-amplitude motions around 5 ns (compared to the 20 ns timescale), which aligns well with the model-free analysis, where the local correlation time is around 6 ns, close to $\tau_2 = 5.02$ ns. Additionally, the calculated tumbling time from the amplitudes A_1 and A_2 ($\tau_m =$

$(A_1 \cdot \tau_1 + A_2 \cdot \tau_2) / (A_1 + A_2)$ shows a similar curve shape as the local tumbling time of the model-free analysis (see Figure S4).

The dynamics of the intrinsically disordered region are dominated by a correlation time of 1.26 ns, with amplitudes of around 0.4-0.5. These amplitudes are smaller for the N-terminal region, consistent with the higher response to the slower correlation times A_1 and A_2 . These findings correlate well with the slow internal correlation time from the model-free analysis, which is around 1 ns for almost all backbone residues in SNAP25a.

For the correlation times τ_4 and τ_5 in the picosecond regime, the N-terminus shows amplitudes smaller than 0.1. Only for the dynamics in the range of 20 ps and faster (τ_6), the N-terminus shows a significant response greater than 0.1. The disordered backbone regions show amplitudes around 0.1 for dynamics in the range of 317 ps and 79.6 ps. For dynamics of 20 ps and faster the amplitude response increases to 0.2. The C-terminus is mostly sensitive to the correlation times $\tau_4 = 317$ ps and $\tau_5 = 79.6$ ps with amplitudes up to 0.4 each, leading to the assumption of a highly flexible C-terminus. Compared to the model-free approach, which showed a fast internal correlation time of 50 ps, the IMPACT analysis can fit these 50 ps dynamics and also faster dynamics using a 20 ps correlation time. The model-free approach only fits the best correlation time and loses its sensitivity to faster dynamics.

Figure S5 underlines that nanosecond dynamics account for up to 80% of the structured N-terminus region. For the IDR, these proportions reduce to 60%. In regions with increased α -helical propensity (E73-K79, D99-K102, E123-M127, T190-A199) it increases to 70%. This 40% contribution from picoseconds dynamics demonstrates the substantial presence of picosecond dynamics in intrinsically disordered protein regions. The highly flexible C-Terminus is dominated by ps dynamics (up to 90%).

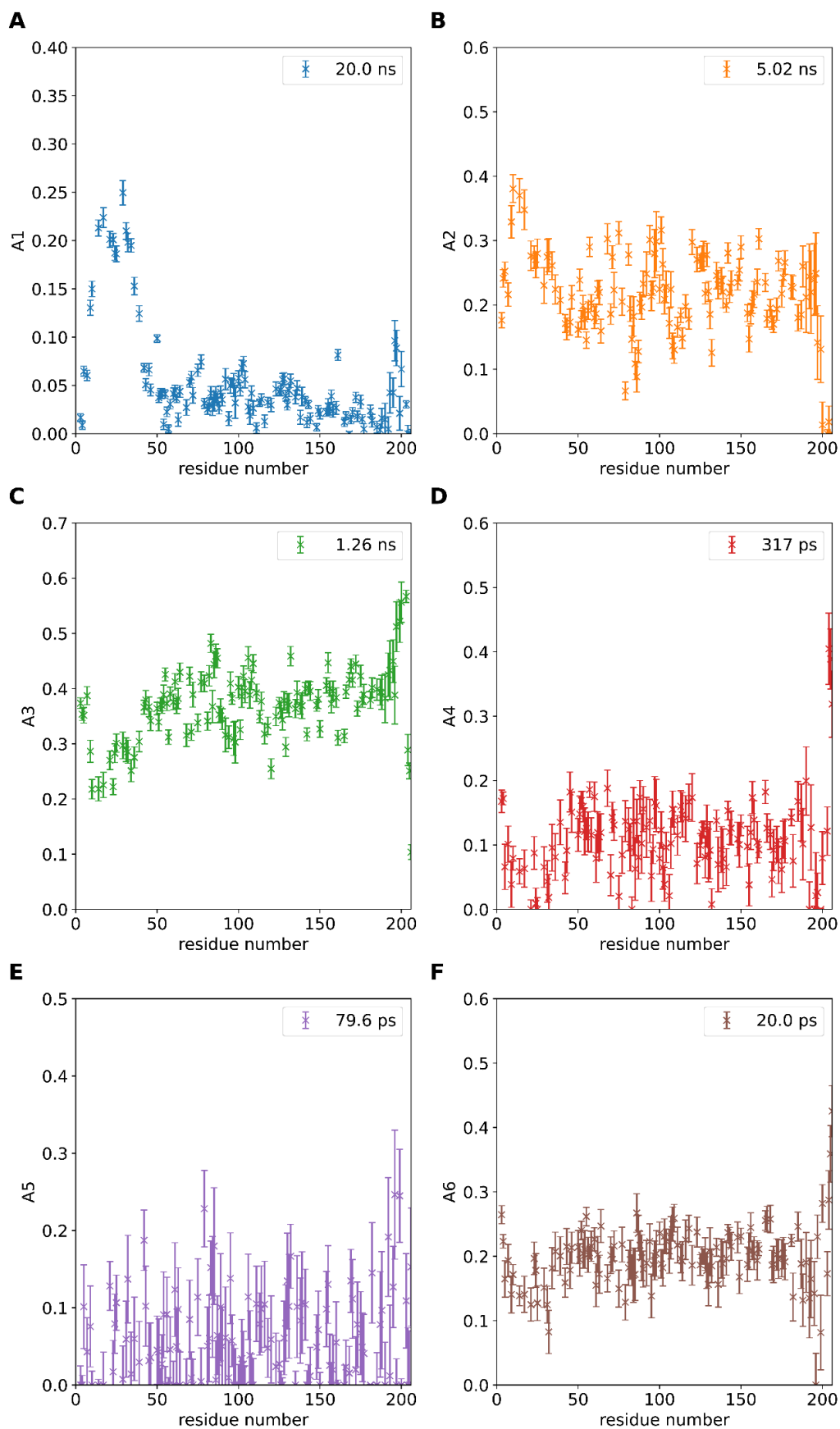


Figure 3: IMPACT analysis of the dynamic motions along the protein backbone of SNAP25a. As experimental input, we used the R_1 and R_2 relaxation rate constants, along with the $^{15}\text{N}\{-^1\text{H}\}$ NOE values at three magnetic fields (600 MHz, 900 MHz, 1200 MHz). The IMPACT analysis was performed with six correlation times: A: 20.0 ns, B: 5.02 ns, C: 1.26 ns, D: 317 ps, E: 79.6 ps, F: 20.0 ps each representing a specific time scale of motion. The amplitudes A: A_1 , B: A_2 , C: A_3 , D: A_4 , E: A_5 and F: A_6 define the response of the individual backbone motions at different time scales to these specific correlation times.

Detectors

The data set used in the Detectors analysis is the same as for the model-free and IMPACT analyses: the ^{15}N relaxation rates R_1 and $R_{1\rho}$ ($\nu = 2$ kHz), as well as hetNOE values measured at three different magnetic fields, 600 MHz, 900 MHz, and 1200 MHz. The detector approach uses a distribution of correlations times, and based on that, the detector response is an indicator for motion in a specific detector window. For the Detectors analysis, we used six detectors to improve comparability with the IMPACT analysis, selecting six correlation times based on an AIC criterion. The correlation times were optimized using the Detectors formalism to account for the sensitivity of the respective ^{15}N rate constants to the magnetic field strengths investigated: [68 ps, 300 ps, 690 ps, 1.7 ns, 7.5 ns, 67 μs]. The detector curves (Figure 4A) and detector responses (Figure 4B) are shown in Figure 4. The dynamic of the α -helical structure at the N-terminus is dominated by dynamics in the range of 7.5 ns with detector responses up to 0.6 (Figure 4B). The calculated average correlation time of A_1 , A_2 , τ_1 , τ_2 of the IMPACT analysis in Figure S4 is around 7.5 ns for the N-terminal regions, which agrees well with the high response of detector ρ_5 for the helical region. For the IDP region of SNAP25a, the detector response ρ_5 is around 0.2. In regions with a slightly increased α -helical propensity (E73-K79, D99-K102, E123-M127, T190-A199), the response increases up to 0.4. The same sensitivity of the regions with increased α -helical propensities was found for the curve of the amplitude A_1 ($\tau_1 = 20.0$ ns) in the IMPACT analysis. The C-terminus of SNAP25a shows no response to the ns dynamics covered by detector ρ_5 . Internal dynamics of 1.7 ns are present over the entire backbone of SNAP25, except for the residues with strong α -helical propensity in the N-terminal region. The detector ρ_4 shows responses between 0.3 and 0.4 for

intrinsically disordered residues, indicating that fast ns dynamics account for a large proportion of the total backbone dynamics in SNAP25a. It is notable that the detector response ρ_4 (Figure 4) shows a pattern similar to the amplitude A_3 (Figure 3C), both of which are related to dynamics in the fast ns range. In contrast to the ρ_5 response, regions with a slightly increased α -helical propensity (E73-K79, D99-K102, E123-M127, T190-A199) show a decreasing response for detector ρ_4 (1.7 ns), whereas neighbored disordered residues show increased responses up to 0.5, indicating the dominance of fast ns dynamics in disordered residues of SNAP25a. The detector ρ_3 shows responses similar to those of detector ρ_4 in the disordered region between 0.3 and 0.4. The N-terminal region, as well as regions with a slightly increased α -helical propensity, also show responses up to 0.4 and cannot be dynamically distinguished from the disordered backbone region. Compared to IMPACT, Detectors here revealed the slow ps dynamic occurring also in α -helical regions. Detectors show low amplitudes to this region for correlation time $\tau_3 = 1.2$ ns, which lies in the middle between detector ρ_4 (1.7 ns) and ρ_3 (690 ps). The response of detector ρ_2 to faster ps dynamic (300 ps) shows less response than the detectors ρ_4 (1.7 ns) and ρ_3 (690 ps). For the α -helical region of the N-terminus, the detector ρ_2 gives no response; for the disordered region, the response is between 0.1 and 0.2. This response is well comparable with the correlation time τ_4 (317 ps) in the IMPACT analysis and shows high agreement in amplitudes between 0.1 and 0.2 as in curvature. The detector ρ_1 (68 ps) also responds in the range between 0.1 and 0.2 for the disordered region of SNAP25a, but slightly higher than detector ρ_2 . The N-terminal regions also show some low response to fast ps dynamics. As a result, intrinsically disordered residues are also substantially influenced by dynamics in the ps regime. Residues with increased α -helical propensities have a small detectable proportion of fast ps dynamics. The response to ρ_2 and ρ_1 is slightly higher (up to 0.3) for the C-terminus compared to the rest of the backbone of SNAP25a. Here, the findings from detectors ρ_2 and ρ_1 align well with the conclusions from amplitudes A_5 and A_6 in the IMPACT analysis. Furthermore, the responses of detector ρ_1 and ρ_2 underscore the fast ps dynamics occurring in the highly flexible C-terminus, IMPACT showed before. Responses of detector ρ_6 are negligibly small compared to the responses of the other detectors.

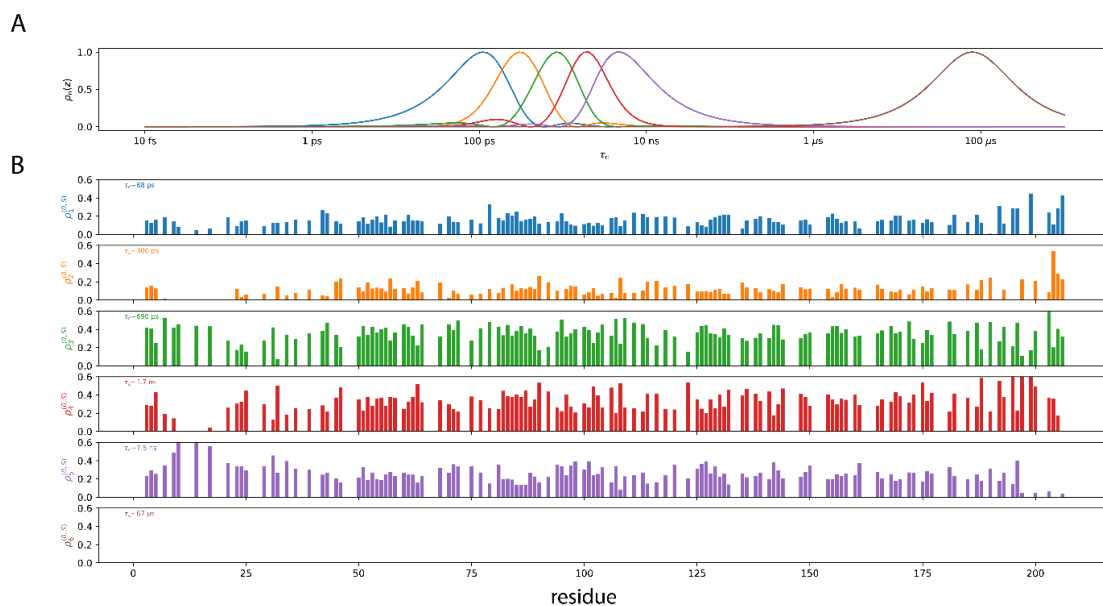


Figure 4: Detectors analysis of the internal backbone motions of SNAP25a. The correlation times of six detectors (68 ps, 300 ps, 690 ps, 1.7 ns, 7.5 ns, 67 μ s) were optimized by Detectors. A: Plot of the detectors $\rho_n(Z)$. B: The detector responses $\rho_n^{(\theta,S)}$ to the backbone dynamics of SNAP25a.

Conclusion

In this work, we analyzed the fast internal backbone dynamics of the intrinsically disordered protein SNAP25a. We extracted picoseconds to nanoseconds backbone dynamics from NMR relaxation ^{15}N R_1 , ^{15}N R_2 , and ^{15}N - $\{^1\text{H}\}$ NOE experiments at three different magnetic fields (600 MHz, 900 MHz, 1200 MHz). Using three independent approaches (Model-free, IMPACT, Detectors) for fitting the data, we found high agreement in amplitudes and timescales across all approaches, indicating high consistency and validity. The analyses revealed dynamics in the 20-ns time window for the helical segment at the N-terminus of SNAP25a. Dynamics in the 1 ns range were present throughout the backbone. In particular, the IDP region of SNAP25a exhibits rapid internal dynamics in the ps regime. Our findings support the rationale of three dynamic modes suggested by Blackledge and co-workers, based on a comparison between the NMR data analysis and molecular dynamics simulations: fast local librational motions (~ 50 ps), conformational sampling of dihedral angles (~ 1 ns), and long ranged chain-like motions (several ns)[26, 53]. In our data analysis, local bond vibrations on a time scale $\tau \leq 50$ ps were

revealed consistently in the EMF, IMPACT, and Detectors approach. Backbone dihedral angle sampling on a 1 ns timescale and segmental motions of several ns, due to the chain-like characteristics of proteins, were also detected by all the methods we used.

Motions of several nanoseconds were mainly observed for the N-terminal region with increased helical propensity, which suggests the restriction of N-H bond vector amplitudes of motion for residues being part of an α -helical formation. Here, the motion of the individual residue is more defined by the motion of the helical construct, a segment in the polypeptide chain, characterized by motions of several ns proven by our NMR relaxation data.

The residues in the intrinsically disordered region also show segmental motions, albeit to a lesser extent, as demonstrated by τ_M of approx. 5 ns in the EMF, by the amplitude A_2 (5.02 ns) and by the detector response ρ_5 (7.5 ns). These residues, as indicated by secondary chemical shifts [3], are in a random-coil conformation, characterized by sampling different dihedral angles over time, resulting in a non-uniform three-dimensional structure. This sampling takes place on the picosecond to nanosecond time scale. The higher the disorder of the residue, meaning a higher degree of freedom in dihedral angles and less dynamic coupling to neighboring amino acids, the faster the timescale of motion τ . This is underlined by higher response of the IDR to fast nanosecond and picosecond correlation times, and lower order parameters in EMF for the IDR compared to the helical N-terminus of SNAP25a (see EMF Figure 2, IMPACT Figure 3 and Detectors Figure 4). Especially the dynamics in the ns range showed strong amplitudes in the IMPACT analysis and high detector responses, while Detectors also revealed slightly faster dynamics ($\rho_3 = 690$ ps), underlining the importance of fast ns dynamics for IDPs. The correlation time τ_s between 1 ns and 2 ns for most disordered residues supports this statement (see EMF Figure 2). Fast picosecond motions (~ 60 ps) were revealed by τ_f in the EMF and by the response to ρ_1 in the Detector analysis. Motions slower than 50 ps are present for all residues (see IMPACT A_6), but the detector ρ_1 and the amplitude A_6 of the correlation time of 20 ps in the IMPACT analysis suggest faster bond librations for highly disordered residues compared to residues with α -helical propensity.

In the IDP SNAP25a, these fast dynamics may play a crucial role in neuronal exocytosis. In Stief et al. 2023 our data suggested that the N-terminus of SNAP25a acts as a nucleation site for the N-to-C-terminal SNARE zippering. The quantitative dynamic analysis of relaxation rates supports this model

of N-terminal nucleation and N-to-C-terminal zippering for the SNARE complex, given the strong presence of ns dynamics at the N-terminus. The disordered region of SNAP25a shows faster ps dynamics, which helps this disordered region adapt its structure during zippering, as some helix-folding occurs on the sub-nanosecond timescale [54, 55]. The regions with slightly increased α -helical propensity (E73-K79, D99-K102, E123-M127, T190-A199) also show slightly increased ns dynamics, eventually supporting the helical transition during the SNARE zippering [56, 57]. Dynamics of several nanoseconds (segmental motions) can involve up to 30 residues [58], supporting the SNARE zippering by reduction of picoseconds dynamics in the disordered part of SNAP25a. This would help reduce the energy barrier to SNARE zippering by limiting the range of motion of SNAP25a's flexible backbone, enabling folding and binding. The helical regions of the SNAP25a backbone may serve as anchor points for SNARE zippering [56, 57].

To conclude, high-field NMR relaxation data from 600 to 1200 MHz and their analysis by three independent approaches (Model-free, IMPACT, Detectors) have provided us with detailed and impressive insights into the residual backbone dynamics of the IDP SNAP25a in the ps and ns range, with a link to biological function.

References

1. Rizo, J., *Molecular Mechanisms Underlying Neurotransmitter Release*. Annu Rev Biophys, 2022. **51**: p. 377-408.
2. Jahn, R., D.C. Cafiso, and L.K. Tamm, *Mechanisms of SNARE proteins in membrane fusion*. Nat Rev Mol Cell Biol, 2024. **25**(2): p. 101-118.
3. Stief, T., et al., *Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its Pre-fusion Conformation*. J Mol Biol, 2023. **435**(10): p. 168069.
4. Dunker, A.K., et al., *Intrinsically disordered protein*. J Mol Graph Model, 2001. **19**(1): p. 26-59.
5. Dyson, H.J. and P.E. Wright, *Intrinsically unstructured proteins and their functions*. Nat Rev Mol Cell Biol, 2005. **6**(3): p. 197-208.
6. Uversky, V.N., *Natively unfolded proteins: a point where biology waits for physics*. Protein Sci, 2002. **11**(4): p. 739-56.
7. van der Lee, R., et al., *Classification of intrinsically disordered regions and proteins*. Chem Rev, 2014. **114**(13): p. 6589-631.
8. Wright, P.E., H.J. Dyson, and R.A. Lerner, *Conformation of peptide fragments of proteins in aqueous solution: implications for initiation of protein folding*. Biochemistry, 1988. **27**(19): p. 7167-75.
9. Dyson, H.J. and P.E. Wright, *Equilibrium NMR studies of unfolded and partially folded proteins*. Nat Struct Biol, 1998. **5 Suppl**: p. 499-503.

10. Smith, L.J., et al., *The concept of a random coil: Residual structure in peptides and denatured proteins*. Folding and Design, 1996. **1**(5): p. R95-R106.
11. Ramachandran, G.N., C. Ramakrishnan, and V. Sasisekharan, *Stereochemistry of polypeptide chain configurations*. Journal of Molecular Biology, 1963. **7**(1): p. 95-99.
12. Tompa, P., et al., *Intrinsically disordered proteins: emerging interaction specialists*. Curr Opin Struct Biol, 2015. **35**: p. 49-59.
13. Salvi, N., A. Abyzov, and M. Blackledge, *Atomic resolution conformational dynamics of intrinsically disordered proteins from NMR spin relaxation*. Prog Nucl Magn Reson Spectrosc, 2017. **102-103**: p. 43-60.
14. Schneider, R., M. Blackledge, and M.R. Jensen, *Elucidating binding mechanisms and dynamics of intrinsically disordered protein complexes using NMR spectroscopy*. Curr Opin Struct Biol, 2019. **54**: p. 10-18.
15. Bah, A., et al., *Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch*. Nature, 2015. **519**(7541): p. 106-9.
16. Rezaei-Ghaleh, N., M. Blackledge, and M. Zweckstetter, *Intrinsically disordered proteins: from sequence and conformational properties toward drug discovery*. Chembiochem, 2012. **13**(7): p. 930-50.
17. Jensen, M.R., et al., *Defining conformational ensembles of intrinsically disordered and partially folded proteins directly from chemical shifts*. J Am Chem Soc, 2010. **132**(4): p. 1270-2.
18. Mittag, T. and J.D. Forman-Kay, *Atomic-level characterization of disordered protein ensembles*. Curr Opin Struct Biol, 2007. **17**(1): p. 3-14.
19. Camacho-Zarco, A.R., et al., *NMR Provides Unique Insight into the Functional Dynamics and Interactions of Intrinsically Disordered Proteins*. Chem Rev, 2022. **122**(10): p. 9331-9356.
20. Sugase, K., H.J. Dyson, and P.E. Wright, *Mechanism of coupled folding and binding of an intrinsically disordered protein*. Nature, 2007. **447**(7147): p. 1021-5.
21. Bessa, L.M., et al., *The intrinsically disordered SARS-CoV-2 nucleoprotein in dynamic complex with its viral partner nsp3a*. Sci Adv, 2022. **8**(3): p. eabm4034.
22. Kay, L.E., D.A. Torchia, and A. Bax, *Backbone dynamics of proteins as studied by ¹⁵N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease*. Biochemistry, 1989. **28**(23): p. 8972-9.
23. Farrow, N.A., et al., *Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by ¹⁵N NMR relaxation*. Biochemistry, 1994. **33**(19): p. 5984-6003.
24. Palmer, A.G., 3rd, *NMR characterization of the dynamics of biomacromolecules*. Chem Rev, 2004. **104**(8): p. 3623-40.
25. Gill, M.L., R.A. Byrd, and A.G. Palmer, III, *Dynamics of GCN4 facilitate DNA interaction: a model-free analysis of an intrinsically disordered region*. Phys Chem Chem Phys, 2016. **18**(8): p. 5839-49.
26. Abyzov, A., et al., *Identification of Dynamic Modes in an Intrinsically Disordered Protein Using Temperature-Dependent NMR Relaxation*. J Am Chem Soc, 2016. **138**(19): p. 6240-51.
27. Salvi, N., A. Abyzov, and M. Blackledge, *Solvent-dependent segmental dynamics in intrinsically disordered proteins*. Sci Adv, 2019. **5**(6): p. eaax2348.
28. Parigi, G., et al., *Long-Range Correlated Dynamics in Intrinsically Disordered Proteins*. Journal of the American Chemical Society, 2014. **136**(46): p. 16201-16209.
29. Lipari, G. and A. Szabo, *Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity*. Journal of the American Chemical Society, 1982. **104**(17): p. 4546-4559.
30. Clore, G.M., et al., *Deviations from the simple two-parameter model-free approach to the interpretation of nitrogen-15 nuclear magnetic relaxation of proteins*. Journal of the American Chemical Society, 1990. **112**(12): p. 4989-4991.
31. Khan, Shahid N., et al., *Distribution of Pico- and Nanosecond Motions in Disordered Proteins from Nuclear Spin Relaxation*. Biophysical Journal, 2015. **109**(5): p. 988-999.
32. Smith, A.A., M. Ernst, and B.H. Meier, *Optimized "detectors" for dynamics analysis in solid-state NMR*. The Journal of Chemical Physics, 2018. **148**(4).
33. Zumpfe, K. and A.A. Smith, *Model-Free or Not?* Front Mol Biosci, 2021. **8**: p. 727553.
34. Palmer, A.G., III, *NMR Characterization of the Dynamics of Biomacromolecules*. Chemical Reviews, 2004. **104**(8): p. 3623-3640.

35. Charlier, C., et al., *Nanosecond Time Scale Motions in Proteins Revealed by High-Resolution NMR Relaxometry*. Journal of the American Chemical Society, 2013. **135**(49): p. 18665-18672.
36. Abyzov, A., et al., *Identification of Dynamic Modes in an Intrinsically Disordered Protein Using Temperature-Dependent NMR Relaxation*. Journal of the American Chemical Society, 2016. **138**(19): p. 6240-6251.
37. Pobbati, A.V., A. Stein, and D. Fasshauer, *N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion*. Science, 2006. **313**(5787): p. 673-676.
38. Stief, T., K. Vormann, and N.A. Lakomek, *Sensitivity-enhanced NMR (^{15}N R(1) and R(1rho) relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields*. Methods, 2024. **223**: p. 1-15.
39. Stief, T., K. Vormann, and N.A. Lakomek, *NMR ^{15}N Relaxation Experiments for the Investigation of Picosecond to Nanoseconds Structural Dynamics of Proteins*. J Vis Exp, 2024(213).
40. Lakomek, N.A., J. Ying, and A. Bax, *Measurement of (1)(5)N relaxation rates in perdeuterated proteins by TROSY-based methods*. J Biomol NMR, 2012. **53**(3): p. 209-21.
41. Delaglio, F., et al., *NMRPipe: A multidimensional spectral processing system based on UNIX pipes*. Journal of Biomolecular NMR, 1995. **6**(3): p. 277-293.
42. d'Auvergne, E.J. and P.R. Gooley, *Optimisation of NMR dynamic models II. A new methodology for the dual optimisation of the model-free parameters and the Brownian rotational diffusion tensor*. Journal of Biomolecular NMR, 2008. **40**(2): p. 121-133.
43. d'Auvergne, E.J. and P.R. Gooley, *Optimisation of NMR dynamic models I. Minimisation algorithms and their performance within the model-free and Brownian rotational diffusion spaces*. Journal of Biomolecular NMR, 2008. **40**(2): p. 107-119.
44. Wales, D.J. and J.P.K. Doye, *Global Optimization by Basin-Hopping and the Lowest Energy Structures of Lennard-Jones Clusters Containing up to 110 Atoms*. The Journal of Physical Chemistry A, 1997. **101**(28): p. 5111-5116.
45. Kraft, D., *A Software Package for Sequential Quadratic Programming*. 1988: Wiss. Berichtswesen d. DFVLR.
46. Virtanen, P., et al., *SciPy 1.0: fundamental algorithms for scientific computing in Python*. Nature Methods, 2020. **17**(3): p. 261-272.
47. Farrow, N.A., et al., *Spectral density function mapping using ^{15}N relaxation data exclusively*. Journal of Biomolecular NMR, 1995. **6**(2): p. 153-162.
48. Abragam, A., *The Principles of Nuclear Magnetism*. 1961: Clarendon Press.
49. Kraichely, K.N., et al., *Functionally distinct SNARE motifs of SNAP25 cooperate in SNARE assembly and membrane fusion*. Biophysical Journal, 2025. **124**(4): p. 637-650.
50. Tanford, C., *Protein denaturation*. Adv Protein Chem, 1968. **23**: p. 121-282.
51. Flory, P.J. and M. Volkenstein, *Statistical mechanics of chain molecules*. Biopolymers, 1969. **8**(5): p. 699-700.
52. Shi, Z., et al., *Conformation of the Backbone in Unfolded Proteins*. Chemical Reviews, 2006. **106**(5): p. 1877-1897.
53. Salvi, N., A. Abyzov, and M. Blackledge, *Analytical Description of NMR Relaxation Highlights Correlated Dynamics in Intrinsically Disordered Proteins*. Angew Chem Int Ed Engl, 2017. **56**(45): p. 14020-14024.
54. Mohammed, O.F., et al., *Primary peptide folding dynamics observed with ultrafast temperature jump*. Angew Chem Int Ed Engl, 2009. **48**(31): p. 5628-32.
55. Tobias, D.J., J.E. Mertz, and C.L. Brooks, 3rd, *Nanosecond time scale folding dynamics of a pentapeptide in water*. Biochemistry, 1991. **30**(24): p. 6054-8.
56. Kim, P.S. and R.L. Baldwin, *Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding*. Annu Rev Biochem, 1982. **51**: p. 459-89.
57. Werner, J.H., et al., *Dynamics of the Primary Processes of Protein Folding: Helix Nucleation*. The Journal of Physical Chemistry B, 2002. **106**(2): p. 487-494.
58. Dey, S., M. MacAinsh, and H.-X. Zhou, *Sequence-Dependent Backbone Dynamics of Intrinsically Disordered Proteins*. Journal of Chemical Theory and Computation, 2022. **18**(10): p. 6310-6323.

11 R2Hahn Echo Experiment for Detection of ms Dynamics

Chemical exchange dynamics in the protein backbone happening on the timescale of microseconds and milliseconds, whereby these dynamics are not detectable by the already presented ^{15}N R_1 and ^{15}N $R_{1\rho}$ relaxation experiments. The ^{15}N Hahn echo experiment is sensitive to exchange contribution occurring on a micro- to milliseconds timescale. Already published ^{15}N R_2 Hahn echo experiments struggle with water suppression, ^{13}C decoupling and were not tested at 1200 MHz.

The author optimized the ^{15}N sensitivity-enhanced Hahn echo pulse sequence for this project by implementing the Hahn echo element in our published ^{15}N relaxation rate pulse sequences with a sensitivity-enhanced readout (chapter 8). Additionally, the author acquired the Hahn echo experiments at two different fields (600 MHz and 1200 MHz). The postprocessing of the spectra and the analysis of the ^{15}N relaxation rates were done by the author. The author contributed text and illustrations to the manuscript. Following further experiments to deepen the discussion section, the corresponding manuscript will be submitted for publication in the next few weeks..

Journal: -

Volume: -

Authors: Tobias Stief
Katharina Vormann
Mirko Kraus
Sophia Werner
Nils-Alexander Lakomek

DOI: -

Abstract

^{15}N NMR relaxation methods are widely used to obtain residue-specific information on protein dynamics. Transverse ^{15}N R_2 relaxation rate constants are sensitive to picoseconds to low nanoseconds dynamics, caused by fast amid bond motions and the overall molecular tumbling, but also sensitive to millisecond and microsecond dynamics, caused by chemical exchange. This exchange contribution to the ^{15}N R_2 relaxation rate constant can be detected by a Hahn-echo experiment. We established a robust ^{15}N R_2 Hahn-echo sequence with a sensitivity-enhanced read out scheme, and tested it both at a 600 MHz and 1200 MHz NMR spectrometers. For human ubiquitin, the R_2 rate constants measured by the Hahn echo at both fields showed a strong correlation with ^{15}N R_2 rate constants extracted from ^{15}N $R_{1\rho}$ experiments. For $^{15}\text{N}^{13}\text{C}$ labeled samples, an adiabatic ^{13}C decoupling scheme was applied in addition. R_2 relaxation rate constant of the double labeled sample showed very high agreement to the ^{15}N labeled sample, as expected. For human ubiquitin, our data did not indicate any substantial $R_{2,\text{ex}}$ exchange contribution at a pH of 6.0 and a sample temperature of 25 °C, underlining that previously identified dynamics must occur on a sub- to low microsecond timescale at room temperature.

Introduction

Structural dynamics of proteins occur over a broad range of timescales, from femtoseconds up to several seconds [1-3]. Nuclear magnetic resonance (NMR) spectroscopy provides a variety of different techniques to reveal insights on protein motions with residue-specific resolution [4-7]. Especially ^{15}N NMR relaxation experiments, developed for labeled protein samples, are indispensable for studies of the protein backbone dynamics. They provide residue-specific insights into protein motions that affect the orientation of the N-H bond in the protein backbone. The transverse ^{15}N relaxation rate constant R_2^0 , extracted from $R_{1\rho}$ experiments, is sensitive to the fast internal dynamics on the picoseconds to low nanoseconds time scale, faster than the overall rotational diffusion of the protein. In contrast, ^{15}N R_2 extracted from Hahn-echo based experiments are sensitive to both, the fast ps-ns timescale (R_2^0) as well as chemical exchange contributions R_{ex} , therefore $R_2 = R_2^0 + R_{\text{ex}}$. The chemical exchange contribution originates from motions that lead to a modulation of the anisotropic ^{15}N chemical shift tensor, typically

occurring on a micro- to milliseconds timescale [8]. This exchange contribution is refocused in spin-lock based experiments, such as the $R_{1\rho}$ experiments [9, 10]. The degree to which the exchange contribution in the $R_{1\rho}$ experiment is refocused depends on the strengths of the spin-lock power [8, 11]. From NMR relaxation experiments, which are sensitive to chemical exchange, detailed information about possible protein function, protein folding, as disorder to order transitions, molecular recognition, and enzyme catalysis can be extracted, emphasizing the biomolecular relevance of the exchange contribution [5]. Further, exchange contributions, such as the deuterium isotope effect, has been revealed previously by using a Hahn echo Sequence with TROSY read out scheme[12].

In this work, we present a ^{15}N Hahn echo sequence, frequently called spin-echo sequence [13], for detection of the transversal ^{15}N relaxation rate constant R_2 sensitive to exchange contributions on the millisecond timescale. Our pulse scheme is based on the improved $R_{1\rho}$ sequence with sensitivity enhanced readout scheme published by Stief et al. 2024[14]. Here, we replaced the spin lock element by a Hahn echo building block[13], which length can be adjusted depending on the length of the super cycle of the applied proton decoupling scheme [15, 16]. We also tested successfully the ^{13}C decoupling during this pulse scheme for the application to ^{15}N - ^{13}C double labeled samples.

Methods

Sample Preparation

The gene encoding the human ubiquitin protein (HuUb; 8.8 kDa, 76 residues) from *Homo sapiens* (UniProt accession number: P62988) was codon-optimized for expression in *Escherichia coli* (*E.coli*) and subcloned into a pET28a(+) vector containing an N-terminal *hexa* histidine tag and a TEV protease cleavage site. Protein expression and purification were carried out according to the protocols described for the SARS-CoV-2 ubiquitin-like domain 1 (Ubl1) by Salvi et al. 2021 [17]. Based on the isoelectric point ($pI = 6.56$) of HuUb, all buffer solutions were prepared at pH 6.0. Uniformly labeled ^1H - ^{15}N and ^{15}N - ^{13}C HuUb samples were concentrated in 50 mM sodium phosphate buffer (pH 6.0) containing 150 mM NaCl. For subsequent NMR measurements, final protein samples (145 μM) were supplemented with 1% (v/v) NaN_3 and 2% (v/v) D_2O .

¹⁵N R₂ Hahn Echo Sequence

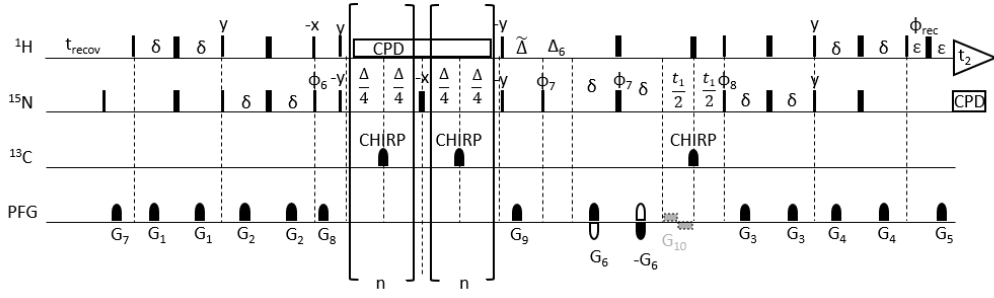


Figure 1: Pulse sequence schemes of ¹⁵N R₂ Hahn echo relaxation experiment using a sensitivity-enhanced HSQC read-out scheme. The following phase cycling is applied: $\phi_6 = y, y, -y, -y$; $\phi_7 = y, -y$, $\phi_{rec} = y, -y, -y, y$. Quadrature detection is implemented by the polarity inversion of the encoding gradient G_6 and the phase cycling of ϕ_8 (Echo/ Anti-Echo detection [18, 19]). Narrow rectangles correspond to 90° hard pulses with phase x, if not marked otherwise. 180° hard pulses are displayed as broad rectangles.

In Figure 1 we present the optimized ¹⁵N R₂ Hahn echo pulse scheme. This pulse sequence is based on a sensitivity-enhanced HSQC detection scheme [20, 21], which has already been used by several groups for ¹⁵N relaxation experiments [22, 23]. The gradients G_5 and G_6 , as the phase cycle of ϕ_8 implement the Echo/ Anti-Echo quadrature detection[18, 19] for a signal-to-noise enhancement compared to traditional quadrature detection schemes [24, 25]. Water suppression is achieved by keeping the water trajectory along the z-direction during the pulse program. Sinc.20 shaped gradients ($G_1, G_2, G_3, G_4, G_8, G_9$) prevent radiation damping[26, 27], a common problem at cryo probes and high magnetic fields, which is solved by the application of already weak gradients that dephase the water magnetization[28]. The gradient G_{10} can be activated as a long, soft gradient ($\leq 1\%$) when radiation damping occurs for long t_1 delays. A short T2-filter of $\tilde{\Delta} =$ of 100 ms was advantageous in terms of water suppression, additionally unwanted coherences are filtered out, e.g. arising from 90° pulse imperfections. The delay Δ_6 compensates the initial ¹⁵N evolution during t_1 and is not incremented. The Hahn echo delay is implemented with two loops, which length is defined by the loop counter n times the delay $\frac{\Delta}{2}$. For preventing systematic errors in ¹⁵N-¹³C labeled samples, occurring from ¹⁵N ¹³C $^{\alpha}$ as well as ¹⁵N ¹³C' J-coupling evolution, and additional cross-correlated relaxation (CCR) terms, such as between the ¹⁵N chemical shift anisotropy (CSA) and the ¹⁵N-¹³C $^{\alpha}$ or dipolar coupling interactions, a 500 μ s long adiabatic CHIRP pulse is placed

in the center of each loop at a ^{13}C carrier frequency of 101 ppm. Between both loops, the 180° ^{15}N pulse refocuses chemical shift evolution, as part of the Hahn echo element. The total lengths of the Hahn echo are n times Δ . In two experiments, the reference experiment has a Hahn echo delay of Δ , and the second experiment has a delay of n times Δ , at which the magnetization should decay by about 37% ($\frac{1}{e}$) compared to the reference experiment. During the whole Hahn echo delay, ^1H decoupling is applied to prevent J-coupling evolution as well as the cross-correlated relaxation between the ^{15}N chemical shift anisotropy (CSA) and the ^1H - ^{15}N dipolar coupling interactions. To prevent disturbed water magnetization and secure a similar course of the water trajectory after the Hahn echo for both relaxation delays, Δ corresponds to the length of the supercycle of the selected decoupling scheme. In the case of a garp decoupling scheme with 70 μs pulse length, this means $\Delta = 126.972 * 70 \mu\text{s} = 8.88804 \text{ ms}$.

Parameters of Relaxation Experiments

For testing and validation of the Hahn echo pulse program, we recorded ^{15}N R_2 rates with the Hahn Echo, as well as ^{15}N R_1 rates and ^{15}N $R_{1\rho}$ rates of the ^{15}N human Ubiquitin sample and of the ^{15}N ^{13}C human Ubiquitin sample both at 600 MHz and a 1200 MHz s. All experiments were implemented as pseudo-3D experiments, with two 2D planes (2-point measurement) in the Hahn Echo experiment and eight 2D planes (8-point measurement) in the R_1 and $R_{1\rho}$ experiment. The 2D-planes were measured with 128 complex points in the ^{15}N dimension at 600 MHz and 256 complex points in the ^{15}N dimension at 1200 MHz. In the ^1H dimension, 1024 complex points were recorded at 600 MHz and 2048 complex points at 1200 MHz. 16.02 ppm were selected for the spectral width in ^1H dimension, corresponding to an acquisition time of 106.5 ms for both 600 MHz and 1200 MHz. The spectral width in the ^{15}N dimension was 33.00 ppm, resulting in acquisition times of 63.8 ms at 600 MHz and 1200 MHz. The carrier frequency in ^1H dimension was set to 4.699 ppm, the ^{13}C carrier to 101 ppm, and ^{15}N to 117.5 ppm. The relaxation delays for the Hahn echo experiment with garp proton decoupling scheme were chosen as follows: [$1 * \Delta = 8.88804 \text{ ms}$, $18 * \Delta = 159.98472 \text{ ms}$] at 600 MHz and [$1 * \Delta = 8.88804 \text{ ms}$, $12 * \Delta = 106.65648 \text{ ms}$] at 1200 MHz. [0 ms, 720 ms, 160 ms, 640 ms, 80ms, 560ms, 320ms, 480 ms] were selected for the relaxation delays in the R_1 experiment at 600 MHz, [0 ms, 800 ms, 240 ms, 80 ms, 160 ms, 640 ms, 320 ms, 480 ms] at the 1200 MHz spectrometer. For the $R_{1\rho}$ experiment the list [5 ms,

65 ms, 25 ms, 45 ms, 15 ms, 55 ms, 35 ms, 10 ms] was used at 600 MHz and 1200 MHz. The interscan delay d_1 was set to 2.0 seconds for every experiment. The resulting experimental time for the Hahn echo was 5 h 32 min at 600 MHz and 10 h 52 min at 1200 MHz, the R_1 experiment had a duration of 11 h 50 min at 600 MHz and 23 h 17 min 1200 MHz, and finally the calculated time for the $R_{1\rho}$ experiment was 10 h 19 min at 600 MHz and 20 h 32 min at 1200 MHz. More parameters of the relaxation experiments on human Ubiquitin, as the detailed parameters of the relaxation experiments for ^{15}N - ^{13}C human Ubiquitin are attached in the SI (Table S1 – S4).

Evaluation of Relaxation Data

For the evaluation of the sensitivity enhanced pseudo-3D HSQC experiments, NMR pipe [29] was used. The scripts are available under the following link: www.ipb.lhu.de/en/teams/team-lakomek/pulsesequences.

The R_2^0 rates were calculated from the R_1 and $R_{1\rho}$ relaxation rates using the following equation [30, 31]:

$$R_2 = \frac{R_{1\rho}}{\sin^2 \theta} - \frac{R_1}{\tan^2 \theta} \quad \text{where } \theta = \arctan\left(\frac{\omega_1}{\Omega}\right)$$

The angle between the axis of the effective magnetic field B_{eff} and the external magnetic field B_0 is defined by θ . Because of the proportionality between the magnetic field and Larmor frequency, the angle θ can be calculated from the RF amplitude of the spin-lock ω_1 and the chemical shift difference Ω between the ^{15}N chemical shift of the respective residue and the ^{15}N carrier frequency [30, 31].

The ^{15}N R_2 rate constant from the Hahn echo was calculated with:

$$R_2 = -\frac{1}{\Delta} \ln\left(\frac{I(\Delta)}{I_0}\right)$$

The peak intensity of the reference experiment is I_0 and the peak intensity of the delay experiment is $I(\Delta)$. Δ is the Hahn echo delay.

For the ^{15}N R_2 Hahn echo experiment (2-point measurement), the error was calculated with a Gaussian error propagation [32]. Due to the 8-point measurement of the ^{15}N R_1 and ^{15}N $R_{1\rho}$ experiments, the error of the R_1 and $R_{1\rho}$ relaxation rates was calculated using a Monte-Carlo-based error analysis[33] provided by NMR pipe [29].

Results

We had tested the Hahn echo pulse scheme on the globular protein human Ubiquitin, which shows small relaxation rates in the range of 6 rad / s-1 at 600 MHz (Figure 2A). Previously, only little chemical exchange was detected for human ubiquitin[11, 34-37], which has the advantage of allowing comparison of R_2 rates from the Hahn echo sequence and the R_2 rates derived from the $R_{1\rho}$ sequence with a 2 kHz spin lock, which averages out dynamics slower than the inverse of the spin-lock frequency, thus slower than about 80 microseconds.

^{15}N R_2 at 600 MHz and 1200 MHz

For evaluating the quality of the R_2 Hahn echo pulse sequence, we compare the R_2 rate constants measured using the Hahn echo based sequence (Figure 1) with the R_2 rate constants (dubbed R_2^0 , see above), derived from $R_{1\rho}$ and R_1 experiments, acquired with established pulse schemes[14].

The R_2 rates from the Hahn echo sequences and the R_2^0 rates derived from the $R_{1\rho}$ and R_1 experiments, both acquired at a ^{15}N labeled sample, are shown in Figure 2. At 600 MHz (A-C), the R_2 dataset and the R_2^0 dataset show high agreement, with a Pearson correlation coefficient R^2 of 0.959 (B). The non-equal distribution of the percentage difference between both data sets around zero (C), indicates an offset between the rate constants. The R_2 rates in mean are 3.58 % higher than the R_2^0 rates, which appears to be systematic, as shown by the equal offset of rates for similar residues (A). Interestingly, the relaxation rate constants at 1200 MHz did not show such differences (D-F). Here, the mean value of the percentage differences of 0.35% is close to zero and the narrow box width (defined by first and third quartile) of the box plot shows almost no offset between the residue specific rate constants (F). The Pearson correlation coefficient R^2 of 0.954 indicates high correlation between both data sets (F).

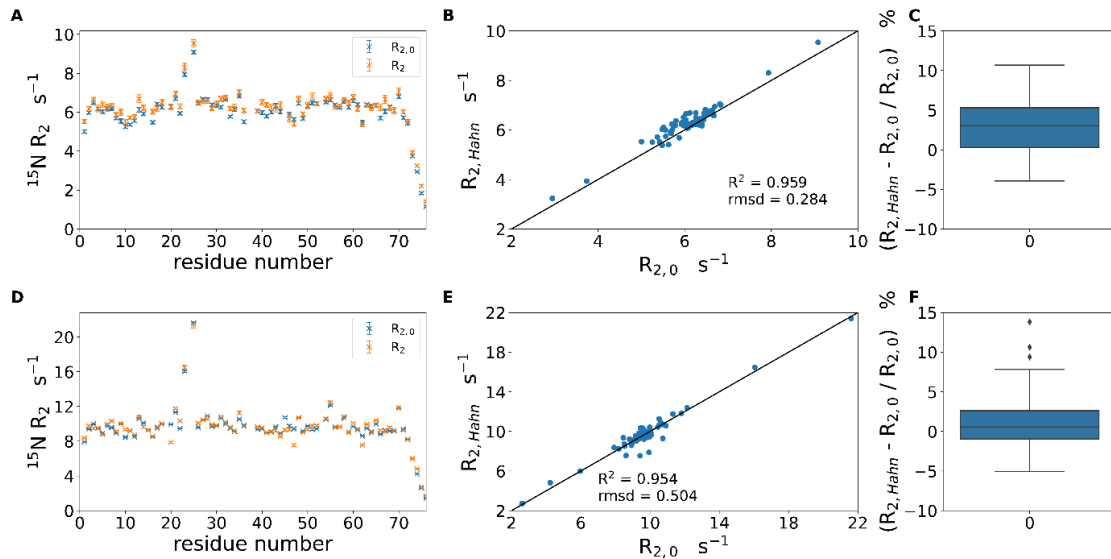


Figure 2: Comparison of R_2 relaxation rates of ^1H - ^{15}N human ubiquitin acquired with the pulse schemes in Figure 1 (Hahn echo) and the sensitivity-enhanced R_1 and $R_{1\rho}$ sequence from Stief et al 2024. A-C: Data from 600 MHz spectrometer at 25 °C A: Residue-specific comparison of relaxation rate R_2 , calculated from the $R_{1\rho}$ and R_1 rate constants (Blue) and the R_2 rate constant detected with the Hahn echo sequence (Orange). B: Correlation plot of the compared R_2 relaxation rate constants. C: Box plot of the residue-specific percentual differences of R_2 relaxation rate constants. D-F: Same plots as in A-C with data from the 1200 MHz (28.2 T) spectrometer.

^{15}N ^{13}C double labeled samples

As many people start their structural dynamic protein analysis with a 3D backbone assignment, for which a double-labeled ^{15}N - ^{13}C sample is necessary, we also included a ^{13}C decoupling. This decoupling is implemented with CHIRP pulses every $d_5/2$ (here: 4.44402 milliseconds) during the Hahn echo and with a single CHIRP pulse during the ^{15}N evolution time.

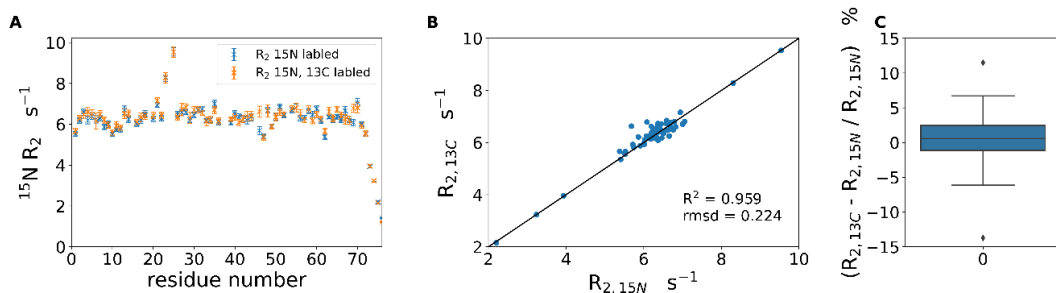


Figure 2: Comparison of R_2 relaxation rates of ^1H - ^{15}N and ^1H - ^{15}N - ^{13}C human ubiquitin acquired with the pulse schemes in Figure 1 (Hahn echo) at 600 MHz and 25°C. A: Residue-specific comparison of relaxation rate R_2 of the ^1H - ^{15}N sample (Blue) and the ^1H - ^{15}N - ^{13}C (Orange). B: Correlation plot of the compared R_2 relaxation rate constants. C: Box plot of the residue-specific percentual differences of R_2 relaxation rate constants.

Figure 3 A-C compares the R_2 rates from the Hahn echo between the ^{15}N human Ubiquitin and $^{15}\text{N}^{13}\text{C}$ human ubiquitin sample at 600 MHz. Both data sets show visually high agreement in the rate constants (A). The high correlation ($R^2 = 0.959$) and small root-mean-square deviation ($\text{rmsd} = 0.224$) in Figure 3 B statistically prove the high consistency of the recorded data. Figure 3 C shows a narrow boxplot with values distributed symmetrically around zero, indicating well matching rate constants. The high agreement between the relaxation rates of both pulse schemes proves the efficiency of the adiabatic decoupling applied during the pulse scheme in Figure 1.

Recommendations

The right choice of the proton decoupling scheme is necessary to average out transversal cross-correlated relaxation, mediated by the ^1H ^{15}N dipolar coupling and the ^{15}N CSA. We recommend the use of the garp pulse scheme, as it has a broader excitation bandwidth compared to a waltz decoupling scheme. For example, at 1200 MHz, a waltz decoupling scheme with 100 ms is not broad enough and leads to off-resonance effects such as a chemical-shift dependence in the relaxation rate constants (Figure S1). We recommend 3.57 kHz ($=70\ \mu\text{s}$) decoupling pulse length with a garp decoupling scheme.

We are using a 2-point measurement for the determination of the R_2 rate constants, which appears statistically unstable at first glance. We tested different relaxation delays ranging from 106.66 ms to 177.76 ms. Across all experiments with different delay lengths, we found a high degree of consistency between the R_2 rate constants (Figure S2). Nevertheless, we recommend using a delay Δ for which the Hahn echo intensity drops to $\sim 37\%$ ($1/e$). This can be tested by comparing the 1D spectrum of the processed first and second FID.

For best water suppression, we strongly recommend the calibration of the ^1H pulse length and the carrier frequency o1p.

Discussion

We tested the R_2 Hahn echo sequence at 600 MHz and 1200 MHz and observed high correlation with the R_2^0 rates of human ubiquitin, extracted from ^{15}N $R_{1\rho}$ experiments. Additionally, high correlation was observed for the comparison of the R_2 rates of a ^{15}N labeled sample and a $^{15}\text{N}^{13}\text{C}$ labeled sample. Therefore, we were able to validate the Hahn echo sequence for the precise detection of the R_2 rate constant ($R_2^0 + R_{\text{ex}}$).

In 2005, Massi et al. determined a chemical exchange rate for the residues I23, N25, T55, and V70 of approximately 25000 s^{-1} at $5\text{ }^\circ\text{C}$ [11]. We were not able to detect any chemical exchange along the backbone of human ubiquitin, but at room temperature, the conformational exchange process for N25 would likely be too fast to lead to a substantial exchange contribution. The offset of the R_2 rate at 600 MHz had a spectrometer-specific reason, as we were not able to see the offset at 1200 MHz although the exchange contribution scales with the square of the external magnetic field. The systematic error is most likely to occur due to an issue directly related to the magnet, the probe or the spectrometer. Otherwise we cannot exclude exchange along human ubiquitin, as we would expect faster exchange than 25 kHz at $25\text{ }^\circ\text{C}$ compared to $5\text{ }^\circ\text{C}$. This temperature difference results in exchange, to which both our Hahn echo and our $R_{1\rho}$ with 2 kHz spin lock are insensitive. Therefore, conformational exchange process in ubiquitin likely occur on a very fast time scale, difficult to catch by standard ^{15}N -based pulse sequences at room temperature. Indeed, high power $R_{1\rho}$ experiments by Griesinger and co-workers identified protein motions on a low-microsecond timescale[37-39].

References

1. Mittermaier, A.K. and L.E. Kay, *Observing biological dynamics at atomic resolution using NMR*. Trends Biochem Sci, 2009. **34**(12): p. 601-11.
2. Palmer, A.G., 3rd, *Enzyme dynamics from NMR spectroscopy*. Acc Chem Res, 2015. **48**(2): p. 457-65.
3. Torchia, D.A., *Dynamics of biomolecules from picoseconds to seconds at atomic resolution*. J Magn Reson, 2011. **212**(1): p. 1-10.
4. Mittermaier, A. and L.E. Kay, *New tools provide new insights in NMR studies of protein dynamics*. Science, 2006. **312**(5771): p. 224-8.
5. Palmer, A.G., 3rd, *NMR characterization of the dynamics of biomacromolecules*. Chem Rev, 2004. **104**(8): p. 3623-40.
6. Ishima, R. and D.A. Torchia, *Protein dynamics from NMR*. Nat Struct Biol, 2000. **7**(9): p. 740-3.
7. Kay, L.E., *Protein dynamics from NMR*. Nature Structural Biology, 1998. **5**(7): p. 513-517.
8. Millet, O., et al., *The Static Magnetic Field Dependence of Chemical Exchange Linebroadening Defines the NMR Chemical Shift Time Scale*. Journal of the American Chemical Society, 2000. **122**(12): p. 2867-2877.
9. Palmer, A.G., C.D. Kroenke, and J. Patrick Loria, *[10] - Nuclear Magnetic Resonance Methods for Quantifying Microsecond-to-Millisecond Motions in Biological Macromolecules*, in *Methods in Enzymology*, T.L. James, V. Dötsch, and U. Schmitz, Editors. 2001, Academic Press. p. 204-238.
10. Palmer, A.G., *Chemical exchange in biomacromolecules: Past, present, and future*. Journal of Magnetic Resonance, 2014. **241**: p. 3-17.
11. Massi, F., M.J. Grey, and A.G. Palmer III, *Microsecond timescale backbone conformational dynamics in ubiquitin studied with NMR R1ρ relaxation experiments*. Protein Science, 2005. **14**(3): p. 735-742.
12. Kumari, P., et al., *(15)N transverse relaxation measurements for the characterization of micro-ms dynamics are deteriorated by the deuterium isotope effect on (15)N resulting from solvent exchange*. J Biomol NMR, 2018. **72**(3-4): p. 125-137.
13. Hahn, E.L., *Spin Echoes*. Physical Review, 1950. **80**(4): p. 580-594.
14. Stief, T., K. Vormann, and N.A. Lakomek, *Sensitivity-enhanced NMR (15)N R(1) and R(1rho) relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields*. Methods, 2024. **223**: p. 1-15.
15. Boyd, J., U. Hommel, and I.D. Campbell, *Influence of cross-correlation between dipolar and anisotropic chemical shift relaxation mechanisms upon longitudinal relaxation rates of 15N in macromolecules*. Chemical Physics Letters, 1990. **175**(5): p. 477-482.
16. John Cavanagh, N.J.S., Wayne J. Fairbrother, Mark Rance, Arthur G. Palmer II, *Protein NMR Spectroscopy: Principles and Practice. 2nd Ed (9780121644918)*. 2007: Academic Press.
17. Salvi, N., et al., *(1)H, (13)C and (15)N backbone chemical shift assignments of SARS-CoV-2 nsp3a*. Biomol NMR Assign, 2021. **15**(1): p. 173-176.
18. Kay, L.E., P. Keifer, and T. Saarinen, *Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity*. Journal of the American Chemical Society, 1992. **114**(26): p. 10663-10665.
19. Palmer, A.G., et al., *Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy*. Journal of Magnetic Resonance (1969), 1991. **93**(1): p. 151-170.
20. Schleucher, J., M. Sattler, and C. Griesinger, *Coherence Selection by Gradients without Signal Attenuation: Application to the Three-Dimensional HNCO Experiment*. Angewandte Chemie International Edition in English, 1993. **32**(10): p. 1489-1491.
21. Sattler, M., et al., *A simultaneous (15)N, (1)H- and (13)C, (1)H-HSQC with sensitivity enhancement and a heteronuclear gradient echo*. J Biomol NMR, 1995. **5**(1): p. 97-102.
22. Lakomek, N.A., J. Ying, and A. Bax, *Measurement of (1)(5)N relaxation rates in perdeuterated proteins by TROSY-based methods*. J Biomol NMR, 2012. **53**(3): p. 209-21.
23. Gairi, M., et al., *An optimized method for 15N R1 relaxation rate measurements in non-deuterated proteins*. Journal of Biomolecular NMR, 2015. **62**(2): p. 209-220.

24. Ernst, R.R. and W.A. Anderson, *Application of Fourier Transform Spectroscopy to Magnetic Resonance*. Review of Scientific Instruments, 1966. **37**(1): p. 93-102.
25. States, D.J., R.A. Haberkorn, and D.J. Ruben, *A two-dimensional nuclear overhauser experiment with pure absorption phase in four quadrants*. Journal of Magnetic Resonance (1969), 1982. **48**(2): p. 286-292.
26. Bloembergen, N. and R.V. Pound, *Radiation Damping in Magnetic Resonance Experiments*. Physical Review, 1954. **95**(1): p. 8-12.
27. Ishima, R., *Effects of radiation damping for biomolecular NMR experiments in solution: a hemisphere concept for water suppression*. Concepts Magn Reson Part A Bridg Educ Res, 2015. **44A**(5): p. 252-262.
28. Sklenar, V., *Suppression of Radiation Damping in Multidimensional NMR Experiments Using Magnetic Field Gradients*. Journal of Magnetic Resonance, Series A, 1995. **114**(1): p. 132-135.
29. Delaglio, F., et al., *NMRPipe: A multidimensional spectral processing system based on UNIX pipes*. Journal of Biomolecular NMR, 1995. **6**(3): p. 277-293.
30. Desvaux, H. and P. Berthault, *Study of dynamic processes in liquids using off-resonance rf irradiation*. Progress in Nuclear Magnetic Resonance Spectroscopy, 1999. **35**(4): p. 295-340.
31. Palmer, A.G., 3rd and F. Massi, *Characterization of the dynamics of biomacromolecules using rotating-frame spin relaxation NMR spectroscopy*. Chem Rev, 2006. **106**(5): p. 1700-19.
32. Taylor, J.R., *An Introduction to Error Analysis: The Study of Uncertainties in Physical Measurements*. 1997: University Science Books.
33. Metropolis, N. and S. Ulam, *The Monte Carlo Method*. Journal of the American Statistical Association, 1949. **44**(247): p. 335-341.
34. Mills, J.L. and T. Szyperski, *Protein dynamics in supercooled water: the search for slow motional modes*. J Biomol NMR, 2002. **23**(1): p. 63-7.
35. Hansen, D.F., et al., *Selective characterization of microsecond motions in proteins by NMR relaxation*. J Am Chem Soc, 2009. **131**(44): p. 16257-65.
36. Tollinger, M., et al., *Site-resolved measurement of microsecond-to-millisecond conformational-exchange processes in proteins by solid-state NMR spectroscopy*. J Am Chem Soc, 2012. **134**(36): p. 14800-7.
37. Ban, D., et al., *Kinetics of conformational sampling in ubiquitin*. Angew Chem Int Ed Engl, 2011. **50**(48): p. 11437-40.
38. Ban, D., et al., *Exceeding the limit of dynamics studies on biomolecules using high spin-lock field strengths with a cryogenically cooled probehead*. J Magn Reson, 2012. **221**: p. 1-4.
39. Smith, C.A., et al., *Population shuffling of protein conformations*. Angew Chem Int Ed Engl, 2015. **54**(1): p. 207-10.

12 Conclusion and Outlook

During my PhD work in the group of Dr. Nils-Alexander Lakomek, I developed and applied several NMR experiments, with a focus on NMR relaxation measurements to study the structure and dynamic of intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs). Currently, three publications originated from that, as well as two more manuscripts that are close to submission. Stief et al., JMB 2023 (presented in chapter 7), Stief et al., Methods 2024 (chapter 8), and Stief et al., Jove 2024 (chapter 9). Chapters 10 and 11 refer to two more first-author manuscripts. The applied NMR methods and complementary biophysical methods can be considered as a workflow for the structural analysis and dynamic investigation of proteins, particularly intrinsically disordered proteins.

In Stief et al. 2023, we began the structural analysis with CD spectroscopy, an established biophysical method for investigating protein secondary structure. In the case of SNAP25a, we found 16.9% helical conformation and at least 50.6% random coil conformation along the backbone of SNAP25a. This result aligns not only with the NMR data, it also agrees with early CD data recorded on SNAP25¹⁹⁶. In that work, Fasshauer et al. investigated the overall protein structure under different buffer conditions and pH values using CD spectroscopy.

SAXS measurements were applied to SNAP25a, which, on the one hand, excluded dimerization and, on the other hand, underlined the chain characteristics of the polypeptide SNAP25a. The R_g/R_h ratio of 0.96 suggested IDP characteristics along the protein backbone of SNAP25a. For comparison, the disordered myelin basic protein, for example, has $R_g/R_h = 1.06$ ¹⁸³. SNAP25a is more compact than a Gaussian coil, but still a random chain as expected for intrinsically disordered proteins.

Residue-specific insights into the intrinsically disordered protein can, however, only be obtained from NMR spectroscopy. Secondary chemical shifts are widely used to determine the secondary structure propensities of individual residues^{197,198}. They were obtained from the (almost complete) backbone assignment of SNAP25a, which we deposited in the BMRB (51598). We used POTENCI¹⁸¹ for the calculation of the random coil chemical shifts, which outperforms the most influential methods at the time¹⁸¹ (in the case of IDPs). The secondary chemical shifts revealed increased α -helical propensities for the N-terminus of SNAP25a and large intrinsically disordered regions along the backbone at 15 °C (Stief et al. 2023). Recently, Kraichely et al. 2025 confirmed our findings for SNAP25a for 25 °C¹⁹⁹.

Comparing to the predictions of different protein structure predictors, the experimentally observed disorder of the second SNARE motif of the monomeric form of SNAP25a was kind of surprising. However, these predictors, such as AlphaFold 2 or 3, are mainly trained using structures from the PDB²⁰⁰ and only structures of SNAP25a in complexes (after the zippering process with SNARE motifs assembling into a coiled coil structure) are deposited in the PDB. Therefore, the incorrect prediction of disorder can be rationalized, underscoring the importance of NMR spectroscopy for the experimental determination of protein secondary structure. As an illustration, we predicted the SNAP25a structure with the current AlphaFold 3 implementation²⁰¹. Figure A1 (Appendix page 219) shows the predicted structure, which is characterized by two long α -helices that overlap well with the SNARE motifs, as well as a largely unstructured linker domain. This structure shows strong similarities with the X-ray structure of the assembled SNARE complex (PDB structure 1N7S). AlphaFold 3 predicted the missing loop region with low confidence, supporting the hypothesis that AlphaFold 3 closely follows the PDB structure.

Disorder predictors also fail to predict the correct secondary structure of SNAP25a (tested with CAID 15/10/2025²⁰²). The reason for this is probably that X-ray crystallography and electron microscopy are not able to acquire residue-specific information about IDRs due to their high flexibility^{1,2,3}, which leads to a diffuse electron density⁴ and an aversion to crystallization¹⁹⁰. Most of the protein structures in the PDB were solved using X-ray crystallography and electron microscopy^{203,204}, such that IDPs and proteins with IDRs are underrepresented²⁰⁵. Consequently, secondary structure predictors are not sufficiently trained to accurately predict IDRs. This concludes that AlphaFold 3 and disorder predictors in general should be used with caution when it comes to predicting disorder. If the underrepresentation of IDPs in the PDB can be eliminated, possibly with support from NMR, disorder predictors, and structure predictors, as AlphaFold continues to improve. Until then, NMR secondary chemical shifts are the safest way to determine residue-specific information of secondary structure of flexible protein region, in particular IDRs and IDPs.

The next step in the workflow for structure and dynamic insights, is the analysis of structural backbone dynamics, based on the ^{15}N relaxation rate constants R_1 and R_2 as hetNOE values. In Stief et al. 2024 we optimized and tailored sensitivity-enhanced NMR ^{15}N R_1 and $R_{1\rho}$ relaxation experiments for highest magnetic fields, improved water-suppression and sensitivity, to allow the detection of reliable relaxation rate constants, even for fully protonated proteins. The usage of a TROSY readout scheme or an HSQC readout scheme was intensively discussed in chapter 8. In the group of NA Lakomek, we mostly study IDPs (SNAP25, Syb-2, α -Synuclein) and small proteins (human ubiquitin, Ubl1), for which the HSQC readout is the preferred pulse scheme. Deuteration of protein samples^{170,171,172} is no longer necessary, as we found only minor differences of the relaxation rate obtained from protonated or deuterated protein samples²⁰⁶.

The acquisition of the ^{15}N relaxation rate experiments, as described in Stief et al. 2024, resulted in a set of three relaxation rate constants (R_1 , R_2 , hetNOE) from three different fields (600 MHz, 900 MHz, 1200 MHz). In the last step of the workflow, we used three evaluation methods, extended model-free, IMPACT and Detectors, for extracting amplitudes and timescales of the backbone motions of SNAP25a. The results of these methods were highly consistent and extracted three dynamic modes: dynamics of several nanoseconds (mainly α -helical N-terminus), dynamics around one nanosecond (dominant for disordered residues), and dynamics of fast picoseconds faster 80 picoseconds (mainly disordered residues). These findings agree well with the work of Blackledge and coworkers, who suggested three dynamic modes in IDPs: segmental motions in the range of several nanoseconds, dihedral angle fluctuation in the range of hundreds of picoseconds up to nanoseconds, and bond librations faster than 50 picoseconds^{191,192}. Uncovering these dynamic modes contributes to a fundamental understanding of protein dynamics.

As an outlook, in the next analytical step, molecular dynamics (MD) simulation could help to identify the type of motion triggering the specific correlation times revealed by the quantitative analysis of the ^{15}N relaxation rates. The various analyses can also be improved in sensitivity by accounting for the specific correlation times in the dynamics of the protein backbone of interest and by modulating the density function. In 2011, Xue et al. already found specific dynamic modes with contributions to the overall dynamics for denatured human ubiquitin: 44 ps (0.30), 1.2 ns (0.42) and 9.4 ns (0.28)¹⁹³. The timescale of the motions and the motional contributions in brackets are in good agreement with the results of Stief et al. 2025 (chapter 10), leading to the optimistic assumption that MD simulations would reproduce the relaxation data of NMR spectroscopy of SNAP25a with high accuracy. Otherwise, the relaxation rates and dynamic modes will contribute to the optimization of force fields for the prediction of IDP dynamics, which would also impact simulation-based drug screening¹⁴.

Nevertheless, these findings of dynamic regimes in intrinsically disordered proteins leave space for speculation. Specifically, for the IDP SNAP25a, we were able to find segmental motions for regions with increased α -helical propensity (E73-K79, D99-K102, E123-M127, T190-A199) in addition to the N-terminus. In Stief et al. 2023 (chapter 7), we already hypothesized that the N-terminal region with its α -helical structure acts as a nucleation site for the SNARE zippering. Besides this, we further speculate based on the segmental motions that the regions E73-K79, D99-K102, E123-M127, and T190-A199 act as anchor points for the SNARE zippering and support the helical transition. Dynamics over several nanoseconds can involve up to 30 adjacent residues along the protein backbone²⁰⁷, coupling segmental movements and potentially reducing fast picosecond dynamics during α -helix transition. Furthermore, Parigi et al. (2014) assume that hydrodynamic couplings between segments with several nanosecond dynamic motions slow down the overall rotational motion of IDPs¹⁹⁴, thereby reducing the energy barrier for protein-protein interactions and favoring the SNARE zippering process. Mind the observation that the Syntaxin-1A:SNAP25 acceptor complex binding to C-terminal fragments of Syb-2 causes well-structured SNARE complexes (N-terminus of Syb-2 is missing)²⁰⁸, which show an accelerated SNARE zippering with full-length Syb-2²⁰⁸ and a reduced energy barrier for the assembly reaction²⁰⁹. The α -helical structured acceptor complex bound to the C-terminus of Syb-2 has to be dominated by segmental motions like the N-terminus of SNAP25a in its pre-fusion conformation. As the SNARE acceptor complex binds to the N-terminal Syb-2, causing only an N-terminally structured SNARE complex (the C-terminus of Syb-2 is missing) that assembles more slowly than the entire SNARE complex²⁰⁸, segmental motions could be essential for rapid SNARE zippering and would be associated with a reduced energy barrier for the reaction.

Indeed, IR spectroscopy studies also detected folding events during the helical formation in the sub-nanosecond range²¹⁰, which fits well with the high proportion of dynamics in the range from hundreds of picoseconds to one nanosecond in disordered regions that we observe. For a clearer picture of the SNARE zippering, the influence of the SNARE partner Synaptobrevin-2 and Syntaxin-1A on the dynamic properties of SNAP25a could proof the assumptions made. Since Syntaxin-1A binds tightly to SNAP25²¹¹, dynamic investigations of the SNARE motif regions are difficult if the corresponding signals are missing in the HSQC spectrum. Future investigations could include the dynamic study of isolated Syntaxin-1A to investigate regions with possible segmental motions to understand the reasons for binding on a dynamic and force driving level. The quantitative dynamic analysis of Synaptobrevin-2 would also help to elucidate the mechanisms and driving forces of SNARE zippering at the atomic level.

In chapter 11, I presented a R_2 Hahn echo pulse scheme with a sensitivity-enhanced readout scheme for the detection of microsecond and millisecond dynamics along the protein backbone. Hahn-echo-based R_2 rate constants are sensitive both to ps-ns dynamics (faster than the rotational correlation of protein) as well as μ s-ms conformational dynamics that lead to a modulation of the isotropic chemical shift. In contrast, R_2 rate constants derived from $R_{1\rho}$ (which have a spin-lock) are sensitive only to ps-ns dynamics faster than the rotational correlation time, as μ s-ms dynamics are refocused by the spin-lock.

To assess the extent of μ s-ms dynamics in ubiquitin, we compared Hahn-echo-based R_2 rate constants with those derived from $R_{1\rho}$ measurements. At 1200 MHz, both rate constants showed very high agreement, indicating little μ s-ms dynamics in the observable time window ($\geq 80 \mu$ s). However, at 600 MHz, the R_2 rates obtained from the Hahn echo sequence showed a systematic offset of approximately 3% relative to the R_2 rate constants derived from the $R_{1\rho}$ measurements. The correlation between the Hahn echo experiment and the spin-lock experiment is sufficiently high to consider the error systematic. As this offset was not observed at 1200 MHz, we can exclude chemical exchange as the cause of the systematic offset, since the chemical exchange contribution would scale with B_0^2 . Previously, chemical exchange was only detected for residues ILE23, ASN25, THR55, and VAL70¹². This exchange happens on a timescale of around 25 kHz at 5°C, a dynamic regime we are not sensitive to using the $R_{1\rho}$ sequence with a 2 kHz spin-lock or the long Hahn echo delays between 90 ms and 180 ms at 25°C.

We performed R_2 Hahn echo experiments with different relaxation delays to test the robustness of the relaxation rate sampling using a two-point measurement. We found no differences in relaxation rates (see Figure S2, page 283), whereby we can exclude the length of relaxation delay as a reason. To resolve the observed discrepancy and locate the cause of the offset, further experiments will be executed using slightly different ^{15}N 180° pulse lengths, as these pulse imperfections could lead to higher relaxation rates. A possible solution to this problem would be to use a composite pulse for the Hahn echo refocusing 180°, which has a broader excitation range. We will test different decoupling schemes and decoupling lengths to assess this variable. We will perform R_2 Hahn echo experiments with a long interscan delay d1 to exclude water saturation during the Hahn echo delay as the cause of increased rates. Saturated water protons would exchange with the amide protons and reduce the magnetization M_0 , since saturated water protons have long T_1 times.

We are optimistic to expand the discussion and resolve this problem after conducting the described experiments and identifying the reason for the systematic offset at 600 MHz. After that, the attached manuscript will be published, including further recommendations for preventing offset effects at 600 MHz.

In the context of this dissertation, the author was able to answer most of the guiding questions. SNAP25a is largely intrinsically disordered and can be termed as IDP. Fast internal dynamics, primarily in the picosecond and low-nanosecond regimes, characterize the backbone dynamics of SNAP25a. Reliable ^{15}N relaxation experiments were optimized to acquire accurate and robust relaxation rates at different magnetic fields. Beyond that, the author used several well-established biophysical methods as a workflow to study the secondary structure and protein backbone dynamics of intrinsically disordered proteins and regions: Stief et al. 2023, chapter 7, Stief et al. 2024, chapter 8, manuscript Stief et al., chapter 10. Most of these methods are also applicable to folded proteins. The author supported the NMR community in making the acquisition and evaluation of advanced state of the art ^{15}N relaxation experiments with sensitivity-enhanced readout easily applicable for beginners (Stief et al. 2024 chapter 9). Additionally, an optimized R_2 Hahn echo pulse program complements the set of ^{15}N NMR relaxation experiments and extends the observable time scale of motion to capture chemical exchange processes in the microsecond-to-millisecond motion regime (manuscript Stief et al. chapter 11).

In conclusion, the author established a robust NMR workflow in the group of Dr. Nils-Alexander Lakomek and applied it to the protein SNAP25a. This work revealed dynamic properties, which are in good agreement with the literature but provide more detailed insights into the dynamic time scales visited by IDPs. In addition, the author was able to support several projects in the group by establishing the underlying NMR experiments. Future projects should extend this work by including computational methods, such as molecular dynamics simulations, to obtain more atomistic insights into the dynamics of SNAP25a revealed by NMR spectroscopy and to gain deeper insights into the backbone dynamics of IDPs on the picoseconds to nanoseconds time scale.

List of Abbreviations

ACF	Auto Correlation Function
AHP	Adiabatic Half passage Pulse
α -Syn	α -Synuclein
BEST	Band Selective Short-Transient
BMRB	Biological Magnetic Resonance Data Bank
BWR	Bloch-Wangsness-Redfield
CCR	Cross Correlated Relaxation
CD-spectroscopy	Circular Dichroism spectroscopy
CEST	Chemical Exchange Saturation Transfer
COVID-19	COronaVirus Disease 2019
CPMG	Carr-Purcell-Meiboom-Gill
CSA	Chemical Shift Anisotropy
CW	Continuous Wave
DD	Dipolar Coupling
DS	DummyScans
EDTA	Ethylenediaminetetraacetic acid
EM	Electron Microscopy
EXSY	EXchange SpectroscopY

FID	Free Induction Decay
hetNOE	heteronuclear Nuclear Overhauser Effect
HMQC	Heteronuclear Single Quantum Coherence
HSQC	Heteronuclear Single Quantum Coherence
IBURP	Inverse Band-selective Uniform-Response Pure-phase
IDP	Intrinsically Disordered Protein
IDR	Intrinsically Disordered Region
IMPACT	Interpretation of Motions by a Projection onto an Array of Correlation Times
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
IPAP	InPhase AntiPhase
IPTG	Isopropyl- β -D-thiogalactopyranosid
MD	Molecular Dynamic
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NS	Number of Scans
PDB	Protein Data Bank
PRE	Paramagnetic Relaxation Enhancement
QCI	Quadruple resonanCe NMR Inverse
RDC	Residual Dipolar Coupling
RF	RadioFrequency
RT	Real-Time

Sars-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SAXS	Small-Angle X-ray Scattering
SNAP25	SyNaptosomal-Associated Protein 25
SNARE	Soluble N-ethylmaleimide-sensitive-factor Attachment REceptor
SNR	Signal-to-Noise Ratio
SW	Spectral Width
SWH	Spectral Width in Hz
Syb-2	Synaptobrevin-2
Syx-1 A	Syntaxin-1A
TCEP	Tris(2-carboxyethyl)phosphin
TCI	Triple resonanCe NMR Inverse
TD	Time Domain
TMD	TransMembrane Domain
TROSY	Transverse Relaxation-Optimized Spectroscopy
tSNARE	target membrane SNARE
UV	UltraViolet
VAMP-2	Vesicle-Associated Membrane protein 2
vSNARE	vesicle SNARE
Ala A	Alanine
Arg R	Arginine

Asn N	Asparagine
Asp D	Aspartic acid
Cys C	Cysteine
Glu E	Glutamic acid
Gln Q	Glutamine
Gly G	Glycine
His H	Histidine
Ile I	Isoleucine
Leu L	Leucine
Lys K	Lysine
Met M	Methionine
Phe F	Phenylalanine
Pro P	Proline
Ser S	Serine
Thr T	Threonine
Trp W	Tryptophan
Tyr Y	Tyrosine
Val V	Valine

Bibliography

- [1] P. Tompa et al. “Intrinsically disordered proteins: emerging interaction specialists”. In: *Curr Opin Struct Biol* 35 (2015), pp. 49–59. DOI: 10.1016/j.sbi.2015.08.009.
- [2] R. Schneider, M. Blackledge, and M. R. Jensen. “Elucidating binding mechanisms and dynamics of intrinsically disordered protein complexes using NMR spectroscopy”. In: *Curr Opin Struct Biol* 54 (2019), pp. 10–18. DOI: 10.1016/j.sbi.2018.09.007.
- [3] N. Salvi, A. Abyzov, and M. Blackledge. “Analytical Description of NMR Relaxation Highlights Correlated Dynamics in Intrinsically Disordered Proteins”. In: *Angew Chem Int Ed Engl* 56.45 (2017), pp. 14020–14024. DOI: 10.1002/anie.201706740.
- [4] A. K. Dunker et al. “Intrinsically disordered protein”. In: *J Mol Graph Model* 19.1 (2001), pp. 26–59. DOI: 10.1016/s1093-3263(00)00138-8.
- [5] P. E. Wright, H. J. Dyson, and R. A. Lerner. “Conformation of peptide fragments of proteins in aqueous solution: implications for initiation of protein folding”. In: *Biochemistry* 27.19 (1988), pp. 7167–75. DOI: 10.1021/bi00419a001.
- [6] H. J. Dyson and P. E. Wright. “Equilibrium NMR studies of unfolded and partially folded proteins”. In: *Nat Struct Biol* 5 Suppl (1998), pp. 499–503. DOI: 10.1038/739.
- [7] S. Mukhopadhyay et al. “A natively unfolded yeast prion monomer adopts an ensemble of collapsed and rapidly fluctuating structures”. In: *Proc Natl Acad Sci U S A* 104.8 (2007), pp. 2649–54. DOI: 10.1073/pnas.0611503104.
- [8] T. Mittag and J. D. Forman-Kay. “Atomic-level characterization of disordered protein ensembles”. In: *Curr Opin Struct Biol* 17.1 (2007), pp. 3–14. DOI: 10.1016/j.sbi.2007.01.009.
- [9] A. K. Dunker et al. “The Protein Trinity: Structure/Function Relationships That Include Intrinsic Disorder”. In: *ScientificWorldJournal* 2 (2002), pp. 49–50. DOI: 10.1100/tsw.2002.25.
- [10] Lewis E Kay, Dennis A Torchia, and Ad Bax. “Backbone dynamics of proteins as studied by nitrogen-15 inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease”. In: *Biochemistry* 28.23 (1989), pp. 8972–8979.

- [11] Giovanni Lipari and Attila Szabo. “Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity”. In: *Journal of the American Chemical Society* 104.17 (1982), pp. 4546–4559. ISSN: 0002-7863. DOI: 10.1021/ja00381a009.
- [12] Francesca Massi, Michael J. Grey, and Arthur G. Palmer III. “Microsecond timescale backbone conformational dynamics in ubiquitin studied with NMR R1p relaxation experiments”. In: *Protein Science* 14.3 (2005), pp. 735–742. ISSN: 0961-8368. DOI: <https://doi.org/10.1110/ps.041139505>.
- [13] N. Salvi, A. Abyzov, and M. Blackledge. “Atomic resolution conformational dynamics of intrinsically disordered proteins from NMR spin relaxation”. In: *Prog Nucl Magn Reson Spectrosc* 102-103 (2017), pp. 43–60. DOI: 10.1016/j.pnmrs.2017.06.001.
- [14] N. Rezaei-Ghaleh, M. Blackledge, and M. Zweckstetter. “Intrinsically disordered proteins: from sequence and conformational properties toward drug discovery”. In: *Chembiochem* 13.7 (2012), pp. 930–50. DOI: 10.1002/cbic.201200093.
- [15] R. Jahn, D. C. Cafiso, and L. K. Tamm. “Mechanisms of SNARE proteins in membrane fusion”. In: *Nat Rev Mol Cell Biol* 25.2 (2024), pp. 101–118. DOI: 10.1038/s41580-023-00668-x.
- [16] J. Rizo. “Molecular Mechanisms Underlying Neurotransmitter Release”. In: *Annu Rev Biophys* 51 (2022), pp. 377–408. DOI: 10.1146/annurev-biophys-111821-104732.
- [17] F. S. Cohen and G. B. Melikyan. “The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement”. In: *J Membr Biol* 199.1 (2004), pp. 1–14. DOI: 10.1007/s00232-004-0669-8.
- [18] R. Jahn and R. H. Scheller. “SNAREs—engines for membrane fusion”. In: *Nat Rev Mol Cell Biol* 7.9 (2006), pp. 631–43. DOI: 10.1038/nrm2002.
- [19] N. Kienle, T. H. Kloepper, and D. Fasshauer. “Phylogeny of the SNARE vesicle fusion machinery yields insights into the conservation of the secretory pathway in fungi”. In: *BMC Evol Biol* 9 (2009), p. 19. DOI: 10.1186/1471-2148-9-19.
- [20] M. A. Poirier et al. “The synaptic SNARE complex is a parallel four-stranded helical bundle”. In: *Nat Struct Biol* 5.9 (1998), pp. 765–9. DOI: 10.1038/1799.
- [21] R. B. Sutton et al. “Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution”. In: *Nature* 395.6700 (1998), pp. 347–53. DOI: 10.1038/26412.
- [22] T. Sollner et al. “A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion”. In: *Cell* 75.3 (1993), pp. 409–18. DOI: 10.1016/0092-8674(93)90376-2.

-
- [23] D. Fasshauer et al. “Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs”. In: *Proc Natl Acad Sci U S A* 95.26 (1998), pp. 15781–6. DOI: 10.1073/pnas.95.26.15781.
- [24] I. Fernandez et al. “Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A”. In: *Cell* 94.6 (1998), pp. 841–9. DOI: 10.1016/s0092-8674(00)81742-0.
- [25] I. Dulubova et al. “A conformational switch in syntaxin during exocytosis: role of munc18”. In: *EMBO J* 18.16 (1999), pp. 4372–82. DOI: 10.1093/emboj/18.16.4372.
- [26] R. Fukuda et al. “Functional architecture of an intracellular membrane t-SNARE”. In: *Nature* 407.6801 (2000), pp. 198–202. DOI: 10.1038/35025084.
- [27] T. Sollner et al. “SNAP receptors implicated in vesicle targeting and fusion”. In: *Nature* 362.6418 (1993), pp. 318–24. DOI: 10.1038/362318a0.
- [28] J. B. Bock et al. “A genomic perspective on membrane compartment organization”. In: *Nature* 409.6822 (2001), pp. 839–841. DOI: 10.1038/35057024.
- [29] Tobias Stief et al. “Intrinsic Disorder of the Neuronal SNARE Protein SNAP25a in its Pre-fusion Conformation”. In: *Journal of Molecular Biology* 435.10 (2023), p. 168069. ISSN: 0022-2836. DOI: <https://doi.org/10.1016/j.jmb.2023.168069>.
- [30] A. Stein et al. “Helical extension of the neuronal SNARE complex into the membrane”. In: *Nature* 460.7254 (2009), pp. 525–8. DOI: 10.1038/nature08156.
- [31] Y. Gao et al. “Single reconstituted neuronal SNARE complexes zipper in three distinct stages”. In: *Science* 337.6100 (2012), pp. 1340–3. DOI: 10.1126/science.1224492.
- [32] F. Li et al. “Energetics and dynamics of SNAREpin folding across lipid bilayers”. In: *Nat Struct Mol Biol* 14.10 (2007), pp. 890–6. DOI: 10.1038/nsmb1310.
- [33] R. Sinha et al. “Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses”. In: *Proc Natl Acad Sci U S A* 108.34 (2011), pp. 14318–23. DOI: 10.1073/pnas.1101818108.
- [34] L. Shi et al. “SNARE proteins: one to fuse and three to keep the nascent fusion pore open”. In: *Science* 335.6074 (2012), pp. 1355–9. DOI: 10.1126/science.1214984.
- [35] G. van den Bogaart et al. “One SNARE complex is sufficient for membrane fusion”. In: *Nat Struct Mol Biol* 17.3 (2010), pp. 358–64. DOI: 10.1038/nsmb.1748.

- [36] R. F. Toonen and M. Verhage. “Vesicle trafficking: pleasure and pain from SM genes”. In: *Trends Cell Biol* 13.4 (2003), pp. 177–86. DOI: 10.1016/s0962-8924(03)00031-x.
- [37] J. K. Archbold et al. “SNARE-ing the structures of Sec1/Munc18 proteins”. In: *Curr Opin Struct Biol* 29 (2014), pp. 44–51. DOI: 10.1016/j.sbi.2014.09.003.
- [38] I. M. Yu and F. M. Hughson. “Tethering factors as organizers of intracellular vesicular traffic”. In: *Annu Rev Cell Dev Biol* 26 (2010), pp. 137–56. DOI: 10.1146/annurev.cellbio.042308.113327.
- [39] C. Santana-Molina, F. Gutierrez, and D. P. Devos. “Homology and Modular Evolution of CATCHR at the Origin of the Eukaryotic Endomembrane System”. In: *Genome Biol Evol* 13.7 (2021). DOI: 10.1093/gbe/evab125.
- [40] Yutaka Hata, Clive A. Slaughter, and Thomas C. Südhof. “Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin”. In: *Nature* 366 (1993), pp. 347–351. DOI: 10.1038/366347a0.
- [41] Y. Xu, L. Su, and J. Rizo. “Binding of Munc18-1 to synaptobrevin and to the SNARE four-helix bundle”. In: *Biochemistry* 49.8 (2010), pp. 1568–76. DOI: 10.1021/bi9021878.
- [42] D. Parisotto et al. “An extended helical conformation in domain 3a of Munc18-1 provides a template for SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly”. In: *J Biol Chem* 289.14 (2014), pp. 9639–50. DOI: 10.1074/jbc.M113.514273.
- [43] X. Chen et al. “NMR analysis of the closed conformation of syntaxin-1”. In: *J Biomol NMR* 41.1 (2008), pp. 43–54. DOI: 10.1007/s10858-008-9239-1.
- [44] Shrutee Jakhanwal et al. “An activated Q‐SNARE/SM protein complex as a possible intermediate in SNARE assembly”. In: *The EMBO Journal* 36.12 (2017), pp. 1788–1802. DOI: <https://doi.org/10.15252/emboj.201696270>.
- [45] C. Ma et al. “Munc13 mediates the transition from the closed syntaxin-Munc18 complex to the SNARE complex”. In: *Nat Struct Mol Biol* 18.5 (2011), pp. 542–9. DOI: 10.1038/nsmb.2047.
- [46] S. Wang et al. “Conformational change of syntaxin linker region induced by Munc13s initiates SNARE complex formation in synaptic exocytosis”. In: *EMBO J* 36.6 (2017), pp. 816–829. DOI: 10.15252/emboj.201695775.
- [47] X. Yang et al. “Syntaxin opening by the MUN domain underlies the function of Munc13 in synaptic-vesicle priming”. In: *Nat Struct Mol Biol* 22.7 (2015), pp. 547–54. DOI: 10.1038/nsmb.3038.
- [48] H. T. McMahon et al. “Complexins cytosolic proteins that regulate SNAP receptor function”. In: *Cell* 83.1 (1995), pp. 111–9. DOI: 10.1016/0092-8674(95)90239-2.

-
- [49] M. Xue et al. “Complexins facilitate neurotransmitter release at excitatory and inhibitory synapses in mammalian central nervous system”. In: *Proc Natl Acad Sci U S A* 105.22 (2008), pp. 7875–80. DOI: 10.1073/pnas.0803012105.
- [50] R. J. Hobson et al. “Complexin maintains vesicles in the primed state in *C. elegans*”. In: *Curr Biol* 21.2 (2011), pp. 106–13. DOI: 10.1016/j.cub.2010.12.015.
- [51] S. Huntwork and J. T. Littleton. “A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth”. In: *Nat Neurosci* 10.10 (2007), pp. 1235–7. DOI: 10.1038/nn1980.
- [52] J. A. Martin et al. “Complexin has opposite effects on two modes of synaptic vesicle fusion”. In: *Curr Biol* 21.2 (2011), pp. 97–105. DOI: 10.1016/j.cub.2010.12.014.
- [53] A. Maximov et al. “Complexin controls the force transfer from SNARE complexes to membranes in fusion”. In: *Science* 323.5913 (2009), pp. 516–21. DOI: 10.1126/science.1166505.
- [54] M. Xue et al. “Distinct domains of complexin I differentially regulate neurotransmitter release”. In: *Nat Struct Mol Biol* 14.10 (2007), pp. 949–58. DOI: 10.1038/nsmb1292.
- [55] J. Rizo and T. C. Sudhof. “C2-domains, structure and function of a universal Ca^{2+} -binding domain”. In: *J Biol Chem* 273.26 (1998), pp. 15879–82. DOI: 10.1074/jbc.273.26.15879.
- [56] R. Fernandez-Chacon et al. “Synaptotagmin I functions as a calcium regulator of release probability”. In: *Nature* 410.6824 (2001), pp. 41–9. DOI: 10.1038/35065004.
- [57] A. S. Holehouse and B. B. Kragelund. “The molecular basis for cellular function of intrinsically disordered protein regions”. In: *Nat Rev Mol Cell Biol* 25.3 (2024), pp. 187–211. DOI: 10.1038/s41580-023-00673-0.
- [58] R. J. Emenecker, D. Griffith, and A. S. Holehouse. “Metapredict: a fast, accurate, and easy-to-use predictor of consensus disorder and structure”. In: *Biophys J* 120.20 (2021), pp. 4312–4319. DOI: 10.1016/j.bpj.2021.08.039.
- [59] V. N. Uversky. “Natively unfolded proteins: a point where biology waits for physics”. In: *Protein Sci* 11.4 (2002), pp. 739–56. DOI: 10.1110/ps.4210102.
- [60] R. van der Lee et al. “Classification of intrinsically disordered regions and proteins”. In: *Chem Rev* 114.13 (2014), pp. 6589–631. DOI: 10.1021/cr400525m.
- [61] H. J. Dyson and P. E. Wright. “Intrinsically unstructured proteins and their functions”. In: *Nat Rev Mol Cell Biol* 6.3 (2005), pp. 197–208. DOI: 10.1038/nrm1589.

- [62] Lorna J. Smith et al. "The concept of a random coil: Residual structure in peptides and denatured proteins". In: *Folding and Design* 1.5 (1996), R95–R106. ISSN: 1359-0278. DOI: [https://doi.org/10.1016/S1359-0278\(96\)00046-6](https://doi.org/10.1016/S1359-0278(96)00046-6).
- [63] G.N. Ramachandran, C. Ramakrishnan, and V. Sasisekharan. "Stereochemistry of polypeptide chain configurations". In: *Journal of Molecular Biology* 7.1 (1963), pp. 95–99. ISSN: 0022-2836. DOI: [https://doi.org/10.1016/S0022-2836\(63\)80023-6](https://doi.org/10.1016/S0022-2836(63)80023-6).
- [64] George D. Rose. *Unfolded Proteins*. 1st ed. Vol. 62. Advances in Protein Chemistry. Academic Press, 2002. ISBN: 9780120342624.
- [65] H. J. Dyson and P. E. Wright. "Insights into the structure and dynamics of unfolded proteins from nuclear magnetic resonance". In: *Adv Protein Chem* 62 (2002), pp. 311–40. DOI: [10.1016/s0065-3233\(02\)62012-1](https://doi.org/10.1016/s0065-3233(02)62012-1).
- [66] T. Flock et al. "Controlling entropy to tune the functions of intrinsically disordered regions". In: *Curr Opin Struct Biol* 26 (2014), pp. 62–72. DOI: [10.1016/j.sbi.2014.05.007](https://doi.org/10.1016/j.sbi.2014.05.007).
- [67] T. R. Jahn and S. E. Radford. "The Yin and Yang of protein folding". In: *FEBS J* 272.23 (2005), pp. 5962–70. DOI: [10.1111/j.1742-4658.2005.05021.x](https://doi.org/10.1111/j.1742-4658.2005.05021.x).
- [68] C. K. Fisher and C. M. Stultz. "Constructing ensembles for intrinsically disordered proteins". In: *Curr Opin Struct Biol* 21.3 (2011), pp. 426–31. DOI: [10.1016/j.sbi.2011.04.001](https://doi.org/10.1016/j.sbi.2011.04.001).
- [69] G. A. Papoian. "Proteins with weakly funneled energy landscapes challenge the classical structure-function paradigm". In: *Proc Natl Acad Sci U S A* 105.38 (2008), pp. 14237–8. DOI: [10.1073/pnas.0807977105](https://doi.org/10.1073/pnas.0807977105).
- [70] P. J. Mitchell and R. Tjian. "Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins". In: *Science* 245.4916 (1989), pp. 371–8. DOI: [10.1126/science.2667136](https://doi.org/10.1126/science.2667136).
- [71] P. Romero et al. "Sequence complexity of disordered protein". In: *Proteins* 42.1 (2001), pp. 38–48. DOI: [10.1002/1097-0134\(20010101\)42:1<38::aid-prot50>3.0.co;2-3](https://doi.org/10.1002/1097-0134(20010101)42:1<38::aid-prot50>3.0.co;2-3).
- [72] S. Vucetic et al. "Flavors of protein disorder". In: *Proteins* 52.4 (2003), pp. 573–84. DOI: [10.1002/prot.10437](https://doi.org/10.1002/prot.10437).
- [73] P. Tompa. "Intrinsically unstructured proteins". In: *Trends Biochem Sci* 27.10 (2002), pp. 527–33. DOI: [10.1016/s0968-0004\(02\)02169-2](https://doi.org/10.1016/s0968-0004(02)02169-2).
- [74] P. Tompa. "The interplay between structure and function in intrinsically unstructured proteins". In: *FEBS Lett* 579.15 (2005), pp. 3346–54. DOI: [10.1016/j.febslet.2005.03.072](https://doi.org/10.1016/j.febslet.2005.03.072).

-
- [75] A. K. Dunker et al. "The unfoldomics decade: an update on intrinsically disordered proteins". In: *BMC Genomics* 9 Suppl 2.Suppl 2 (2008), S1. DOI: 10.1186/1471-2164-9-S2-S1.
- [76] G. W. Daughdrill et al. "Dynamic behavior of an intrinsically unstructured linker domain is conserved in the face of negligible amino acid sequence conservation". In: *J Mol Evol* 65.3 (2007), pp. 277–88. DOI: 10.1007/s00239-007-9011-2.
- [77] P. Tompa. "Intrinsically unstructured proteins evolve by repeat expansion". In: *Bioessays* 25.9 (2003), pp. 847–55. DOI: 10.1002/bies.10324.
- [78] L. Tskhovrebova and J. Trinick. "Titin: properties and family relationships". In: *Nat Rev Mol Cell Biol* 4.9 (2003), pp. 679–89. DOI: 10.1038/nrm1198.
- [79] F. Diella et al. "Understanding eukaryotic linear motifs and their role in cell signaling and regulation". In: *Front Biosci* 13 (2008), pp. 6580–603. DOI: 10.2741/3175.
- [80] M. Fuxreiter, P. Tompa, and I. Simon. "Local structural disorder imparts plasticity on linear motifs". In: *Bioinformatics* 23.8 (2007), pp. 950–6. DOI: 10.1093/bioinformatics/btm035.
- [81] T. J. Gibson. "Cell regulation: determined to signal discrete cooperation". In: *Trends Biochem Sci* 34.10 (2009), pp. 471–82. DOI: 10.1016/j.tibs.2009.06.007.
- [82] J. R. Perkins et al. "Transient protein-protein interactions: structural, functional, and network properties". In: *Structure* 18.10 (2010), pp. 1233–43. DOI: 10.1016/j.str.2010.08.007.
- [83] L. M. Iakoucheva et al. "The importance of intrinsic disorder for protein phosphorylation". In: *Nucleic Acids Res* 32.3 (2004), pp. 1037–49. DOI: 10.1093/nar/gkh253.
- [84] M. O. Collins et al. "Phosphoproteomic analysis of the mouse brain cytosol reveals a predominance of protein phosphorylation in regions of intrinsic sequence disorder". In: *Mol Cell Proteomics* 7.7 (2008), pp. 1331–48. DOI: 10.1074/mcp.M700564-MCP200.
- [85] C. A. Galea et al. "Regulation of cell division by intrinsically unstructured proteins: intrinsic flexibility, modularity, and signaling conduits". In: *Biochemistry* 47.29 (2008), pp. 7598–609. DOI: 10.1021/bi8006803.
- [86] L. J. Holt et al. "Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution". In: *Science* 325.5948 (2009), pp. 1682–6. DOI: 10.1126/science.1172867.
- [87] J. J. Ward et al. "Prediction and functional analysis of native disorder in proteins from the three kingdoms of life". In: *J Mol Biol* 337.3 (2004), pp. 635–45. DOI: 10.1016/j.jmb.2004.02.002.

- [88] J. Gsponer and M. M. Babu. “The rules of disorder or why disorder rules”. In: *Prog Biophys Mol Biol* 99.2-3 (2009), pp. 94–103. DOI: 10.1016/j.pbiomolbio.2009.03.001.
- [89] S. Vucetic et al. “Functional anthology of intrinsic disorder. 2. Cellular components, domains, technical terms, developmental processes, and coding sequence diversities correlated with long disordered regions”. In: *J Proteome Res* 6.5 (2007), pp. 1899–916. DOI: 10.1021/pr060393m.
- [90] V. N. Uversky. “Intrinsic disorder-based protein interactions and their modulators”. In: *Curr Pharm Des* 19.23 (2013), pp. 4191–213. DOI: 10.2174/1381612811319230005.
- [91] R. Schroeder, A. Barta, and K. Semrad. “Strategies for RNA folding and assembly”. In: *Nat Rev Mol Cell Biol* 5.11 (2004), pp. 908–19. DOI: 10.1038/nrm1497.
- [92] J. C. Young et al. “Pathways of chaperone-mediated protein folding in the cytosol”. In: *Nat Rev Mol Cell Biol* 5.10 (2004), pp. 781–91. DOI: 10.1038/nrm1492.
- [93] B. W. Pontius. “Close encounters: why unstructured, polymeric domains can increase rates of specific macromolecular association”. In: *Trends Biochem Sci* 18.5 (1993), pp. 181–6. DOI: 10.1016/0968-0004(93)90111-y.
- [94] K. Sugase, H. J. Dyson, and P. E. Wright. “Mechanism of coupled folding and binding of an intrinsically disordered protein”. In: *Nature* 447.7147 (2007), pp. 1021–5. DOI: 10.1038/nature05858.
- [95] P. E. Wright and H. J. Dyson. “Linking folding and binding”. In: *Curr Opin Struct Biol* 19.1 (2009), pp. 31–8. DOI: 10.1016/j.sbi.2008.12.003.
- [96] Z. Mucsi et al. “Binding-induced folding transitions in calpastatin subdomains A and C”. In: *Protein Sci* 12.10 (2003), pp. 2327–36. DOI: 10.1110/ps.03138803.
- [97] T. Trudeau et al. “Structure and intrinsic disorder in protein autoinhibition”. In: *Structure* 21.3 (2013), pp. 332–41. DOI: 10.1016/j.str.2012.12.013.
- [98] A. C. Ferreon et al. “Modulation of allostery by protein intrinsic disorder”. In: *Nature* 498.7454 (2013), pp. 390–4. DOI: 10.1038/nature12294.
- [99] A. Cumberworth et al. “Promiscuity as a functional trait: intrinsically disordered regions as central players of interactomes”. In: *Biochem J* 454.3 (2013), pp. 361–9. DOI: 10.1042/BJ20130545.
- [100] H. Wu. “Higher-order assemblies in a new paradigm of signal transduction”. In: *Cell* 153.2 (2013), pp. 287–92. DOI: 10.1016/j.cell.2013.03.013.
- [101] Z. Peng et al. “A creature with a hundred waggly tails: intrinsically disordered proteins in the ribosome”. In: *Cell Mol Life Sci* 71.8 (2014), pp. 1477–504. DOI: 10.1007/s00018-013-1446-6.

-
- [102] A. J. Daniels, R. J. Williams, and P. E. Wright. “The character of the stored molecules in chromaffin granules of the adrenal medulla: a nuclear magnetic resonance study”. In: *Neuroscience* 3.6 (1978), pp. 573–85. DOI: 10.1016/0306-4522(78)90022-2.
- [103] C. Holt. “Unfolded phosphopeptides enable soft and hard tissues to coexist in the same organism with relative ease”. In: *Curr Opin Struct Biol* 23.3 (2013), pp. 420–5. DOI: 10.1016/j.sbi.2013.02.010.
- [104] A. Abyzov, M. Blackledge, and M. Zweckstetter. “Conformational Dynamics of Intrinsically Disordered Proteins Regulate Biomolecular Condensate Chemistry”. In: *Chem Rev* 122.6 (2022), pp. 6719–6748. DOI: 10.1021/acs.chemrev.1c00774.
- [105] A. Bah et al. “Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch”. In: *Nature* 519.7541 (2015), pp. 106–9. DOI: 10.1038/nature13999.
- [106] H. J. Dyson and P. E. Wright. “Perspective: the essential role of NMR in the discovery and characterization of intrinsically disordered proteins”. In: *J Biomol NMR* 73.12 (2019), pp. 651–659. DOI: 10.1007/s10858-019-00280-2.
- [107] M. R. Jensen et al. “Defining conformational ensembles of intrinsically disordered and partially folded proteins directly from chemical shifts”. In: *J Am Chem Soc* 132.4 (2010), pp. 1270–2. DOI: 10.1021/ja909973n.
- [108] A. R. Camacho-Zarco et al. “NMR Provides Unique Insight into the Functional Dynamics and Interactions of Intrinsically Disordered Proteins”. In: *Chem Rev* 122.10 (2022), pp. 9331–9356. DOI: 10.1021/acs.chemrev.1c01023.
- [109] H. J. Dyson and P. E. Wright. “Unfolded proteins and protein folding studied by NMR”. In: *Chem Rev* 104.8 (2004), pp. 3607–22. DOI: 10.1021/cr030403s.
- [110] H. J. Dyson and P. E. Wright. “NMR illuminates intrinsic disorder”. In: *Curr Opin Struct Biol* 70 (2021), pp. 44–52. DOI: 10.1016/j.sbi.2021.03.015.
- [111] L. M. Bessa et al. “The intrinsically disordered SARS-CoV-2 nucleoprotein in dynamic complex with its viral partner nsp3a”. In: *Sci Adv* 8.3 (2022), eabm4034. DOI: 10.1126/sciadv.abm4034.
- [112] S. Milles et al. “Characterization of intrinsically disordered proteins and their dynamic complexes: From in vitro to cell-like environments”. In: *Prog Nucl Magn Reson Spectrosc* 109 (2018), pp. 79–100. DOI: 10.1016/j.pnmrs.2018.07.001.
- [113] H. Günther. *NMR Spectroscopy: Basic Principles, Concepts and Applications in Chemistry*. Wiley, 2013. ISBN: 9783527674770.

- [114] E. Rutherford. “LIV Collision of a particles with light atoms IV An anomalous effect in nitrogen”. In: *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science* 37.222 (1919), pp. 581–587. DOI: 10.1080/14786440608635919.
- [115] Walther Gerlach and Otto Stern. “Der experimentelle Nachweis der Richtungsquantelung im Magnetfeld”. In: *Zeitschrift für Physik* 9.1 (1922), pp. 349–352. ISSN: 0044-3328. DOI: 10.1007/BF01326983.
- [116] W. Pauli. “Über den Zusammenhang des Abschlusses der Elektronengruppen im Atom mit der Komplexstruktur der Spektren”. In: *Zeitschrift für Physik* 31.1 (1925), pp. 765–783. ISSN: 0044-3328. DOI: 10.1007/BF02980631.
- [117] G. E. Uhlenbeck and S. Goudsmit. “Spinning Electrons and the Structure of Spectra”. In: *Nature* 117.2938 (1926), pp. 264–265. ISSN: 1476-4687. DOI: 10.1038/117264a0.
- [118] I. Estermann, R. Frisch, and O. Stern. “Magnetic Moment of the Proton”. In: *Nature* 132.3326 (1933), pp. 169–170. ISSN: 1476-4687. DOI: 10.1038/132169a0.
- [119] I. I. Rabi et al. “A New Method of Measuring Nuclear Magnetic Moment”. In: *Physical Review* 53.4 (1938), pp. 318–318. DOI: 10.1103/PhysRev.53.318.
- [120] J. Cavanagh. *Protein NMR Spectroscopy: Principles and Practice. 2nd Ed (9780121644918)*. Academic Press, 2007.
- [121] P.A.M. Dirac. *The Principles of Quantum Mechanics*. International series of monographs on physics. Clarendon Press, 1930.
- [122] M.H. Levitt. *Spin Dynamics: Basics of Nuclear Magnetic Resonance*. Wiley, 2013. ISBN: 9781118681848.
- [123] F. Bloch. “Nuclear Induction”. In: *Physical Review* 70.7-8 (1946), pp. 460–474. DOI: 10.1103/PhysRev.70.460.
- [124] N. Bloembergen, E. M. Purcell, and R. V. Pound. “Relaxation Effects in Nuclear Magnetic Resonance Absorption”. In: *Physical Review* 73.7 (1948), pp. 679–712. DOI: 10.1103/PhysRev.73.679.
- [125] R. K. Wangsness and F. Bloch. “The Dynamical Theory of Nuclear Induction”. In: *Physical Review* 89.4 (1953), pp. 728–739. DOI: 10.1103/PhysRev.89.728.
- [126] F. Bloch. “Dynamical Theory of Nuclear Induction. II”. In: *Physical Review* 102.1 (1956), pp. 104–135. DOI: 10.1103/PhysRev.102.104.
- [127] I. Solomon. “Relaxation Processes in a System of Two Spins”. In: *Physical Review* 99.2 (1955), pp. 559–565. DOI: 10.1103/PhysRev.99.559.
- [128] A. Abragam. *The Principles of Nuclear Magnetism*. Clarendon Press, 1961. ISBN: 9780198520146.

-
- [129] A. G. Redfield. “On the Theory of Relaxation Processes”. In: *IBM Journal of Research and Development* 1.1 (1957), pp. 19–31. ISSN: 0018-8646. DOI: 10.1147/rd.11.0019.
- [130] A. G. Redfield. “The Theory of Relaxation Processes”. In: *Advances in Magnetic and Optical Resonance*. Ed. by John S. Waugh. Vol. 1. Academic Press, 1965, pp. 1–32. ISBN: 1057-2732. DOI: <https://doi.org/10.1016/B978-1-4832-3114-3.50007-6>.
- [131] F. Bloch. “Generalized Theory of Relaxation”. In: *Physical Review* 105.4 (1957), pp. 1206–1222. DOI: 10.1103/PhysRev.105.1206.
- [132] Kazuhisa Tomita. “A General Theory of Magnetic Resonance Saturation”. In: *Progress of Theoretical Physics* 19.5 (1958), pp. 541–580. ISSN: 0033-068X. DOI: 10.1143/ptp.19.541.
- [133] M. Goldman. “Formal theory of spin–lattice relaxation”. In: *J Magn Reson* 149.2 (2001), pp. 160–87. ISSN: 1090-7807 (Print) 1090-7807 (Linking). DOI: 10.1006/jmre.2000.2239.
- [134] J. Von Neumann. *Mathematische Grundlagen der Quantenmechanik*. Die Grundlehren der mathematischen Wissenschaften : in Einzeldarstellungen mit besonderer Berücksichtigung der Anwendungsgebiete. Julius Springer, 1932.
- [135] Neil A. Farrow et al. “Spectral density function mapping using ¹⁵N relaxation data exclusively”. In: *Journal of Biomolecular NMR* 6 (Sept. 1995), pp. 153–162. DOI: 10.1007/BF00211779.
- [136] G. Marius Clore et al. “Deviations from the simple two-parameter model-free approach to the interpretation of nitrogen-15 nuclear magnetic relaxation of proteins”. In: *Journal of the American Chemical Society* 112.12 (1990), pp. 4989–4991. ISSN: 0002-7863. DOI: 10.1021/ja00168a070.
- [137] Shahid N Khan et al. “Distribution of Pico- and Nanosecond Motions in Disordered Proteins from Nuclear Spin Relaxation”. In: *Biophysical Journal* 109.5 (2015), pp. 988–999. ISSN: 0006-3495. DOI: <https://doi.org/10.1016/j.bpj.2015.06.069>.
- [138] Peter A. Beckmann. “Spectral densities and nuclear spin relaxation in solids”. In: *Physics Reports* 171.3 (1988), pp. 85–128. ISSN: 0370-1573. DOI: [https://doi.org/10.1016/0370-1573\(88\)90073-7](https://doi.org/10.1016/0370-1573(88)90073-7).
- [139] Albert A. Smith, Matthias Ernst, and Beat H. Meier. “Optimized “detectors” for dynamics analysis in solid-state NMR”. In: *The Journal of Chemical Physics* 148.4 (2018). ISSN: 0021-9606. DOI: 10.1063/1.5013316.
- [140] David S. Wishart and Brian D. Sykes. “The ¹³C Chemical-Shift Index: A simple method for the identification of protein secondary structure using ¹³C chemical-shift data”. In: *Journal of Biomolecular NMR* 4 (1994), pp. 171–180. DOI: 10.1007/BF00175245.

- [141] Arthur G Palmer and Francesca Massi. “Characterization of the dynamics of biomacromolecules using rotating-frame spin relaxation NMR spectroscopy”. In: *Chemical reviews* 106.5 (2006), pp. 1700–1719.
- [142] Neil A Farrow et al. “Backbone dynamics of a free and a phosphopeptide-complexed Src homology 2 domain studied by ^{15}N NMR relaxation”. In: *Biochemistry* 33.19 (1994), pp. 5984–6003.
- [143] Joel R Tolman and Ke Ruan. “NMR residual dipolar couplings as probes of biomolecular dynamics”. In: *Chemical reviews* 106.5 (2006), pp. 1720–1736.
- [144] Frans AA Mulder et al. “Measurement of slow (μs - ms) time scale dynamics in protein side chains by ^{15}N relaxation dispersion NMR spectroscopy: application to Asn and Gln residues in a cavity mutant of T4 lysozyme”. In: *Journal of the American Chemical Society* 123.5 (2001), pp. 967–975.
- [145] Mikael Akke and Arthur G Palmer. “Monitoring macromolecular motions on microsecond to millisecond time scales by $\text{R}1\rho$ - $\text{R}1$ constant relaxation time NMR spectroscopy”. In: *Journal of the American Chemical Society* 118.4 (1996), pp. 911–912.
- [146] Pramodh Vallurupalli, Guillaume Bouvignies, and Lewis E Kay. “Studying “invisible” excited protein states in slow exchange with a major state conformation”. In: *Journal of the American Chemical Society* 134.19 (2012), pp. 8148–8161.
- [147] J Jeneer et al. “Investigation of exchange processes by two-dimensional NMR spectroscopy”. In: *J. chem. Phys* 71 (1979), pp. 4546–4553.
- [148] G Marius Clore and Junji Iwahara. “Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes”. In: *Chemical reviews* 109.9 (2009), pp. 4108–4139.
- [149] Nils-Alexander Lakomek et al. “Residual dipolar couplings as a tool to study molecular recognition of ubiquitin”. In: *Biochemical Society Transactions* 36.6 (Nov. 2008), pp. 1433–1437. ISSN: 0300-5127. DOI: 10.1042/BST0361433.
- [150] Junji Iwahara, Chun Tang, and G. Marius Clore. “Practical aspects of ^1H transverse paramagnetic relaxation enhancement measurements on macromolecules”. In: *Journal of Magnetic Resonance* 184.2 (2007), pp. 185–195. ISSN: 1090-7807. DOI: <https://doi.org/10.1016/j.jmr.2006.10.003>.
- [151] N. A. Farrow et al. “Comparison of the backbone dynamics of a folded and an unfolded SH3 domain existing in equilibrium in aqueous buffer”. In: *Biochemistry* 34.3 (1995), pp. 868–78. ISSN: 0006-2960 (Print) 0006-2960 (Linking). DOI: 10.1021/bi00003a021.
- [152] Maria Grazia Spillantini et al. “ α -Synuclein in Lewy bodies”. In: *Nature* 388 (1997), pp. 839–840. DOI: 10.1038/42166.

-
- [153] Mihael H. Polymeropoulos et al. "Mutation in the α -Synuclein Gene Identified in Families with Parkinson's Disease". In: *Science* 276.5321 (1997), pp. 2045–2047. DOI: 10.1126/science.276.5321.2045.
- [154] Yongdae Shin and Clifford P. Brangwynne. "Liquid phase condensation in cell physiology and disease". In: *Science* 357.6357 (2017), eaaf4382. DOI: 10.1126/science.aaf4382.
- [155] Shana Elbaum-Garfinkle et al. "The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics". In: *Proceedings of the National Academy of Sciences* 112.23 (2015), pp. 7189–7194. DOI: 10.1073/pnas.1504822112.
- [156] Marina Feric et al. "Coexisting Liquid Phases Underlie Nucleolar Subcompartments". In: *Cell* 165 (2016), pp. 1686–1697. DOI: 10.1016/j.cell.2016.04.047.
- [157] *The Nobel Prize in Physiology or Medicine 2013*. NobelPrize.org. <https://www.nobelprize.org/prizes/medicine/2013/summary/>. Accessed: 2025-10-16.
- [158] Andrei T. Alexandrescu, Chitrananda Abeygunawardana, and David Shortle. "Structure and Dynamics of a Denatured 131-Residue Fragment of Staphylococcal Nuclease: A Heteronuclear NMR Study". In: *Biochemistry* 33.5 (1994), pp. 1063–1072. DOI: 10.1021/bi00171a004.
- [159] Bernhard Brutscher, Rafael Brueschweiler, and Richard R. Ernst. "Backbone Dynamics and Structural Characterization of the Partially Folded A State of Ubiquitin by ^1H , ^{13}C , and ^{15}N Nuclear Magnetic Resonance Spectroscopy". In: *Biochemistry* 36.42 (1997), pp. 13043–13053. DOI: 10.1021/bi971538t.
- [160] {Alexei V.} Buevich and Jean Baum. "Dynamics of unfolded proteins: Incorporation of distributions of correlation times in the model free analysis of NMR relaxation data". English (US). In: *Journal of the American Chemical Society* 121.37 (Sept. 1999), pp. 8671–8672. ISSN: 0002-7863. DOI: 10.1021/ja9910412.
- [161] Jian Yao et al. "NMR Structural and Dynamic Characterization of the Acid-Unfolded State of Apomyoglobin Provides Insights into the Early Events in Protein Folding". In: *Biochemistry* 40.12 (2001), pp. 3561–3571. DOI: 10.1021/bi002776i.
- [162] Judith Klein-Seetharaman et al. "Long-Range Interactions Within a Nonnative Protein". In: *Science* 295.5560 (2002), pp. 1719–1722. DOI: 10.1126/science.1067680.
- [163] Cecile S. (Le Duff) et al. "Characterisation of the Conformational Properties of Urea-unfolded Im7: Implications for the Early Stages of Protein Folding". In: *Journal of Molecular Biology* 364.4 (2006), pp. 824–835. ISSN: 0022-2836. DOI: <https://doi.org/10.1016/j.jmb.2006.09.037>.

- [164] Julia Wirmer, Wolfgang Peti, and Harald Schwalbe. “Motional properties of unfolded ubiquitin: a model for a random coil protein”. In: *Journal of Biomolecular NMR* 35 (2006), pp. 175–186. DOI: 10.1007/s10858-006-9026-9.
- [165] Klaartje Houben et al. “Intrinsic Dynamics of the Partly Unstructured PX Domain from the Sendai Virus RNA Polymerase Cofactor P”. In: *Biophysical Journal* 93.8 (2007), pp. 2830–2844. ISSN: 0006-3495. DOI: <https://doi.org/10.1529/biophysj.107.108829>.
- [166] Dennis Kurzbach et al. “Compensatory Adaptations of Structural Dynamics in an Intrinsically Disordered Protein Complex”. In: *Angewandte Chemie International Edition* 53.15 (2014), pp. 3840–3843. DOI: <https://doi.org/10.1002/anie.201308389>.
- [167] Robert Silvers et al. “Modulation of Structure and Dynamics by Disulfide Bond Formation in Unfolded States”. In: *Journal of the American Chemical Society* 134.15 (2012), pp. 6846–6854. DOI: 10.1021/ja3009506.
- [168] Marc-Olivier Ebert et al. “NMR Relaxation Study of the Complex Formed Between CBP and the Activation Domain of the Nuclear Hormone Receptor Coactivator ACTR”. In: *Biochemistry* 47.5 (2008), pp. 1299–1308. DOI: 10.1021/bi701767j.
- [169] Francoise Ochsenbein et al. “Dynamical characterization of residual and non-native structures in a partially folded protein by ¹⁵N NMR relaxation using a model based on a distribution of correlation times”. In: *Protein Science* 11.4 (2002), pp. 957–964. DOI: <https://doi.org/10.1110/ps.4000102>.
- [170] David M. LeMaster and Frederic M. Richards. “NMR sequential assignment of Escherichia coli thioredoxin utilizing random fractional deuteration”. In: *Biochemistry* 27.1 (1988), pp. 142–150. DOI: 10.1021/bi00401a022.
- [171] Dennis A Torchia, Steven W Sparks, and AD Bax. “Delineation of alpha-helical domains in deuteriated Staphylococcal nuclease by 2D NOE NMR spectroscopy”. In: *Journal of the American Chemical Society* 110.7 (1988), pp. 2320–2321.
- [172] Michael Sattler and Stephen W Fesik. “Use of deuterium labeling in NMR: overcoming a sizeable problem”. In: *Structure* 4 (1996), pp. 1245–1249. DOI: [doi:10.1016/S0969-2126\(96\)00133-5](https://doi.org/10.1016/S0969-2126(96)00133-5).
- [173] N. A. Lakomek, J. Ying, and A. Bax. “Measurement of (1)(5)N relaxation rates in perdeuterated proteins by TROSY-based methods”. In: *J Biomol NMR* 53.3 (2012), pp. 209–21. ISSN: 1573-5001 (Electronic) 0925-2738 (Print) 0925-2738 (Linking). DOI: 10.1007/s10858-012-9626-5.
- [174] Jeffrey W Peng and Gerhard Wagner. “Mapping of spectral density functions using heteronuclear NMR relaxation measurements”. In: *Journal of Magnetic Resonance (1969)* 98.2 (1992), pp. 308–332. ISSN: 0022-2364. DOI: [https://doi.org/10.1016/0022-2364\(92\)90135-T](https://doi.org/10.1016/0022-2364(92)90135-T).

-
- [175] Anthony K Mittermaier and Lewis E Kay. “Observing biological dynamics at atomic resolution using NMR”. In: *Trends in biochemical sciences* 34.12 (2009), pp. 601–611.
- [176] Arthur G Palmer III. “Enzyme dynamics from NMR spectroscopy”. In: *Accounts of Chemical Research* 48.2 (2015), pp. 457–465.
- [177] Dennis A Torchia. “Dynamics of biomolecules from picoseconds to seconds at atomic resolution”. In: *Journal of Magnetic Resonance* 212.1 (2011), pp. 1–10.
- [178] Philipp Neudecker, Patrik Lundström, and Lewis E Kay. “Relaxation dispersion NMR spectroscopy as a tool for detailed studies of protein folding”. In: *Biophysical journal* 96.6 (2009), pp. 2045–2054.
- [179] Ashok Sekhar and Lewis E Kay. “An NMR view of protein dynamics in health and disease”. In: *Annual review of biophysics* 48.1 (2019), pp. 297–319.
- [180] András Micsonai et al. “BeStSel: webserver for secondary structure and fold prediction for protein CD spectroscopy”. In: *Nucleic acids research* 50.W1 (2022), W90–W98.
- [181] Jakob Toudahl Nielsen and Frans AA Mulder. “POTENCI: prediction of temperature, neighbor and pH-corrected chemical shifts for intrinsically disordered proteins”. In: *Journal of Biomolecular NMR* 70.3 (2018), pp. 141–165.
- [182] Jakob Toudahl Nielsen and Frans AA Mulder. “CheSPI: chemical shift secondary structure population inference”. In: *Journal of Biomolecular NMR* 75.6 (2021), pp. 273–291.
- [183] Andreas M. Stadler et al. “Internal Nanosecond Dynamics in the Intrinsically Disordered Myelin Basic Protein”. In: *Journal of the American Chemical Society* 136.19 (2014), pp. 6987–6994. DOI: 10.1021/ja502343b.
- [184] Lewis E. Kay, Paul Keifer, and Tim Saarinen. “Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity”. In: *Journal of the American Chemical Society* 114.26 (1992), pp. 10663–10665. DOI: 10.1021/ja00052a088.
- [185] Nils-Alexander Lakomek et al. “Structural dynamics and transient lipid binding of synaptobrevin-2 tune SNARE assembly and membrane fusion”. In: *Proceedings of the National Academy of Sciences* 116.18 (2019), pp. 8699–8708. DOI: 10.1073/pnas.1813194116.
- [186] Albert A. Smith et al. “Reducing bias in the analysis of solution-state NMR data with dynamics detectors”. In: *The Journal of Chemical Physics* 151.3 (2019), p. 034102. ISSN: 0021-9606. DOI: 10.1063/1.5111081.

- [187] Edward J. d’Auvergne and Paul R. Gooley. “Optimisation of NMR dynamic models I. Minimisation algorithms and their performance within the model-free and Brownian rotational diffusion spaces”. In: *Journal of Biomolecular NMR* 40.2 (2008), pp. 107–119. ISSN: 1573-5001. DOI: 10.1007/s10858-007-9214-2.
- [188] Edward J. d’Auvergne and Paul R. Gooley. “Optimisation of NMR dynamic models II. A new methodology for the dual optimisation of the model-free parameters and the Brownian rotational diffusion tensor”. In: *Journal of Biomolecular NMR* 40.2 (2008), pp. 121–133. ISSN: 1573-5001. DOI: 10.1007/s10858-007-9213-3.
- [189] Michael Bieri, Edward J. d’Auvergne, and Paul R. Gooley. “relaxGUI: a new software for fast and simple NMR relaxation data analysis and calculation of ps-ns and us motion of proteins”. In: *Journal of Biomolecular NMR* 50.2 (2011), pp. 147–155. ISSN: 1573-5001. DOI: 10.1007/s10858-011-9509-1.
- [190] M. L. Gill, R. A. Byrd, and III Palmer A. G. “Dynamics of GCN4 facilitate DNA interaction: a model-free analysis of an intrinsically disordered region”. In: *Phys Chem Chem Phys* 18.8 (2016), pp. 5839–49. ISSN: 1463-9076 (Print) 1463-9076. DOI: 10.1039/c5cp06197k.
- [191] A. Abyzov et al. “Identification of Dynamic Modes in an Intrinsically Disordered Protein Using Temperature-Dependent NMR Relaxation”. In: *J Am Chem Soc* 138.19 (2016), pp. 6240–51. ISSN: 0002-7863. DOI: 10.1021/jacs.6b02424.
- [192] N. Salvi, A. Abyzov, and M. Blackledge. “Solvent-dependent segmental dynamics in intrinsically disordered proteins”. In: *Sci Adv* 5.6 (2019), eaax2348. ISSN: 2375-2548. DOI: 10.1126/sciadv.aax2348.
- [193] Yi Xue and Nikolai R. Skrynnikov. “Motion of a Disordered Polypeptide Chain as Studied by Paramagnetic Relaxation Enhancements, ¹⁵N Relaxation, and Molecular Dynamics Simulations: How Fast Is Segmental Diffusion in Denatured Ubiquitin”. In: *Journal of the American Chemical Society* 133 (2011), pp. 14614–14628. DOI: 10.1021/ja201605c.
- [194] Giacomo Parigi et al. “Long-range correlated dynamics in intrinsically disordered proteins”. In: *Journal of the American Chemical Society* 136 (2014), pp. 16201–9. DOI: 10.1021/ja506820r.
- [195] Pratibha Kumari et al. “¹⁵N transverse relaxation measurements for the characterization of us–ms dynamics are deteriorated by the deuterium isotope effect on ¹⁵N resulting from solvent exchange”. In: *ournal of Biomolecular NMR* 72 (2018), pp. 125–137. DOI: 10.1007/s10858-018-0211-4.
- [196] Dirk Fasshauer et al. “A Structural Change Occurs upon Binding of Syntaxin to SNAP-25”. In: *Journal of Biological Chemistry* 272.7 (1997), pp. 4582–4590. ISSN: 0021-9258. DOI: <https://doi.org/10.1074/jbc.272.7.4582>.

-
- [197] D.S. Wishart, B.D. Sykes, and F.M. Richards. “Relationship between nuclear magnetic resonance chemical shift and protein secondary structure”. In: *Journal of Molecular Biology* 222.2 (1991), pp. 311–333. ISSN: 0022-2836. DOI: [https://doi.org/10.1016/0022-2836\(91\)90214-Q](https://doi.org/10.1016/0022-2836(91)90214-Q).
- [198] D. S. Wishart, B. D. Sykes, and F. M. Richards. “The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy”. In: *Biochemistry* 31.6 (1992), pp. 1647–1651. DOI: 10.1021/bi00121a010.
- [199] Katelyn N. Kraichely et al. “Functionally distinct SNARE motifs of SNAP25 cooperate in SNARE assembly and membrane fusion”. In: *Biophysical Journal* 124.4 (2025), pp. 637–650. ISSN: 0006-3495. DOI: <https://doi.org/10.1016/j.bpj.2024.12.034>.
- [200] John Jumper et al. “Highly accurate protein structure prediction with AlphaFold”. In: *Nature* 596.7873 (2021), pp. 583–589. ISSN: 1476-4687. DOI: 10.1038/s41586-021-03819-2.
- [201] Josh Abramson and et al. “Accurate structure prediction of biomolecular interactions with AlphaFold 3”. In: *Nature* 630 (2024), pp. 493–500. DOI: 10.1038/s41586-024-07487-w.
- [202] Alessio Del Conte et al. “CAID prediction portal: a comprehensive service for predicting intrinsic disorder and binding regions in proteins”. In: *Nucleic Acids Research* 51.W1 (May 2023), W62–W69. ISSN: 0305-1048. DOI: 10.1093/nar/gkad430.
- [203] *PDB Data Growth by Experimental Method*. 2025. URL: <https://www.rcsb.org/statistics/growth/experimental-method> (visited on 09/19/2025).
- [204] Helen M. Berman et al. “The Protein Data Bank”. In: *Nucleic Acids Research* 28.1 (Jan. 2000), pp. 235–242. ISSN: 0305-1048. DOI: 10.1093/nar/28.1.235.
- [205] Tanguy Le Gall et al. “Intrinsic Disorder in the Protein Data Bank”. In: *Journal of Biomolecular Structure and Dynamics* 24.4 (2007), pp. 325–341. DOI: 10.1080/07391102.2007.10507123.
- [206] Tobias Stief, Katharina Vormann, and Nils-Alexander Lakomek. “Sensitivity-enhanced NMR ^{15}N R1 and R1 ρ relaxation experiments for the investigation of intrinsically disordered proteins at high magnetic fields”. In: *Methods* 223 (2024), pp. 1–15. ISSN: 1046-2023. DOI: <https://doi.org/10.1016/j.ymeth.2024.01.008>.
- [207] Souvik Dey, Matthew MacAinsh, and Huan-Xiang Zhou. “Sequence-Dependent Backbone Dynamics of Intrinsically Disordered Proteins”. In: *J. Chem. Theory Comput.* 18 (2022), pp. 6310–6323. DOI: 10.1021/acs.jctc.2c00328.
- [208] Ajaybabu V. Pobbati, Alexander Stein, and Dirk Fasshauer. “N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion”. In: *Science* 313.5787 (2006), pp. 673–676. DOI: 10.1126/science.1129486.

- [209] Feng Li et al. “Kinetic barriers to SNAREpin assembly in the regulation of membrane docking/priming and fusion”. In: *Proceedings of the National Academy of Sciences* 113.38 (2016), pp. 10536–10541. DOI: 10.1073/pnas.1604000113.
- [210] Jens Bredenbeck et al. “ α -Helix formation in a photoswitchable peptide tracked from picoseconds to microseconds by time-resolved IR spectroscopy”. In: *Proceedings of the National Academy of Sciences* 102.7 (2005), pp. 2379–2384. DOI: 10.1073/pnas.0406948102.
- [211] Dirk Fasshauer et al. “Structural Changes Are Associated with Soluble N-Ethylmaleimide-sensitive Fusion Protein Attachment Protein Receptor Complex Formation”. In: *Journal of Biological Chemistry* 272 (1997), pp. 28036–28041. DOI: 10.1074/jbc.272.44.28036.

Eidesstattliche Versicherung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, den 16. Dezember 2025

(Tobias Stief)

Danksagung

In diesem letzten Kapitel meiner Dissertation möchte ich mich gerne auf deutscher Sprache bei den Personen bedanken, die mich auf meinem Weg zu dieser Dissertation begleitet und unterstützt haben. Ich beginne mit meinem Doktorvater Nils-Alexander Lakomek, der mich am Spektrometer eingearbeitet und sich für meine Projekte und entstandenen Fragen immer Zeit genommen hat. Katharina Vormann möchte ich für die kooperative Zusammenarbeit danken, durch die diese Projekte in dem Umfang nicht umsetzbar gewesen wären. Des Weiteren möchte ich Mirko Kraus und Sophia Werner für die Diskussionsbereitschaft in der Arbeitsgruppe danken. Für die respektvolle und positive Arbeitsatmosphäre im Institut für physikalische Biologie und dem IBI-7 möchte ich allen Kollegen der letzten Jahre meinen Dank aussprechen. Meinen Freunden aus dem Fussballverein, sowie aus dem Musikverein, aus der Schule, als auch aus dem Studium sage ich: "Danke für die tollen Freizeitmomente". Für eine unvergessene Zeit und die bunten Pyramiden danke ich vor allem meinem Bruder und meiner Freundin. Zuletzt möchte ich mich bei meinen Eltern bedanken. Ohne ihre offene und unterstützende Art für das Studium, wäre es mir nicht möglich gewesen diese Dissertation zu beginnen und zu beenden. DANKE!

Appendix

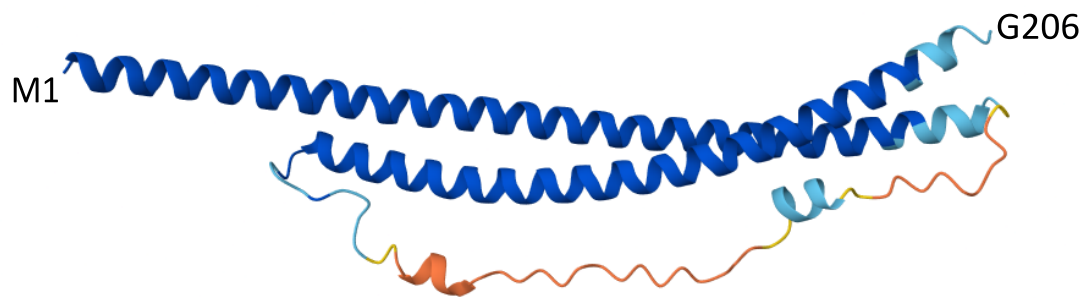


Figure A1: Predicted structure of SNAP25a using AlphaFold 3²⁰¹ on December 14, 2025. The confidence level is color-coded. Very high (pLDDT >90): dark blue, confident (90 > pLDDT >70): light blue, low (70 > pLDDT >50): yellow, very low (pLDDT <50): orange.

Chapter 7: Intrinsic Disorder of SNAP25a

Table S1: Experimental parameters of three-dimensional NMR backbone assignment experiments.

	HNCO	HNCA	HN(CO)CA	HNCACB	HN(CO)CACB	HN(CA)CO
Field / T	17.6	17.6	17.6	17.6	17.6	17.6
¹ H Larmor frequency/ MHz	750	750	750	750	750	750
Bruker pulse program	hncogp3d	hncagp3d	hncocagp3d	hncacbgp3d	hncocacbgp3d	hncacogp3d
Inter-scan recovery delay / s	1	1	1	1	1	1
Number of scans	16	16	16	16	16	16
Total experimental time	2d	2d	2d	5d	5d	2d
Direct dimension (¹ H)						
¹ H carrier/ppm	4.70	4.70	4.70	4.70	4.70	4.70
Spectral width/ppm	16.02	16.02	16.02	16.02	16.02	16.02
Data points (TD)	2048	2048	2048	2048	2048	2048
Time/ms	85.2	85.2	85.2	85.2	85.2	85.2
Indirect dimension (¹⁵ N)						
¹⁵ N carrier/ppm	117.00	117.00	117.00	117.00	117.00	117.00
Spectral width/ppm	35.00	35.00	35.00	35.00	35.00	35.00
Data points (TD)	100	100	100	100	100	100
Acquisition time/ms	18.8	18.8	18.8	18.8	18.8	18.8
Indirect dimension (¹³ C)						
¹³ C carrier/ppm	173.50	53.20	53.20	43.00	43.00	173.50
Spectral width/ppm	10.00	31.18	31.18	70.68	70.68	10.00
Data points (TD)	90	90	90	220	220	90
Acquisition time/ms	23.9	7.7	7.7	8.3	8.3	23.9
Assignment statistics						
# visible resonances	204	204	204	204	204	204
# assigned resonances	204	204	204	204	204	204

Table S2: Experimental parameters of two-dimensional ^1H - ^{15}N HSQC experiments.

	HSQC	HSQC	HSQC
Field / T	14.1	17.6	21.15 T
^1H Larmor frequency / MHz	600	750	900
Bruker pulse program	fhsqcf3gpqh	fhsqcf3gpqh	fhsqcf3gpqh
Inter-scan recovery delay / s	1	1	2
Number of scans	8	8	8
Total experimental time	80 min	80min	145 min
Direct dimension (^1H)			
^1H carrier/ppm	4.70	4.694	4.70
Spectral width/ppm	16.03	16.02	16.34
Data points	2048	2048	2048
Acquisition time/ms	106.6	85.2	69.7
Indirect dimension (^{15}N)			
^{15}N carrier/ppm	117.00	117.00	117.00
Spectral width/ppm	35.00	35.00	35.00
Data points	512	512	512
Acquisition time /ms	120.3	96.0	80.1

Table S3: Experimental parameters of ^{15}N NMR relaxation experiments.

	R1	R1rho	{ ^1H } - ^{15}N NOE	R2 (Hahn-echo)	R2 (Hahn-echo)
Field / T	14.1	14.1	14.1	14.1	21.15
^1H Larmor frequency / MHz	600	600	600	600	900
pulse program	Lakomek et al., JBNMR 2012	Lakomek et al., JBNMR 2012	Lakomek et al., JBNMR 2012	see Figure S8	see Figure S8
Inter-scan recovery delay / s	2	2	2	2	3
Number of scans	8	8	64	16	16
Total experimental time	24h	21h	5d 13h	10.5h	15h
Direct dimension (^1H)					
^1H carrier/ppm	4.700	4.696	4.700	4.700	4.700
Spectral width/ppm	16.03	16.03	16.03	16.03	16.03
Data points	2048	2048	2048	2048	2048
Acquisition time	106.5	106.5	106.5	106.5	106.5
Indirect dimension (^{15}N)					
^{15}N carrier/ppm	117.00	117.00	117.00	117.00	117.00
Spectral width/ppm	29.91	35.00	35.00	29.91	30.00
Data points (total)	4096	4096	1024	1024	1024
Acquisition time/ms	140.8	120.3	120.3	140.8	93.6
Relaxation period					
# relaxation delays	8	8	2	2	2
Time points (ms)	0, 720, 320, 480, 80, 640, 160, 560	5,85, 15, 65, 35, 25, 45, 75	0, 5000	1, 111	1, 111

Backbone chemical shift assignments have been submitted to the Biological Magnetic Resonance Bank (BMRB ID 51598).

```

    10    20    30    40    50    60
MAEDADMRNE LEEMQRRADQ LADESLESTR RMLQLVEESK DAGIRTLVML DEQGEQLDRV

    70    80    90   100   110   120
EEGMNHINQD MKEAEKNLKD LGKCCGLFIC PCNKLKSSDA YKKA WGNNQD GVVASQPARV

   130   140   150   160   170   180
VDEREQMAIS GGFIRRV TND ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR

   190   200
IMEKADSNKT RIDEANQRAT KMLGSG

```

Figure S1. The sequence of SNAP25a (isoform 2), organism: *Rattus norvegicus*, Uniprot accession number: P60881-2, 206 amino acids, 23.3 kDa. The two SNARE motifs, SN1 (L11 to L81) and SN2 (N1444 to A199), as observed in the post-fusion cis-SNARE complexes [1, 2], are color-coded in green.

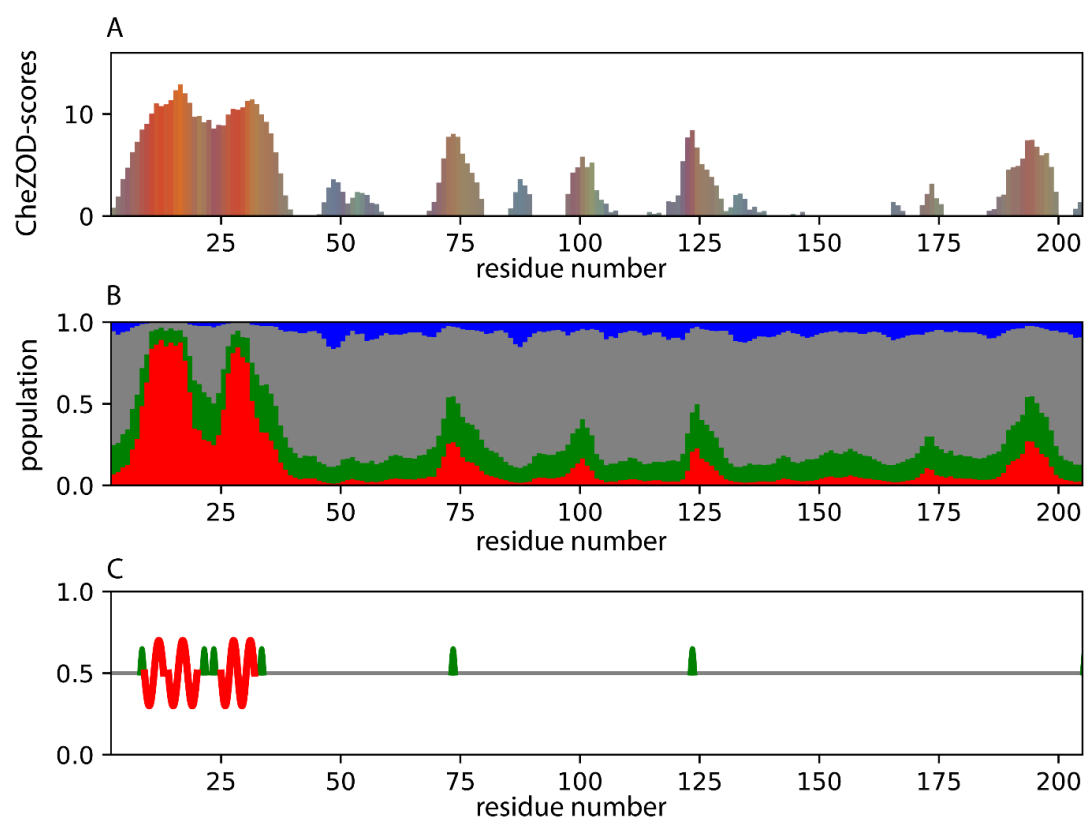


Figure S2. Output multi-panel plot generated by the CheSPI software [3] using the obtained NMR chemical shifts as input. (A) Bar plot with bar height corresponding to the so-called CheZOD Z-scores. These scores are translated to a color scheme in which blue colors stand for sheets, red for helices, and green colors for turns, while disordered states with principal components close to zero are represented

in grey. (B) Stacked bar plot of populations of “extended” (blue), “helical” (red), “turn” (green), and “non-folded” (grey) local structures (corresponding to DSSP classes), as derived by the CheSPI software. (C) Representation of the most likely predictions for secondary structure separated into eight DSSP classes : α -helix (curved red lines), turns (green arcs), and coil (grey lines) [3].

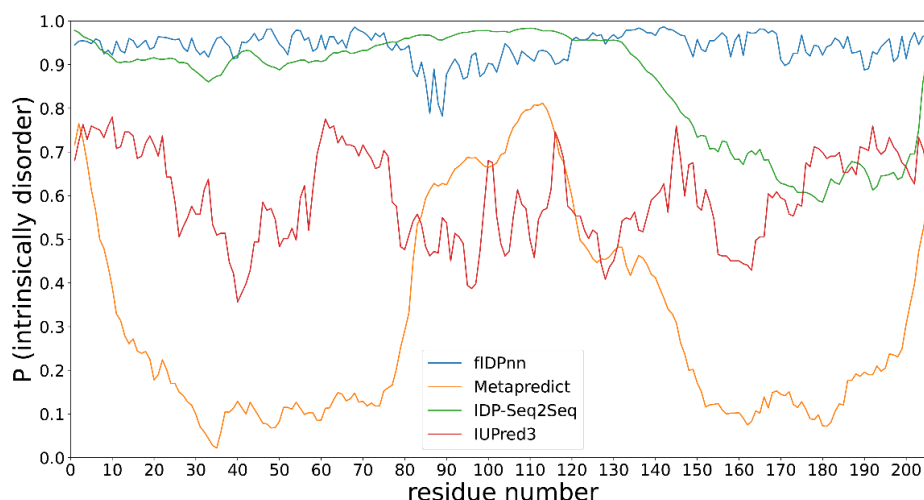


Figure S3. Computational disorder predictions of SNAP25a using different disorder predictors. A score of 1 means 100% probability to be intrinsically disordered, a score of 0 means 0%. Disorder prediction by four different disorder predictors fIDPnn [4], Metapredict [5], IDP-Seq2Seq [6], IUPred3 [7] are shown.

Table S4. Backbone chemical shift assignments of SNAP25a. The columns contain the following information for each residue (rows): # (residue number), amino acid residue type (1-letter code), $\delta\text{H}(\text{N})$ chemical shift / ppm, $\delta(\text{N})$ / ppm, $\delta(\text{C}')$ / ppm, $\delta(\text{C}\alpha)$ / ppm, $\delta(\text{C}\beta)$ / ppm.

1	M	8.44	122.0	176.2	55.6	32.6
2	A	8.32	124.5	178.0	52.8	18.9
3	E	8.44	119.7	176.6	56.9	29.8
4	D	8.32	120.7	176.5	54.5	40.9
5	A	8.27	124.1	178.2	53.7	18.8
6	D	8.33	118.5	177.2	55.2	40.4
7	M	8.21	120.6	177.1	56.2	32.3
8	R	8.26	120.8	177.1	57.7	30.0
9	N	8.50	119.0	176.3	54.3	38.4
10	E	8.44	121.6	178.0	58.5	29.4

11	L	8.21	120.7	179.2	57.1	41.5
12	E	8.25	119.8	178.6	58.6	29.2
13	E	8.25	120.1	178.3	58.4	29.0
14	M	8.25	119.1	178.5	57.8	32.0
15	Q	8.17	120.3	177.1	57.6	28.3
16	R	8.16	120.3	178.4	58.2	30.0
17	R	8.16	119.7	177.7	57.8	30.1
18	A	8.13	123.2	179.4	54.1	18.0
19	D	8.41	119.0	177.3	55.7	40.2
20	Q	8.06	119.7	177.1	57.1	28.6
21	L	8.12	121.2	178.2	56.1	41.7
22	A	8.13	123.4	178.4	53.3	18.5
23	D	8.21	119.5	177.4	55.2	40.7
24	E	8.49	121.6	177.7	57.7	29.4
25	S	8.38	116.1	175.7	59.6	63.3
26	L	8.12	123.5	178.6	56.6	41.7
27	E	8.37	120.2	178.0	58.2	29.0
28	S	8.31	115.2	176.4	60.4	62.7
29	T	8.07	117.0	175.6	64.6	68.9
30	R	8.17	122.0	177.9	58.3	29.9
31	R	8.21	119.9	177.6	57.7	30.1
32	M	8.12	119.7	177.4	56.9	31.9
33	L	8.12	121.1	178.1	56.2	41.7
34	Q	8.13	119.8	176.5	56.7	28.6
35	L	8.11	121.8	178.3	56.0	41.8
36	V	8.06	120.6	177.0	63.5	32.3
37	E	8.36	123.5	177.1	57.2	29.8
38	E	8.49	121.2	177.0	57.0	29.9
39	S	8.30	116.2	175.1	58.7	63.5
40	K	8.30	123.2	176.7	56.7	32.5
41	D	8.29	120.5	176.2	54.5	40.9
42	A	8.26	124.4	178.5	53.0	18.8
43	G	8.44	107.3	174.3	45.3	n
44	I	7.94	119.9	176.5	61.3	38.3
45	R	8.45	124.8	176.3	56.1	30.5
46	T	8.22	116.1	174.2	62.0	69.5
47	L	8.31	124.9	177.0	55.2	42.1
48	V	8.17	121.6	175.8	62.4	32.5
49	M	8.50	124.6	175.8	55.1	32.6
50	L	8.35	123.5	177.1	54.9	42.5
51	D	8.46	121.0	176.6	54.0	40.9
52	E	8.49	120.8	176.8	57.1	29.7
53	Q	8.43	119.8	176.6	56.0	29.1
54	G	8.35	109.2	174.0	45.3	n
55	E	8.41	120.5	176.4	56.3	30.1
56	Q	8.58	121.3	176.1	55.7	28.8
57	L	8.38	123.8	177.1	55.2	42.2

58	D	8.44	120.5	176.0	54.2	40.7
59	R	8.19	121.1	176.3	56.0	30.5
60	V	8.29	122.2	176.4	62.6	32.4
61	E	8.59	124.6	176.6	56.5	29.9
62	E	8.53	122.1	177.2	57.0	29.9
63	G	8.54	109.6	174.5	45.4	n
64	M	8.20	119.1	176.1	55.5	32.3
65	N	8.45	118.8	174.9	53.3	38.4
66	H	8.42	119.1	174.6	55.7	29.0
67	I	8.14	121.8	176.0	61.3	38.4
68	N	8.60	122.4	175.5	53.4	38.4
69	Q	8.49	121.2	175.9	56.3	29.0
70	D	8.44	120.9	176.8	54.9	40.6
71	M	8.32	121.0	176.9	56.1	32.3
72	K	8.32	121.4	177.6	57.1	32.3
73	E	8.40	121.3	177.1	57.3	29.6
74	A	8.26	123.3	178.7	53.4	18.6
75	E	8.23	118.9	177.3	57.3	29.6
76	K	8.13	121.1	176.8	57.2	32.5
77	N	8.39	118.3	175.7	53.6	38.4
78	L	8.13	121.8	178.0	55.9	41.8
79	K	8.19	120.5	176.7	57.0	32.4
80	D	8.25	120.3	176.7	54.5	40.7
81	L	8.20	122.4	178.4	55.7	41.7
82	G	8.46	108.4	174.6	45.6	n
83	K	8.09	120.3	177.0	56.4	32.7
84	S	8.42	116.2	174.7	58.4	63.5
85	S	8.39	117.5	175.0	58.6	63.6
86	G	8.43	110.3	173.8	45.3	n
87	L	7.98	121.0	176.7	55.0	42.2
88	F	8.29	120.9	175.0	57.5	39.3
89	I	8.03	124.0	175.4	60.2	38.6
90	S	8.48	121.8	173.0	56.3	63.2
91	P	n	n	n	n	n
92	S	8.36	114.9	174.7	58.6	63.4
93	N	8.38	120.8	175.4	53.3	38.4
94	K	8.23	121.3	176.7	56.7	32.6
95	L	8.16	122.2	177.6	55.3	41.9
96	K	8.32	121.7	176.8	56.3	32.6
97	S	8.32	116.4	174.9	58.6	63.5
98	S	8.41	117.5	174.8	58.7	63.6
99	D	8.40	122.3	176.7	55.2	40.7
100	A	8.15	123.0	178.2	53.5	18.5
101	Y	8.00	118.2	176.0	58.6	38.1
102	K	7.87	121.7	176.7	56.8	32.6
103	K	8.12	121.5	176.5	56.7	32.6
104	A	8.09	124.0	177.5	52.5	18.8

105	W	8.09	119.6	176.8	57.2	29.5
106	G	8.22	109.7	173.8	45.3	n
107	N	8.30	118.3	175.1	53.1	38.6
108	N	8.50	118.8	175.3	53.4	38.4
109	Q	8.46	120.1	175.8	56.0	28.9
110	D	8.37	120.7	176.7	54.7	40.8
111	G	8.31	108.9	174.0	45.3	n
112	V	7.94	120.0	176.3	62.5	32.3
113	V	8.32	124.8	175.9	62.2	32.4
114	A	8.48	128.2	177.5	52.4	19.0
115	S	8.31	115.3	174.1	58.1	63.6
116	Q	8.37	122.6	173.8	53.5	28.7
117	P	n	n	n	n	n
118	A	8.47	124.4	177.6	52.3	18.8
119	R	8.36	120.7	176.1	55.9	30.7
120	V	8.35	123.2	176.1	62.4	32.3
121	V	8.33	124.2	175.6	62.3	32.5
122	D	8.48	124.7	176.4	53.9	41.2
123	E	8.67	122.9	177.2	57.6	29.6
124	R	8.40	120.3	177.5	57.4	30.0
125	E	8.29	120.7	177.1	57.3	29.6
126	Q	8.32	119.9	176.7	56.5	28.9
127	M	8.30	120.3	176.4	56.0	32.6
128	A	8.20	124.2	178.2	52.9	18.7
129	I	8.12	119.2	176.9	61.6	38.5
130	S	8.37	118.4	175.2	58.7	63.5
131	G	8.42	110.8	174.5	45.4	n
132	G	8.21	107.9	173.5	45.0	n
133	F	8.08	119.6	175.3	57.7	39.5
134	I	8.13	123.2	175.5	60.7	38.6
135	R	8.41	125.9	175.8	55.8	30.6
136	R	8.51	123.9	176.1	55.9	30.7
137	V	8.42	122.1	176.3	62.3	32.6
138	T	8.30	117.5	174.1	61.4	69.7
139	N	8.57	120.8	174.8	53.2	38.6
140	D	8.37	120.7	175.9	54.4	40.9
141	A	8.28	124.3	177.9	52.8	18.8
142	R	8.35	119.6	176.7	56.2	30.3
143	E	8.49	121.0	176.4	57.1	29.8
144	N	8.45	118.3	175.2	53.4	38.6
145	E	8.38	120.8	176.5	56.8	29.8
146	M	8.38	120.2	175.9	55.5	32.6
147	D	8.34	121.6	176.5	54.4	40.9
148	E	8.53	121.6	176.3	57.0	29.8
149	N	8.58	119.0	175.3	53.4	38.5
150	L	8.19	122.1	177.7	55.5	41.9
151	E	8.39	120.8	176.6	56.9	29.8

152	Q	8.35	120.7	176.2	55.9	29.0
153	V	8.22	120.7	176.4	62.5	32.3
154	S	8.45	118.6	175.1	58.6	63.7
155	G	8.44	110.7	174.0	45.3	n
156	I	8.00	120.0	176.5	61.3	38.3
157	I	8.26	124.8	176.9	61.4	38.1
158	G	8.49	112.3	173.9	45.3	n
159	N	8.33	118.6	175.4	53.2	38.6
160	L	8.25	121.9	177.5	55.5	41.8
161	R	8.28	120.5	176.2	56.3	30.1
162	H	8.43	119.4	174.8	55.8	29.2
163	M	8.34	121.6	175.7	55.5	32.6
164	A	8.35	124.9	177.6	52.5	18.9
165	L	8.24	121.0	177.1	55.1	42.2
166	D	8.35	120.5	176.3	54.1	40.8
167	M	8.40	120.9	177.0	55.5	32.1
168	G	8.55	109.2	174.0	45.5	n
169	N	8.35	118.3	175.3	53.1	38.8
170	E	8.56	121.1	176.6	57.0	29.7
171	I	8.20	121.0	176.1	61.4	38.4
172	D	8.45	123.7	177.0	54.4	40.9
173	T	8.18	114.5	175.3	62.6	69.1
174	Q	8.39	121.3	176.1	56.5	28.8
175	N	8.39	118.5	175.2	53.5	38.3
176	R	8.27	121.0	176.3	56.3	30.4
177	Q	8.46	121.4	176.2	56.1	28.8
178	I	8.18	121.5	175.9	61.4	38.5
179	D	8.38	123.5	176.1	54.5	40.7
180	R	8.20	121.1	176.4	56.2	30.4
181	I	8.23	122.1	176.6	61.6	37.9
182	M	8.46	123.9	176.3	55.3	32.3
183	E	8.40	122.3	176.5	56.7	29.9
184	K	8.40	122.5	176.5	56.3	32.7
185	A	8.42	125.2	177.7	52.7	18.9
186	D	8.41	119.4	176.7	54.2	40.9
187	S	8.34	116.7	174.8	59.1	63.4
188	N	8.52	120.2	175.5	53.8	38.4
189	K	8.17	121.1	177.0	57.0	32.6
190	T	8.18	114.9	174.7	62.9	69.3
191	R	8.30	123.3	176.9	56.7	30.4
192	I	8.26	122.4	176.3	61.8	38.3
193	D	8.47	123.5	177.1	54.7	40.7
194	E	8.40	121.6	177.4	57.7	29.6
195	A	8.30	123.1	178.7	53.7	18.3
196	N	8.29	116.8	176.0	53.9	38.3
197	Q	8.23	120.0	176.7	56.7	28.7
198	R	8.23	120.7	176.6	56.8	30.3

199	A	8.22	123.7	178.3	53.0	18.8
200	T	8.10	113.2	174.8	62.5	69.6
201	K	8.24	123.3	176.6	56.5	32.6
202	M	8.38	121.3	176.3	55.5	32.3
203	L	8.33	123.5	177.9	55.4	42.0
204	G	8.48	109.8	174.2	45.2	n
205	S	8.26	115.4	174.1	58.2	64.0
206	G	8.20	116.8	179.1	46.1	n

Table S5. Secondary chemical shift data for SNAP25a. Secondary chemical shift data have been calculated from the $\delta(\text{C}\alpha)$ and $\delta(\text{C}\beta)$ chemical shifts assignments minus random coil chemical shifts predicted from the amino acid sequence of SNAP25a and the experimental buffer conditions using the POTENCI software [8]. The columns contain the following information for each residue (rows): # (residue number), amino acid residue type (1-letter code), secondary chemical shifts $\Delta\delta\text{C}\alpha$ / ppm, $\Delta\delta\text{C}\beta$ / ppm, $\Delta\delta\text{C}\alpha - \Delta\delta\text{C}\beta$ / ppm.

2	A	0.1	-0.4	0.6
3	E	0.2	-0.5	0.7
4	D	0.2	-0.3	0.4
5	A	0.8	-0.4	1.1
6	D	0.7	-0.5	1.2
7	M	0.5	-0.3	0.8
8	R	1.3	-0.6	1.9
9	N	1.0	-0.5	1.5
10	E	1.7	-0.7	2.3
11	L	1.7	-0.7	2.5
12	E	1.8	-1.1	2.9
13	E	1.7	-1.2	2.9
14	M	2.1	-0.6	2.8
15	Q	1.6	-1.1	2.7
16	R	2.0	-0.7	2.8
17	R	1.7	-0.7	2.5
18	A	1.5	-1.3	2.7
19	D	1.1	-0.9	2.1
20	Q	1.1	-0.7	1.8
21	L	0.7	-0.4	1.2
22	A	0.7	-0.7	1.4
23	D	0.8	-0.5	1.3
24	E	0.8	-0.9	1.7
25	S	1.0	-0.4	1.4
26	L	1.2	-0.5	1.7
27	E	1.6	-1.2	2.8
28	S	1.9	-1.1	3.0
29	T	2.5	-0.8	3.4
30	R	2.1	-0.8	2.9

31	R	1.4	-0.6	2.1
32	M	1.3	-0.8	2.2
33	L	0.8	-0.5	1.3
34	Q	1.0	-0.8	1.8
35	L	0.8	-0.5	1.2
36	V	1.2	-0.5	1.7
37	E	0.6	-0.5	1.1
38	E	0.4	-0.5	0.9
39	S	0.3	-0.3	0.6
40	K	0.2	-0.5	0.7
41	D	0.2	-0.2	0.4
42	A	0.1	-0.3	0.4
43	G	-0.1	0.0	-0.1
44	I	0.2	-0.5	0.6
45	R	-0.1	-0.3	0.2
46	T	0.2	-0.3	0.5
47	L	0.0	-0.2	0.1
48	V	-0.1	-0.2	0.1
49	M	-0.4	-0.1	-0.2
50	L	-0.3	0.2	-0.5
51	D	-0.4	-0.3	-0.1
52	E	0.3	-0.5	0.8
53	Q	-0.2	-0.3	0.1
54	G	-0.1	0.0	-0.1
55	E	-0.6	-0.2	-0.4
56	Q	-0.1	-0.6	0.5
57	L	-0.1	-0.1	0.0
58	D	-0.2	-0.4	0.2
59	R	-0.3	-0.2	-0.1
60	V	0.1	-0.4	0.5
61	E	0.0	-0.5	0.5
62	E	0.2	-0.5	0.6
63	G	0.0	0.0	0.0
64	M	-0.1	-0.5	0.4
65	N	0.0	-0.3	0.3
66	H	-0.1	-0.5	0.5
67	I	0.0	-0.3	0.2
68	N	0.2	-0.4	0.6
69	Q	0.2	-0.4	0.7
70	D	0.3	-0.3	0.6
71	M	0.3	-0.4	0.7
72	K	0.5	-0.5	1.0
73	E	0.7	-0.7	1.4
74	A	0.7	-0.7	1.3
75	E	0.7	-0.6	1.3
76	K	0.7	-0.4	1.2
77	N	0.3	-0.4	0.7

78	L	0.5	-0.4	0.9
79	K	0.5	-0.5	1.0
80	D	0.2	-0.4	0.6
81	L	0.1	-0.5	0.6
82	G	0.1	0.0	0.1
83	K	0.1	-0.4	0.5
84	S	0.0	-0.3	0.3
85	S	-0.1	-0.1	0.1
86	G	-0.1	0.0	-0.1
87	L	-0.2	-0.1	-0.1
88	F	-0.2	-0.2	-0.1
89	I	-0.5	-0.4	-0.1
90	S	0.0	-0.3	0.3
91	P	n	n	n
92	S	0.1	-0.4	0.5
93	N	0.0	-0.4	0.4
94	K	0.1	-0.3	0.4
95	L	0.0	-0.3	0.2
96	K	0.0	-0.4	0.4
97	S	0.2	-0.4	0.5
98	S	0.2	-0.2	0.4
99	D	0.7	-0.4	1.0
100	A	0.7	-0.6	1.2
101	Y	0.4	-0.6	1.0
102	K	0.6	-0.6	1.2
103	K	0.3	-0.4	0.7
104	A	-0.1	-0.3	0.1
105	W	-0.2	-0.1	-0.2
106	G	-0.1	0.0	-0.1
107	N	-0.3	-0.2	-0.1
108	N	0.0	-0.3	0.3
109	Q	-0.2	-0.4	0.2
110	D	0.1	-0.2	0.3
111	G	-0.1	0.0	-0.1
112	V	0.1	-0.4	0.5
113	V	0.0	-0.4	0.4
114	A	-0.2	-0.3	0.1
115	S	-0.1	-0.2	0.1
116	Q	-0.3	-0.3	0.0
117	P	n	n	n
118	A	-0.3	-0.3	0.0
119	R	-0.1	-0.2	0.1
120	V	0.1	-0.5	0.6
121	V	0.1	-0.4	0.4
122	D	-0.4	0.0	-0.3
123	E	0.8	-0.6	1.4
124	R	0.9	-0.6	1.6

125	E	0.4	-0.7	1.1
126	Q	0.5	-0.5	1.0
127	M	0.5	-0.2	0.8
128	A	0.2	-0.4	0.6
129	I	0.5	-0.2	0.7
130	S	0.2	-0.3	0.5
131	G	-0.1	0.0	-0.1
132	G	-0.2	0.0	-0.2
133	F	-0.3	0.0	-0.3
134	I	-0.4	-0.2	-0.2
135	R	-0.1	-0.3	0.1
136	R	-0.1	-0.3	0.1
137	V	-0.1	-0.3	0.2
138	T	-0.4	-0.1	-0.3
139	N	-0.1	-0.3	0.2
140	D	-0.2	-0.2	0.0
141	A	-0.2	-0.2	0.0
142	R	-0.1	-0.3	0.2
143	E	0.3	-0.4	0.7
144	N	0.0	-0.2	0.2
145	E	-0.1	-0.3	0.2
146	M	-0.2	-0.1	-0.1
147	D	0.0	-0.2	0.2
148	E	0.1	-0.4	0.5
149	N	-0.1	-0.3	0.2
150	L	0.0	-0.3	0.3
151	E	0.1	-0.3	0.3
152	Q	0.1	-0.4	0.5
153	V	0.1	-0.5	0.6
154	S	0.1	-0.2	0.4
155	G	0.0	0.0	0.0
156	I	0.2	-0.4	0.5
157	I	0.2	-0.5	0.7
158	G	0.1	0.0	0.1
159	N	0.0	-0.3	0.3
160	L	0.1	-0.3	0.4
161	R	0.0	-0.5	0.5
162	H	-0.1	-0.6	0.4
163	M	-0.1	-0.3	0.2
164	A	-0.2	-0.2	0.0
165	L	-0.2	-0.1	0.0
166	D	-0.2	-0.2	0.0
167	M	-0.4	-0.5	0.1
168	G	-0.1	0.0	-0.1
169	N	-0.1	-0.2	0.1
170	E	0.1	-0.3	0.4
171	I	0.2	-0.3	0.4

172	D	0.1	-0.3	0.4
173	T	0.5	-0.6	1.1
174	Q	0.4	-0.6	1.0
175	N	0.1	-0.5	0.5
176	R	-0.1	-0.3	0.2
177	Q	0.3	-0.6	0.8
178	I	0.2	-0.3	0.4
179	D	0.1	-0.4	0.5
180	R	-0.1	-0.2	0.1
181	I	0.2	-0.5	0.7
182	M	-0.1	-0.6	0.4
183	E	-0.1	-0.3	0.2
184	K	0.0	-0.4	0.3
185	A	0.0	-0.4	0.4
186	D	-0.1	-0.2	0.2
187	S	0.4	-0.3	0.8
188	N	0.2	-0.3	0.6
189	K	0.3	-0.3	0.6
190	T	0.8	-0.4	1.3
191	R	0.6	-0.4	1.1
192	I	0.6	-0.4	1.1
193	D	0.4	-0.5	0.9
194	E	1.1	-0.7	1.8
195	A	0.7	-0.7	1.5
196	N	0.5	-0.4	0.9
197	Q	0.7	-0.6	1.2
198	R	0.6	-0.5	1.1
199	A	0.3	-0.5	0.8
200	T	0.5	-0.2	0.7
201	K	0.0	-0.4	0.3
202	M	-0.1	-0.4	0.3
203	L	0.0	-0.1	0.1
204	G	0.0	0.0	0.0
205	S	-0.3	0.0	-0.3

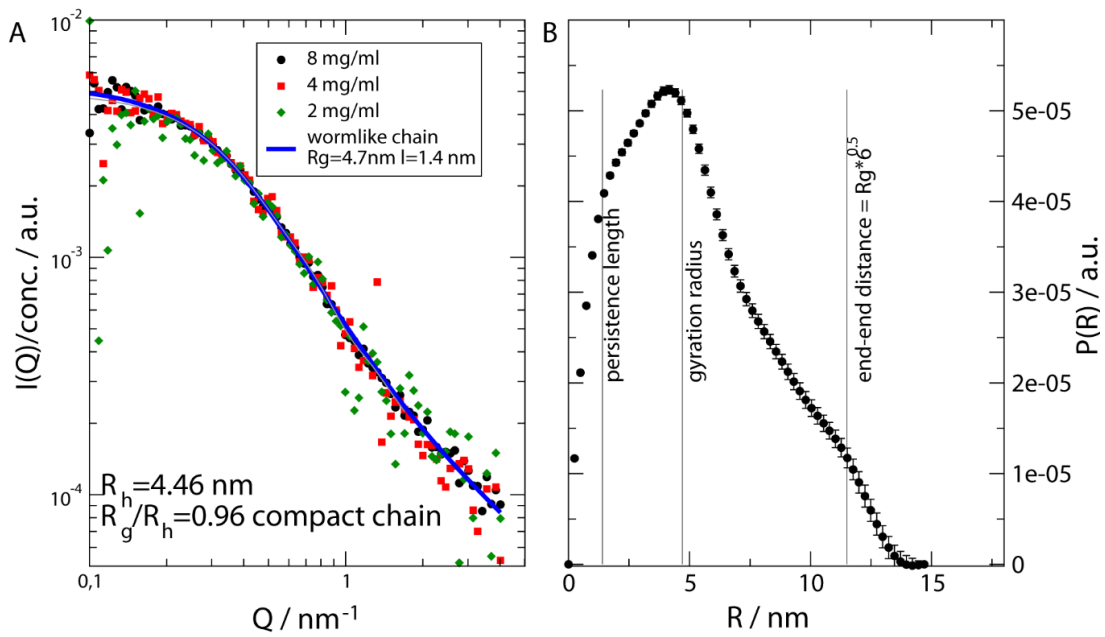


Figure S4. SAXS analysis of SNAP25a. (A) Scattering intensity $I(Q)$ of SNAP25a as a function of the scattering vector Q . The scattering intensity is scaled by concentration after background subtraction. (B) Pair distance distribution function, $P(r)$, of SNAP25a. The first shoulder indicates the persistence length and the maximum corresponds to the radius of gyration. The end-to-end distance results in the shoulder around 12.5 nm as the maximum extension of the chain is a bit larger than $R_e=6^{0.5}R_g$ of a Gaussian chain.

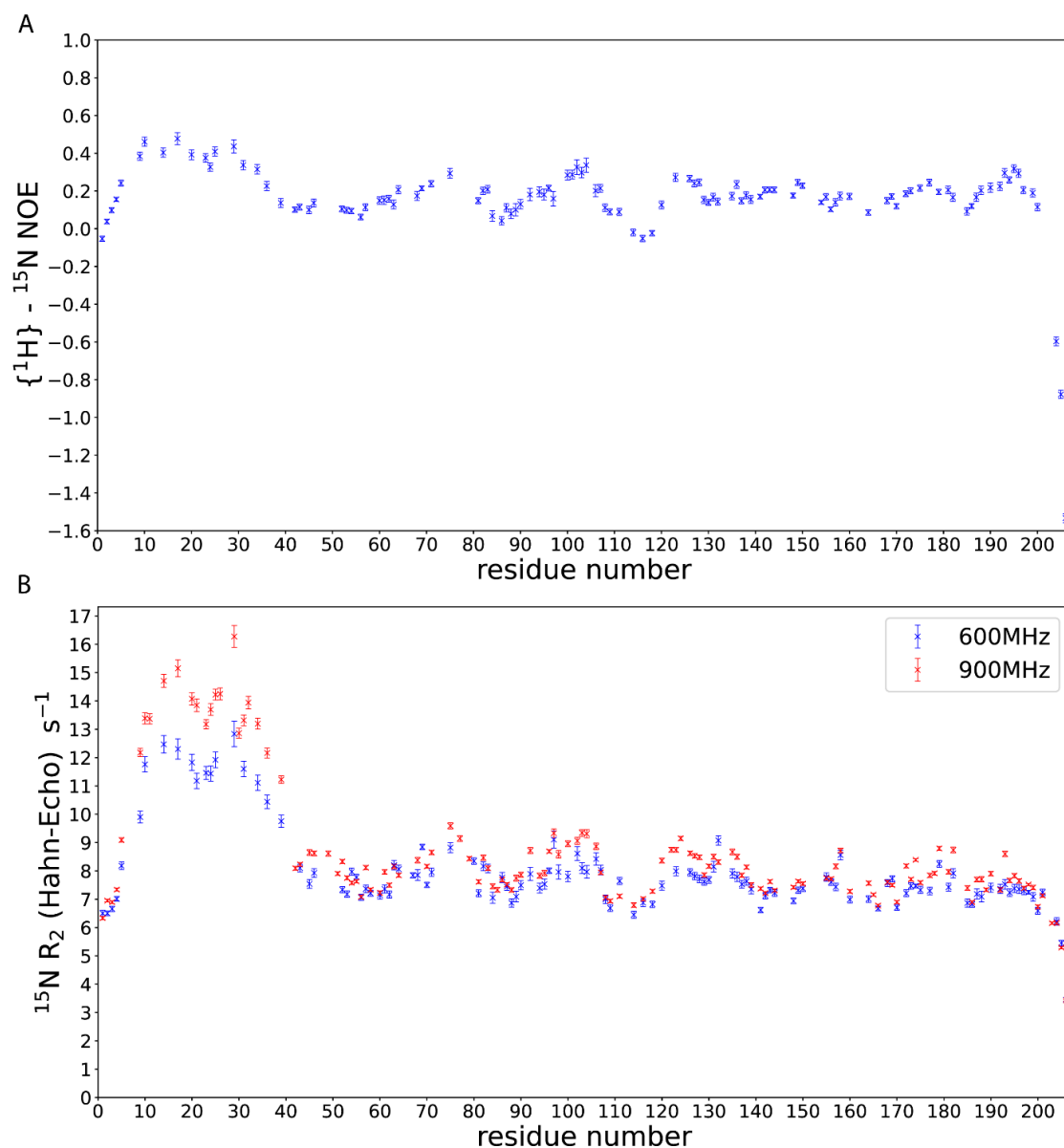


Figure S5. Additions on the NMR relaxation data on SNAP25 shown in Figure 4 of the main manuscript. (A) $\{^1\text{H}\}$ - ^{15}N NOE values indicate a high degree of internal dynamics on the fast ps timescale. (B) Individual ^{15}N R_2 (900 MHz) and R_2 (600 MHz) rate constants. In Figure 4D their difference is shown.

Table S6. NMR relaxation data. The columns contain the following information for each residue (rows): # (residue number) + AA type (1-letter code), longitudinal relaxation rate constants ^{15}N R_1 / s^{-1} (600 MHz), experimental error $\delta(^{15}\text{N}$ R_1) / s^{-1} (600 MHz); transverse relaxation rate constants (derived from $R_{1\rho}$) ^{15}N $R_2(R_{1\rho})$ / s^{-1} (600 MHz), experimental error $\delta(^{15}\text{N}$ $R_2(R_{1\rho}))$ / s^{-1} (600 MHz); heteronuclear NOE $\{^1\text{H}\}$ - ^{15}N values (I / I_0) (600 MHz), experimental error $\delta(I / I_0)$ (600 MHz); transverse relaxation

rate constants (600 MHz) (derived from Hahn-echo experiment) $^{15}\text{N } R_2 / \text{s}^{-1}$ (600 MHz), experimental error $\delta(^{15}\text{N } R_2) / \text{s}^{-1}$ (600 MHz), $^{15}\text{N } R_2 / \text{s}^{-1}$ (900 MHz), $\delta(^{15}\text{N } R_2) / \text{s}^{-1}$ (900 MHz), difference $^{15}\text{N } R_2 / \text{s}^{-1}$ (900 MHz) minus $^{15}\text{N } R_2 / \text{s}^{-1}$ (600 MHz), propagated experimental error $\delta(R_2 / \text{s}^{-1} (900 \text{ MHz}) - R_2 / \text{s}^{-1} (600 \text{ MHz}))$.

1M	1.60	0.01	3.17	0.04	-0.05	0.02	6.50	0.11	6.33	0.06	-0.17	0.13
2A	1.63	0.01	3.67	0.03	0.04	0.02	6.50	0.09	6.95	0.05	0.45	0.10
3E	1.63	0.01	3.52	0.04	0.10	0.02	6.66	0.10	6.89	0.05	0.24	0.11
4D	1.69	0.01	4.07	0.04	0.16	0.02	7.01	0.09	7.34	0.05	0.33	0.10
5A	1.67	0.02	5.22	0.06	0.24	0.02	8.18	0.14	9.09	0.09	0.91	0.16
6D	n	n	n	n	n	n	n	n	n	n	n	n
7M	n	n	n	n	n	n	n	n	n	n	n	n
8R	n	n	n	n	n	n	n	n	n	n	n	n
9N	1.67	0.02	6.82	0.09	0.38	0.03	9.90	0.22	12.18	0.15	2.28	0.26
10E	1.69	0.02	8.37	0.10	0.46	0.03	11.76	0.27	13.39	0.20	1.63	0.34
11L	n	n	n	n	n	n	n	n	13.37	0.18	n	n
12E	n	n	n	n	n	n	n	n	n	n	n	n
13E	n	n	n	n	n	n	n	n	n	n	n	n
14M	1.68	0.02	9.57	0.13	0.40	0.03	12.47	0.31	14.71	0.23	2.24	0.39
15Q	n	n	n	n	n	n	n	n	n	n	n	n
16R	n	n	n	n	n	n	n	n	n	n	n	n
17R	1.63	0.02	10.16	0.15	0.48	0.04	12.30	0.36	15.15	0.30	2.85	0.47
18A	n	n	n	n	n	n	n	n	n	n	n	n
19D	n	n	n	n	n	n	n	n	n	n	n	n
20Q	n	n	n	n	0.39	0.03	11.83	0.29	14.08	0.22	2.25	0.37
21L	1.55	0.02	8.85	0.11	n	n	11.18	0.28	13.85	0.23	2.67	0.36
22A	n	n	n	n	n	n	n	n	n	n	n	n
23D	1.50	0.02	8.47	0.10	0.38	0.03	11.46	0.24	13.18	0.17	1.72	0.29
24E	1.58	0.02	8.65	0.11	0.33	0.03	11.43	0.27	13.69	0.21	2.26	0.34
25S	1.58	0.02	9.11	0.12	0.41	0.03	11.92	0.29	14.22	0.20	2.30	0.35
26L	n	n	n	n	n	n	n	n	14.25	0.22	n	n
27E	n	n	n	n	n	n	n	n	n	n	n	n
28S	n	n	n	n	n	n	n	n	n	n	n	n
29T	1.63	0.03	10.42	0.20	0.44	0.04	12.83	0.45	16.28	0.39	3.44	0.60
30R	n	n	n	n	n	n	n	n	12.86	0.18	n	n
31R	1.67	0.02	8.70	0.11	0.34	0.03	11.60	0.28	13.32	0.19	1.72	0.33
32M	n	n	n	n	n	n	n	n	13.94	0.23	n	n
33L	n	n	n	n	n	n	n	n	n	n	n	n
34Q	1.60	0.02	8.62	0.11	0.31	0.03	11.11	0.28	13.20	0.20	2.09	0.34
35L	n	n	n	n	n	n	n	n	n	n	n	n
36V	1.53	0.02	7.63	0.10	0.23	0.03	10.44	0.25	12.16	0.18	1.72	0.30
37E	n	n	n	n	n	n	n	n	n	n	n	n
38E	n	n	n	n	n	n	n	n	n	n	n	n
39S	1.55	0.02	6.17	0.09	0.14	0.03	9.75	0.22	11.23	0.14	1.47	0.26
40K	n	n	n	n	n	n	n	n	n	n	n	n
41D	n	n	n	n	n	n	n	n	n	n	n	n

42A	n	n	n	n	0.10	0.02	n	n	8.09	0.07	n	n
43G	1.57	0.02	4.05	0.06	0.11	0.02	8.10	0.14	8.23	0.08	0.12	0.16
44I	n	n	n	n	n	n	n	n	n	n	n	n
45R	1.66	0.02	4.77	0.07	0.10	0.02	7.54	0.16	8.65	0.10	1.11	0.19
46T	1.63	0.02	4.54	0.06	0.14	0.03	7.93	0.16	8.62	0.08	0.70	0.18
47L	n	n	n	n	n	n	n	n	n	n	n	n
48V	n	n	n	n	n	n	n	n	n	n	n	n
49M	n	n	n	n	n	n	n	n	8.61	0.10	n	n
50L	n	n	n	n	n	n	n	n	n	n	n	n
51D	n	n	n	n	n	n	n	n	7.91	0.07	n	n
52E	1.64	0.01	4.38	0.05	0.11	0.02	7.33	0.12	8.33	0.08	1.00	0.14
53Q	1.67	0.02	4.20	0.05	0.10	0.02	7.18	0.12	7.75	0.07	0.57	0.14
54G	1.63	0.02	3.41	0.05	0.09	0.02	7.97	0.13	7.58	0.07	-0.39	0.14
55E	1.67	0.01	3.79	0.04	n	n	7.78	0.10	7.63	0.06	-0.15	0.12
56Q	1.64	0.02	3.82	0.05	0.06	0.02	7.06	0.12	7.08	0.06	0.02	0.13
57L	1.68	0.02	4.10	0.05	0.11	0.02	7.37	0.14	8.11	0.08	0.75	0.16
58D	n	n	n	n	n	n	7.21	0.11	7.34	0.07	0.12	0.13
59R	n	n	n	n	n	n	n	n	n	n	n	n
60V	1.68	0.02	4.07	0.06	0.15	0.02	7.14	0.15	7.22	0.08	0.08	0.16
61E	1.71	0.02	4.15	0.06	0.15	0.03	7.36	0.15	7.96	0.09	0.60	0.17
62E	1.70	0.02	4.14	0.05	0.16	0.02	7.17	0.13	7.50	0.08	0.33	0.15
63G	1.68	0.02	3.72	0.06	0.13	0.03	8.20	0.17	8.16	0.09	-0.04	0.19
64M	1.74	0.02	4.10	0.06	0.21	0.03	8.06	0.15	7.85	0.07	-0.22	0.16
65N	n	n	n	n	n	n	n	n	n	n	n	n
66H	n	n	n	n	n	n	n	n	n	n	n	n
67I	1.71	0.01	5.00	0.04	n	n	7.84	0.10	n	n	n	n
68N	1.71	0.02	4.84	0.08	0.17	0.03	7.85	0.19	8.38	0.10	0.52	0.22
69Q	1.66	0.01	5.82	0.04	0.21	0.02	8.85	0.11	n	n	n	n
70D	1.69	0.01	4.36	0.04	n	n	7.50	0.10	8.16	0.07	0.66	0.12
71M	1.70	0.02	4.63	0.05	0.24	0.02	7.95	0.14	8.65	0.09	0.70	0.17
72K	n	n	n	n	n	n	n	n	n	n	n	n
73E	n	n	n	n	n	n	n	n	n	n	n	n
74A	n	n	n	n	n	n	n	n	n	n	n	n
75E	1.72	0.02	5.64	0.07	0.29	0.03	8.82	0.18	9.59	0.11	0.77	0.21
76K	n	n	n	n	n	n	n	n	n	n	n	n
77N	n	n	n	n	n	n	n	n	9.14	0.11	n	n
78L	n	n	n	n	n	n	n	n	n	n	n	n
79K	n	n	n	n	n	n	n	n	8.43	0.08	n	n
80D	1.73	0.01	5.74	0.05	n	n	8.35	0.12	n	n	n	n
81L	1.72	0.02	4.20	0.05	0.15	0.02	7.21	0.13	7.62	0.07	0.41	0.14
82G	1.79	0.02	4.22	0.06	0.20	0.02	8.17	0.16	8.47	0.09	0.30	0.19
83K	1.76	0.02	4.19	0.06	0.21	0.02	8.09	0.16	8.09	0.08	0.00	0.18
84S	1.64	0.02	3.73	0.08	0.07	0.03	7.05	0.20	7.46	0.10	0.40	0.22
85S	n	n	n	n	n	n	n	n	7.33	0.09	n	n
86G	1.61	0.02	3.55	0.07	0.04	0.03	7.77	0.18	7.69	0.09	-0.07	0.20
87L	1.63	0.02	3.74	0.05	0.11	0.03	7.45	0.14	7.51	0.08	0.07	0.16
88F	1.62	0.02	4.01	0.06	0.08	0.03	6.87	0.15	7.33	0.09	0.46	0.17

89I	1.62	0.02	3.96	0.08	0.10	0.04	7.09	0.20	7.75	0.12	0.66	0.23
90S	1.63	0.02	4.45	0.07	0.13	0.03	7.49	0.17	7.86	0.09	0.37	0.19
91P	n	n	n	n	n	n	n	n	n	n	n	n
92S	1.63	0.02	4.65	0.09	0.18	0.04	7.89	0.23	8.72	0.12	0.82	0.26
93N	n	n	n	n	n	n	n	n	n	n	n	n
94K	1.68	0.02	4.45	0.08	0.20	0.03	7.39	0.18	7.83	0.10	0.43	0.20
95L	1.71	0.02	4.72	0.07	0.18	0.03	7.53	0.18	7.92	0.10	0.39	0.21
96K	1.72	0.01	4.83	0.04	0.22	0.02	8.01	0.11	8.69	0.06	0.68	0.12
97S	1.67	0.03	4.53	0.10	0.16	0.04	9.10	0.31	9.33	0.16	0.23	0.35
98S	n	n	n	n	n	n	7.96	0.26	8.58	0.14	0.62	0.29
99D	n	n	n	n	n	n	n	n	n	n	n	n
100A	1.76	0.02	5.23	0.07	0.28	0.03	7.80	0.19	8.96	0.11	1.16	0.22
101Y	n	n	n	n	0.29	0.03	n	n	n	n	n	n
102K	1.79	0.03	5.47	0.09	0.33	0.04	8.61	0.25	9.06	0.13	0.45	0.28
103K	1.74	0.02	5.58	0.08	0.30	0.03	8.10	0.20	9.34	0.13	1.24	0.23
104A	1.75	0.03	5.13	0.09	0.34	0.04	7.97	0.23	9.31	0.15	1.34	0.27
105W	n	n	n	n	n	n	n	n	n	n	n	n
106G	1.74	0.02	4.13	0.08	0.20	0.04	8.42	0.22	8.87	0.12	0.45	0.25
107N	1.74	0.02	4.04	0.06	0.21	0.03	8.03	0.17	7.94	0.08	-0.09	0.19
108N	1.69	0.02	3.77	0.06	0.11	0.02	7.00	0.16	7.06	0.08	0.06	0.17
109Q	1.66	0.02	3.57	0.05	0.09	0.02	6.69	0.13	6.94	0.07	0.25	0.14
110D	n	n	n	n	n	n	n	n	n	n	n	n
111G	1.60	0.02	3.35	0.05	0.09	0.02	7.64	0.13	7.10	0.07	-0.54	0.14
112V	n	n	n	n	n	n	n	n	n	n	n	n
113V	n	n	n	n	n	n	n	n	n	n	n	n
114A	1.55	0.02	3.42	0.06	-0.02	0.02	6.45	0.13	6.79	0.09	0.34	0.16
115S	n	n	n	n	n	n	n	n	n	n	n	n
116Q	1.52	0.02	3.89	0.06	-0.05	0.02	6.88	0.14	7.01	0.07	0.13	0.16
117P	n	n	n	n	n	n	n	n	n	n	n	n
118A	1.50	0.01	3.64	0.05	-0.02	0.02	6.82	0.12	7.28	0.07	0.46	0.14
119R	n	n	n	n	n	n	n	n	n	n	n	n
120V	1.56	0.02	4.47	0.07	0.13	0.03	7.48	0.17	8.36	0.09	0.88	0.19
121V	n	n	n	n	n	n	n	n	n	n	n	n
122D	n	n	n	n	n	n	n	n	8.75	0.09	n	n
123E	1.72	0.02	5.18	0.07	0.27	0.03	7.99	0.16	8.74	0.09	0.75	0.18
124R	n	n	n	n	n	n	n	n	9.15	0.08	n	n
125E	n	n	n	n	n	n	n	n	n	n	n	n
126Q	1.76	0.02	4.84	0.06	0.27	0.02	7.95	0.14	8.62	0.08	0.67	0.16
127M	1.73	0.02	4.88	0.06	0.24	0.02	7.82	0.14	8.53	0.08	0.71	0.16
128A	1.69	0.02	4.69	0.06	0.25	0.02	7.71	0.14	8.48	0.09	0.77	0.16
129I	1.61	0.02	4.51	0.06	0.15	0.02	7.62	0.13	7.85	0.07	0.24	0.15
130S	1.72	0.02	4.46	0.05	0.14	0.02	7.69	0.13	8.17	0.07	0.48	0.15
131G	1.67	0.02	3.96	0.07	0.17	0.03	8.15	0.19	8.50	0.09	0.35	0.21
132G	1.62	0.02	3.98	0.06	0.14	0.02	9.08	0.17	8.31	0.08	-0.76	0.19
133F	n	n	n	n	n	n	n	n	n	n	n	n
134I	n	n	n	n	n	n	n	n	n	n	n	n
135R	1.75	0.02	4.76	0.07	0.17	0.02	7.91	0.17	8.67	0.10	0.76	0.19

136R	1.73	0.02	4.49	0.07	0.24	0.03	7.79	0.17	8.50	0.09	0.71	0.19
137V	1.68	0.02	4.07	0.05	0.15	0.02	7.53	0.14	7.84	0.08	0.32	0.16
138T	1.75	0.02	4.32	0.06	0.18	0.02	7.63	0.15	8.13	0.08	0.50	0.16
139N	1.72	0.02	4.17	0.07	0.16	0.03	7.35	0.17	7.50	0.08	0.15	0.19
140D	n	n	n	n	n	n	n	n	n	n	n	n
141A	1.65	0.01	3.92	0.04	0.17	0.02	6.62	0.09	7.38	0.06	0.76	0.11
142R	1.67	0.01	3.99	0.05	0.21	0.02	7.12	0.11	7.22	0.06	0.10	0.12
143E	1.75	0.01	4.26	0.05	0.21	0.02	7.32	0.11	7.62	0.06	0.30	0.12
144N	1.75	0.02	4.00	0.05	0.21	0.02	7.22	0.12	7.31	0.06	0.09	0.13
145E	n	n	n	n	n	n	n	n	n	n	n	n
146M	n	n	n	n	n	n	n	n	n	n	n	n
147D	n	n	n	n	n	n	n	n	n	n	n	n
148E	1.71	0.01	4.03	0.05	0.18	0.02	6.94	0.11	7.42	0.06	0.48	0.12
149N	1.76	0.02	4.15	0.05	0.25	0.02	7.32	0.12	7.63	0.06	0.32	0.13
150L	1.73	0.02	4.33	0.05	0.23	0.02	7.38	0.12	7.55	0.06	0.17	0.13
151E	n	n	n	n	n	n	n	n	n	n	n	n
152Q	n	n	n	n	n	n	n	n	n	n	n	n
153V	n	n	n	n	n	n	n	n	n	n	n	n
154S	n	n	n	n	0.14	0.02	n	n	n	n	n	n
155G	1.66	0.02	3.88	0.06	0.17	0.02	7.78	0.15	7.74	0.07	-0.03	0.17
156I	1.67	0.02	4.00	0.05	0.10	0.02	7.60	0.12	7.71	0.07	0.11	0.14
157I	1.67	0.02	4.45	0.06	0.14	0.02	7.43	0.14	8.16	0.09	0.73	0.17
158G	1.72	0.02	4.50	0.07	0.17	0.03	8.56	0.17	8.72	0.09	0.16	0.19
159N	n	n	n	n	n	n	n	n	n	n	n	n
160L	1.74	0.02	3.93	0.05	0.17	0.02	6.99	0.13	7.28	0.07	0.29	0.14
161R	n	n	n	n	n	n	n	n	n	n	n	n
162H	n	n	n	n	n	n	n	n	n	n	n	n
163M	n	n	n	n	n	n	n	n	n	n	n	n
164A	1.65	0.01	4.09	0.05	0.09	0.02	7.01	0.12	7.57	0.07	0.56	0.14
165L	n	n	n	n	n	n	n	n	7.16	0.05	n	n
166D	n	n	n	n	n	n	6.67	0.09	6.78	0.05	0.10	0.10
167M	n	n	n	n	n	n	n	n	n	n	n	n
168G	1.71	0.02	3.47	0.05	0.15	0.02	7.57	0.13	7.60	0.07	0.02	0.14
169N	1.72	0.01	3.63	0.04	0.17	0.02	7.72	0.12	7.50	0.06	-0.23	0.13
170E	1.68	0.01	3.64	0.05	0.12	0.02	6.70	0.10	6.90	0.06	0.19	0.12
171I	n	n	n	n	n	n	n	n	n	n	n	n
172D	1.71	0.02	4.17	0.05	0.19	0.02	7.21	0.13	8.18	0.07	0.97	0.15
173T	1.71	0.02	3.99	0.05	0.20	0.02	7.47	0.12	7.70	0.06	0.23	0.13
174Q	1.76	0.01	4.63	0.03	n	n	7.47	0.07	8.39	0.05	0.92	0.08
175N	1.73	0.02	4.38	0.06	0.22	0.02	7.33	0.14	7.58	0.07	0.26	0.15
176R	n	n	n	n	n	n	n	n	n	n	n	n
177Q	1.78	0.02	4.25	0.06	0.24	0.02	7.29	0.14	7.84	0.08	0.55	0.16
178I	n	n	n	n	n	n	n	n	7.91	0.07	n	n
179D	n	n	n	n	0.19	0.02	8.24	0.13	8.79	0.08	0.55	0.15
180R	n	n	n	n	n	n	n	n	n	n	n	n
181I	1.73	0.02	4.38	0.06	0.21	0.03	7.43	0.15	7.97	0.08	0.54	0.17
182M	1.73	0.02	4.52	0.07	0.17	0.03	7.92	0.17	8.74	0.11	0.82	0.20

183E	n	n	n	n	n	n	n	n	n	n	n	n
184K	n	n	n	n	n	n	n	n	n	n	n	n
185A	1.68	0.02	3.90	0.06	0.09	0.02	6.87	0.15	7.40	0.09	0.53	0.17
186D	1.60	0.01	3.85	0.04	0.12	0.02	6.82	0.11	6.89	0.06	0.07	0.12
187S	1.68	0.02	3.86	0.07	0.17	0.03	7.18	0.18	7.69	0.09	0.51	0.20
188N	1.76	0.02	4.16	0.08	0.20	0.03	7.09	0.20	7.70	0.10	0.61	0.22
189K	n	n	n	n	n	n	n	n	7.33	0.06	n	n
190T	1.73	0.02	4.05	0.07	0.22	0.03	7.41	0.17	7.90	0.09	0.49	0.19
191R	n	n	n	n	n	n	n	n	n	n	n	n
192I	1.81	0.02	4.18	0.07	0.22	0.03	7.36	0.17	7.33	0.09	-0.03	0.19
193D	1.80	0.02	4.48	0.07	0.30	0.03	7.53	0.18	8.60	0.10	1.07	0.20
194E	1.80	0.02	3.96	0.05	0.26	0.02	7.23	0.13	7.66	0.08	0.43	0.15
195A	1.82	0.02	4.30	0.06	0.32	0.03	7.38	0.15	7.83	0.08	0.45	0.17
196N	1.79	0.02	4.36	0.07	0.29	0.03	7.35	0.17	7.65	0.08	0.29	0.18
197Q	1.80	0.02	4.22	0.06	0.20	0.02	7.30	0.14	7.39	0.07	0.09	0.16
198R	1.72	0.01	4.23	0.03	n	n	7.25	0.07	7.52	0.04	0.27	0.08
199A	1.76	0.02	3.74	0.06	0.19	0.03	7.09	0.15	7.41	0.08	0.32	0.17
200T	1.67	0.02	3.05	0.06	0.11	0.02	6.59	0.13	6.73	0.07	0.14	0.15
201K	n	n	n	n	n	n	7.24	0.12	7.13	0.06	-0.12	0.13
202M	n	n	n	n	n	n	n	n	n	n	n	n
203L	n	n	n	n	n	n	n	n	6.16	0.05	n	n
204G	1.29	0.01	2.01	0.06	-0.60	0.03	6.21	0.14	6.16	0.07	-0.05	0.15
205S	1.09	0.01	1.60	0.05	-0.88	0.03	5.45	0.11	5.29	0.05	-0.16	0.12
206G	0.74	0.01	0.81	0.04	-1.53	0.03	3.45	0.07	3.43	0.03	-0.02	0.07

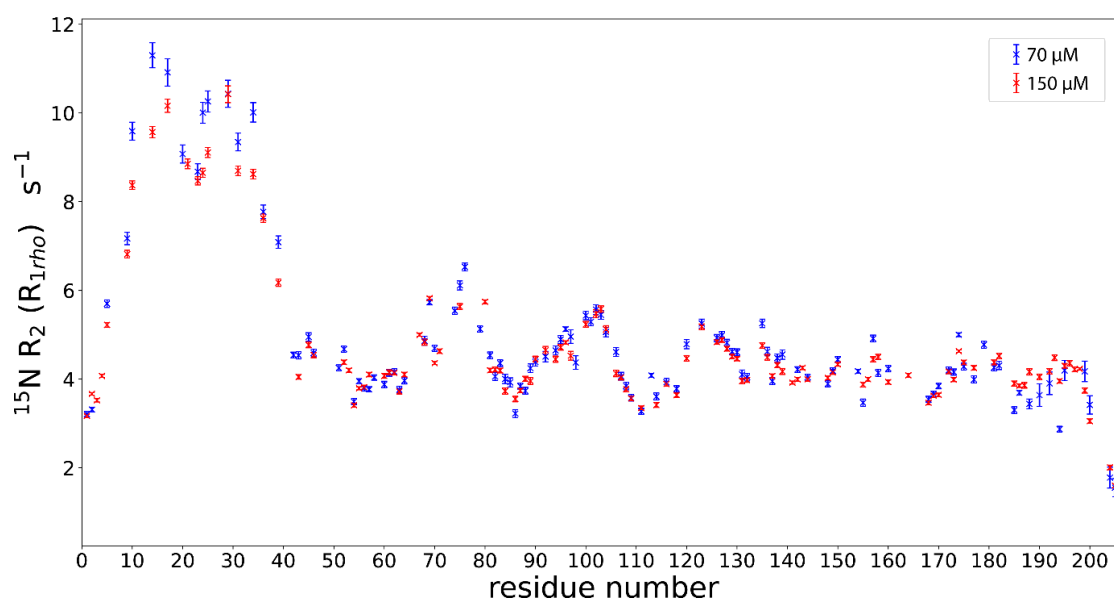


Figure S6. Comparison of ^{15}N R_2 ($R_{1\rho}$) rate constants of two SNAP25a samples at different concentrations of 150 μM and 70 μM .

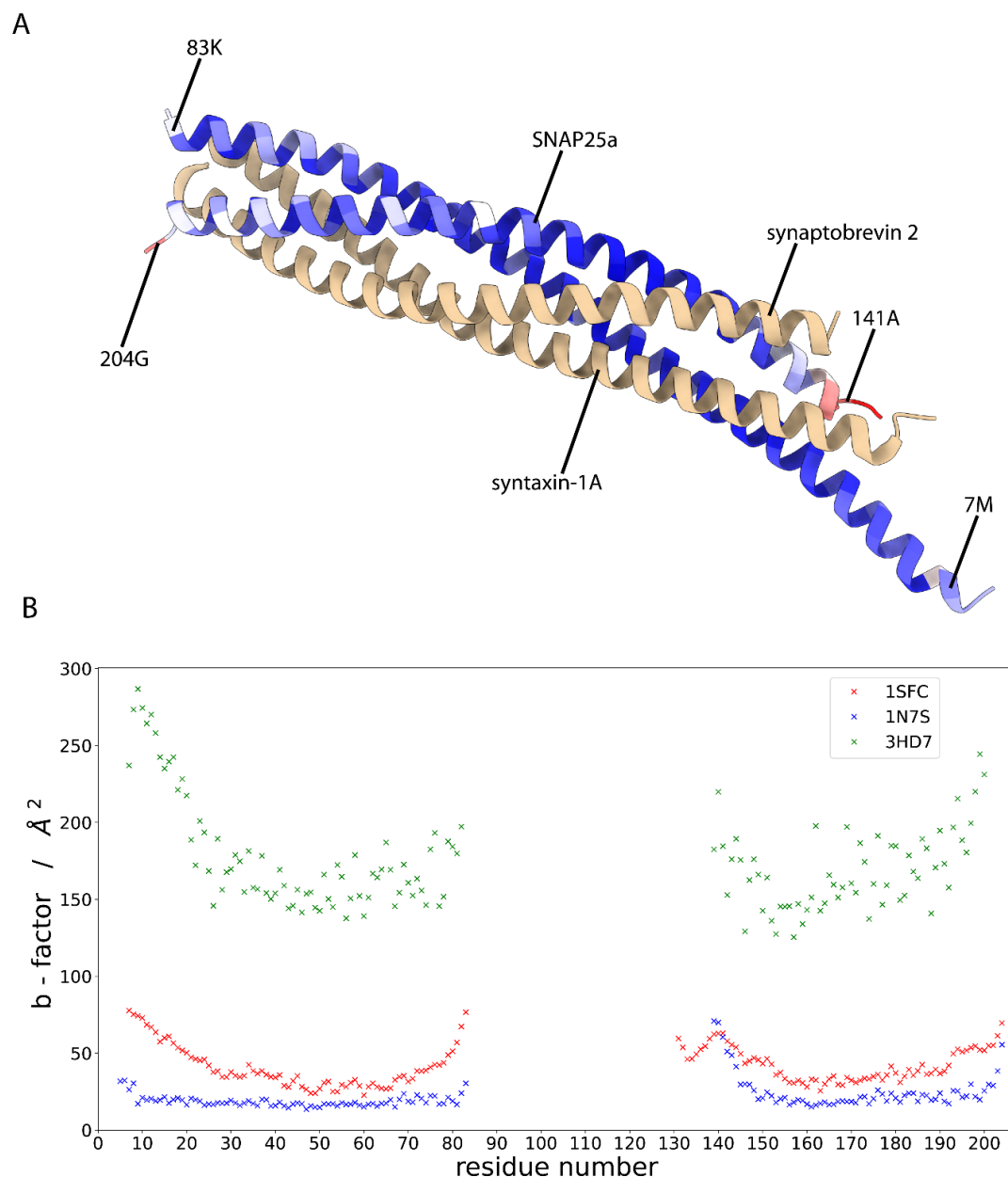


Figure S7. Post-fusion cis-SNARE complex. (A) Post-fusion structure of SNAP25a in the assembled cis-SNARE complex composed of synaptobrevin-2, syntaxin-1a and SNAP25 (PDB code 1N7S). Syntaxin-1a and synaptobrevin-2 contribute one helix each and SNAP25a two helices (SN1) and (SN2). B-factors/temperature factors are plotted onto the structure, blue indicates a low B-factor, white medium and red a high B-factor. (B) Comparison of B-factors of SNAP25a within three different X-ray structures (PDB codes: 1SFC, 1N7S, 3HD7) of the post-fusion cis-SNARE complex.

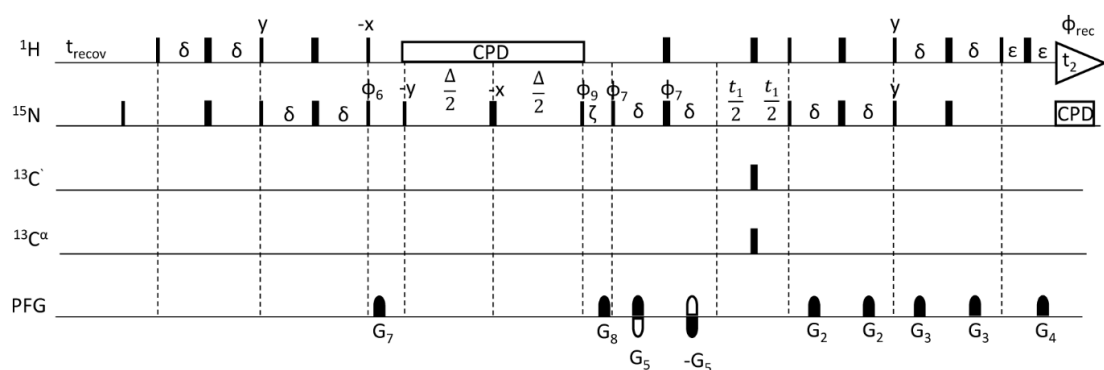


Figure S8. Pulse sequence scheme of the used Hahn-echo based ^{15}N R_2 experiment. 90° hard pulses with phase x if not marked otherwise are illustrated as narrow rectangular and 180° hard pulses as broad rectangular. The durations of the implemented delays are $\delta = 2.65\text{ms}$ and ϵ is equivalent to the duration of the decoding gradient G4 ($201\mu\text{s}$). With this offset it is possible to insert the decoding gradient without producing a linear phase error in proton dimension. Adjusting the delay ζ can improve the overall water-suppression; we used a delay of 20ms . The variable delay Δ corresponds to the relaxation period. The Gradients G2 (2.65 ms , -2.7 G/cm), G3 (2.65 ms , -0.3 G/cm) are rectangular shaped. Gradients G4 ($201\mu\text{s}$, 21.4 G/cm), G5 (1 ms , 21.4 G/cm), G7 ($200\mu\text{s}$; -26.8 G/cm) and G8 (1 ms , 5.4 G/cm) are sine-bell shaped. Phase cycling: $\phi_6 = y, y, -y, -y$; $\phi_7 = y, -y, \phi_9 = 4(-y), 4(y)$, $\phi_{\text{rec}} = y, -y, -y, y, -y, y, y, -y$. The quadrature detection is implemented with the polarity inversion of the encoding gradient G5 and the phase cycling of ϕ_7 [9]. For the decoupling in the proton dimension a waltz16 decoupling scheme was used. In the ^{15}N dimension a garp decoupling scheme was used.

References

- [1] Stein A, Weber G, Wahl MC, Jahn R. Helical extension of the neuronal SNARE complex into the membrane. *Nature*. 2009;460:525-8.
- [2] Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at $2.4\text{ \AA}$ resolution. *Nature*. 1998;395:347-53.
- [3] Nielsen JT, Mulder FAA. CheSPI: chemical shift secondary structure population inference. *J Biomol Nmr*. 2021;75:273-91.
- [4] Hu G, Katuwawala A, Wang K, Wu Z, Ghadermarzi S, Gao J, et al. fIDPnn: Accurate intrinsic disorder prediction with putative propensities of disorder functions. *Nat Commun*. 2021;12:4438.
- [5] Emenecker RJ, Griffith D, Holehouse AS. Metapredict: a fast, accurate, and easy-to-use predictor of consensus disorder and structure. *Biophys J*. 2021;120:4312-9.
- [6] Tang YJ, Pang YH, Liu B. IDP-Seq2Seq: identification of intrinsically disordered regions based on sequence to sequence learning. *Bioinformatics*. 2021;36:5177-86.
- [7] Erdos G, Pajkos M, Dosztanyi Z. IUPred3: prediction of protein disorder enhanced with unambiguous experimental annotation and visualization of evolutionary conservation. *Nucleic Acids Res*. 2021;49:W297-W303.
- [8] Nielsen JT, Mulder FAA. POTENCI: prediction of temperature, neighbor and pH-corrected chemical shifts for intrinsically disordered proteins. *J Biomol Nmr*. 2018;70:141-65.
- [9] Kay LE, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J Am Chem Soc*. 1992;114:10663-5.

Chapter 8: Improved R_1 and $R_{1\rho}$ Relaxation Experiments**Table S1:** Experimental parameters of ^{15}N NMR relaxation experiments at 1200 MHz.

	R_1	$R_{1\rho}$
Field / T	28.2	28.2
^1H Larmor frequency / MHz	1200	1200
Pulse program	Figure 1	Figure 1
Interscan recovery delay / s	2	2
Number of scans	4	4
Total experimental time	12 h 12 min	10 h 19 min
Direct dimension (^1H)		
^1H carrier / ppm	4.690	4.693
Spectral width / ppm	16.03	16.03
Data points	4096	4096
Acquisition time / ms	106.5	106.5
90° (^1H) pulse length / μs	8.0	8.0
Indirect dimension (^{15}N)		
^{15}N carrier / ppm	117.00	117.00
Spectral width / ppm	30.02	30.02
Data points (total)	4096	4096
Acquisition time / ms	70.14	70.14
90° (^{15}N) pulse length / μs	34.8	34.8
Relaxation period		
# relaxation delays	8	8
Time points / ms	0, 960, 240, 800, 160, 640, 320, 480	5, 65, 25, 45, 15, 55, 35, 10

Table S2: Experimental parameters of ^{15}N NMR relaxation experiments at 600 MHz.

	R_1	$R_{1\rho}$
Field / T	14.1	14.1
^1H Larmor frequency / MHz	600	600
pulse program	Figure 1	Figure 1
Interscan recovery delay / s	2	2
Number of scans	4	4
Total experimental time	12 h 23 min	10 h 29 min
Direct dimension (^1H)		
^1H carrier / ppm	4.692	4.692
Spectral width / ppm	16.02	16.02
Data points	2048	2048
Acquisition time / ms	106.50	106.50
90° (^1H) pulse length / μs	8.75	8.75
Indirect dimension (^{15}N)		
^{15}N carrier/ppm	117.00	117.00
Spectral width / ppm	30.00	30.00
Data points (total)	4096	4096
Acquisition time / ms	140.29	140.29
90° (^{15}N) pulse length / μs	43	43
Relaxation period		
# relaxation delays	8	8
Time points / ms	0, 960, 240, 800, 160, 640, 320, 480	5, 65, 25, 45, 15, 55, 35, 10

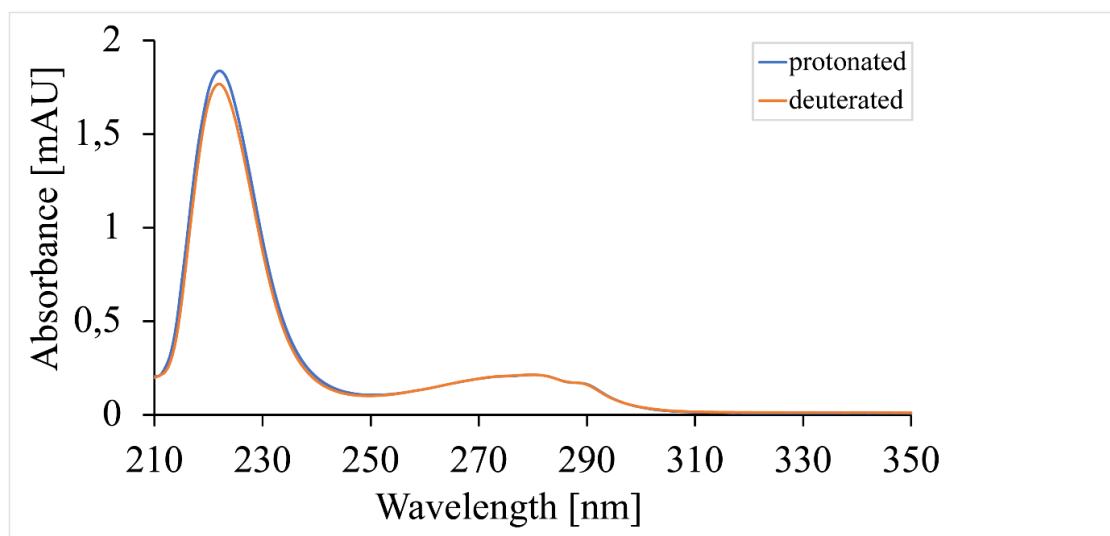


Figure S1: UV-vis spectrum of deuterated ^2H - ^{15}N - ^{13}C Syb-2 (1-96) and protonated ^1H - ^{15}N Syb-2 (1-96). Protein concentrations were calculated using the Beer-Lambert's Law (Molar extinction coefficient: $12490 \text{ M}^{-1}\text{cm}^{-1}$; path length: 1 cm; dilution factor: 10; A_{280} of deuterated ^2H - ^{15}N Syb-2 (1-96): 0.21441475; A_{280} of protonated ^1H - ^{15}N Syb-2 (1-96): 0.2137355). According to the equation, the final sample concentrations have been 171.7 μM of deuterated ^2H - ^{15}N Syb-2 (1-96) and 171.1 μM of protonated ^1H - ^{15}N Syb-2 (1-96) in 50 mM MES (pH 6.0), 150 mM NaCl, 1 mM EDTA and 0.1 mM TCEP.

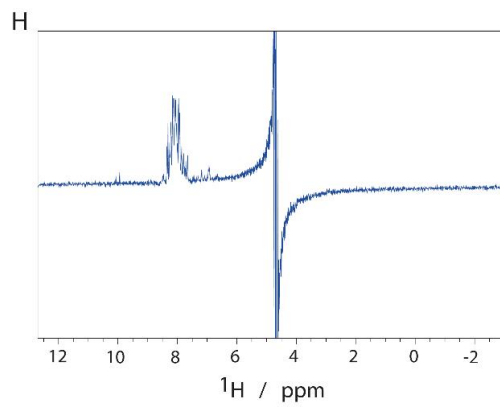
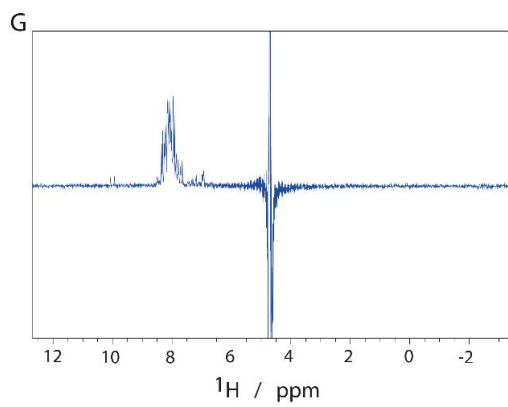
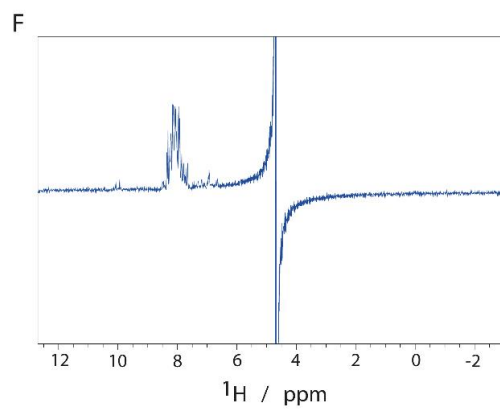
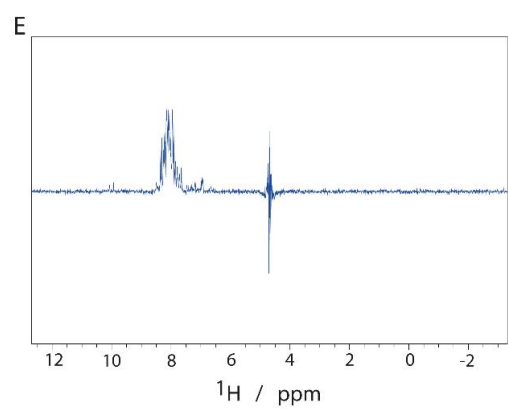
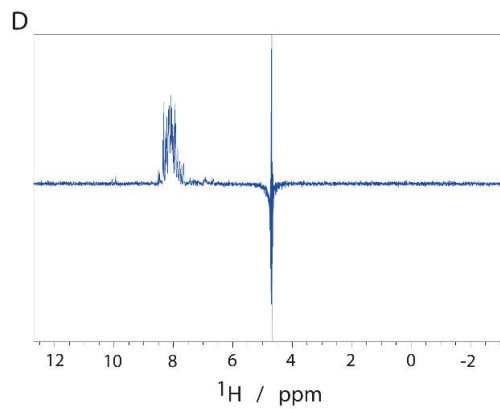
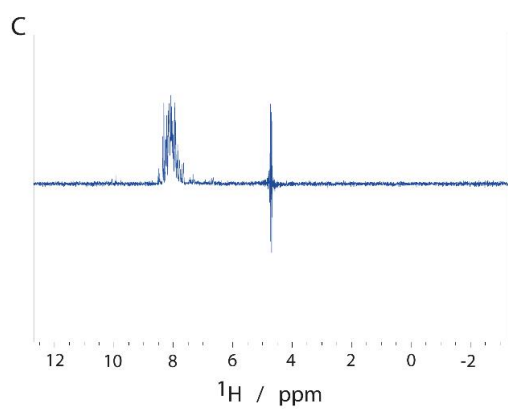
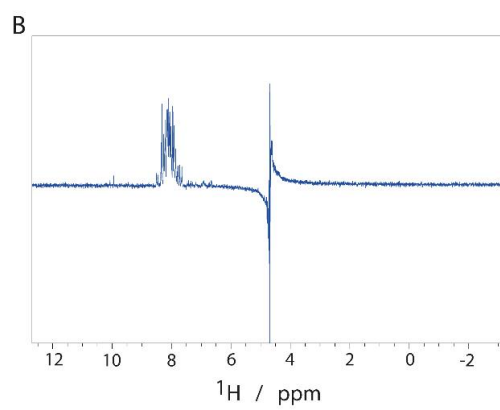
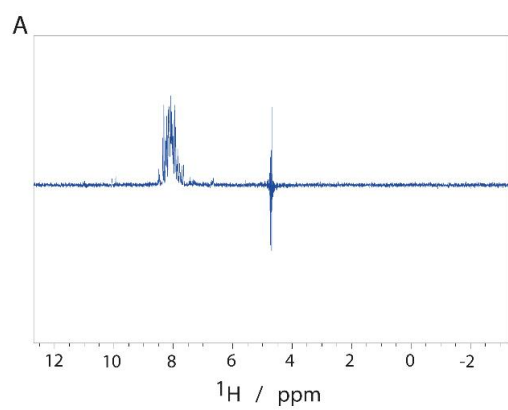


Figure S2: Improvement of water suppression in ^{15}N R_1 and ^{15}N $R_{1\rho}$ NMR relaxation experiments with sensitivity-enhanced HSQC-detection using the new implementation (left, see also Figure 1) and compared to the old implementation (right, SI Figure S1 in [1]). Shown is the 1D spectrum obtained from the first FID recorded on the **deuterated** ^{15}N Syb-2 sample. (A) Optimized ^{15}N $R_{1\rho}$ experiment at 1200 MHz compared to (B) previous implementation. ^{15}N R_1 experiment at 1200 MHz: (C) New and (D) old. ^{15}N $R_{1\rho}$ experiment at 600 MHz: (E) new and (F) old. ^{15}N R_1 experiment at 600 MHz: (G) new and (H) old.

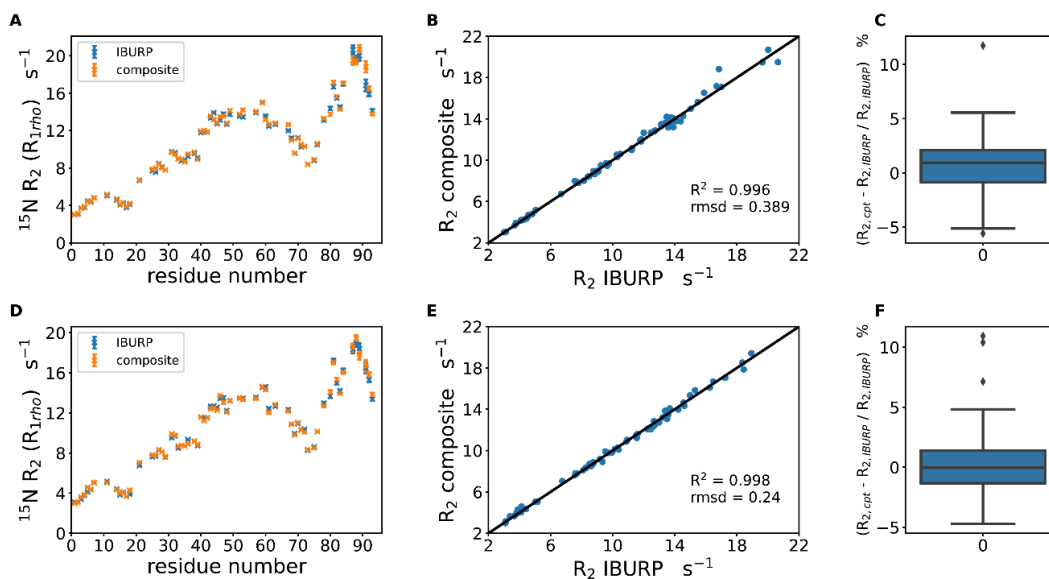


Figure S3: Comparison of R_2 relaxation rate constants of the protonated ^1H - ^{15}N Syb-2 (1-96) at 1200 MHz, acquired with the pulse scheme in Figure 1B (IBURP) and the previous implementation to refocus cross-correlation relaxation during the $R_{1\rho}$ relaxation period, where composite pulses were used plus and additional weak gradient in between (that alternated its phase half-way), to prevent radiation damping, as described for the TROSY-based sequence [1]. **A-C:** Protonated sample. **D-F:** Deuterated sample.

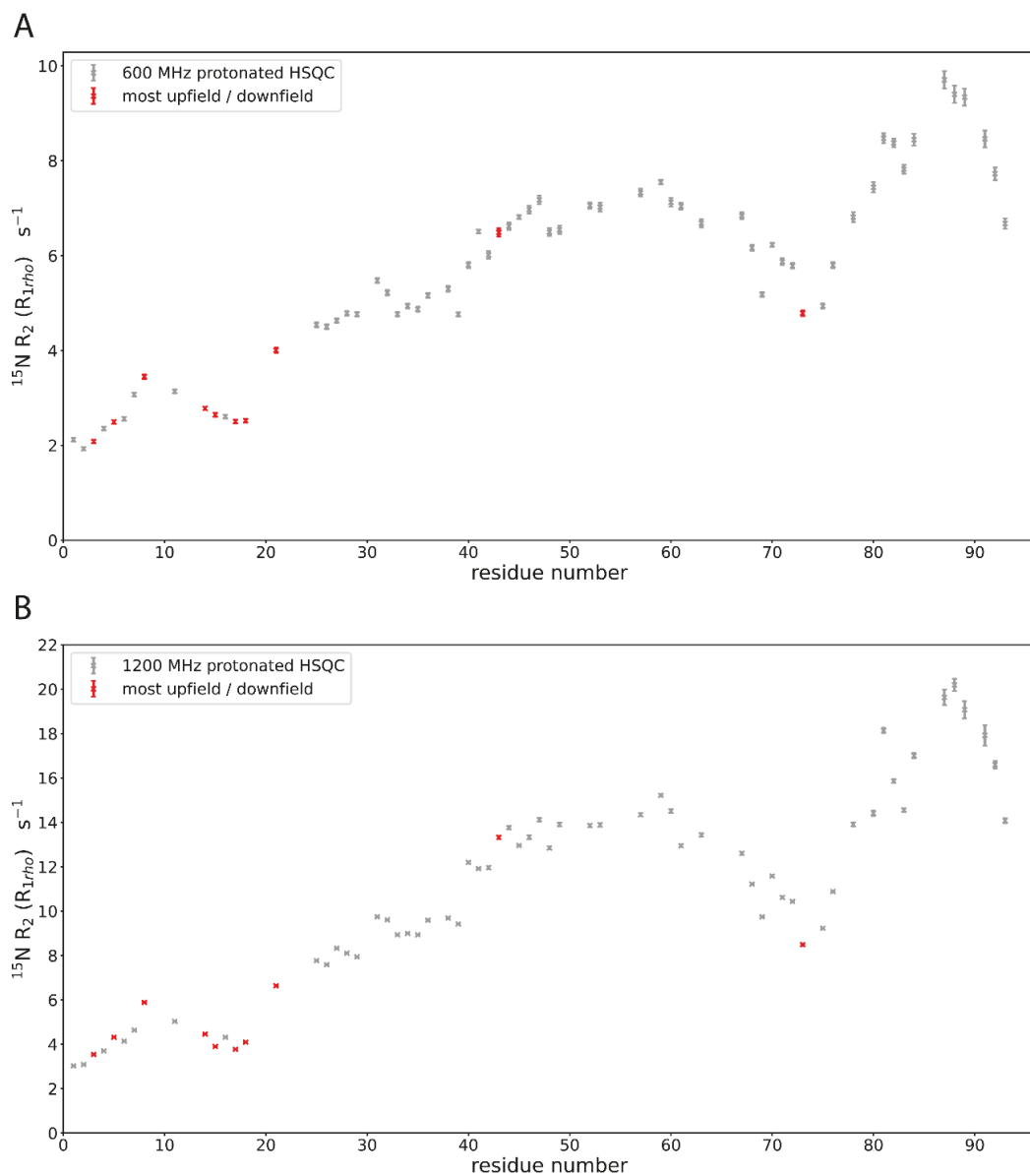


Figure S4: ^{15}N R_2 rate constants recorded at 600 MHz and 1200 MHz using the optimized sensitivity-enhanced HSQC detection scheme and a 2 kHz RF amplitude for the spinlock. Rate constants for residues with the most ^{15}N upfield and downfield resonances are colored in red: (A) 600 MHz and (B) 1200 MHz for comparison.

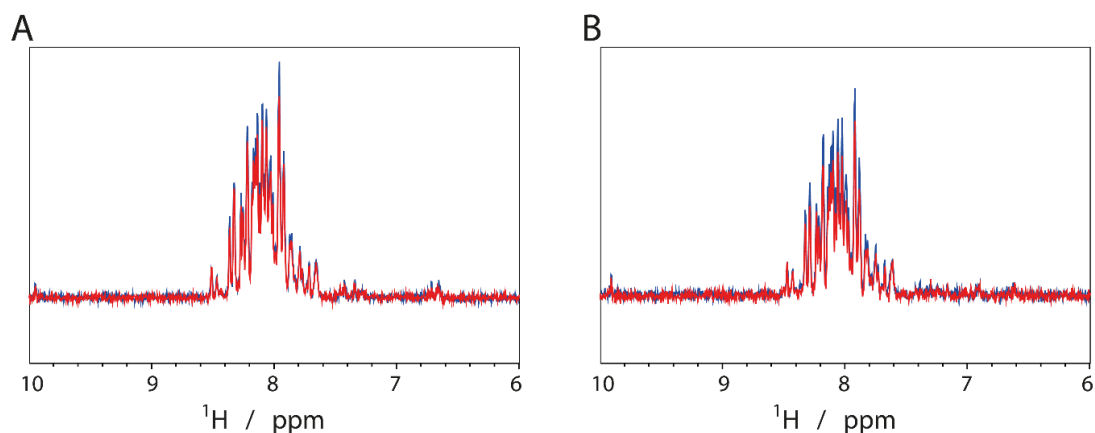


Figure S5: Intensity comparisons of the 1D spectrum obtained from the first FID of the ^{15}N R_1 experiments recorded on the protonated sample ^{15}N Syb-2 sample at 1200 MHz: (A) HSQC-detected experiment vs (B) TROSY detected experiment. The red spectrum indicates the intensity after $d_1=2\text{s}$ recovery delay, the blue spectrum after 4s recovery delay.

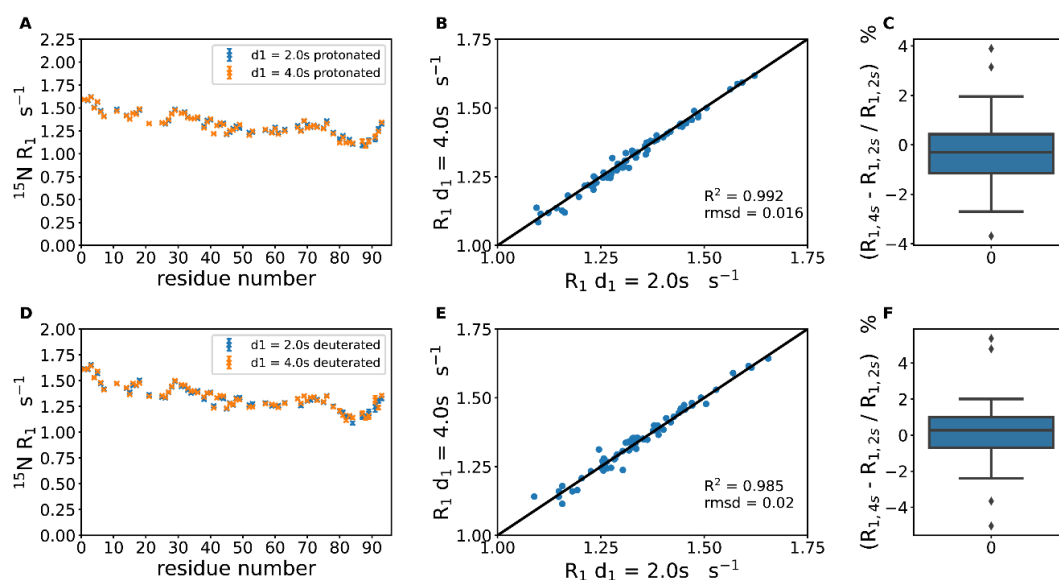


Figure S6: No impact of longer inter-scan recovery times on the derived R_1 relaxation rate constants. Experiments were conducted at 1200 MHz and 5°C , using the pulse scheme shown in Figure 1A with an interscan recovery delay of 2.0 s and 4.0 s. **A:** Residue-specific R_1 relaxation rate comparison. **B:** Correlation plot of the R_1 relaxation rate constants. **C:** Box plot of the residue-specific percentual differences of R_1 relaxation rates. In **A-C**, the data for protonated ^1H - ^{15}N Syb-2 (1-96) are shown, and in **D-F**, the data for deuterated ^2H - ^{15}N - ^{13}C Syb-2 (1-96).

Table S3. NMR relaxation data of ^{15}N Syb-2 measured at 1200 MHz and 5°C (compare Figure 4). The columns contain the following information for each residue (rows): # (residue number), AA type (1-letter code), transverse relaxation rate constants (derived from $R_{1\rho}$) ^{15}N $R_2(R_{1\rho})/s^{-1}$ (1200 MHz), experimental error $\delta(^{15}\text{N } R_2(R_{1\rho}))/s^{-1}$ (1200 MHz); longitudinal relaxation rate constants ^{15}N R_1/s^{-1} (1200 MHz), experimental error $\delta(^{15}\text{N } R_1)/s^{-1}$ (1200 MHz).

1	M	3.027	0.018	1.574	0.003
2	S	3.085	0.013	1.570	0.002
3	A	3.540	0.021	1.617	0.003
4	T	3.703	0.019	1.499	0.003
5	A	4.314	0.025	1.560	0.004
6	A	4.138	0.020	1.459	0.003
7	T	4.638	0.020	1.418	0.003
8	A	5.885	0.031	1.484	0.004
11	A	5.030	0.024	1.464	0.003
14	A	4.458	0.021	1.414	0.003
15	G	3.902	0.028	1.370	0.003
16	E	4.316	0.020	1.450	0.003
17	G	3.771	0.022	1.438	0.003
18	G	4.095	0.027	1.479	0.004
21	A	6.634	0.036	1.326	0.004
25	N	7.769	0.026	1.333	0.004
26	L	7.585	0.032	1.323	0.004
27	T	8.326	0.028	1.367	0.004
28	S	8.103	0.024	1.425	0.004
29	N	7.941	0.025	1.470	0.004
31	R	9.742	0.031	1.460	0.004
32	L	9.607	0.036	1.442	0.005
33	Q	8.934	0.028	1.406	0.004
34	Q	8.991	0.029	1.389	0.004
35	T	8.931	0.028	1.388	0.004
36	Q	9.589	0.036	1.382	0.005
38	Q	9.684	0.042	1.311	0.006
39	V	9.419	0.035	1.333	0.005
40	D	12.20	0.05	1.369	0.005
41	E	11.92	0.03	1.221	0.003
42	V	11.96	0.07	1.313	0.007
43	V	13.32	0.07	1.315	0.007
44	D	13.76	0.07	1.301	0.006
45	I	12.96	0.03	1.200	0.003
46	M	13.33	0.09	1.253	0.009
47	R	14.12	0.08	1.278	0.007
48	V	12.84	0.07	1.280	0.006
49	N	13.90	0.09	1.279	0.008
52	K	13.86	0.07	1.231	0.006
53	V	13.89	0.08	1.252	0.007
57	D	14.34	0.07	1.264	0.006
59	K	15.22	0.05	1.244	0.004

60	L	14.51	0.08	1.255	0.007
61	S	12.95	0.06	1.224	0.006
63	L	13.43	0.08	1.260	0.007
67	A	12.60	0.06	1.296	0.006
68	D	11.22	0.05	1.249	0.005
69	A	9.744	0.035	1.334	0.004
70	L	11.58	0.05	1.300	0.005
71	Q	10.62	0.05	1.295	0.006
72	A	10.44	0.04	1.321	0.005
73	G	8.491	0.048	1.290	0.005
75	S	9.228	0.030	1.275	0.004
76	Q	10.88	0.05	1.334	0.005
78	E	13.91	0.10	1.248	0.008
80	S	14.42	0.12	1.177	0.010
81	A	18.14	0.12	1.154	0.008
82	A	15.86	0.09	1.119	0.006
83	K	14.55	0.08	1.132	0.006
84	L	17.00	0.13	1.124	0.008
87	K	19.63	0.34	1.166	0.021
88	Y	20.19	0.27	1.088	0.016
89	W	19.07	0.39	1.116	0.024
91	K	17.92	0.46	1.176	0.031
92	N	16.59	0.17	1.243	0.013
93	L	14.07	0.11	1.345	0.010

Table S4. NMR relaxation data of ^{15}N Syb-2 measured at 600 MHz and 5°C (compare Figure 5). The columns contain the following information for each residue (rows): # (residue number), AA type (1-letter code), transverse relaxation rate constants (derived from $R_{1\rho}$) ^{15}N $R_2(R_{1\rho}) / \text{s}^{-1}$ (600 MHz), experimental error $\delta(^{15}\text{N } R_2(R_{1\rho})) / \text{s}^{-1}$ (600 MHz); longitudinal relaxation rate constants ^{15}N R_1 / s^{-1} (600 MHz), experimental error $\delta(^{15}\text{N } R_1) / \text{s}^{-1}$ (600 MHz).

1	M	2.121	0.039	1.335	0.006
2	S	1.929	0.032	1.386	0.005
3	A	2.084	0.034	1.455	0.005
4	T	2.356	0.038	1.420	0.006
5	A	2.494	0.039	1.515	0.006
6	A	2.560	0.039	1.425	0.006
7	T	3.072	0.039	1.453	0.006
8	A	3.448	0.051	1.551	0.009
11	A	3.139	0.045	1.496	0.007
14	A	2.780	0.036	1.439	0.005
15	G	2.644	0.041	1.378	0.006
16	E	2.607	0.037	1.443	0.006
17	G	2.502	0.037	1.389	0.005
18	G	2.522	0.040	1.369	0.006
21	A	4.006	0.055	1.459	0.008

25	N	4.542	0.052	1.555	0.009
26	L	4.500	0.054	1.566	0.010
27	T	4.630	0.052	1.596	0.009
28	S	4.787	0.049	1.615	0.009
29	N	4.765	0.049	1.661	0.010
31	R	5.474	0.051	1.710	0.010
32	L	5.217	0.057	1.707	0.011
33	Q	4.766	0.050	1.667	0.010
34	Q	4.938	0.051	1.620	0.010
35	T	4.870	0.053	1.588	0.010
36	Q	5.161	0.053	1.589	0.010
38	Q	5.300	0.060	1.525	0.010
39	V	4.760	0.046	1.573	0.008
40	D	5.803	0.062	1.582	0.011
41	E	6.510	0.038	1.566	0.007
42	V	6.016	0.078	1.568	0.013
43	V	6.490	0.085	1.569	0.014
44	D	6.622	0.079	1.590	0.014
45	I	6.815	0.041	1.526	0.007
46	M	6.969	0.085	1.591	0.015
47	R	7.176	0.091	1.581	0.015
48	V	6.495	0.084	1.551	0.014
49	N	6.545	0.087	1.596	0.015
52	K	7.058	0.066	1.579	0.011
53	V	7.023	0.097	1.560	0.016
57	D	7.328	0.082	1.629	0.015
59	K	7.548	0.049	1.586	0.009
60	L	7.125	0.087	1.586	0.015
61	S	7.042	0.075	1.577	0.013
63	L	6.683	0.089	1.619	0.016
67	A	6.842	0.073	1.658	0.013
68	D	6.164	0.062	1.616	0.011
69	A	5.180	0.054	1.587	0.009
70	L	6.229	0.043	1.671	0.008
71	Q	5.873	0.068	1.644	0.013
72	A	5.786	0.059	1.609	0.010
73	G	4.786	0.061	1.551	0.010
75	S	4.937	0.054	1.541	0.009
76	Q	5.800	0.063	1.687	0.012
78	E	6.812	0.104	1.650	0.019
80	S	7.441	0.110	1.657	0.020
81	A	8.475	0.104	1.582	0.017
82	A	8.374	0.090	1.631	0.016
83	K	7.820	0.092	1.662	0.017
84	L	8.445	0.124	1.685	0.023
87	K	9.707	0.186	1.699	0.033
88	Y	9.402	0.179	1.688	0.032

89	W	9.341	0.179	1.694	0.033
91	K	8.461	0.181	1.833	0.038
92	N	7.727	0.133	1.721	0.026
93	L	6.676	0.107	1.708	0.021

References

[1] N.A. Lakomek, J. Ying, A. Bax, Measurement of $(1)(5)\text{N}$ relaxation rates in perdeuterated proteins by TROSY-based methods, *J Biomol NMR* 53(3) (2012) 209-21.

^{15}N R_1 relaxation experiment with sensitivity-enhanced HSQC detection

```
15N_T1_Relaxation
; 15N-T1 relaxation experiment with sensitivity enhanced (Rance-Kay) HSQC read-out
; for 15N, 2H15N, 15N13C and 2H15N13C labelled proteins
; built on a sequence by Lakomek et al., JBNMR 2012
; new version by Tobias Stief October 2022
;last update December 2023
```

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
;;#define LABEL_CN ; switch on for 13C labelled samples
define list<loopcounter> vclist = <$VCLIST>
#define TEMP_COMPENSATION
```

```
"acqt0=0"
```

```
"in0=inf1*0.5"
```

```
"d0=10u"
```

```
"d11=30m"
```

```
"d26=p7-p1"
```

```
"DELTA=2.65m"
```

```
"DELTA1=2.65m-200u-p21"
```

```
"DELTA2=2.65m-200u-p22"
```

```
"DELTA3=2.65m-200u-p23"
```

```
"DELTA4=p25-p19*0.63"
```

```
"DELTA5=2.65m-p26-200u"
```

```
"DELTA7=2.65m"
```

```
"DELTA8=2.65m-200u-p24"
```

```
"l1=1"
```

```
"l2=1"
```

```
# ifdef LABEL_CN
```

```
"DELTA6=d0*2+26u+p4*4";+10u if gp10 active
```

```
"d25=20m-p15*0.5-p4*4-30u"
```

```
# else
```

```
"DELTA6=d0*2+p1*2" ;+10u if gp10 active
```

```

"d25=20m-p15*0.5"
# endif

"cnst21=176"
"cnst22=56"
"cnst18=800"

"spoff4=bf2*((cnst22-cnst21)/1000000)"

1      ze
      1m
2      2m do:f3
      1m do:f2
      1m
      d1 l
3      3m
4      1m
5      1m do:f3
      1m do:f2
      1m BLKGRAD
      10u p11:f1
      10u p14:f2
      10u fq=cnst21(bf ppm):f2

;;-----temperature compensation and d1 recovery delay-----
# ifdef TEMP_COMPENSATION

"d17=d1-p18"

10u fq=cnst18(bf ppm):f3
10u p18:f3
(p18 ph0):f3 ; 15N pulse is applied far off-resonance
10u
10u fq=0:f3
d17
# else
d1
# endif
1m UNBLKGRAD
10u p17:f3

;----- kill steady state 15N -----
      (p7 ph0):f3
      5u
      p27:gp7
      200u

;----- first INEPT Hz-> 2HxNz -----
      (p1 ph0):f1
      5u
      p21:gp1
      200u
      DELTA1
      (center(p1*2 ph0):f1 (p7*2 ph0):f3)
      DELTA1
      5u
      p21:gp1
      200u

;----- rephase 2HxNz to Nz-----

```

```

(center(p1 ph5):f1 (p7 ph0):f3)
5u
p22:gp2
200u
DELTA2
(center (p1*2 ph0):f1 (p7*2 ph0):f3)
DELTA2
5u
p22:gp2
200u
(p7 ph6):f3 ; phase-cycle Nz, -Nz for Freeman-Hill decay
5u
;-----
(p1 ph2):f1 ; purge pulse to kill any residual HzNz

5u
p28:gp8
100u
100u pl0:f1
;-----15N T1 relaxation period-----

if "l2==1" goto 77

70      d25*0.5

#      ifdef LABEL_CN
4u pl4:f2
(p4*2 ph0 3u 10u fq=cnst22(bf ppm) 3u p4*2 ph0 10u fq=cnst21(bf ppm)):f2
#      endif

d25*0.5
(p15:sp5 ph0):f1
d25*0.5

#      ifdef LABEL_CN
4u pl4:f2
(p4*2 ph0 3u 10u fq=cnst22(bf ppm) 3u p4*2 ph0 10u fq=cnst21(bf ppm)):f2
#      endif

d25*0.5

lo to 70 times vclist ; delay=vclist*d25 (20ms)

;-----Gradient-----

77      5u
p29:gp9
200u

;----- Echo- Antiecho encoding-----

10u pl1:f1
5u pl4:f2
5u pl7:f3
(p7 ph7):f3

DELTA6 ;compensation for d0 15N evolution
DELTA5
190u
p26:gp6*EA
10u
(center (p1*2 ph0):f1 (p7*2 ph7):f3)
10u
p26:gp6*EA*-1
190u
DELTA5

```

```

;----t1 evolution -----
      d0*0.5 ;gron10 5u groff
      d0*0.5 ;gron10*-1 5u groff

#      ifdef LABEL_CN
      (center (p1*2 ph0):f1 (p4*2 ph0 3u 10u fq=cnst22(bf ppm) 3u p4*2 ph0 10u fq=cnst21(bf ppm)):f2)

#      else
      (p1*2 ph0):f1
#      endif

      d0

;----first INEPT for sensitivity-enhanced back transfer -----

      (center (p1 ph0):f1 (p7 ph8):f3)
      5u
      p23:gp3
      200u
      DELTA3
      (center(p1*2 ph0):f1 (p7*2 ph0):f3)
      DELTA3
      5u
      p23:gp3
      200u

; ---second INEPT -----
      (center (p1 ph1):f1 (p7 ph1):f3)
      5u
      p24:gp4
      200u
      DELTA8
      (center(p1*2 ph0):f1 (p7*2 ph0):f3)
      DELTA8
      5u
      p24:gp4
      200u
      (d26*0.5 p19 ph0:r):f1
;---spin-echo with decoding gradient EA-----
      245u
      DELTA4
      (p1*2 ph0):f1
      5u
      p25:gp5
      200u
      5u
      5u pl30:f2
      10u pl31:f3
      20u BLKGRAD
      go=2 ph31 cpds3:f3 ;cpd2:f2
      200u
      400u do:f3
      400u do:f2
      1m
      d11 wr #0 if #0 zd
      1m vclist.inc
      1m iu2
      lo to 3 times l6
      1m ip8*2
      1m igrad EA
      1m ru2
      lo to 4 times 2
      1m id0
      lo to 5 times l3
      1m do:f3
      1m BLKGRAD
      exit

```

```
ph0=0
ph1=1
ph2=2
ph5=1 1 1 1
ph6=1 1 3 3
ph7= 1 3
ph8= 0
ph31=1 3 3 1
```

```
;-----NOTES-----
```

```
;o1p = 4.7 ppm
;o2p=176 ppm (CO)
;o3p=119 ppm
```

```
;NS=4*n
;in0=inf/2
;SW=1/(2*in0)
;echo-antiecho in N15 (process as Complex in NmrDraw before splitting the spectra)
```

```
; 1H pulses
```

```
;p1: 90 deg hard 1H pulse @p11
;p19 last 90 deg hard 1H pulse @p11, can be adjusted for improving water-supression
;p11: 1H 90 deg
;p10: 1000 dB
;p15: 1000u (@ 1200 MHz) 180 deg IBurp2 pulse on 1H (sp15)
;sp5: 180 deg IBurp2 pulse on 1H (sp15)
;spnam5: IBurp2
;spoffs5: 4680Hz @ 1200 MHz (8.6 ppm) , should be centered in amide region but not touch the water
```

```
; 13C pulses
```

```
;p4: 13CO selective 180 deg (11.850*2us @ 1200 MHz) @p14
;p14: 13C 90 deg
;sp4: 13CA selective 180 deg (11.850*2us @ 1200 MHz)
```

```
;15N pulses
```

```
;p7 : 90 deg hard 15N pulse @p17
;p17 :15N 90 deg
;CPDPRG3: garp (15N decoupling)
;pcpd3: 15N decoupling (200u @p131)
;p131: 15N decoupling power
```

```
; gradients
```

```
;p21: 200u
;p22: 200u
;p23: 800u
;p24: 200u
;p25: 201u Echo/Anti-echo decoding gradient
;p26: 1000u Echo/Anti-echo half-encoding gradient
;p27: 200u
;p28: 200u
;p29: 200u
```

```
;for z-only gradients
```

```
;gpz1: 1%
;gpz2: 2%
;gpz3: 31%
;gpz4: 11%
;gpz5: 50%
;gpz6: 50%
```

;gpz7: 50%
;gpz8: 5%
;gpz9: 14%, optional
;gpz10: 0.5% ;optional to prevent radiation damping during t1

;gpnam1 SINE.20
;gpnam2 SINE.20
;gpnam3 SINE.20
;gpnam4 SINE.20
;gpnam5 SINE.20
;gpnam6 SINE.20
;gpnam7 SINE.50
;gpnam8 SINE.20
;gpnam9 SINE.20

15N R_{1ρ} relaxation experiment with sensitivity-enhanced HSQC detection

```
15N T1rho Relaxation
; 15N-T1rho relaxation experiment with sensitivity enhanced (Rance-Kay) HSQC read-out
; for 15N, 2H15N, 15N13C and 2H15N13C labelled proteins
; built on a sequence by Lakomek et al., JBNMR 2012
; new version by Tobias Stief October 2022
; last update Dec 2023
```

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
;;define LABEL_CN ; switch on for 13C labelled samples
define list<pulse> vplist = <$VPLIST>
#define TEMP_COMPENSATION
```

```
"acqt0=0"
```

```
"in0=inf1*0.5"
```

```
"d0=10u"
"d11=30m"
"d26=p7-p1"
"DELTA=2.65m"
"DELTA1=2.65m-200u-p21"
"DELTA2=2.65m-200u-p22"
"DELTA3=2.65m-200u-p23"
"DELTA4=p25-p19*0.63"
"DELTA5=2.65m-p26-200u"
"DELTA8=2.65m-200u-p24"
"l1=1"
"l2=1"
```

```
# ifdef LABEL_CN
"DELTA6=d0*2+26u+p4*4";+10u if gp10 active
# else
"DELTA6=d0*2+p1*2" ;+10u if gp10 active
# endif
```

```
"cnst21=176"
"cnst22=56"
"cnst18=-800"
```

```
"spoff4=bf2*((cnst22-cnst21)/1000000)"
```

```
1      ze
      1m
2      2m do:f3
      1m do:f2
      1m
      d11
3      3m
4      1m
5      1m do:f3
      1m do:f2
      1m BLKGRAD
      10u pl1:f1
      10u pl4:f2
```

```

;-----temperature compensation-----
# ifdef TEMP_COMPENSATION
"p17=p18+1m-vplist"
"d17=d1-p17"
# endif

;-----delays for spin lock relaxation period-----
"d8=p8+vplist*0.25-p15*0.5+1u"
"d9=vplist*0.5-p15+2u"
"d29=p8+vplist*0.5+2u-p4*2-8u"

;----- Temperature compensation and d1 recovery delay-----
#ifdef TEMP_COMPENSATION
    10u fq=cnst18(bf ppm):f3
    10u p18:f3
    (p17 ph0):f3 ; 15N pulse is applied far off-resonance
    10u
    10u fq=0:f3
    d17
#else
    d1
#endif

    10u fq=cnst21(bf ppm):f2
    1m UNBLKGRAD
    10u p17:f3

;----- kill steady state 15N -----
    (p7 ph0):f3
    5u
    p27:gp7
    200u

;----- first INEPT Hz-> 2HxNz -----
    (p1 ph0):f1
    5u
    p21:gp1
    200u
    DELTA1
    (center(p1*2 ph0):f1 (p7*2 ph0):f3)
    DELTA1
    5u
    p21:gp1
    200u

;----- rephase 2HxNz to Nz-----
    (p1 ph5):f1 (p7 ph0):f3
    5u
    p22:gp2
    200u
    DELTA2
    (center (p1*2 ph0):f1 (p7*2 ph0):f3)
    DELTA2
    5u
    p22:gp2
    200u

    (p7 ph6):f3 ; phase-cycle Nz, -Nz for Freeman-Hill decay
    5u
;-----

```

```

(p1 ph2):f1 ; purge pulse to kill any residual HzNz
5u
p28:gp8
100u
50u p18:f3 ; change to pl0:120dB for Avance I or DRX systems
50u pl0:f1

;----- N15 T1rho relaxation period with shaped 180 H pulses for CCR compensation -----
# ifdef LABEL_CN
    (d8 p15:sp5 ph0 d9 p15:sp5 ph0 d8):f1 (p8:sp8 ph0 4u pl8 vplist ph0 p8:sp9 ph0):f3 (d29 p4*2 ph0 3u 10u
fq=cnst22(bf ppm) 3u p4*2 ph0 10u fq=cnst21(bf ppm)):f2

#else
    (d8 p15:sp5 ph0 d9 p15:sp5 ph0 d8):f1 (p8:sp8 ph0 4u pl8 vplist ph0 p8:sp9 ph0):f3
#endif

;-----Cleaning Gradient-----
5u
p29:gp9
100u
100u ;fq=0:f1
;----- Echo- Antiecho encoding-----

77    3u pl7:f3
        3u pl4:f2
        3u pl1:f1
        (p7 ph7):f3

        DELTA6 ;compensation for d0 15N evolution
        DELTA5
        190u
        p26:gp6*EA
        10u
        (center (p1*2 ph0):f1 (p7*2 ph7):f3)
        5u
        p26:gp6*EA*-1
        195u
        DELTA5

; t1 evolution -----

        d0*0.5 ;gron10 5u groff
        d0*0.5 ;gron10*-1 5u groff

#ifdef LABEL_CN
    (center (p1*2 ph0):f1 (p4*2 ph0 3u 10u fq=cnst22(bf ppm) 3u p4*2 ph0 10u fq=cnst21(bf ppm)):f2 )

#else
    (p1*2 ph0):f1
#endif

d0
;goto 999
;-----first INEPT for sensitivity-enhanced back transfer-----

    (center (p1 ph0):f1 (p7 ph8):f3)
    5u
    p23:gp3
    200u
    DELTA3
    (center(p1*2 ph0):f1 (p7*2 ph0):f3)
    DELTA3
    5u
    p23:gp3
    200u

; ---second INEPT -----
    (center (p1 ph1):f1 (p7 ph1):f3)

```

```

5u
p24:gp4
200u
DELTA8
(center(p1*2 ph0):f1 (p7*2 ph0):f3)
DELTA8
5u
p24:gp4
200u
(d26*0.5 p19 ph0:r):f1
;-----spin-echo with decoding gradient EA
998 245u
DELTA4
(p1*2 ph0):f1
5u
p25:gp5
200u
999 5u
5u pl30:f2
10u pl31:f3
20u BLKGRAD
go=2 ph31 cpds3:f3 ;cpd2:f2
200u
400u do:f3
400u do:f2
d11 wr #0 if #0 zd
1m
2m vplist.inc
lo to 3 times l6
1m ip8*2
1m igrad EA
1m ru2
lo to 4 times 2
1m id0
lo to 5 times l3
1m do:f3
1m BLKGRAD
exit

ph0=0
ph1=1
ph2=2
ph5=1
ph6=1 1 3 3
ph7= 1 3
ph8= 0
ph31=1 3 3 1

;-----NOTES-----

;o1p = 4.7 ppm
;o2p=176 ppm (CO)
;o3p=119 ppm

;NS=4*n
;in0=inf/2
;SW=1/(2*in0)
;echo-antiecho in N15 (process as Complex in NmrDraw before splitting the spectra)

; 1H pulses
;p1: 90 deg hard 1H pulse @p11
;p19 last 90 deg hard 1H pulse @p11, can be adjusted for improving water-supression
;p11: 1H 90 deg
;p10: 1000 dB
;p15: 1000u (@ 1200 MHz) 180 deg IBurp2 pulse on 1H (sp15)
;sp5: 180 deg IBurp2 pulse on 1H (sp15)
;spnam5: IBurp2

```

;spoffs5: 4680Hz @ 1200 MHz (8.6 ppm) , should be centered in amide region but not touch the water

; 13C pulses
;p4: 13CO selective 180 deg (11.850*2us @ 1200 MHz) @pl4
;pl4: 13C 90 deg
;sp4: 13CA selective 180 deg (11.850*2us @ 1200 MHz)

;15N pulses
;p7 : 90 deg hard 15N pulse @pl7
;pl7: 15N 90 deg
;pl8: 15N spin-lock power
;pl0 1000 dB
;sp8 @pl8 spin-lock power
;sp9 @pl8 spin-lock power
;spnam8 AHP TanhTan (1st half)
;spnam9 AHP TanhTan (2nd half)
;spoal8 1
;spoal9 0
;CPDPRG3: garp (15N decoupling)
;pcpd3: 15N decoupling (200u @pl31)
;pl31: 15N decoupling power

; gradients
;p21: 200u
;p22: 200u
;p23: 800u
;p24: 200u
;p25: 201u Echo/Anti-echo decoding gradient
;p26: 1000u Echo/Anti-echo half-encoding gradient
;p27: 200u
;p28: 200u
;p29: 200u

;for z-only gradients
;gpz1: 1%
;gpz2: 2%
;gpz3: 31%
;gpz4: 11%
;gpz5: 50%
;gpz6: 50%
;gpz7: 50%
;gpz8: 5%
;gpz9: 10 % optional
;gpz10: 0.5% optional prevent t1 radiation damping

;gpnam1 SINE.20
;gpnam2 SINE.20
;gpnam3 SINE.20
;gpnam4 SINE.20
;gpnam5 SINE.20
;gpnam6 SINE.20
;gpnam7 SINE.50
;gpnam8 SINE.20
;gpnam9 SINE.20

Chapter 10: Internal Backbone Dynamics of SNAP25a

Table S1: Experimental parameters of ^{15}N -SNAP25a NMR relaxation experiments at 1200 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	$\{^1\text{H}\}$ - ^{15}N NOE
Field [T]	28.2	28.2	28.2
^1H Larmor frequency [MHz]	1199.85	1199.85	1199.85
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Stief et al., JMB 2023
Interscan recovery delay [s]	2.00	2.00	2.00
Number of scans	8	8	32
Total experimental time [h]	24.4	20.6	66.7
Direct dimension (^1H)			
^1H carrier [ppm]	4.695	4.695	4.695
Spectral width [ppm]	16.03	16.03	16.03
Data points	4096	4096	4096
Acquisition time [ms]	106.50	106.50	106.50
90° (^1H) pulse length [μs]	9.4	9.4	9.4
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.00	117.00	117.00
Spectral width [ppm]	35.00	35.00	35.00
Data points (total)	8	8	2
Acquisition time [ms]	60.16	60.16	60.16
90° (^{15}N) pulse length [μs]	36.2	36.2	36.2
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 960, 240, 800, 160, 640, 320, 480	5, 65, 25, 45, 15, 55, 35, 10	5 s saturation on ^1H

Table S2: Experimental parameters of ^{15}N -SNAP25a NMR relaxation experiments at 900 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	$\{^1\text{H}\}$ - ^{15}N NOE
Field [T]	21.1	21.1	21.1
^1H Larmor frequency [MHz]	899.84	899.84	899.84
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Stief et al., JMB 2023
Inter-scan recovery delay [s]	3.00	3.00	3.00
Number of scans	4	4	16
Total experimental time [h]	16.4	14.7	33.2
Direct dimension (^1H)			
^1H carrier [ppm]	4.695	4.695	4.695
Spectral width [ppm]	16.34	16.34	16.34
Data points	2048	2048	2048
Acquisition time [ms]	69.90	69.90	69.90
90° (^1H) pulse length [μs]	7.7	7.7	7.7
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.00	117.00	117.00
Spectral width [ppm]	36.55	36.55	36.55
Data points (total)	8	8	2
Acquisition time [ms]	76.80	76.80	76.80
90° (^{15}N) pulse length [μs]	42.60	42.60	42.60
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 800, 80, 720, 160, 640, 320, 480	5, 55, 25, 20, 15, 45, 35, 10	5 s saturation on ^1H

Table S3: Experimental parameters of ^{15}N -SNAP25a NMR relaxation experiments at 600 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	$\{^1\text{H}\}$ - ^{15}N NOE
Field [T]	14.1	14.1	14.1
^1H Larmor frequency [MHz]	600.19	600.19	600.19
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Stief et al., JMB 2023
Interscan recovery delay [s]	2.00	2.00	2.00
Number of scans	4	4	32
Total experimental time [h]	12.0	10.4	66.6
Direct dimension (^1H)			
^1H carrier [ppm]	4.695	4.695	4.695
Spectral width [ppm]	16.02	16.02	16.02
Data points	2048	2048	2048
Acquisition time [ms]	106.91	106.91	106.91
90° (^1H) pulse length [μs]	8.9	8.9	8.9
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.00	117.00	117.00
Spectral width [ppm]	34.98	34.98	34.98
Data points (total)	8	8	2
Acquisition time [ms]	120.32	120.32	120.32
90° (^{15}N) pulse length [μs]	42.50	42.50	42.50
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 720, 320, 480, 80, 640, 160, 560	5, 65, 25, 40, 15, 50, 35, 10	5 s saturation on ^1H

Table S4: NMR ^{15}N relaxation rate constants. # (residue number) + AA type (1-letter code), transverse relaxation rate constants (derived from $R_{1\rho}$) ^{15}N $R_2(R_{1\rho}) / \text{s}^{-1}$ (600 MHz), experimental error $\delta(^{15}\text{N } R_2(R_{1\rho})) / \text{s}^{-1}$ (600 MHz), longitudinal relaxation rate constants ^{15}N R_1 / s^{-1} (600 MHz), $\delta(^{15}\text{N } R_1) / \text{s}^{-1}$ (600 MHz), heteronuclear NOE $\{^1\text{H}\}$ - ^{15}N values (I / I_0) (600 MHz), $\delta(I / I_0)$ (600 MHz), ^{15}N $R_2(R_{1\rho}) / \text{s}^{-1}$ (900 MHz), $\delta(^{15}\text{N } R_2(R_{1\rho})) / \text{s}^{-1}$ (900 MHz), ^{15}N R_1 / s^{-1} (900 MHz), $\delta(^{15}\text{N } R_1) / \text{s}^{-1}$ (900 MHz), hetNOE $\{^1\text{H}\}$ - ^{15}N values (I / I_0) (900 MHz), $\delta(I / I_0)$ (900 MHz), ^{15}N $R_2(R_{1\rho}) / \text{s}^{-1}$ (1200 MHz), $\delta(^{15}\text{N } R_2(R_{1\rho})) / \text{s}^{-1}$ (1200 MHz), ^{15}N R_1 / s^{-1} (1200 MHz), $\delta(^{15}\text{N } R_1) / \text{s}^{-1}$ (1200 MHz), hetNOE $\{^1\text{H}\}$ - ^{15}N values (I / I_0) (1200 MHz), $\delta(I / I_0)$ (1200 MHz)

3	3.34	0.08	1.56	0.01	0.01	0.02	4.03	0.05	1.43	0.01	0.39	0.02	4.99	0.06	1.47	0.01	0.61	0.02
4	3.60	0.08	1.66	0.01	0.07	0.02	4.82	0.05	1.48	0.01	0.43	0.02	6.07	0.06	1.50	0.01	0.62	0.02
5	5.20	0.11	1.62	0.01	0.19	0.02	6.68	0.07	1.45	0.01	0.48	0.02	8.41	0.09	1.40	0.01	0.61	0.03
7	4.91	0.10	1.66	0.01	0.18	0.02	6.05	0.07	1.47	0.01	0.48	0.02	8.06	0.08	1.52	0.01	0.64	0.03
9	7.49	0.15	1.67	0.02	0.35	0.03	8.80	0.09	1.37	0.01	0.60	0.03	12.47	0.14	1.34	0.02	0.62	0.03
10	8.30	0.16	1.67	0.02	0.38	0.03	10.55	0.12	1.30	0.01	0.64	0.03	14.46	0.19	1.28	0.02	0.68	0.04
14	10.00	0.19	1.67	0.02	0.45	0.05	12.06	0.14	1.28	0.01	0.65	0.03	16.96	0.23	1.25	0.02	0.77	0.05
17	10.15	0.21	1.64	0.02	0.46	0.05	13.27	0.18	1.28	0.01	0.63	0.04	18.51	0.32	1.28	0.03	0.72	0.05
21	9.01	0.18	1.52	0.02	0.40	0.04	11.76	0.17	1.26	0.01	0.55	0.03	16.32	0.24	1.22	0.02	0.59	0.04
23	8.90	0.15	1.46	0.02	0.32	0.04	9.90	0.11	1.21	0.01	0.48	0.03	14.07	0.17	1.13	0.02	0.70	0.04
24	8.52	0.17	1.50	0.02	0.38	0.04	11.35	0.13	1.27	0.01	0.56	0.03	15.72	0.21	1.20	0.02	0.67	0.04
25	8.69	0.18	1.56	0.02	0.38	0.04	10.83	0.13	1.34	0.01	0.59	0.03	15.18	0.19	1.23	0.02	0.63	0.04
29	10.13	0.27	1.54	0.03	0.34	0.05	13.66	0.22	1.30	0.01	0.58	0.04	17.72	0.30	1.25	0.02	0.71	0.06
31	9.33	0.17	1.60	0.02	0.39	0.04	11.51	0.13	1.32	0.01	0.56	0.03	16.29	0.20	1.32	0.02	0.69	0.04
32	8.99	0.20	1.56	0.02	0.31	0.04	12.22	0.16	1.34	0.01	0.54	0.03	16.28	0.27	1.21	0.03	0.61	0.05
34	8.72	0.17	1.51	0.02	0.33	0.04	10.81	0.14	1.25	0.01	0.47	0.03	14.69	0.22	1.24	0.02	0.74	0.05
36	7.28	0.18	1.45	0.02	0.19	0.03	10.07	0.13	1.25	0.01	0.47	0.03	13.76	0.18	1.22	0.02	0.58	0.04
39	6.46	0.16	1.51	0.02	0.11	0.03	7.70	0.10	1.33	0.01	0.42	0.03	10.68	0.14	1.32	0.02	0.60	0.04
42	4.69	0.10	1.50	0.01	0.08	0.02	5.52	0.07	1.37	0.01	0.37	0.02	7.45	0.09	1.38	0.01	0.52	0.02
43	4.17	0.12	1.52	0.01	0.06	0.03	4.56	0.08	1.39	0.01	0.40	0.02	6.52	0.11	1.44	0.02	0.54	0.03
45	4.75	0.14	1.59	0.02	0.01	0.03	6.13	0.09	1.46	0.01	0.34	0.02	7.86	0.10	1.48	0.02	0.64	0.03
46	4.35	0.14	1.56	0.02	0.05	0.03	5.68	0.08	1.43	0.01	0.37	0.02	7.20	0.08	1.39	0.01	0.62	0.03
50	5.50	0.09	1.51	0.01	0.09	0.02	6.14	0.07	1.38	0.01	0.41	0.02	7.33	0.09	1.39	0.01	0.62	0.03
51	4.34	0.09	1.63	0.01	0.10	0.02	5.53	0.06	1.44	0.01	0.41	0.02	6.43	0.08	1.47	0.01	0.60	0.03
52	4.06	0.10	1.57	0.01	0.01	0.02	5.62	0.06	1.45	0.01	0.40	0.02	7.82	0.07	1.45	0.01	0.62	0.03
53	4.23	0.10	1.61	0.01	0.04	0.02	5.20	0.06	1.47	0.01	0.42	0.02	7.29	0.08	1.49	0.01	0.59	0.03
54	3.18	0.11	1.57	0.01	0.03	0.02	4.34	0.07	1.46	0.01	0.37	0.02	5.49	0.09	1.48	0.01	0.57	0.02
55	3.89	0.08	1.57	0.01	0.06	0.02	4.55	0.05	1.48	0.01	0.47	0.02	5.89	0.06	1.50	0.01	0.62	0.02
56	3.61	0.10	1.57	0.01	0.05	0.02	4.31	0.06	1.44	0.01	0.37	0.02	6.05	0.07	1.46	0.01	0.57	0.02
57	3.78	0.09	1.68	0.01	0.09	0.02	5.16	0.06	1.48	0.01	0.41	0.02	7.47	0.08	1.44	0.01	0.66	0.03
58	3.97	0.09	1.62	0.01	0.10	0.02	4.61	0.06	1.48	0.01	0.45	0.02	6.24	0.06	1.48	0.01	0.61	0.02
60	4.10	0.11	1.60	0.01	0.03	0.02	4.88	0.07	1.46	0.01	0.39	0.02	6.25	0.08	1.50	0.01	0.63	0.03
61	4.29	0.11	1.63	0.01	0.11	0.03	5.32	0.08	1.50	0.01	0.43	0.02	7.17	0.09	1.52	0.01	0.59	0.03
62	4.25	0.10	1.63	0.01	0.11	0.02	5.29	0.06	1.47	0.01	0.44	0.02	6.67	0.07	1.46	0.01	0.61	0.02
63	3.62	0.13	1.63	0.02	0.08	0.03	4.30	0.08	1.51	0.01	0.39	0.02	5.87	0.09	1.47	0.01	0.62	0.03
64	4.09	0.12	1.62	0.01	0.12	0.03	4.83	0.07	1.50	0.01	0.46	0.02	6.13	0.07	1.54	0.01	0.67	0.03
68	4.53	0.16	1.73	0.02	0.13	0.03	5.79	0.09	1.51	0.01	0.42	0.02	7.87	0.11	1.48	0.02	0.65	0.03
70	4.51	0.10	1.62	0.01	0.18	0.02	5.80	0.06	1.48	0.01	0.49	0.02	7.98	0.07	1.50	0.01	0.62	0.02
71	5.17	0.12	1.66	0.02	0.16	0.03	5.84	0.08	1.44	0.01	0.48	0.02	8.19	0.08	1.43	0.01	0.62	0.03
72	4.39	0.16	1.69	0.02	0.15	0.03	5.70	0.09	1.50	0.01	0.49	0.03	6.92	0.11	1.54	0.02	0.65	0.04

75	5.63	0.12	1.68	0.02	0.32	0.03	6.66	0.08	1.45	0.01	0.54	0.03	8.90	0.08	1.39	0.01	0.65	0.03
77	5.26	0.14	1.66	0.02	0.23	0.03	5.99	0.08	1.50	0.01	0.52	0.02	8.71	0.09	1.50	0.02	0.71	0.03
79	3.01	0.10	1.45	0.01	-0.24	0.02	2.83	0.06	1.41	0.01	0.19	0.02	2.81	0.07	1.51	0.01	0.44	0.03
81	4.30	0.11	1.69	0.01	0.17	0.02	5.12	0.07	1.48	0.01	0.45	0.02	7.37	0.08	1.48	0.01	0.62	0.03
82	3.97	0.13	1.66	0.02	0.14	0.03	5.35	0.08	1.53	0.01	0.45	0.02	6.33	0.11	1.54	0.02	0.66	0.03
83	4.01	0.13	1.63	0.02	0.20	0.03	4.93	0.08	1.53	0.01	0.48	0.02	6.54	0.08	1.56	0.02	0.63	0.03
84	3.71	0.21	1.56	0.02	0.00	0.04	4.14	0.10	1.44	0.01	0.33	0.03	5.34	0.11	1.45	0.02	0.56	0.04
85	3.42	0.20	1.52	0.02	-0.01	0.04	4.46	0.10	1.46	0.01	0.37	0.03	5.18	0.12	1.50	0.02	0.53	0.04
86	3.53	0.16	1.54	0.02	0.00	0.03	4.14	0.09	1.49	0.01	0.41	0.02	5.36	0.12	1.54	0.02	0.63	0.03
87	3.64	0.12	1.57	0.01	0.09	0.03	4.29	0.07	1.50	0.01	0.44	0.02	5.50	0.07	1.52	0.01	0.64	0.03
88	3.80	0.12	1.59	0.01	0.00	0.03	4.88	0.07	1.44	0.01	0.39	0.02	6.18	0.08	1.47	0.01	0.56	0.03
89	4.19	0.16	1.55	0.02	0.06	0.03	5.63	0.10	1.41	0.01	0.41	0.03	6.79	0.11	1.41	0.02	0.55	0.03
90	4.25	0.13	1.61	0.02	0.04	0.03	5.75	0.08	1.48	0.01	0.40	0.02	6.91	0.08	1.42	0.01	0.60	0.03
92	4.88	0.21	1.57	0.03	0.12	0.04	5.84	0.11	1.41	0.01	0.40	0.03	7.07	0.12	1.36	0.02	0.61	0.04
94	4.13	0.16	1.67	0.02	0.20	0.03	5.30	0.10	1.44	0.01	0.45	0.03	6.76	0.11	1.43	0.02	0.65	0.04
95	4.62	0.14	1.68	0.02	0.17	0.03	5.48	0.09	1.50	0.01	0.48	0.03	7.35	0.09	1.53	0.02	0.58	0.03
96	4.76	0.07	1.64	0.01	0.17	0.02	6.00	0.05	1.49	0.00	0.47	0.01	7.96	0.05	1.47	0.01	0.65	0.02
97	4.97	0.26	1.67	0.03	0.13	0.06	6.55	0.16	1.44	0.02	0.45	0.04	7.53	0.17	1.43	0.03	0.64	0.05
98	4.57	0.33	1.68	0.04	0.12	0.06	5.68	0.16	1.44	0.02	0.47	0.04	7.36	0.19	1.43	0.04	0.63	0.05
100	4.90	0.14	1.67	0.02	0.23	0.03	6.46	0.09	1.49	0.01	0.56	0.03	8.66	0.10	1.49	0.02	0.68	0.03
101	5.15	0.14	1.74	0.02	0.26	0.03	6.52	0.08	1.48	0.01	0.51	0.02	8.60	0.08	1.46	0.01	0.68	0.03
102	5.47	0.16	1.70	0.02	0.26	0.03	6.37	0.10	1.52	0.01	0.56	0.03	9.13	0.10	1.46	0.02	0.69	0.04
103	5.23	0.15	1.65	0.02	0.27	0.03	6.77	0.10	1.49	0.01	0.59	0.03	8.71	0.10	1.47	0.02	0.67	0.04
104	4.88	0.18	1.68	0.02	0.21	0.04	6.35	0.12	1.49	0.01	0.53	0.03	8.52	0.13	1.51	0.02	0.70	0.04
106	3.74	0.16	1.62	0.02	0.19	0.03	4.91	0.10	1.52	0.01	0.49	0.03	6.80	0.11	1.51	0.02	0.64	0.03
107	3.79	0.17	1.66	0.02	0.17	0.04	4.31	0.09	1.48	0.01	0.47	0.03	6.05	0.10	1.51	0.02	0.63	0.04
108	3.84	0.16	1.55	0.02	-0.03	0.04	4.48	0.08	1.49	0.01	0.43	0.02	5.45	0.09	1.47	0.02	0.60	0.03
109	3.43	0.12	1.60	0.02	0.03	0.03	4.24	0.07	1.50	0.01	0.46	0.02	5.20	0.08	1.57	0.02	0.61	0.03
111	2.97	0.11	1.55	0.01	0.01	0.02	3.59	0.07	1.44	0.01	0.36	0.02	4.84	0.09	1.49	0.01	0.55	0.02
113	3.79	0.08	1.55	0.01	-0.02	0.02	5.37	0.06	1.41	0.00	0.34	0.01	6.17	0.08	1.47	0.01	0.53	0.02
114	3.67	0.12	1.52	0.01	-0.06	0.02	4.53	0.10	1.43	0.01	0.32	0.02	5.61	0.11	1.44	0.01	0.55	0.03
116	3.24	0.14	1.47	0.01	-0.04	0.03	4.38	0.08	1.35	0.01	0.30	0.02	6.18	0.09	1.33	0.01	0.52	0.03
118	3.70	0.11	1.49	0.01	-0.05	0.02	4.69	0.07	1.36	0.01	0.35	0.02	6.19	0.08	1.39	0.01	0.52	0.02
120	4.39	0.15	1.59	0.02	0.06	0.03	6.14	0.08	1.36	0.01	0.37	0.02	7.28	0.10	1.35	0.02	0.52	0.03
123	4.79	0.14	1.62	0.02	0.24	0.03	6.20	0.08	1.45	0.01	0.53	0.02	8.82	0.11	1.35	0.02	0.67	0.03
125	4.80	0.06	1.72	0.01	0.23	0.01	5.89	0.04	1.52	0.00	0.50	0.01	7.55	0.05	1.50	0.01	0.68	0.02
126	4.87	0.11	1.72	0.02	0.20	0.03	6.12	0.07	1.50	0.01	0.52	0.02	7.84	0.08	1.50	0.02	0.68	0.03
127	5.15	0.12	1.70	0.02	0.20	0.03	6.02	0.07	1.46	0.01	0.51	0.02	8.31	0.09	1.47	0.02	0.70	0.03
128	4.77	0.12	1.61	0.01	0.18	0.03	5.74	0.08	1.45	0.01	0.47	0.02	8.15	0.10	1.44	0.01	0.64	0.03
129	4.57	0.11	1.60	0.01	0.13	0.03	5.55	0.07	1.39	0.01	0.39	0.02	7.04	0.07	1.38	0.01	0.58	0.03
130	4.04	0.12	1.61	0.01	0.14	0.03	5.37	0.06	1.47	0.01	0.37	0.02	7.26	0.08	1.46	0.01	0.64	0.03
131	4.16	0.17	1.54	0.02	0.08	0.03	4.99	0.09	1.40	0.01	0.41	0.02	6.37	0.13	1.43	0.02	0.55	0.03
132	4.15	0.14	1.54	0.02	0.15	0.03	4.37	0.09	1.47	0.01	0.42	0.02	6.33	0.12	1.48	0.02	0.63	0.03
135	4.46	0.14	1.67	0.02	0.14	0.03	5.65	0.09	1.49	0.01	0.47	0.02	8.08	0.12	1.46	0.02	0.69	0.03
136	4.65	0.14	1.66	0.02	0.19	0.03	5.62	0.08	1.51	0.01	0.45	0.02	7.58	0.10	1.48	0.02	0.66	0.03
138	3.79	0.13	1.63	0.02	0.14	0.03	5.52	0.07	1.47	0.01	0.44	0.02	6.95	0.08	1.45	0.01	0.62	0.03
139	4.25	0.18	1.60	0.02	0.13	0.04	5.34	0.10	1.44	0.01	0.44	0.03	6.05	0.11	1.44	0.02	0.58	0.03

141	4.04	0.09	1.60	0.01	0.14	0.02	5.03	0.06	1.47	0.01	0.45	0.02	6.37	0.07	1.45	0.01	0.61	0.02
142	3.87	0.10	1.65	0.01	0.13	0.02	4.71	0.05	1.43	0.01	0.45	0.02	6.05	0.06	1.45	0.01	0.63	0.02
143	4.11	0.09	1.68	0.01	0.17	0.02	5.02	0.06	1.51	0.01	0.48	0.02	6.28	0.07	1.54	0.01	0.68	0.02
144	3.81	0.10	1.69	0.01	0.15	0.02	4.69	0.06	1.54	0.01	0.46	0.02	5.87	0.07	1.51	0.01	0.68	0.03
148	3.47	0.09	1.62	0.01	0.14	0.02	4.97	0.05	1.47	0.01	0.42	0.02	6.08	0.07	1.46	0.01	0.65	0.02
149	4.16	0.11	1.70	0.01	0.16	0.03	4.82	0.06	1.53	0.01	0.47	0.02	6.34	0.07	1.51	0.01	0.63	0.03
150	4.21	0.10	1.67	0.01	0.18	0.02	4.86	0.06	1.45	0.01	0.49	0.02	6.65	0.08	1.43	0.01	0.66	0.03
154	3.64	0.09	1.61	0.01	0.09	0.02	5.28	0.05	1.49	0.01	0.40	0.01	6.17	0.06	1.50	0.01	0.61	0.02
155	3.57	0.13	1.57	0.02	0.14	0.03	4.63	0.07	1.48	0.01	0.43	0.02	5.66	0.09	1.52	0.02	0.62	0.03
156	3.71	0.11	1.60	0.01	0.10	0.02	4.87	0.06	1.47	0.01	0.45	0.02	5.96	0.07	1.50	0.01	0.61	0.03
157	4.21	0.12	1.64	0.01	0.06	0.02	5.93	0.08	1.49	0.01	0.40	0.02	7.39	0.10	1.50	0.02	0.64	0.03
158	3.91	0.12	1.68	0.02	0.12	0.03	5.27	0.07	1.52	0.01	0.44	0.02	6.75	0.08	1.54	0.02	0.65	0.03
160	4.09	0.11	1.66	0.01	0.15	0.03	4.72	0.06	1.51	0.01	0.47	0.02	6.54	0.08	1.48	0.01	0.63	0.03
161	5.97	0.11	1.66	0.01	0.26	0.02	8.01	0.08	1.41	0.01	0.57	0.02	11.29	0.11	1.36	0.01	0.69	0.03
165	3.53	0.09	1.56	0.01	0.03	0.02	4.53	0.05	1.39	0.01	0.38	0.02	5.60	0.06	1.40	0.01	0.58	0.02
166	3.43	0.08	1.57	0.01	0.08	0.02	4.37	0.05	1.45	0.01	0.41	0.02	5.84	0.06	1.47	0.01	0.64	0.02
168	3.05	0.10	1.64	0.01	0.11	0.02	4.07	0.07	1.51	0.01	0.47	0.02	5.31	0.08	1.53	0.01	0.62	0.03
169	3.39	0.10	1.62	0.01	0.13	0.02	3.97	0.05	1.52	0.01	0.45	0.02	5.23	0.06	1.52	0.01	0.60	0.02
170	3.53	0.09	1.60	0.01	0.07	0.02	4.29	0.06	1.49	0.01	0.40	0.02	5.51	0.06	1.51	0.01	0.59	0.02
172	4.23	0.11	1.71	0.01	0.18	0.02	5.08	0.06	1.57	0.01	0.49	0.02	7.12	0.08	1.60	0.02	0.69	0.03
173	4.03	0.11	1.66	0.01	0.20	0.03	4.79	0.06	1.48	0.01	0.48	0.02	6.33	0.07	1.44	0.01	0.64	0.03
175	4.19	0.13	1.67	0.02	0.21	0.03	5.02	0.07	1.54	0.01	0.48	0.02	6.22	0.08	1.50	0.02	0.69	0.03
176	3.81	0.10	1.67	0.01	0.16	0.02	4.87	0.06	1.50	0.01	0.45	0.02	6.28	0.07	1.50	0.01	0.65	0.03
177	3.73	0.12	1.73	0.02	0.20	0.03	5.09	0.07	1.55	0.01	0.46	0.02	6.61	0.08	1.53	0.02	0.69	0.03
181	3.96	0.12	1.68	0.02	0.12	0.03	5.34	0.07	1.49	0.01	0.46	0.02	7.03	0.09	1.53	0.02	0.68	0.03
182	3.69	0.14	1.68	0.02	0.14	0.03	5.55	0.09	1.53	0.01	0.40	0.02	7.48	0.11	1.52	0.02	0.62	0.03
185	3.16	0.12	1.61	0.01	0.02	0.03	4.19	0.08	1.50	0.01	0.42	0.02	5.63	0.10	1.52	0.02	0.61	0.03
187	3.11	0.17	1.63	0.02	0.02	0.03	3.94	0.09	1.49	0.01	0.34	0.03	4.81	0.10	1.55	0.02	0.60	0.04
188	4.02	0.21	1.73	0.03	0.16	0.05	4.43	0.11	1.58	0.01	0.43	0.03	4.89	0.13	1.52	0.03	0.66	0.04
190	3.64	0.43	1.71	0.06	0.02	0.09	4.62	0.23	1.52	0.03	0.37	0.07	6.14	0.27	1.58	0.06	0.79	0.11
192	3.58	0.42	1.68	0.05	0.24	0.08	5.36	0.23	1.56	0.03	0.51	0.07	6.38	0.31	1.51	0.05	0.52	0.09
193	4.57	0.44	1.76	0.06	0.15	0.11	5.75	0.22	1.61	0.03	0.50	0.07	8.83	0.31	1.61	0.06	0.67	0.10
195	4.66	0.33	1.71	0.05	0.39	0.08	4.96	0.22	1.61	0.03	0.49	0.06	7.19	0.28	1.54	0.05	0.68	0.09
196	6.21	0.44	1.74	0.06	0.26	0.10	4.60	0.22	1.52	0.03	0.41	0.06	6.57	0.27	1.55	0.06	0.60	0.10
197	5.37	0.35	1.67	0.04	0.25	0.09	4.63	0.20	1.61	0.03	0.60	0.07	5.56	0.26	1.52	0.05	0.78	0.11
199	3.40	0.42	1.63	0.05	0.25	0.09	4.54	0.24	1.70	0.03	0.35	0.06	5.83	0.30	1.59	0.05	0.57	0.08
200	3.88	0.39	1.55	0.04	-0.04	0.09	3.75	0.21	1.55	0.03	0.56	0.08	4.99	0.24	1.57	0.05	0.77	0.09
203	2.84	0.10	1.60	0.01	-0.10	0.02	3.07	0.06	1.63	0.01	0.35	0.02	3.47	0.08	1.76	0.02	0.60	0.03
204	1.40	0.47	1.25	0.03	-0.85	0.11	1.90	0.20	1.28	0.02	-0.09	0.06	2.49	0.28	1.29	0.04	0.40	0.08
205	0.76	0.35	1.06	0.03	-0.94	0.11	2.05	0.16	1.13	0.02	-0.18	0.06	1.91	0.21	1.26	0.03	0.29	0.07
206	0.84	0.27	0.72	0.02	-1.60	0.10	0.29	0.11	0.75	0.01	-0.73	0.05	0.59	0.17	0.88	0.02	-0.18	0.05

Table S5: Extended model-free analysis of NMR ^{15}N relaxation data of SNAP25a. # (residue number), order parameter S_f^2 , error of order parameter S_f^2 , fast internal correlation time τ_f / ps, error of τ_f / ps, order parameter S_s^2 , error of S_s^2 , slow internal correlation time τ_s / ns, error of τ_s / ns, order parameter S^2 , error of S^2 , local correlation time τ_m / ns, error of τ_m / ns, internal correlation time τ_e / ns, error of τ_e / ns

3	0.71	0.02	38.39	4.68	0.37	0.01	1.00	0.06	0.27	0.01	4.88	0.14	n	n
4	0.77	0.02	41.61	6.14	0.43	0.01	0.95	0.06	0.33	0.01	5.12	0.14	n	n
5	0.73	0.02	62.46	4.27	0.46	0.03	1.46	0.13	0.34	0.03	7.52	0.52	n	n
7	0.79	0.02	48.20	7.45	0.44	0.02	1.10	0.08	0.35	0.02	6.76	0.26	n	n
9	n	n	n	n	n	n	n	n	0.62	0.01	6.92	0.12	0.62	0.02
10	0.93	0.02	37.05	24.98	0.67	0.02	0.91	0.12	0.63	0.02	7.98	0.22	n	n
14	n	n	n	n	n	n	n	n	0.70	0.01	8.46	0.16	0.82	0.02
17	n	n	n	n	n	n	n	n	0.68	0.01	9.40	0.21	0.92	0.03
21	n	n	n	n	n	n	n	n	0.65	0.01	8.83	0.17	0.70	0.02
23	0.81	0.03	55.65	8.00	0.66	0.02	1.17	0.19	0.53	0.03	9.09	0.45	n	n
24	n	n	n	n	n	n	n	n	0.64	0.01	8.63	0.14	0.66	0.01
25	n	n	n	n	n	n	n	n	0.64	0.01	8.28	0.15	0.73	0.02
29	0.90	0.04	91.87	53.96	0.62	0.04	1.30	0.37	0.55	0.06	11.33	1.22	n	n
31	0.92	0.03	80.75	35.02	0.63	0.02	1.11	0.17	0.58	0.03	9.59	0.47	n	n
32	0.76	0.06	73.35	19.16	0.49	0.10	2.11	0.29	0.37	0.10	14.21	5.06	n	n
34	n	n	n	n	n	n	n	n	0.62	0.01	8.48	0.16	0.61	0.01
36	0.79	0.03	64.95	7.67	0.58	0.02	1.19	0.17	0.46	0.03	9.98	0.64	n	n
39	0.79	0.03	59.87	9.63	0.52	0.02	1.01	0.11	0.41	0.02	8.35	0.37	n	n
42	0.68	0.02	59.76	3.55	0.42	0.02	1.26	0.10	0.29	0.02	7.46	0.42	n	n
43	0.70	0.02	47.61	4.64	0.39	0.02	1.13	0.08	0.27	0.02	6.35	0.31	n	n
45	0.78	0.02	71.31	8.50	0.42	0.02	1.01	0.08	0.32	0.02	7.23	0.34	n	n
46	0.71	0.02	62.41	5.10	0.44	0.02	1.20	0.12	0.31	0.02	6.74	0.42	n	n
50	0.74	0.02	49.03	4.83	0.43	0.01	1.10	0.07	0.32	0.01	7.43	0.26	n	n
51	0.76	0.02	52.16	5.94	0.45	0.01	1.01	0.07	0.34	0.01	5.75	0.15	n	n
52	0.77	0.02	54.25	6.10	0.40	0.01	1.03	0.06	0.31	0.01	7.12	0.24	n	n
53	0.77	0.02	55.78	6.35	0.41	0.01	1.04	0.07	0.32	0.01	6.36	0.23	n	n
54	0.69	0.02	53.86	4.47	0.37	0.02	1.16	0.10	0.25	0.02	5.50	0.29	n	n
55	0.72	0.01	39.74	4.41	0.34	0.01	1.15	0.06	0.25	0.01	6.11	0.25	n	n
56	0.70	0.02	52.65	4.28	0.40	0.02	1.11	0.08	0.28	0.01	5.58	0.22	n	n
57	0.80	0.02	49.85	7.90	0.48	0.01	0.91	0.07	0.38	0.01	5.47	0.14	n	n
58	0.72	0.02	48.09	4.70	0.40	0.02	1.18	0.09	0.29	0.02	5.67	0.23	n	n
60	0.77	0.02	43.85	6.96	0.39	0.01	0.97	0.06	0.30	0.01	5.81	0.20	n	n
61	0.74	0.02	61.14	5.10	0.39	0.02	1.24	0.10	0.29	0.02	6.72	0.38	n	n
62	0.73	0.02	53.16	5.05	0.43	0.02	1.18	0.09	0.32	0.02	5.98	0.24	n	n
63	0.69	0.02	60.63	4.55	0.39	0.03	1.33	0.14	0.27	0.03	5.38	0.38	n	n
64	0.75	0.02	38.40	6.44	0.36	0.02	1.15	0.09	0.27	0.02	6.03	0.33	n	n
68	0.82	0.03	64.92	12.78	0.50	0.02	0.96	0.11	0.41	0.02	5.72	0.21	n	n
70	0.75	0.02	48.70	4.78	0.39	0.02	1.29	0.08	0.30	0.02	7.46	0.40	n	n
71	0.80	0.02	48.92	8.24	0.50	0.01	1.01	0.09	0.40	0.02	6.15	0.20	n	n
72	0.80	0.03	38.47	12.01	0.43	0.02	1.03	0.11	0.35	0.02	5.94	0.29	n	n
75	0.75	0.02	53.21	5.29	0.51	0.04	1.60	0.21	0.38	0.04	7.05	0.64	n	n
77	0.79	0.03	40.85	8.47	0.43	0.03	1.23	0.12	0.34	0.03	7.27	0.50	n	n
79	0.61	0.02	61.00	4.39	0.27	0.03	1.10	0.09	0.17	0.02	3.47	0.25	n	n

81	0.77	0.02	51.43	6.53	0.48	0.02	1.06	0.09	0.37	0.02	5.57	0.19	n	n
82	0.74	0.02	53.08	6.03	0.38	0.02	1.26	0.11	0.28	0.02	6.27	0.43	n	n
83	0.70	0.03	47.18	4.87	0.29	0.05	1.48	0.12	0.21	0.04	7.64	1.76	n	n
84	0.69	0.03	57.26	7.56	0.39	0.03	1.07	0.15	0.27	0.03	5.10	0.51	n	n
85	0.65	0.03	59.30	5.71	0.27	0.05	1.35	0.14	0.18	0.04	7.04	2.93	n	n
86	0.71	0.02	40.86	6.93	0.28	0.03	1.13	0.09	0.20	0.02	6.35	0.69	n	n
87	0.68	0.02	45.49	4.35	0.29	0.03	1.35	0.10	0.20	0.03	6.45	0.69	n	n
88	0.74	0.02	51.80	6.40	0.41	0.01	0.99	0.07	0.31	0.01	5.65	0.19	n	n
89	0.70	0.02	58.47	6.05	0.42	0.03	1.21	0.13	0.30	0.03	6.71	0.49	n	n
90	0.71	0.02	71.61	4.65	0.43	0.03	1.33	0.14	0.30	0.03	6.58	0.49	n	n
92	0.72	0.03	57.50	7.79	0.49	0.04	1.15	0.20	0.35	0.04	6.18	0.57	n	n
94	0.76	0.03	41.81	9.21	0.51	0.02	1.01	0.13	0.38	0.02	5.25	0.25	n	n
95	0.76	0.02	56.57	7.08	0.42	0.03	1.22	0.13	0.32	0.03	6.38	0.42	n	n
96	0.75	0.01	57.11	3.51	0.44	0.01	1.28	0.07	0.33	0.01	6.96	0.23	n	n
97	0.82	0.04	47.27	18.26	0.51	0.03	0.91	0.15	0.41	0.03	5.98	0.34	n	n
98	0.82	0.04	34.55	19.24	0.50	0.04	0.85	0.15	0.41	0.03	5.51	0.36	n	n
100	0.80	0.02	37.04	8.52	0.45	0.02	1.20	0.11	0.36	0.03	7.16	0.42	n	n
101	0.82	0.02	49.21	10.82	0.53	0.02	1.07	0.12	0.44	0.02	6.13	0.23	n	n
102	n	n	n	n	n	n	n	n	0.52	0.01	5.70	0.11	0.60	0.01
103	0.76	0.03	39.87	6.84	0.40	0.04	1.45	0.15	0.31	0.04	8.23	1.10	n	n
104	0.82	0.03	34.24	13.44	0.45	0.02	1.08	0.12	0.37	0.03	6.80	0.39	n	n
106	0.69	0.03	46.95	5.23	0.29	0.06	1.56	0.14	0.20	0.05	8.01	2.77	n	n
107	0.74	0.03	38.10	7.84	0.42	0.03	1.12	0.14	0.31	0.03	5.09	0.35	n	n
108	0.69	0.03	49.91	5.88	0.32	0.03	1.23	0.12	0.22	0.03	6.18	0.93	n	n
109	0.74	0.02	36.43	6.60	0.32	0.02	1.09	0.08	0.24	0.02	5.45	0.32	n	n
111	0.67	0.02	47.62	4.57	0.36	0.02	1.12	0.09	0.24	0.02	4.70	0.24	n	n
113	0.73	0.01	60.00	4.42	0.40	0.01	1.00	0.05	0.29	0.01	6.29	0.17	n	n
114	0.69	0.02	59.60	5.10	0.36	0.02	1.08	0.09	0.25	0.02	6.03	0.30	n	n
116	0.65	0.02	59.22	4.34	0.42	0.02	1.13	0.11	0.27	0.02	5.99	0.35	n	n
118	0.71	0.02	50.02	5.01	0.41	0.01	0.97	0.07	0.29	0.01	6.04	0.19	n	n
120	0.75	0.02	62.29	7.33	0.53	0.02	0.90	0.09	0.40	0.02	5.93	0.19	n	n
123	0.70	0.03	49.25	4.13	0.41	0.06	1.74	0.18	0.29	0.05	8.28	2.52	n	n
125	0.79	0.01	48.35	4.15	0.47	0.01	1.19	0.07	0.37	0.01	6.06	0.16	n	n
126	0.83	0.02	36.26	9.82	0.48	0.02	1.02	0.09	0.40	0.02	6.00	0.20	n	n
127	0.84	0.02	29.27	12.77	0.50	0.01	0.94	0.08	0.42	0.02	6.06	0.18	n	n
128	0.75	0.02	50.44	5.74	0.44	0.02	1.24	0.11	0.33	0.02	6.99	0.38	n	n
129	0.74	0.02	55.53	5.66	0.51	0.01	1.02	0.09	0.38	0.01	5.73	0.17	n	n
130	0.71	0.02	65.54	4.45	0.42	0.03	1.30	0.13	0.30	0.03	6.62	0.46	n	n
131	0.70	0.02	50.57	5.76	0.41	0.03	1.16	0.13	0.29	0.03	6.25	0.49	n	n
132	0.66	0.02	47.84	4.01	0.28	0.04	1.48	0.12	0.19	0.03	7.78	1.83	n	n
135	0.81	0.02	42.61	10.18	0.46	0.02	1.01	0.09	0.37	0.02	6.20	0.24	n	n
136	0.73	0.03	60.15	5.74	0.42	0.04	1.40	0.17	0.31	0.04	6.80	0.72	n	n
138	0.72	0.02	58.45	4.86	0.42	0.03	1.33	0.14	0.30	0.03	6.43	0.49	n	n
139	0.70	0.03	51.78	5.52	0.44	0.03	1.25	0.17	0.31	0.03	5.84	0.48	n	n
141	0.69	0.02	52.53	3.29	0.38	0.02	1.40	0.10	0.26	0.02	6.45	0.40	n	n
142	0.76	0.02	31.88	6.34	0.48	0.01	0.91	0.06	0.36	0.01	4.83	0.11	n	n
143	0.77	0.02	36.76	5.97	0.41	0.01	1.10	0.07	0.32	0.01	5.50	0.18	n	n

144	0.74	0.02	47.03	5.70	0.41	0.02	1.23	0.11	0.30	0.02	5.12	0.26	n	n
148	0.71	0.02	49.72	4.04	0.42	0.02	1.24	0.09	0.30	0.02	5.60	0.23	n	n
149	0.74	0.02	52.50	5.18	0.44	0.02	1.22	0.12	0.33	0.02	5.24	0.24	n	n
150	0.77	0.02	32.52	6.21	0.49	0.01	0.98	0.07	0.38	0.01	5.05	0.13	n	n
154	0.71	0.02	59.02	3.94	0.37	0.02	1.27	0.09	0.27	0.02	6.36	0.32	n	n
155	0.68	0.02	46.35	4.45	0.32	0.03	1.35	0.11	0.22	0.03	6.45	0.74	n	n
156	0.73	0.02	43.49	4.70	0.38	0.02	1.15	0.08	0.28	0.02	5.82	0.25	n	n
157	0.79	0.02	58.09	8.12	0.42	0.01	1.02	0.08	0.33	0.02	6.56	0.25	n	n
158	0.77	0.02	52.19	7.30	0.41	0.02	1.12	0.10	0.32	0.02	5.91	0.30	n	n
160	0.73	0.02	50.40	5.76	0.43	0.02	1.24	0.12	0.31	0.02	5.55	0.29	n	n
161	n	n	n	n	n	n	n	n	0.57	0.01	6.65	0.10	0.60	0.01
165	0.72	0.02	40.92	4.91	0.44	0.01	0.92	0.06	0.32	0.01	5.01	0.12	n	n
166	0.71	0.01	43.18	4.34	0.37	0.01	1.15	0.07	0.26	0.01	5.65	0.20	n	n
168	0.72	0.02	38.06	5.63	0.37	0.02	1.17	0.09	0.27	0.02	4.76	0.23	n	n
169	0.67	0.02	49.96	3.23	0.32	0.04	1.48	0.11	0.21	0.03	5.47	0.67	n	n
170	0.69	0.02	53.53	3.89	0.35	0.02	1.25	0.09	0.24	0.02	5.57	0.28	n	n
172	0.79	0.02	43.27	7.49	0.38	0.02	1.19	0.09	0.30	0.02	6.19	0.32	n	n
173	0.71	0.02	48.04	4.56	0.45	0.03	1.34	0.14	0.32	0.02	5.34	0.30	n	n
175	0.70	0.02	49.22	4.46	0.35	0.05	1.58	0.15	0.24	0.04	6.42	1.79	n	n
176	0.74	0.02	47.08	5.48	0.43	0.02	1.17	0.10	0.32	0.02	5.37	0.22	n	n
177	0.76	0.02	51.34	6.79	0.43	0.02	1.24	0.13	0.33	0.02	5.42	0.30	n	n
181	0.81	0.02	32.72	10.22	0.43	0.01	0.96	0.07	0.35	0.02	5.78	0.21	n	n
182	0.73	0.03	71.92	6.30	0.40	0.03	1.34	0.14	0.29	0.03	6.77	0.65	n	n
185	0.74	0.02	45.11	6.68	0.36	0.02	1.06	0.09	0.26	0.02	5.21	0.25	n	n
187	0.71	0.03	51.35	9.01	0.37	0.03	1.05	0.13	0.27	0.03	4.50	0.35	n	n
188	0.72	0.04	54.91	9.15	0.46	0.06	1.27	0.29	0.33	0.06	4.14	0.62	n	n
190	0.87	0.05	4.98	25.64	0.39	0.05	0.77	0.17	0.33	0.05	4.99	0.70	n	n
192	0.56	0.05	47.37	13.72	0.01	0.13	1.84	0.31	0.01	0.10	200.00	9.65	n	n
193	0.83	0.07	65.16	35.63	0.40	0.08	1.20	0.31	0.33	0.08	6.90	3.56	n	n
195	0.59	0.05	39.97	11.70	0.01	0.10	1.84	0.27	0.01	0.09	200.00	6.90	n	n
196	0.74	0.08	67.88	23.04	0.49	0.12	1.25	0.48	0.36	0.10	5.04	4.17	n	n
197	0.62	0.04	30.35	13.54	0.04	0.10	1.78	0.24	0.03	0.08	36.65	6.48	n	n
199	0.59	0.06	59.23	13.82	0.01	0.10	1.60	0.25	0.00	0.08	200.00	11.72	n	n
200	0.78	0.04	0.00	13.44	0.24	0.05	1.01	0.12	0.19	0.04	5.88	2.15	n	n
203	0.70	0.02	54.64	5.14	0.12	0.03	1.23	0.06	0.09	0.02	5.38	1.12	n	n
204	0.76	0.05	0.00	18.49	0.36	0.09	0.38	0.13	0.27	0.07	1.57	0.45	n	n
205	0.54	0.05	49.48	10.57	0.07	0.05	0.75	0.10	0.04	0.03	5.63	4.74	n	n
206	n	n	n	n	n	n	n	n	0.35	0.03	0.54	0.05	0.05	0.01

Table S6: IMPACT analysis of NMR ^{15}N relaxation data of SNAP25a. # (residue number), amplitude A₁, amplitude A₂, amplitude A₃, amplitude A₄, amplitude A₅, amplitude A₆, error of A₁, error of A₂, error of A₃, error of A₄, error of A₅, error of A₆

3	0.02	0.18	0.37	0.17	0	0.26	0	0.01	0.01	0.02	0.02	0.01
4	0.01	0.24	0.35	0.17	0	0.22	0	0.01	0.01	0.01	0.01	0.01
5	0.06	0.25	0.35	0.07	0.1	0.16	0.01	0.01	0.01	0.04	0.05	0.03
7	0.06	0.22	0.39	0.1	0.04	0.19	0.01	0.02	0.02	0.03	0.04	0.03
9	0.13	0.33	0.29	0.04	0.08	0.14	0.01	0.03	0.02	0.04	0.05	0.03
10	0.15	0.38	0.22	0.08	0	0.17	0.01	0.02	0.02	0.02	0.02	0.02
14	0.21	0.37	0.22	0.06	0	0.14	0.01	0.03	0.02	0.02	0.01	0.02
17	0.22	0.35	0.23	0.06	0	0.14	0.01	0.03	0.02	0.03	0.04	0.03
21	0.2	0.28	0.27	0	0.13	0.13	0.01	0.02	0.02	0.01	0.03	0.03
23	0.2	0.27	0.22	0.09	0.02	0.2	0.01	0.02	0.01	0.03	0.04	0.03
24	0.19	0.26	0.28	0.01	0.08	0.18	0.01	0.02	0.02	0.02	0.04	0.03
25	0.19	0.28	0.3	0	0.11	0.13	0.01	0.02	0.02	0.02	0.04	0.03
29	0.25	0.23	0.3	0.07	0.01	0.15	0.01	0.03	0.02	0.03	0.04	0.03
31	0.21	0.27	0.29	0.04	0.06	0.12	0.01	0.03	0.02	0.03	0.05	0.03
32	0.2	0.28	0.29	0.02	0.14	0.08	0.01	0.03	0.02	0.04	0.06	0.03
34	0.19	0.26	0.25	0.1	0.01	0.18	0.01	0.02	0.02	0.04	0.05	0.03
36	0.15	0.22	0.28	0.08	0.06	0.21	0.01	0.02	0.02	0.04	0.05	0.03
39	0.12	0.21	0.3	0.14	0.03	0.2	0.01	0.02	0.02	0.03	0.05	0.03
42	0.07	0.17	0.37	0.05	0.19	0.16	0	0.01	0.01	0.02	0.04	0.02
43	0.05	0.16	0.38	0.09	0.1	0.22	0.01	0.02	0.02	0.03	0.05	0.03
45	0.07	0.17	0.37	0.18	0.03	0.18	0.01	0.02	0.02	0.03	0.05	0.03
46	0.05	0.21	0.34	0.15	0.04	0.21	0.01	0.02	0.02	0.03	0.04	0.02
50	0.1	0.16	0.36	0.12	0.05	0.21	0	0.01	0.01	0.03	0.04	0.03
51	0.04	0.24	0.34	0.15	0.04	0.19	0.01	0.02	0.02	0.03	0.04	0.03
52	0.04	0.18	0.37	0.17	0	0.24	0.01	0.01	0.01	0.01	0.02	0.01
53	0.04	0.2	0.37	0.16	0.03	0.2	0.01	0.02	0.01	0.02	0.04	0.02
54	0.01	0.18	0.39	0.12	0.09	0.21	0.01	0.02	0.02	0.04	0.06	0.03
55	0.04	0.15	0.43	0.13	0	0.26	0	0.01	0.01	0.02	0.02	0.01
56	0.02	0.19	0.37	0.12	0.09	0.2	0.01	0.01	0.01	0.03	0.05	0.03
57	0	0.29	0.31	0.19	0	0.21	0	0.02	0.01	0.01	0.01	0.01
58	0.03	0.2	0.39	0.12	0.05	0.22	0	0.01	0.01	0.03	0.04	0.02
60	0.04	0.18	0.38	0.18	0	0.23	0.01	0.02	0.01	0.02	0.03	0.02
61	0.04	0.19	0.41	0.08	0.12	0.16	0.01	0.02	0.01	0.04	0.06	0.03
62	0.04	0.22	0.37	0.12	0.05	0.2	0	0.01	0.01	0.03	0.05	0.03
63	0.01	0.22	0.38	0.11	0.1	0.17	0.01	0.02	0.02	0.04	0.05	0.03
64	0.04	0.16	0.43	0.12	0	0.25	0.01	0.02	0.02	0.03	0.04	0.03
68	0.03	0.3	0.32	0.19	0	0.17	0.01	0.02	0.02	0.03	0.03	0.02
70	0.05	0.19	0.42	0.05	0.08	0.2	0	0.02	0.02	0.03	0.05	0.03
71	0.06	0.27	0.32	0.14	0.01	0.2	0.01	0.02	0.02	0.02	0.03	0.02
72	0.04	0.22	0.39	0.13	0	0.22	0.01	0.03	0.02	0.02	0.03	0.02
75	0.07	0.31	0.34	0.02	0.11	0.15	0.01	0.02	0.02	0.03	0.05	0.03
77	0.07	0.21	0.41	0.08	0	0.22	0.01	0.02	0.02	0.03	0.04	0.03
79	0.03	0.07	0.41	0.14	0.23	0.13	0.01	0.01	0.02	0.03	0.05	0.03
81	0.03	0.28	0.34	0.12	0.05	0.18	0.01	0.02	0.02	0.03	0.05	0.03
82	0.03	0.19	0.42	0.1	0.06	0.19	0.01	0.02	0.02	0.04	0.06	0.03

83	0.04	0.15	0.48	0	0.15	0.18	0.01	0.02	0.02	0.02	0.03	0.02
84	0.03	0.18	0.37	0.14	0.11	0.17	0.01	0.03	0.03	0.05	0.08	0.04
85	0.03	0.11	0.44	0.06	0.18	0.17	0.01	0.02	0.02	0.05	0.08	0.04
86	0.04	0.09	0.46	0.14	0.01	0.27	0.01	0.02	0.02	0.03	0.05	0.03
87	0.04	0.13	0.46	0.08	0.06	0.24	0.01	0.02	0.02	0.03	0.05	0.03
88	0.03	0.2	0.35	0.17	0.03	0.22	0.01	0.02	0.01	0.03	0.04	0.02
89	0.04	0.2	0.36	0.1	0.1	0.2	0.01	0.02	0.02	0.05	0.07	0.04
90	0.04	0.23	0.34	0.16	0.05	0.18	0.01	0.02	0.02	0.04	0.05	0.03
92	0.06	0.25	0.32	0.13	0.06	0.19	0.01	0.03	0.03	0.05	0.07	0.04
94	0.02	0.3	0.31	0.14	0.01	0.22	0.01	0.02	0.02	0.03	0.05	0.03
95	0.05	0.21	0.41	0.05	0.14	0.14	0.01	0.02	0.02	0.04	0.06	0.03
96	0.05	0.23	0.38	0.09	0.06	0.19	0	0.01	0.01	0.02	0.03	0.02
97	0.05	0.28	0.31	0.17	0	0.19	0.01	0.04	0.03	0.04	0.05	0.03
98	0.03	0.3	0.3	0.16	0	0.2	0.02	0.05	0.04	0.04	0.05	0.04
100	0.06	0.22	0.4	0.08	0	0.24	0.01	0.02	0.02	0.02	0.03	0.02
101	0.05	0.32	0.33	0.12	0	0.19	0.01	0.02	0.02	0.03	0.04	0.02
102	0.07	0.26	0.39	0.07	0.03	0.18	0.01	0.02	0.02	0.03	0.05	0.03
103	0.07	0.21	0.42	0.04	0.03	0.23	0.01	0.02	0.02	0.03	0.04	0.03
104	0.06	0.23	0.4	0.1	0	0.22	0.01	0.02	0.02	0.02	0.02	0.02
106	0.03	0.17	0.46	0.02	0.11	0.21	0.01	0.02	0.02	0.03	0.06	0.03
107	0.02	0.22	0.39	0.1	0.04	0.23	0.01	0.03	0.02	0.04	0.06	0.03
108	0.04	0.14	0.42	0.16	0	0.25	0.01	0.02	0.02	0.03	0.04	0.03
109	0.03	0.13	0.45	0.14	0	0.26	0.01	0.02	0.02	0.02	0.03	0.02
111	0.01	0.17	0.39	0.12	0.11	0.22	0.01	0.02	0.02	0.03	0.05	0.03
113	0.03	0.18	0.35	0.16	0.08	0.2	0	0.01	0.01	0.02	0.04	0.02
114	0.04	0.15	0.38	0.15	0.1	0.19	0.01	0.02	0.02	0.03	0.06	0.03
116	0.01	0.2	0.32	0.14	0.1	0.23	0.01	0.02	0.02	0.04	0.06	0.03
118	0.03	0.18	0.33	0.17	0.05	0.24	0.01	0.02	0.02	0.03	0.04	0.02
120	0.03	0.3	0.25	0.17	0.06	0.19	0.01	0.02	0.02	0.04	0.06	0.03
123	0.05	0.27	0.35	0.07	0.02	0.24	0.01	0.02	0.02	0.03	0.04	0.02
125	0.04	0.27	0.37	0.1	0.03	0.19	0	0.01	0.01	0.02	0.03	0.02
126	0.05	0.27	0.36	0.12	0	0.2	0.01	0.02	0.01	0.02	0.02	0.01
127	0.06	0.28	0.34	0.12	0	0.2	0.01	0.02	0.02	0.01	0.01	0.01
128	0.06	0.22	0.38	0.08	0.06	0.2	0.01	0.02	0.02	0.03	0.05	0.03
129	0.04	0.28	0.29	0.13	0.08	0.18	0.01	0.02	0.02	0.03	0.05	0.03
130	0.03	0.22	0.37	0.08	0.13	0.16	0.01	0.02	0.02	0.04	0.06	0.03
131	0.04	0.19	0.37	0.09	0.1	0.21	0.01	0.02	0.02	0.04	0.07	0.04
132	0.05	0.13	0.46	0.01	0.17	0.19	0.01	0.02	0.02	0.02	0.04	0.03
135	0.04	0.25	0.36	0.14	0	0.21	0.01	0.02	0.02	0.02	0.02	0.01
136	0.05	0.23	0.39	0.07	0.1	0.16	0.01	0.02	0.02	0.04	0.07	0.04
138	0.02	0.24	0.37	0.09	0.08	0.2	0.01	0.02	0.02	0.04	0.06	0.03
139	0.04	0.22	0.37	0.08	0.11	0.19	0.01	0.02	0.02	0.05	0.07	0.03
141	0.03	0.2	0.4	0.07	0.1	0.2	0	0.01	0.01	0.03	0.05	0.03
142	0.01	0.28	0.32	0.15	0	0.24	0.01	0.02	0.01	0.01	0.02	0.01
143	0.03	0.22	0.4	0.12	0	0.23	0	0.01	0.01	0.02	0.03	0.02
144	0.02	0.23	0.4	0.13	0.01	0.22	0.01	0.02	0.02	0.03	0.04	0.02
148	0.01	0.23	0.37	0.11	0.05	0.23	0	0.01	0.01	0.03	0.04	0.02

149	0.02	0.25	0.38	0.1	0.07	0.17	0.01	0.02	0.02	0.03	0.05	0.03
150	0.02	0.29	0.33	0.13	0	0.23	0.01	0.02	0.02	0.02	0.02	0.01
154	0.02	0.19	0.41	0.1	0.1	0.19	0	0.01	0.01	0.03	0.05	0.03
155	0.03	0.15	0.45	0.04	0.13	0.21	0.01	0.02	0.02	0.03	0.05	0.03
156	0.03	0.19	0.4	0.12	0.03	0.24	0.01	0.02	0.01	0.03	0.04	0.02
157	0.04	0.2	0.37	0.17	0	0.21	0.01	0.02	0.02	0.02	0.03	0.01
158	0.02	0.22	0.4	0.14	0.02	0.2	0.01	0.02	0.02	0.03	0.04	0.03
160	0.03	0.24	0.38	0.1	0.06	0.19	0.01	0.02	0.02	0.03	0.05	0.03
161	0.08	0.3	0.31	0.09	0	0.21	0.01	0.02	0.01	0.01	0.01	0.01
165	0.01	0.23	0.31	0.18	0	0.26	0	0.01	0.01	0.02	0.03	0.02
166	0.02	0.18	0.39	0.13	0.02	0.26	0	0.01	0.01	0.02	0.03	0.02
168	0	0.2	0.42	0.12	0.02	0.26	0	0.01	0.01	0.02	0.03	0.02
169	0.01	0.18	0.44	0.05	0.13	0.19	0	0.01	0.01	0.03	0.04	0.02
170	0.02	0.17	0.42	0.09	0.11	0.19	0	0.01	0.01	0.03	0.05	0.03
172	0.04	0.19	0.44	0.1	0.02	0.2	0.01	0.02	0.02	0.03	0.04	0.02
173	0.02	0.27	0.36	0.07	0.08	0.2	0.01	0.02	0.01	0.03	0.05	0.03
175	0.03	0.22	0.42	0.06	0.07	0.2	0.01	0.02	0.02	0.04	0.05	0.03
176	0.02	0.24	0.38	0.11	0.05	0.2	0	0.01	0.01	0.03	0.04	0.02
177	0	0.27	0.39	0.11	0.04	0.19	0.01	0.02	0.02	0.03	0.05	0.03
181	0.02	0.23	0.38	0.14	0	0.22	0.01	0.02	0.02	0.01	0	0.01
182	0.01	0.23	0.4	0.08	0.15	0.14	0.01	0.02	0.02	0.04	0.07	0.04
185	0.01	0.18	0.4	0.17	0	0.25	0.01	0.02	0.02	0.03	0.04	0.02
187	0	0.18	0.39	0.15	0.08	0.19	0.01	0.02	0.02	0.04	0.07	0.04
188	0.02	0.26	0.39	0.1	0.1	0.13	0.01	0.03	0.03	0.06	0.08	0.04
190	0.01	0.21	0.38	0.2	0	0.19	0.02	0.05	0.04	0.05	0.06	0.05
192	0	0.24	0.43	0	0.19	0.14	0.01	0.05	0.04	0.04	0.08	0.06
193	0.04	0.22	0.43	0.13	0.01	0.16	0.02	0.06	0.05	0.07	0.09	0.06
195	0.04	0.24	0.45	0	0.13	0.14	0.02	0.06	0.04	0.01	0.05	0.05
196	0.1	0.25	0.39	0.02	0.25	0	0.02	0.06	0.05	0.07	0.08	0.05
197	0.09	0.14	0.51	0.03	0	0.23	0.02	0.06	0.05	0.03	0.03	0.03
199	0.02	0.13	0.52	0	0.24	0.08	0.02	0.05	0.04	0	0.06	0.06
200	0.07	0.01	0.56	0.08	0	0.28	0.02	0.04	0.04	0.04	0.01	0.03
203	0.03	0	0.57	0.12	0.11	0.17	0	0.01	0.01	0.04	0.06	0.03
204	0	0.02	0.29	0.4	0	0.29	0.01	0.02	0.03	0.06	0.07	0.05
205	0	0	0.25	0.39	0	0.36	0	0	0.01	0.05	0.07	0.04
206	0	0	0.1	0.32	0.15	0.43	0	0	0.01	0.05	0.08	0.04

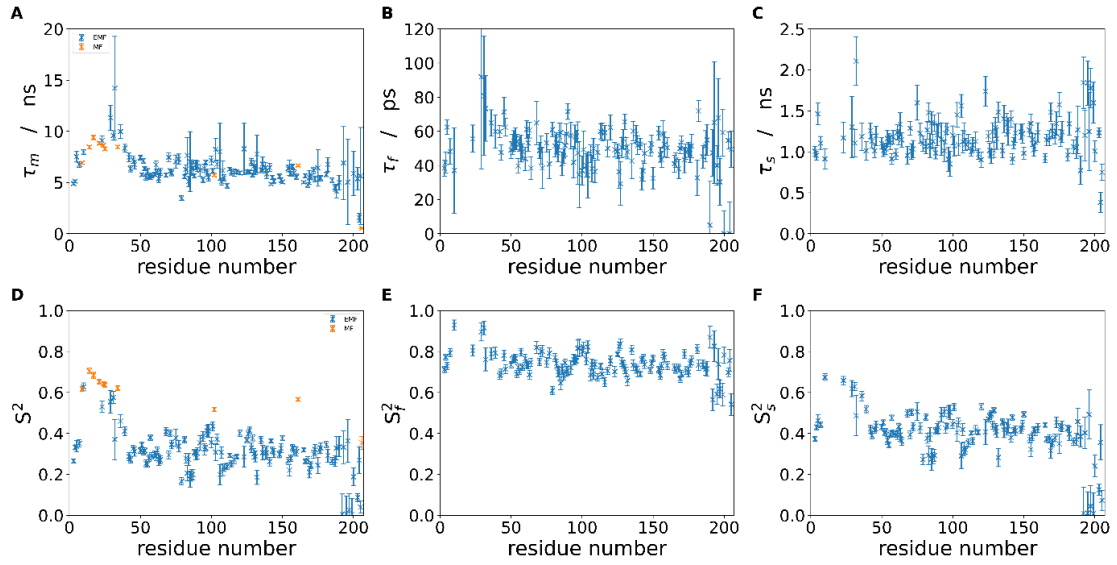


Figure S1: Model Free parameter of the model free analysis of SNAP25. Residues that were fitted using the original model-free approach are orange, while residues for which the extended model-free approach was most suitable are blue. **(A)** Local correlation time τ_m for each residue. **(B)** Fast internal correlation time τ_f represents the timescale of the faster of two independent internal motions. **(C)** The timescale of the slower internal motions is modelled with the slow internal correlation time τ_s . **(D)** Order parameter $S^2 = S_f^2 * S_s^2$. **(E)** Order parameter S_f^2 of fast internal motions. **(F)** Order parameter S_s^2 of slow internal motions.

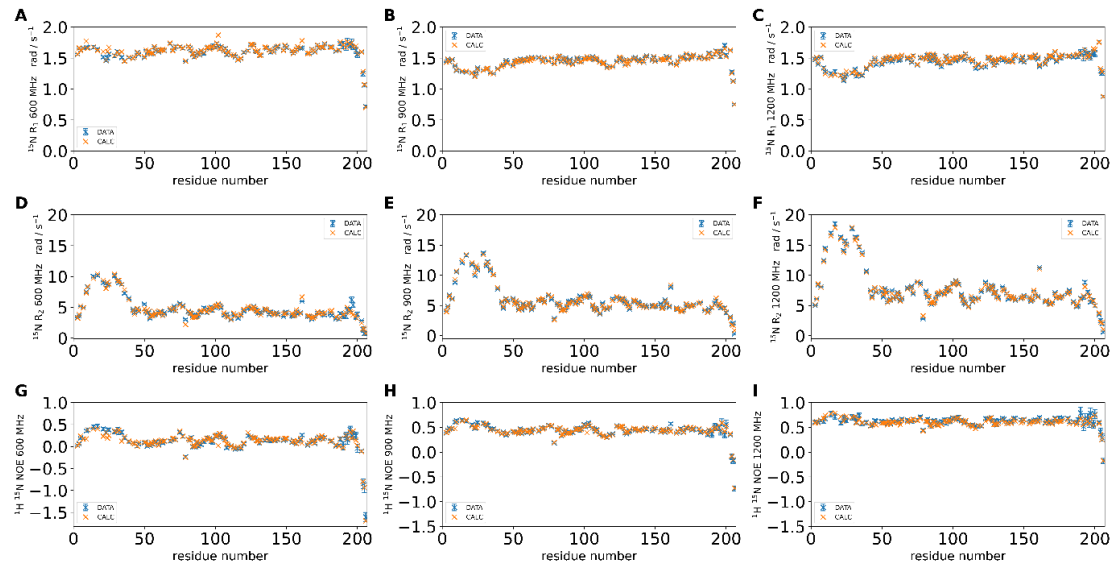


Figure S2: Back-calculated NMR ^{15}N relaxation rate constants based on the model free parameters of SNAP25a. Experimental data are colored in orange, back-calculated data in blue color. **(A)** R_1 relaxation rates at 600 MHz. **(B)** R_2 relaxation rates at 600 MHz. **(C)** hetNOE values at 600 MHz. **(D)** R_1 relaxation rates at 900 MHz. **(E)** R_2 relaxation rates at 900 MHz. **(F)** hetNOE values at 900 MHz. **(G)** R_1 relaxation rates at 1200 MHz. **(H)** R_2 relaxation rates at 1200 MHz. **(I)** hetNOE values at 1200 MHz.

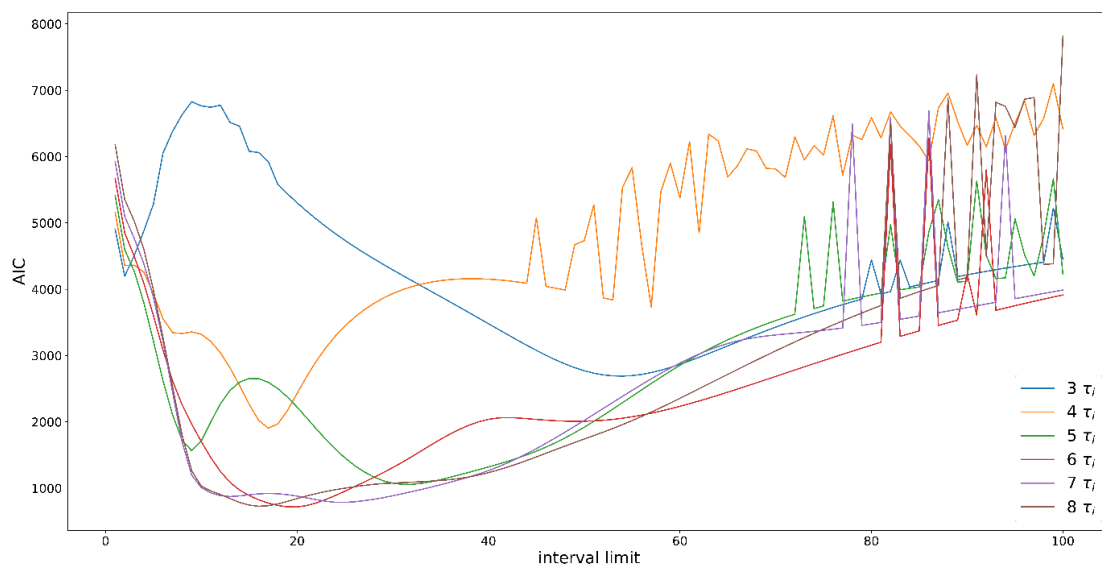


Figure S3: AIC criterion of the IMPACT analysis for different correlation time intervals and different number of correlation times. The number of correlation times is color coded. The x-axis represents the interval limits, which are used to calculate the logarithmic scaled correlation times between x ps and x ns.

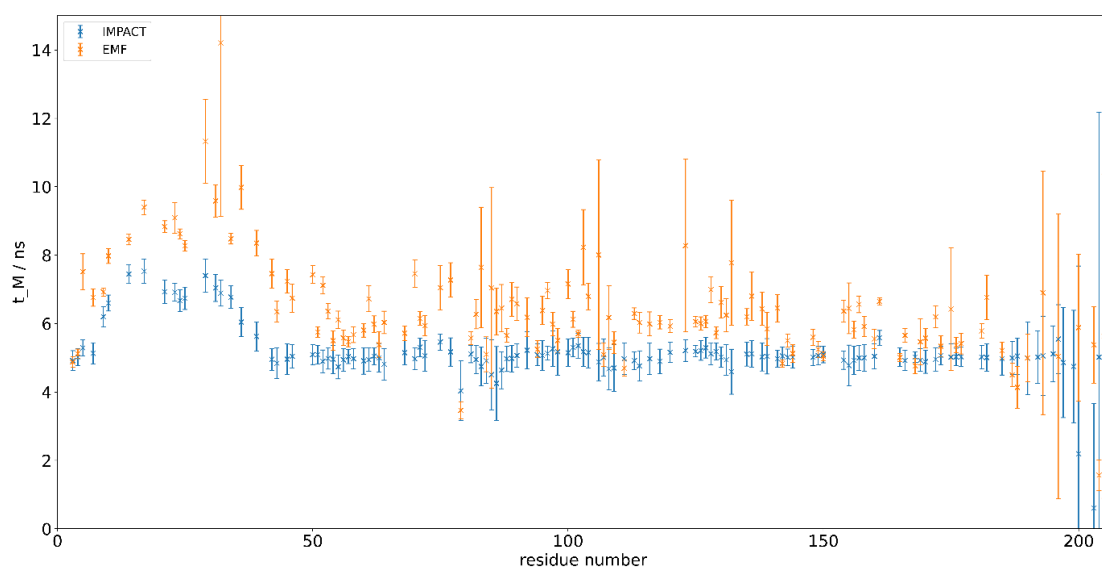


Figure S4: Local correlation time τ_{loc} (τ_m in the paper) of the Model Free approach compared to the tumbling time τ_m calculated from the IMPACT analysis. The tumbling time τ_m was calculated by the following equation: $\tau_m = (A_1 \cdot \tau_1 + A_2 \cdot \tau_2) / (A_1 + A_2)$. Blue: tumbling time τ_m . Orange: local correlation time τ_{loc} of the model free analysis.

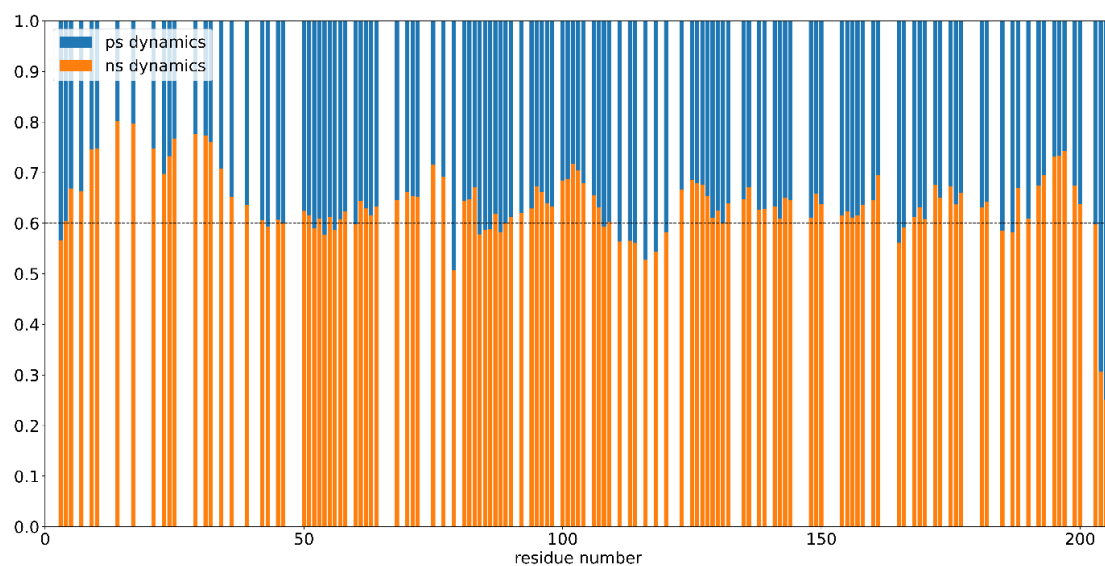


Figure S5: Distribution of ps and ns dynamics along the backbone of SNAP25a. In Blue proportion of ps dynamics, in orange proportion of ns dynamics.

Chapter 11: R2Hahn Echo Experiment for Detection of ms Dynamics

Table S1: Experimental parameters of ^{15}N human ubiquitin NMR relaxation experiments at 1200 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	^{15}N Hahn Echo
Field [T]	28.2	28.2	28.2
^1H Larmor frequency [MHz]	1199.92	1199.92	1199.92
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Figure 1
Interscan recovery delay [s]	2.00	2.00	2.00
Number of scans	8	8	16
Total experimental time [h]	23.3	20.5	10.9
Direct dimension (^1H)			
^1H carrier [ppm]	4.699	4.699	4.699
Spectral width [ppm]	16.03	16.03	16.03
Data points	4096	4096	4096
Acquisition time [ms]	106.50	106.50	106.50
90° (^1H) pulse length [μs]	9.200	9.200	9.200
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.500	117.500	117.500
Spectral width [ppm]	33.00	33.00	33.00
Data points (total)	8	8	2
Acquisition time [ms]	63.8	63.8	63.8
90° (^{15}N) pulse length [μs]	35.600	35.600	35.600
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 800, 240, 80, 160, 640, 320, 480	5, 65, 25, 45, 15, 55, 35, 10	8.88804, 106.65648

Table S2: Experimental parameters of ^{15}N human ubiquitin NMR relaxation experiments at 600 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	^{15}N Hahn Echo
Field [T]	14.1	14.1	14.1
^1H Larmor frequency [MHz]	600.19	600.19	600.19
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Figure 1
Interscan recovery delay [s]	2.00	2.00	2.00
Number of scans	8	8	16
Total experimental time [h]	10.3	11.8	5.5
Direct dimension (^1H)			
^1H carrier [ppm]	4.699	4.699	4.699
Spectral width [ppm]	16.02	16.02	16.02
Data points	2048	2048	2048
Acquisition time [ms]	106.5	106.5	106.5
90° (^1H) pulse length [μs]	9.000	9.000	9.000
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.500	117.500	117.500
Spectral width [ppm]	33.00	33.00	33.00
Data points (total)	8	8	2
Acquisition time [ms]	63.8	63.8	63.8
90° (^{15}N) pulse length [μs]	41.500	41.500	41.500
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 720, 160, 640, 80, 560, 320, 480	5, 65, 25, 45, 15, 55, 35, 10	8.88804, 159.98472

Table S3: Experimental parameters of ^{15}N ^{13}C human ubiquitin NMR relaxation experiments at 600 MHz.

	^{15}N R_1	^{15}N $R_{1\rho}$	^{15}N Hahn Echo
Field [T]	14.1	14.1	14.1
^1H Larmor frequency [MHz]	600.19	600.19	600.19
Pulse program	Stief et al., Methods 2024	Stief et al., Methods 2024	Figure 1
Interscan recovery delay [s]	2.00	2.00	2.00
Number of scans	8	8	16
Total experimental time [h]	10.3	11.8	5.5
Direct dimension (^1H)			
^1H carrier [ppm]	4.699	4.699	4.699
Spectral width [ppm]	16.02	16.02	16.02
Data points	2048	2048	2048
Acquisition time [ms]	106.5	106.5	106.5
90° (^1H) pulse length [μs]	9.000	9.000	9.250
Indirect dimension (^{15}N)			
^{15}N carrier [ppm]	117.500	117.500	117.500
Spectral width [ppm]	33.00	33.00	33.00
Data points (total)	8	8	2
Acquisition time [ms]	63.8	63.8	63.8
90° (^{15}N) pulse length [μs]	41.500	41.500	44.000
Relaxation period			
# relaxation delays	8	8	2
Time points [ms]	0, 720, 160, 640, 80, 560, 320, 480	5, 65, 25, 45, 15, 55, 35, 10	8.88804, 159.98472

Table S4: NMR ^{15}N relaxation rate constants. The columns contain the following information for each residue (rows): # (residue number) + AA type (1-letter code), transverse relaxation rate constants (derived from $R_{1\rho}$) ^{15}N $R_2(R_{1\rho})/s^{-1}$ (600 MHz), experimental error $\delta(^{15}\text{N } R_2(R_{1\rho}))/s^{-1}$ (600 MHz); ^{15}N $R_2(\text{Hahn echo})/s^{-1}$ (600 MHz), $\delta(^{15}\text{N } R_2(\text{Hahn echo}))/s^{-1}$ (600 MHz), ^{15}N $R_2(\text{Hahn echo})/s^{-1}$ (^{15}N - ^{13}C sample) (600 MHz), $\delta(^{15}\text{N } R_2(\text{Hahn echo}))/s^{-1}$ (^{15}N - ^{13}C sample) (600 MHz), ^{15}N $R_2(R_{1\rho})/s^{-1}$ (1200 MHz), $\delta(^{15}\text{N } R_2(R_{1\rho}))/s^{-1}$ (1200 MHz); ^{15}N $R_2(\text{Hahn echo})/s^{-1}$ (1200 MHz), $\delta(^{15}\text{N } R_2(\text{Hahn echo}))/s^{-1}$ (1200 MHz).

1M	5.00	0.08	5.53	0.12	5.49	0.15	7.93	0.04	8.37	0.09
2Q	5.97	0.08	6.18	0.13	6.29	0.14	9.39	0.04	9.76	0.10
3I	6.48	0.09	6.61	0.16	6.39	0.18	9.97	0.04	9.47	0.11
4F	6.17	0.10	6.20	0.18	6.25	0.20	9.48	0.05	9.49	0.13
5V	6.03	0.09	6.40	0.16	6.29	0.20	8.95	0.05	8.78	0.12
6K	6.18	0.11	6.24	0.18	6.69	0.40	9.74	0.07	9.93	0.15
7T	6.19	0.08	6.31	0.14	6.29	0.16	9.58	0.04	10.33	0.10
8L	5.69	0.08	5.86	0.12	6.02	0.15	8.93	0.03	9.14	0.08
9T	5.55	0.12	6.01	0.17	6.08	0.17	10.04	0.08	10.00	0.14
10G	5.25	0.07	5.50	0.11	5.68	0.12	8.46	0.04	9.36	0.08
11K	5.37	0.06	5.72	0.08	5.86	0.19	9.18	0.02	9.22	0.05
12T	5.56	0.08	5.75	0.13	5.91	0.14	8.52	0.03	8.74	0.08
13I	6.12	0.11	6.69	0.20	6.63	0.23	10.61	0.08	10.80	0.18
14T	5.93	0.09	6.24	0.15	6.32	0.17	10.09	0.04	9.67	0.11
16E	5.48	0.08	6.03	0.13	6.10	0.14	8.53	0.04	8.51	0.08
17V	6.41	0.10	6.21	0.17	6.15	0.21	9.52	0.05	9.63	0.12
18E	6.26	0.10	6.49	0.17	6.43	0.18	10.02	0.05	9.99	0.11
20S	6.28	0.09	6.27	0.14	6.40	0.16	9.94	0.06	7.88	0.10
21D	6.69	0.07	6.94	0.13	7.12	0.15	11.32	0.04	11.76	0.11
22T	5.94	0.08	6.30	0.14	6.39	0.17	9.45	0.05	10.34	0.12
23I	7.94	0.10	8.31	0.20	8.12	0.25	16.05	0.09	16.47	0.30
25N	9.08	0.08	9.54	0.20	9.48	0.25	21.62	0.09	21.41	0.39
26V	6.43	0.08	6.49	0.13	6.26	0.16	9.61	0.04	10.07	0.09
27K	6.67	0.08	6.58	0.14	6.58	0.30	10.46	0.04	10.46	0.10
28A	6.66	0.07	6.59	0.12	6.66	0.16	10.92	0.04	10.58	0.10
29K	6.38	0.08	6.21	0.13	6.55	0.29	9.70	0.04	9.89	0.09
30I	6.35	0.08	6.45	0.14	6.52	0.16	9.77	0.04	9.80	0.10
31Q	6.26	0.08	6.69	0.13	6.33	0.15	10.64	0.04	10.81	0.12
32D	6.65	0.06	6.75	0.11	6.49	0.13	10.01	0.03	10.19	0.08
33K	5.78	0.06	6.18	0.11	6.15	0.23	9.51	0.03	9.59	0.07
34E	6.13	0.10	6.24	0.17	6.24	0.18	9.13	0.05	9.17	0.14
35G	6.84	0.09	7.00	0.16	6.96	0.18	10.53	0.06	11.26	0.18
36I	5.51	0.08	6.10	0.12	6.11	0.14	9.84	0.04	9.71	0.10
39D	6.06	0.06	6.07	0.09	6.08	0.11	9.35	0.03	9.65	0.07
40Q	5.98	0.09	6.51	0.16	6.40	0.17	9.42	0.04	9.53	0.11
41Q	5.80	0.08	6.37	0.13	6.12	0.14	9.01	0.03	8.99	0.09
42R	6.01	0.09	6.25	0.15	6.08	0.17	9.06	0.05	9.13	0.11
43L	6.33	0.11	6.21	0.19	6.28	0.23	9.65	0.08	9.22	0.18
44I	6.03	0.11	6.35	0.18	6.33	0.21	8.93	0.05	8.60	0.13
46A	5.88	0.14	5.68	0.19	6.50	0.25	9.76	0.11	9.35	0.15
47G	5.63	0.09	5.41	0.13	4.97	0.15	9.43	0.09	7.54	0.14

48K	6.45	0.08	6.68	0.13	6.50	0.27	10.73	0.04	10.70	0.11
49Q	5.67	0.07	5.91	0.10	6.02	0.12	9.18	0.03	9.01	0.08
50L	6.32	0.10	6.34	0.16	6.60	0.19	9.75	0.06	9.40	0.14
51E	6.48	0.10	6.82	0.17	6.79	0.19	9.34	0.06	9.77	0.15
52D	6.44	0.06	6.43	0.10	6.49	0.12	9.45	0.03	9.81	0.08
54R	6.51	0.08	6.68	0.13	6.96	0.15	10.53	0.04	10.62	0.10
55T	6.63	0.11	6.79	0.19	6.72	0.21	12.14	0.09	12.39	0.25
56L	6.38	0.08	6.31	0.14	6.36	0.18	9.63	0.04	9.58	0.10
57S	6.24	0.06	6.32	0.10	6.45	0.13	9.67	0.03	9.67	0.07
58D	6.55	0.07	6.64	0.13	6.67	0.15	10.66	0.04	10.83	0.10
59Y	6.26	0.09	6.47	0.15	6.31	0.16	9.05	0.04	9.00	0.10
60N	6.58	0.08	6.76	0.14	6.37	0.15	9.70	0.04	10.08	0.10
61I	6.40	0.08	6.17	0.13	6.06	0.15	9.30	0.04	9.39	0.10
62Q	5.48	0.09	5.38	0.13	5.33	0.15	8.62	0.04	7.56	0.08
63K	6.40	0.06	6.36	0.10	6.74	0.23	9.50	0.03	9.95	0.08
64E	6.31	0.09	6.25	0.16	6.29	0.17	9.00	0.05	9.14	0.13
65S	6.35	0.06	6.37	0.11	6.51	0.13	9.99	0.03	10.41	0.08
66T	5.70	0.09	6.24	0.16	6.23	0.18	8.81	0.04	9.22	0.11
67L	6.03	0.12	6.66	0.21	6.09	0.23	9.27	0.08	9.18	0.17
68H	6.24	0.09	6.23	0.16	6.18	0.19	9.17	0.05	9.56	0.13
69L	6.08	0.09	6.08	0.16	6.18	0.18	9.24	0.05	9.14	0.11
70V	6.81	0.11	7.06	0.20	6.83	0.24	11.81	0.08	11.83	0.19
71L	5.72	0.07	6.02	0.12	6.08	0.14	9.41	0.04	9.28	0.08
72R	5.42	0.07	5.52	0.10	5.40	0.12	8.20	0.03	8.24	0.08
73L	3.74	0.06	3.94	0.07	3.94	0.08	5.97	0.02	6.00	0.04
74R	2.95	0.05	3.24	0.05	3.32	0.06	4.24	0.02	4.82	0.03
75G	1.83	0.06	2.20	0.06	2.11	0.06	2.62	0.02	2.73	0.03
76G	1.17	0.04	1.40	0.03	1.22	0.03	1.45	0.01	1.77	0.02

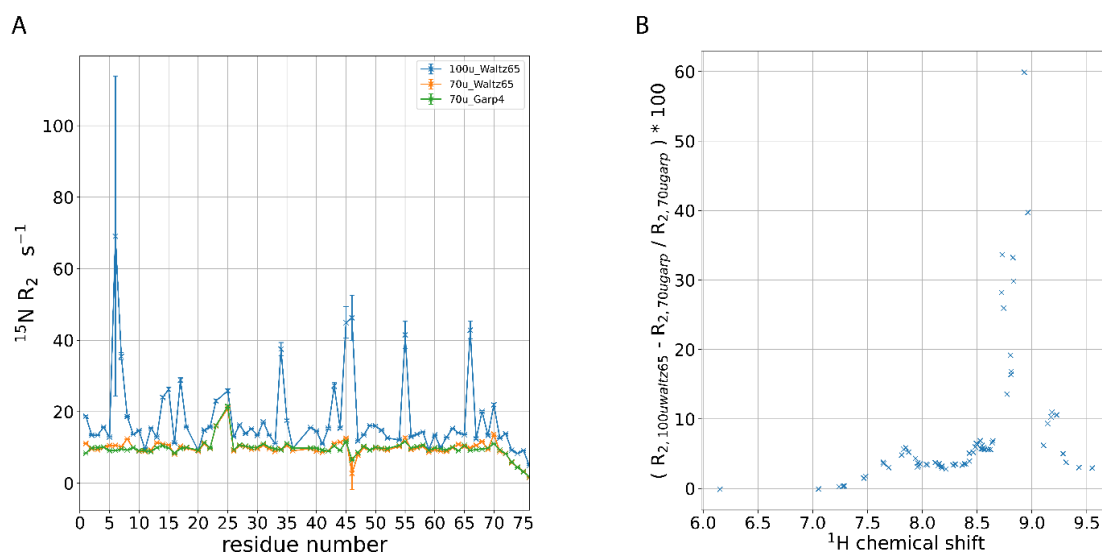
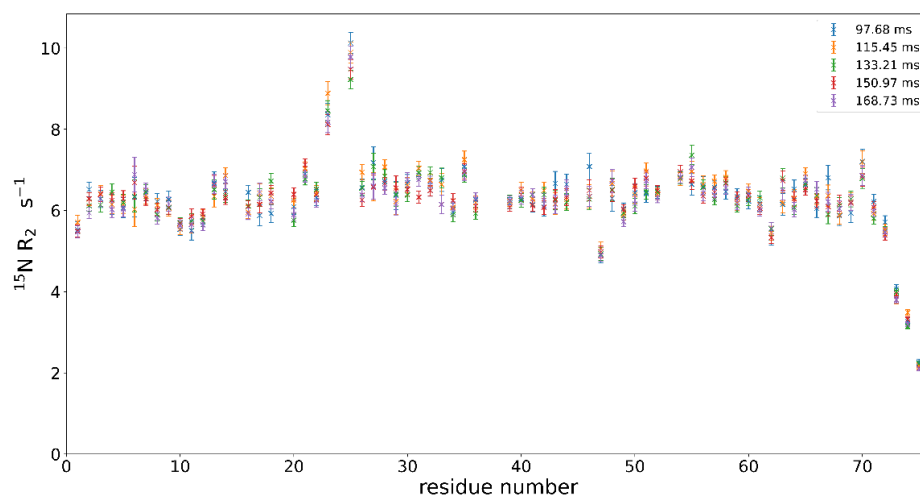


Figure S1: (A) Comparison of ^{15}N R_2 relaxation rates on human ubiquitin at 1200 MHz using three different decoupling schemes: 100 μs waltz65, 70 μs waltz65, 70 μs garp4. (B) Percentual difference between the ^{15}N R_2 relaxation rate data sets acquired with a 100 μs waltz65 decoupling scheme and a 70 μs garp4 decoupling scheme.



FigureS2: Comparison of the ^{15}N R_2 relaxation rates of human ubiquitin at 25 $^{\circ}\text{C}$, acquired with different Hahn echo delays at 600 MHz.