Compatible solute induced stabilisation of membrane proteins studied by single molecule force spectroscopy

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die hier vorgelegte Dissertation eigenständig und ohne unerlaubte Hilfe angefertigt habe. Es wurden keinerlei andere Quellen und Hilfsmittel, außer den angegebenen, benutzt. Zitate aus anderen Arbeiten wurden kenntlich gemacht. Diese Dissertation wurde noch bei keiner anderen Institution eingereicht und es wurden bisher keine Promotionsversuche von mir unternommen.

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To my family

Summary

Compatible solutes are small organic molecules produced and accumulated by microorganisms inside their cell to counteract different kinds of environmental stress. These are known for their uncharged, zwitter ionic and osmotically active nature. Compatible solutes show osmotic activity which even at molar concentrations do not interfere with cell metabolism and rather protect the cell against osmotic imbalance. It has also been shown that oral doses of some of the osmolytes monitored in patients with hepatic disorders have shown positive responses.

These molecules significantly affect the thermodynamic equilibrium properties of proteins and are often used for studying the structure and dynamics of protein folding mechanism playing a vital role in determining the stability of the protein. But most of such studies involved ensemble measurements and only very recently single molecules techniques are being used for investigating protein stability. Mechanical single molecule techniques offer exciting possibilities for investigating protein folding and stability in native environments at sub-nanometer resolutions. The single molecules without inherent symmetry can directly be monitored in their physiological conditions using atomic force microscopy (AFM).

The aim of this work was to investigate the stabilising effects of compatible solutes, ectoine, betaine and taurine on the membrane protein Bacteriorhodopsin at different concentrations. Using Atomic Force Microscopy based Force Spectroscopy their impact was quantified by measuring the forces required to pull the protein out of the membrane and the change in the persistence length of the unfolded polypeptide chain. Increasing unfolding forces were observed indicating the strengthening of intra-molecular interactions, which are vital for the protein stability. A decrease in persistence length was recorded showing the increasing tendencies of the polypeptide strand to coil up. Interestingly, it was revealed that these molecules have different stabilising effects on protein unfolding at different concentrations.

This allows us to predict the mechanism of interaction between the unfolded polypeptide chain of Bacteriorhodopsin and the osmolyte. The osmolytes are expelled from the protein surface due to the increase in chemical potential of the exposed stretched state forcing the protein to acquire a more compact structure.

This information is aimed to achieve a better understanding of the interaction between the unfolded protein and the solutes. The results show that the unfolding of single proteins provides in-depth and crucial information about their structure-dynamic relationship at sub-nanometer scale and will open new paths for our further studies regarding the effects of

compatible solutes on other membrane proteins.

We expect that these results can further provide information which can directly resolve the transient intermediate states and multiple reaction pathways. Additionally, the results can be useful for studies related to the characterization of the complex dynamics of protein folding. Thus, this study is set to provide exciting possibilities in the field of drug development for liver diseases including *in vitro* rescue of the misfolded proteins and to directly analyze and correlate their structural and functional properties at the sub-molecular level.

Zussamenfassung

Kompatible Solute sind kleine organische Moleküle, die von Mikroorganismen in der Zelle produziert und akkumuliert werden, um verschiedene Arten von umweltbedingtem Stress entgegenzuwirken. Diese Moleküle sind für ihre ungeladene, zwitterionische und osmotisch-aktive Natur bekannt. Des Weiteren weisen sie eine osmotische Aktivität auf, die selbst bei molaren Konzentrationen nicht den Zellmetabolismus beeinträchtigt und stattdessen die Zelle gegen osmotisches Ungleichgewicht schützt. Es wurde gezeigt, dass oral verabreichte Dosen von einigen Osmolyten an Patienten mit hepatischen Funktionsstörungen positive Reaktionen hervorriefen.

Diese Moleküle beeinflussen signifikant die Eigenschaften des thermischen Gleichgewichts von Proteinen und werden oft verwendet, um die Struktur und die Dynamik des Proteinfaltungsmechanismus, der eine lebensnotwendige Rolle in der Bestimmung der Proteinstabilität spielt, zu erforschen. Allerdings beinhalten die meisten dieser Studien Ensemble-Messungen und erst in letzterer Zeit werden Einzelmolekültechniken zur Erforschung der Stabilität von Proteinen verwendet. Mechanische Einzelmolekültechniken ermöglichen faszinierende Möglichkeiten zur Erforschung der Proteinfaltung und der Proteinstabilität in ihrer nativen Umgebung mit einer Auflösung im Nanometerbereich. Einzelne Moleküle ohne eine inhärente Symmetrie können direkt unter physiologischen Bedingungen durch das Rasterkraftmikroskop (englisch: Atomic Force Microscope, AFM) beobachtet werden.

Das Ziel dieser Arbeit war die Erforschung des stabilisierenden Effekts von kompatiblen Soluten, nämlich Ectoine, Betaine und Taurine auf das Membranprotein Bacteriorhodopsin bei verschiedenen Konzentrationen. Die Kraftspektroskopie basierend auf dem Rasterkraftmikroskop wurde verwendet, um den Einfluss dieser Moleküle zu quantifizieren, indem die Kräfte gemessen wurden, die erforderlich waren, um ein Protein aus der Membran zu entfalten, sowie die Veränderung in der Persistenzlänge der gefalteten Polypeptidkette. Es wurden ansteigende Entfaltungskräfte beobachtet, die auf eine Verstärkung der intramolekularen Wechselwirkungen, welche essenziell sind für die Stabilität des Proteins, hinweisen. Des Weiteren wurde eine Abnahme in der Persistenzlänge festgestellt, was auf eine steigende Tendenz des Polypeptids sich zusammen zu knäulen hindeutet. Interessanterweise konnte gezeigt werden, dass die untersuchten Moleküle verschiedene Einflüsse auf die Proteinentfaltung bei unterschiedlichen Konzentrationen haben.

Das erlaubt uns eine Vorhersage des Mechanismus der Wechselwirkung zwischen der

gefalteten Polypeptidkette des Bacteriorhodopsins und den Osmolyten zu machen. Die Osmolyte werden von der Proteinoberfläche des freiliegenden, gedehnten Zustands aufgrund des steigenden chemischen Potentials ausgeschlossen, was das Protein zwingt eine kompaktere Struktur anzunehmen.

Diese Informationen zielen auf ein besseres Verständnis der Wechselwirkung zwischen dem entfalteten Protein und den kompatiblen Soluten. Außerdem zeigen die Ergebnisse, dass die Entfaltung von einzelnen Proteinen detaillierte und entscheidende Informationen über den strukturdynamischen Zusammenhang im Nanometerbereich liefert und neue Wege für weitere Studien hinsichtlich des Effekts von kompatiblen Soluten auf weitere zu untersuchende Membranproteine erschließt.

Wir nehmen an, dass diese Ergebnisse ferner Informationen liefern können, welche die kurzlebigen Zwischenentfaltungsschritte und multiplen Reaktionswege der Proteine direkt klären können. Außerdem können die Resultate nützlich für Studien bezogen auf die Charakterisierung der komplexen Dynamik der Proteinfaltung sein. Deshalb liefert unsere Studie spannende Möglichkeiten in dem Bereich der Arzneimittelentwicklung gegen Lebererkrankungen, einschließlich der *in vitro*-Rettung von fehlgefalteten Proteinen, und um die strukturellen und funktionellen Eigenschaften von Proteinen auf der submolekularen Ebene zu analysieren und in Beziehung zu setzen.

Publications

1. **Studying Bacterial Membrane Proteins with Single-Molecule Atomic Force Spectroscopy**
   Roychoudhury, A. and Oesterhelt, F., Microscopy and Analysis (2011) *25*, 15-17.

2. **Effect of the Compatible Solute Ectoine on the Stability of the Membrane Proteins**
   Roychoudhury, A., Häussinger, D., Oesterhelt, F. Protein Pept. Lett. (2012) *19*, 791-794.

3. **Concentration dependent Membrane Protein Stability under the Influence of Different Compatible Solutes Studied by Single Molecule Force Spectroscopy**
   Roychoudhury, A., Bieker, A., Häussinger, D., and F. Oesterhelt, Biol. Chem
   (Under revision)

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Chapter 1 Introduction

The earliest form of rich life on our planet earth is recorded to have begun almost 4 billion years ago. Today, about 1.75 million species of animals and plants exhibit the complexity of life to survive under the very favorable as well as extreme and hostile environmental conditions. Despite the complexity and diversity of nature, the numbers of building blocks used are minimal. Of over the hundreds of chemicals known, biomolecules are composed of only six elements (C, H, O, N, S, P) which represent the atomic raw materials in the living matter according to the principles of molecular recognition and self-assembly. Different inter and intra molecular interactions between the individual biomolecular building blocks lead to the formation of simple to complex systems such as single cells as well as higher organisms. The understanding of life processes at molecular level as an area of research in classic science has gained a lot of importance recently. Inter and multi-disciplinary interactions between various sciences enable the full exploration of complex biomolecules and their interaction.

The biomolecules take up about 30% - 40% of the interior volume of a cell which leads to a crowded environment inside the cell. Life is established through the specific intra and intermolecular interactions in an aquous environment. Understanding and comprehending the effects of additives from external source or synthesised internally in a cell is very important for broader scientific research because of the diverse kinetic and thermodynamic nature of the biomolecules (Minton, 2000; Ellis, 2001). The knowledge and information gained from such studies prove to be very vital for medical, biolochemical and other scientific fields.

The stressed conditions in a system can be mimicked by adding different kinds and concentrations of osmolytes into the aquous buffer which retain the physiological properties as well as create a crowded environment. One of the most important biological macromolecules acting as the building block in living organisms is protein.

Proteins are hetero-polymers composed by twenty different naturally occurring monomers, known as amino acids. These amino acids are bound together by peptide bonds and for this reason they are also known as polypeptides, which define the primary structure of a protein. The amino acids have two main components, namely

(i) a backbone forming region, identical for all amino acids which, by binding to the previous and to the following monomer, is forming the polymer chain, and

(ii) a side chain.

These lateral groups interact with each other along the same polypeptide. Functionally folded

conformation and structure is essential for proteins to carry out crucial functions inside a cell. These interactions between different domains in a polypeptide chain are the driving forces that fold the chain in a unique three dimensional conformation commonly called as the folded conformation (Bowie *et al*., 1991). The information of the linear amino acid sequence is thus transferred into a more complex interaction pattern producing the final functional protein form. An important detail to outline is that, except for some particular cases (disulfide bonds), these interactions are weak, i.e., non-covalent. The hydrogen bonds and the hydrophobic interactions are the two key interactions playing vital role in folding of proteins. The former allows the chain to form locally ordered structures (alpha -helices, beta-sheets) via hydrogen bonds between nearest neighboring amino acids and the latter, due to the burying of the hydrophobic residues inside the structure to exclude water. Hydrophobic interaction helps in keeping the protein alive and retain its biological activity by allowing the protein to decrease its surface area which results in exclusion of water. This reduces the undesirable interactions with water and stabilises the whole structure (Atkins *et al*., 2006).

The energy of these interactions is in the form of thermal energy, making the proteins susceptible to external stress. It is a well known fact that proteins, if subjected to very high temperature, high solute concentration or exposed to extreme pH, unfold and often alter their three dimensional structure to another one which is no longer biologically functional, denatured or dead, or, even toxic for the cell.

The high efficiency of the proteins in performing their biological functions depends on the structural flexibility of the protein which allows them to change and acquire different conformations to carry out different kinds of biochemical reactions inside the cell. A covalently stabilised compact protein structure would be for example, much more thermally stable but, very rigid making it inefficient in performing any other function than scaffolding.

A wide class of different proteins exists in the cell accomplishing much diverse range of tasks round the clock like cell signaling, catalysis of enzymes, in DNA replication, ATP synthesis, transportation of molecules to different parts of the cell and also to other neighbouring cells, refolding other proteins or degrading them etc. In all these reactions, the structural flexibility plays a vital role. Physical and chemical stability is also very important for retaining the dynamic behaviour of proteins by which they change the conformation. By doing this, proteins can convert chemical energy into mechanical energy required for carrying out different biochemical pathways. Furthermore, they also use their dynamic behaviour and structural flexibility to regulate their own activity.

Protein studies have been in the focus of research interest in natural sciences because of their

importance as the molecular machine of life, the molecules life form is made up of and we are made up of. The interesting characteristics and dynamics of protein behavior can be studied by various biophysical techniques and methods. With the advent of nanotechnological advances various new techniques were developed and eventually their application is increasing.

Nanotechnology is considered one of the key technologies of the 21st century. The first definition of nanotechnology given by Taniguchi in 1974 was, *"Nanotechnology mainly consists of the processing, separation, consolidation, and deformation of materials by one atom or by one molecule"* (Taniguchi, 1974). It is not an individual or independent research area but a generalised and broader technology which encompasses and includes close cooperation with varied fields of natural science. Although nanotechnology is commonly associated with electronics and physics, it is getting more popular also in other fields of fundamental researches involving increasing shelf life of food items, developing make up items and so on. The study of small structure at micro and nano level has gained much curiosity and been proven of economic importance as well. The application and scope of nanotechnology ranges from nanoelectronics to semiconductor devices, to medical applications and high end gadgets to name a few. Nanotechnology is a multidisciplinary technology encompassing various disciplines of science and technology. For the widespread and most efficient use of the developments in this field a very close collaboration of different branches of research is required (Drexler, 1986).

In the field of nanotechnology, the single-molecule visualisation techniques that are used to determine protein structure and dynamics have very recently evolved and are being extensively exploited by scientists. One of the widely used instruments is the Atomic Force Microscope (AFM). It is an extensively used tool for imaging, measuring and manipulating matter at nanoscale and in turn has inspired other scanning probe techniques. AFM is also expected to be one of the major approaches to play an important part in enabling the wide application of nanobiotechnology and medicinal studies, in particular to membrane proteins which form the largest class of drug targets.

For studying the structure and dynamics of biological macromolecules in solution, scanning probe microscopy is considered a very powerful technique. This technique can be used in varied ranges of biology, from visualization of transcription in real time to mechanical manipulation of a single molecule. Through imaging or forced spectroscopy, single molecule methods provide a wider view of biological properties leading to a variety of property options to investigate. These techniques help in the determination of forces, elasticity, adhesion,

dynamics and motion of molecules. Single molecule methods may reveal the mechanics of unfolding proteins as influenced by adding co-solutes to the system preventing at the same time, the natural tendency of proteins to aggregate in crowded environments because of low the low concentration of protein molecules used in the solutions (Ellis and Minton, 2006). Such measurements at molecular level help in the deeper understanding of the physio-chemical principles governing the macromolecular interactions with the small molecules present in the surrounding inside the cells.

The primary objective of this project was to characterize the effects of compatible solutes on the unfolding of individual protein molecules using single molecule methods. In order to ensure that the induced effect is the result of interaction among protein, particular osmolyte and water, correlating with the nature and concentration dependency, the aqueous standard buffer mimicking the physiological condition was used with a single type of non-interacting osmolyte for every measurement (in this case referred to as compatible solutes) at different concentrations. The next chapter henceforth deals with objective, study and motivation behind this project.

Chapter 2 Biological Motivation

Osmolytes that are compatible with metabolic activities in a cell are known as compatible solutes. This study focuses on the effects, which compatible solutes have in membrane protein stabilisation. Compatible solutes are also known to stabilise protein structures and support protein folding. These molecules displace the solvent molecules, thereby affecting protein folding. We aimed to understand whether biological effects on the cellular level induced by an increase or decrease in the solute level, respectively, may be explained by the direct interaction of the compatible solutes with the membrane proteins under investigation. To accomplish this we decided to test the effect of compatible solutes, betaine, taurine and ectoine on stability and folding of different membrane proteins like Bacteriorhodopsin. Using Atomic Force Microscopy and Force Spectroscopy we investigated the effects in presence and absence of the osmolytes. First, the stabilisation of secondary structure elements of the membrane proteins by forced unfolding experiments and second, stiffening of the unfolded amino acid chains due to solvent activity by measuring the polymer elasticity. In this chapter we shortly look at the biological role of osmolytes in general which is discussed in detail in the next chapter. We also take an elaborate look at the immediate objective as well as long term and detailed goals of this study and the significance of undertaking this particular research project.

2.1 Introducing the role of osmolytes in protein stabilization

Proteins play a key role in maintaining life processes and hence understanding their function is crucial to unravel the mechanism of the biological processes carried out by cells and also the diseases caused by them when they do not function well. Yet the molecular functions of most proteins that determine the origin and cause of various diseases have only been partially understood. Membrane proteins are of great interest for scientists because they constitute about 30% of all proteins in eukaryotic cells. However, the mechanism by which membrane proteins insert and fold into the membranes is not well understood.

Most of the techniques available protein structure determination and characterization like chromatography, capillary electrophoresis, PAGE electrophoresis, x-ray crystallography and electrospray mass spectrometry, NMR which characterize proteins are mostly designed to study soluble proteins. But membrane proteins are not water soluble, and they denature outside the membrane which make them difficult to handle. So far, these techniques have been adapted to membrane proteins with only limited success. New techniques designed from

the ground up for membrane proteins are needed, which brings chemistry and biology together. Being responsible for membrane related functions, for example the interaction between different cells or the cell and its environment, make the study of membrane proteins highly important as well as challenging. Mutations or the misfolding of membrane proteins resulting in a malfunction can be related to neurodegenerative diseases like Alzheimer's disease (Song *et al*. 2007; Song *et al*. 2009), making knowledge about their functionality and constitution even more valuable. Due to their high sensitivity and selective nature, membrane proteins are also often used as biosensors (Atanasov *et al*. 2005).

Since membrane proteins are harder to express and handle than soluble proteins, specialized expression, solubilisation, and stabilisation techniques are required with the focus on the biochemistry and biophysics of membrane proteins and membranes. Our main interest is to examine folding and stability of membrane proteins in membranes, i.e. the mechanisms and physical principles of membrane protein folding in presence of different compatible solutes.

Besides studying the stabilising properties of osmolytes on membrane proteins using Bacteriorhodopsin as the model protein, this study also interestingly throws light on the prokaryotic stress management mechanisms during environmental shocks.

The characterization of macromolecular effects has gained attention and awareness in the scientific community producing numerous results which can be used in different fields of natural science and beyond. This study was set to characterize the mechanical properties of membrane proteins in aqueous solution with osmolytes of different types at different concentrations, analysing the effect of the relative concentration of the solutes on the unfolding forces as well as induced changes in the free energy of the membrane protein Bacteriorhodopsin.

Previous studies have established that osmolytes modulate the activity of molecular chaperones (heat-shock proteins) probably by promoting the local refolding within the chaperone protein molecules, suggesting a link between the chemical and molecular chaperones in regulation of protein folding *in vivo* (Diamant *et al.,* 2001). So, from these studies it is understood that various biological processes including protein-protein interaction, folding and refolding of the protein as well as disaggregation of protein are regulated directly or indirectly by accumulation of specific osmolytes inside the cell. But the mechanism by which these osmolytes act and modulate protein functions is still not clear. In the recent years, the mechanism of osmolyte action and their compatibility with cellular metabolism at molar concentration and the ability to induce stabilization of protein has attracted considerable attention of the scientific community in general.

Furthermore, the fact that Bacteriorhodopsin is a bacterial protein present in the class of halo-bacteria that is found in high saline regions also makes it interesting to study the stability of this protein under the influence of compatible solutes as it could also be interesting to see how bacterial cells neutralize and counteract the negative effects of environmental stress.

2.2 Goal and Significance of this Project

The long term goals of this project are to elucidate the structural properties, quantitatively examine the mechanical and biophysical properties of the membrane protein stabilization taking Bacteriorhodopsin as the model membrane protein and to understand the interplay between protein and protecting osmolytes on Bacteriorhodopsin's mechanical function in different environments that mimic *in vivo* conditions. These information and approaches provide basis for our further studies regarding the effects of compatible solutes on other membrane proteins of medical importance particularly in case of liver diseases, which can directly resolve transient intermediate states and multiple reaction pathways, and thus are uniquely powerful in characterizing the complex dynamics of protein folding. Thus, this study, furthermore, has the potential to provide new therapeutic approaches (using compatible solutes and organic osmolytes) and is set to provide exciting possibilities in the field of drug development for liver diseases including in vitro rescue of the misfolded proteins and to directly analyse and correlate their structural and functional properties at the sub-molecular level.

This project on a broader perspective mainly aims:

(i) **To determine the effects of naturally occurring osmolytes (compatible solutes) on the stability of membrane protein Bacteriorhodopsin.** The results are described in the Chapter 8.

(ii) **To understand the mechanism by which organic osmolytes affect the mechanical properties of individual Bacteriorhodopsin molecule and establish the system for other membrane proteins of medical relevance.** The results are described in the Chapter 8.

To investigate the influence of the osmolytes, the non-interacting naturally occurring osmolytes were chosen so that they do not interfere with the cell metabolism even at molar concentrations. To perform these extensive investigations and initial characterization of the protein stability, the membrane protein was chosen as the sample to be studied because it is one of the most studied membrane protein and represents this class of protein very efficiently. Thus, the experiments aimed to probe mechanical stability of bacteriorhodopsin and to

determine its unfolding events were designed and conducted.

The big advantage of single molecule techniques over other techniques used in such works is that, looking at one molecule at a time this technique helps analyzing behaviour of every single molecule, while in case of ensemble experiments, the measured properties are averaged over a huge amount of molecules like in classical bulk techniques. Thus, the interpretation of the results is not as strongly dependent on assumptions as it is often the case for bulk measurements.

Several methods have been used to address the study of macromolecular and co-solute effects, from traditional bulk to recently developed single molecule methods. Bulk methods explore the average properties of the whole ensemble of biomolecules, extracting information about the status of a biochemical process. These methods answer questions, for example, on folding and refolding rates of proteins using chemical denaturants, like urea, or physical denaturants, such as temperature. But there are other properties that are waiting to be explored. Mechanical properties of proteins, elastic properties of cellular membranes and many others are not measurable with the usual experimental methods. Measurements of this effect at the molecular level could lead to a deeper understanding of the physical principles governing the molecular interactions in cells.

Our study involving single molecule methods hence aims to understand and to reveal how the mechanics of unfolding proteins are influenced by adding compatible solutes to the system preventing at the same time, the natural tendency of proteins to aggregate in crowded environment.

So, by detailing and breaking down long term goals into short term objectives carried out each year, we designed experiments that use osmolytes–protein (Bacteriorhodopsin) mixtures in a threefold agenda, which are:

(i) to understand and describe the molecular mechanism for osmolyte action on biologically relevant macromolecules;

(ii) to show the use of osmolytes in affecting macromolecular (membrane protein) stability;

(iii) and to demonstrate how osmolytes also serve as probes of the forces acting at and between macromolecular interfaces.

However, due to the interactions among the protein and water, protein and osmolyte, and osmolyte and water within the system, this is a very complicated mechanism and phenomenon to understand and interprete. Hence it is still unclear and various hypotheses prevail.

This dissertation discusses briefly the introduction to the work in Chapter 1. The motivation

behind taking up this project and the goal set for this PhD study is discussed in Chapter 2. An overview of the osmolytes and their applications are discussed in Chapter 3. Chapter 4 focuses on membrane proteins, their structure and composition with special reference to Bacteriorhodopsin and its properties. An elaborate description of the theory and principle of Atomic force microscopy and imaging modes are discussed and illustrated in Chapter 5. In Chapter 6 emphasis is given on Single Molecule Force Spectroscopy and the underlining principle. Materials, methods, experimental procedures and data analysis using the software Igor are described in Chapter 7. Chapter 8 shows the results obtained during the course of this work. Chapter 9 discusses the results obtained and their probable explanations based on established theories and new findings drawing a general conclusion, respectively. The final chapter Chapter 10 summarizes the whole work, infers a general conclusion from the findings of this work and also gives an outlook and perspectives of future research in this field of work.

Bolen and co-workers, one of the pioneers in the field of osmolyte studies presented a general mechanism of stabilising as well as destabilising osmolytes by expanding the underlying principles behind the Tanford's transfer model (Tanford, 1964) by classifying solvents as good and bad solvent (Bolen and Rose, 2008). This accounts for the thermodynamic stabilisation caused by the both side chains and backbone of the protein during a crowded and stressed condition (Zhou, 2008a; Zhou, 2008b; Linhananta *et al*, 2011).

We hope that the work carried out during this project associate and expand some of the theories stating the mechanism of protein stabilisation and bring together the different concepts existing in the field of protein folding, impact of osmolytes on it as well as stress mechanism in living organisms as well.

Chapter 3 Osmolytes and Compatible Solutes

Some organic osmolytes are called compatible solutes since these solutes (except urea) do not interfere with macromolecules in harmful and detrimental ways, and hence they can be safely up- and down regulated with negligible impact on cellular structure and functions (Brown and Simpson, 1972; Yancey *et al*., 1982).

3.1 Introduction

Microorganisms produce and accumulate osmolytes in the cytoplasm to protect themselves from environmental stress. Osmolytes are small molecules having slow molecular weight are mostly either uncharged or zwitterionic organic molecules including polyols, sugars, methylamines, urea, amino acids and their derivatives (Yancey, 2001). These types of solutes are soluble in water and help maintaining osmotic balance without interacting with the essential cellular processes. Since they have relatively little effect on the cytosolic ionic strength, no special adaptation of the molecular pathways is required even at molar concentrations (Za´vodszky *et al.,* 1995 and Yancey *et al.,* 1982).

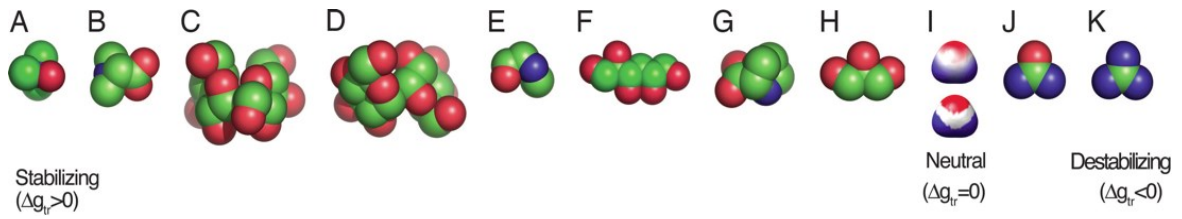

Figure 3-1: Molecular Structure of different osmolytes.

Protecting osmolytes are TMAO, betaine, sucrose, trehalose, sarcosine, sorbitol, proline, and glycerol (A–H), and denaturants are urea and guanidine (J–K). Compounds are ordered by their measured Δgtr values (the free energy change that accompanies the transfer of a backbone unit from water to a 1 M osmolyte solution.) (Taken from Street *et al*., 2006).

Glycine betaine, proline, trehalose, mannitol are some of the abundantly occurring osmolytes present in plants (Tarczynski *et al.*, 1993). Trimethylamine-N oxide has exhibited an extraordinary capability to fold denatured proteins to native-like species, and is found in sharks, presumably there to keep proteins in the functionally folded states in the presence of the high tissue urea levels found in such creatures (Yancey, 2004). Certain classes of osmolytes, found in some organisms and in mammalian kidney cells, known as counteracting osmolytes, are known to counteract the effects of high intracellular concentrations of urea (Garcia-Perez *et al.,* 1990). Human kidney contains several osmolytes, including glycine

betaine, glycerophosphoclidine, sorbitol, inositol, and taurine (Burg, 1996; Kumar *et al*., 2001). Out of these, because of their relevance in medical studies and human diseases, we used betaine and taurine in our experimental work. Betaine occurs almost in every realm of life, and taurine is used vastly by marine animals and some mammalian organs. But also in addition to these uses there is a multitude of organs and cells which use a combination of different classes of osmolytes. For example, taurine, betaine as well as polyols like sorbitol and myo-ionositol as well as high concentration of urea co-exist inside the mammalian kidney and play role in exclusion of waste products and concentrating urine in the body.

This group of osmolytes works by counteracting the effects of denaturing osmolytes like urea on the biological processes and functions. For example, urea tends to decrease the catalytic rate constant, $\boldsymbol{k}_{cat}$ and increase the Michaelis – Menten constant, $\boldsymbol{K}_m$ of enzymatic reactions, while the counteracting osmolyte tends to have the opposite effect (Wang *et al*., 1997). Under stressed conditions, the concentration of osmolytes reach very high level inside certain cell compartments (Baptista, *et al.,* 2008; Burg *et al*., 1996). For example, in extreme physiological or environmental conditions, the desert mouse can accumulate counteracting osmolytes inside its kidney up to a concentration of 2.5M to counteract the effects of urea, which may reach up to 5M concentration under such conditions. This allows the retention of structure and function of essential proteins (MacMillen *et al.,* 1967). Depending upon the requirements of the cell and the environmental conditions, some tissues and cells have higher osmolyte concentrations than others (Wang *et al*., 1996). In the field of biology of adaptation, the common agreement is that when osmolyte-induced folding is cooperative in nature, protein dramatically adopts native-folded structure (Baldwin *et al.* 1999**,** Street *et al.* 2006), and are also known to be widely used by cells and organisms to participate in cell volume regulation (Khan *et al*., 2010).

Some of these osmolytes have been shown to have a destabilizing effect (such as urea). Denaturing osmolytes, as its name indicates, favors the unfolded state by lowering the free energy whereas others are known as protecting osmolytes (such methylamines and polyols), because they counteract the destabilizing effect of urea or other osmolytes. Protecting osmolytes raise the free-energy of the unfolded state favoring the folded or native state.

Urea is an organic osmolyte found in nature, mostly in kidney of humans and other mammals. It has been extensively studied for its denaturing properties on proteins (Tanford 1968; Greene *et al.* 1974; Santoro *et al.* 1988; Schellman 2002). Urea has been found to be directly or indirectly affecting the protein structure by destabilising it. Hence it is also known as denaturing osmolyte (Caballero-Herrera *et al.* 2005). It can indirectly modulate the protein

structure by interacting with the water structure and altering it. This is done through hydrophobic interactions (Bennion *et al.* 2003; Idrissi 2005; Daggett 2006). The second possibility is to directly interact with the hydrogen bonding of both the backbone and side chains of the polypeptide. This facilitates the unfolding of protein by contributing to negative free-energy (Wu *et al.* 1999; Mountain *et al.* 2003; Auton *et al.* 2005). In 2007, Beck *et al*., showed that urea-induced denaturation may occur through a combination of both indirect and direct actions on proteins (Beck *et al.* 2007; Ma *et al*., 2010).

Stabilising or protecting osmolytes on the other hand are known to stabilise and enhance protein folding thermodynamically and protect the protein against denaturing effects of urea

It has been shown that protecting osmolytes, such as TMAO, sorbitol, sarcosine and glycerol, can stabilize the native state (Auton *et al.* 2005; Street *et al.* 2006; Garcia-Manyes *et al.* 2009); however, the molecular origin of these effects still remains fairly very little understood.

By accumulating high concentrations of osmolytes, organisms adapt to perturbations that can cause structural changes in their cellular proteins. Osmolytes shift the equilibrium towards natively-folded conformations by raising the free energy of the unfolded state.

The stabilising osmolytes exert a force that causes the protein to co-operatively fold into a native-like functional conformation from an unstructured conformation in aqueous environment (Schellman, 2002; Baskakov *et al.,* 1998). Several studies have demonstrated that the stabilizing property of naturally occurring osmolytes correlate with the preferential exclusion of these osmolytes from the vicinity of unfolded or denatured protein which result in the formation of a denser and more structured water shell (preferential hydration) around the stretched protein (Xie *et al*., 1997a; Xie *et al*., 1997b).

Naturally occurring osmolytes are one of the most potent stabilizers for many proteins and are capable of reversing protein misfolding and or aggregation, which are known to be underlying cause of several diseases (Lamitina *et al*., 2006). Osmolytes have also been found to modulate activity of molecular chaperones (heat-shock proteins) probably because of the promotion of local refolding within the chaperone protein molecules, suggesting a link between the chemical and molecular chaperones in regulation of protein folding *in vivo* (Diamant *et al.,* 2001). Therefore, it is seen that by accumulating specific osmolytes at various concentrations cells regulate the multitude of biological processes like protein folding pathways, protein-protein interactions, aggregating mechanisms of protein and so on.

In recent years, the mechanism of osmolyte compatibility and osmolyte-induced stability has attracted considerable attention. Regulation of cell volume significantly contributes to the

pathophysiology of several disorders, and cells respond to these changes by importing, exporting, exchanging or synthesizing osmolytes to maintain volume balance and homeostasis.

Compatible solutes are the osmolytes that are organic molecules that affect the stability properties of proteins either by pushing towards the native state or favoring the denaturalized state. These types of solutes help maintaining osmotic balance without interfering with the essential cellular processes. Since they have relatively little effect on the cytosolic ionic strength, no special adaptation of the molecular pathways is required even at molar concentrations (Brown, 1990, Oren, 1999).

3.1.1 Compatible solutes under study

In our studies, we used three different compatible solutes namely, ectoine, betaine and taurine and studied their effect on the mechanical stabilization of the membrane protein Bacteriorhodopsin using unfolding experiments carried out by Atomic force microscopy based force spectroscopy.

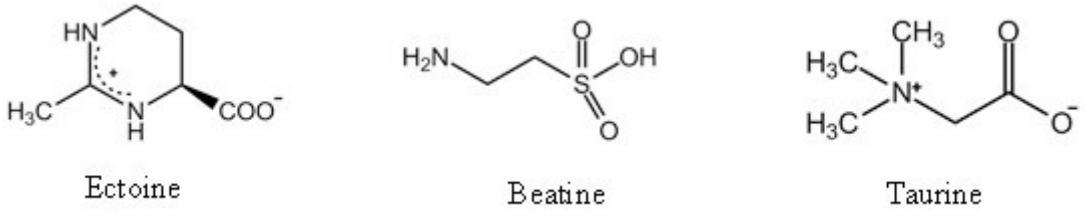

Figure 3-2: Chemical structures of osmolytes (Compatible solutes) used in this study.

Ectoine is a heterocyclic amino acid that serves as a protective substance in many bacterial cells (Galinski *et al*., 1985; Galinski, 1995; Knapp, 1999). It allows microorganisms to resist extreme living conditions like drastic temperature variations (Oren, 1999; Zhang *et al*., 2006; Calderon *et al*., 2004), high salinity (Yancey *et al*., 1982) and osmotic shocks (Bolen, 2001; Roessler *et al*., 2001). Hence it is also known as an osmoprotectant, which is obtained from halophototrophic bacteria. These effects are also based on the direct stabilisation of cytoplasmic proteins against denaturation (Arakawa and Timasheff, 1985; Oberdörfer *et al*., 2003; Göller and Galinski, 1999). However little is known about the stabilising effects of ectoine on membrane proteins.

Betaine is a medically relevant compatible solute which is also present in human body and is zwitter ionic at neutral pH. It has been of greater medical importance because of its role in

osmoregulation in humans and mammals. Many naturally occurring betaines help biological systems to protect themselves against osmotic stress, drought, high salinity or high temperature. Betaine plays a vital role in regulation of liver function, maintaining protein structure and membrane integrity, cellular reproduction and also permits water retention in the cells under high osmotic pressure thus protecting the cell from dehydration. It is medically approved to be administered orally to treat heart and liver diseases.

Taurine is a major constituent of bile and was first isolated from ox bile. It is a derivative of the sulfur-containing (sulfhydryl) amino acid cysteine. It is present in mammalian milk and in food, especially in seafood and meat, to name a few sources of it. Taurine is also known for its myriad of role in regulating cardiac functions, blood pressure, cell metabolism, body temperature, synthesis of bile acid and salts to name a few. Some of these activities are believed to be caused due to taurine's effects on membranes as well as on phosphorylation of protein. Hence it was considered and chosen to be one of the osmolytes in our work to study its effect on membrane protein stabilization (Schaffer *et al*., 2000).

3.2 Applications and Uses

Many remarkable applications, uses and effects of osmolytes have been reported so far. Most of the protecting osmolytes are known to stabilise the folded state of protein, counteract osmotic stress and denaturing effects etc. Although the molecular details and insights of the stabilising phenomena are still inadequate, these small molecules are of much interest and are in use both in scientific research as well as commercially. In this section, we take a look at their varied applications and uses.

3.2.1 To study the physiological role of osmolytes

To study the physiological and biochemical effects of osmolytes, their solvation properties are investigated. This probing of solvation properties is useful because the effects of osmolytes on other molecules in-vivo and in-vitro are governed by their solvation behaviour. In humans there are various instances where osmolytes play an important pathological role. Best known example is the kidney where it is known that kidney cells accumulate organic osmolytes in order to protect themselves from the high concentrations of NaCl and urea (Garcia-perez and Brug 1991). Furthermore, role of a certain osmolyte (TMAO) in Alzheimer's disease was found (Scaramozzino *et. al*. 2006) was also studied.

3.2.2 To test the molecular properties of biological macromolecules

Osmolytes are very well known for their application in probing molecular properties. The

resence of these molecules in an environment where the biomolecules are present has been found to be beneficial for the bio macromolecules because of the stabilising properties of osmolytes. But in special cases, if destabilising osmolytes are present or the stabilising osmolytes are not used properly, the effects may not be as positive. One of the best-known examples is the use of urea-induced unfolding to determine the stability of proteins (Pace and Shaw, 2000).

3.2.3 Influence on protein folding

More recently, osmolytes were used for the reverse, osmolyte-induced forced folding of intrinsically unstable proteins. This procedure also allows one to determine the stability of proteins (Baskakov and Bolen, 1998). Other uses of osmolytes include probing solvation properties of macromolecules and to measure forces within and between large macromolecular aggregates such as DNA and stacks of lipid membranes (Parsegian *et al.,* 1995).

3.2.4 As additive molecules in different buffers

Osmolytes are often used as an additive to solubilize protein. Salts are most commonly used for this purpose.

3.2.5 Osmolytes as stabilizers, precipitants, solubilizers, or denaturants

Osmolytes' usage is diverse. It varies from stabilizers to denaturants. This depends on the balance between osmolyte's interactions with the protein's side-chain and backbone respectively. Urea for e.g. acts favourably with both peptide backbone and amino acid side-chains. In urea solution, the proteins tend to maintain a state with increased surface exposure: the denatured state, thus exemplifying Le Chatelier's principle. In fact, urea normally solubilizes proteins (Bolen, 2004), because dissolved proteins expose more surface compared to precipitated proteins. Another osmolyte namely guanidine hydrochloride (GdnHCl) is both a denaturant and solubilizer of proteins. As would be obvious these dual effects of osmolytes to denature and solubilize proteins become a problem. However there is another alternative to GdnHcl namely arginine. Arginine solubilizes proteins without strongly denaturing them (Arakawa and Tsumoto, 2003). Proline is another such example of a solubilizer since it favourably interacts with the side-chain and unfavourably with the backbone.

Stabilising osmolytes also tend to be precipitants due to the overcompensation of the osmolyte-side chain interactions over osmolyte-backbone interactions (Auton and Bolen, 2005; Bolen, 2004). These effects of stabilising osmolytes can however be partially overcome

by mixing with a solubilizer like proline that does not denature proteins (Kumar *et al*., 2001; Harries and Rösgen, 2008).

3.2.6 Commercial uses

The addition of betaine to polymerase chain reaction (PCR) enhances the amplification of DNA by reducing the formation of secondary structure in GC-rich regions. The addition of betaine has been reported to enhance the specificity of the polymerase chain reaction by eliminating the base pair composition dependence of DNA melting (Henke *et al*., 1997). Osmolytes like ectoine, taurine etc. are also known for their industrial applications. Of late, ectoine has been used as a protective substance in sunscreen lotions and also in some other cosmetic products. Taurine has also been introduced as a composition in some cosmetic items. This is possible due to their protective and antifibrotic properties. They have been shown to help maintain skin dehydration (Janeke *et al*., 2003). Taurine is also used in many infant food products and some contact lens solutions (James *et al*., 2004).

3.3 Mechanism of action of osmolytes and previous works

The folding and arrangement of a protein into its proper three dimensional native structures, the formation and assembly of complexes containing various macromolecules are very crucial for proper functioning of macromolecules. Macromolecular interactions in proteins are controlled and determined by not only the amino acid sequences but largely also by the small molecules and particles around the protein (Yancey, 2004; Yancey, 2001). Osmolytes are not of one kind. There are various classes of such small molecules which are accumulated inside the cell at low to moderate and more commonly at very high concentrations which without interfering with the biochemical processes of the cell protects the cell against different kinds of environmental stresses (Yancey, 2005).

The magnitude of interaction of osmolytes and water with protein and other macromolecules, whether weak or strong can be determined by their exclusion or inclusion from the macromolecular surfaces in the solution. The excluded volume is although is mostly limited to the immediate proximity of the macromolecular surface but can also perturb many solvation layers in bulk solution. The burial of the polypeptide backbone further enhances the compactness of the conformation of the macromolecule resulting in its stronger stabilisation by reducing the interfacial surface area. This can be explained as a consequence of Le Chatelier's principle. Solute addition destabilizes the protein by increasing its chemical potential, so that burial of those destabilizing surfaces is favored. And if we think about the cosolute: the burial of surfaces will cause less osmolyte exclusion, and again the perturbation

is reduced. As much the folded *versus* denatured state of a protein is stabilized, binding to proteins is strengthened by excluded solutes, too. Destabilising osmolytes like urea push the protein towards denaturation where the protein shows a favourable preferential interaction with the osmolyte molecules (Harries and Rösgen, 2008).

Asakura and Oosawa in 1954 and Vrij in 1976 were among the firsts to describe the entropy induced depletion responsible of energetically shifting the equilibrium towards the native state (Asakura and Oosawa, 1954; Vrij, 1976). The loss of entropy by the unfolded state drive the protein to its native conformation. The entropy loss mechanism is consistent with experimental works that observed increased compactness in unfolded states of cutinase (Bapista *et al.,* 2008), protein S6 (Chen *et al.,* 2001), and Rnase S (Ratnaparkhi *et al.,* 2001) due to osmolytes. This would imply that even if an osmolyte interacts with a protein with the same energetics as water, it would still stabilize the protein for entropic reasons (Hadizadeh, 2010).

Bolen's approach results in a solvent quality paradigm which classifies solvents as good or poor. In a poor solvent (solvophobic), intramolecular interactions of the protein dominate, which favors a compact folded native state that minimizes the solvent exposed protein surface area. In a good solvent (solvophilic), protein-solvent interactions dominate, which favors an unfolded state that maximizes the protein-solvent contacts. At the middle of the solvent quality scale is the neutral solvent that favours neither native nor unfolded states. Water is a poor solvent for proteins since the effective water-protein interactions lead to hydrophobic effects, one of the major forces that folds proteins. Aqueous osmolytes and aqueous urea solutions are poor and good solvents, respectively. The solvent quality paradigm has led to several molecular free energy transfer models, which are phenomenological models that utilize as input, the experimentally measured change in free energy of proteins on transfer from pure water to aqueous osmolyte or urea solutions (Street *et al*., 2006; Rosgen *et al*., 2005). The protein acquires a nearly natively folded structure from the unstructured and unfolded form because of the reduction in the area of accessible protein parts. This reduction is due to excluded volume upon addition of osmolytes (Bapista *et al.,* 2008; Chen *et al.,* 2001; Ratnaparkhi *et al.,* 2001).

The protein backbone is osmophobic in nature, so when the concentration or number of compatible solute molecules increases in the solvent accessible area of the protein, the protein acquires a more compact conformation by hiding the backbone into the core of folded proteins in order to reduce its exposure to the added solutes (Bolen, 2001; Yancey, 2005; Baskakov *et al*., 1998). This raises the enthalpy of the system and as a result the unfolded

state of the protein will be more destabilised due to its greater solvent exposed area in turn resulting in stabilisation of the folded conformation.

3.4 Salts as osmotic stressors : Salting-in, salting-out and Hofmeister series

It is well known for a long time now that salts alter the solubility of proteins. Over more than 125 years ago, Franz Hofmeister had studied and investigated the effect of various salt concentrations on protein solubility (Hofmeister, 1888; Lewith, 1888). Around similar time, Setschenow also made quantitative solubility studies albeit not on proteins (Setschenow, 1889, 1892. The outcome of these experiments and investigations led to the creation of “The Hofmeister series" and "Setschenow coefficients" which are regarded as key milestones in understanding protein solubility (Arakawa and Timasheff, 1985) The Hofmeister series ranks various ions (cations and anions) by their ability to precipitate proteins. Compared to cations, this series is particularly robust for anions and seem to correlate well with ion polarizability or size. For instance, the halide ions ranked by salting out tendency as, $F^- > Cl^- > Br^- > I^- >$ (Collins and Washabaugh, 1985; Hofmeister, 1888; Kunz *et al*., 2004). Very interestingly, Hoffmeister and his co-workers also found that this ranking was insensitive to the type of macromolecules used for study.

Salts can be regarded as special osmolytes because of their role in osmosis. Most biological macromolecules such as proteins, DNA, lipid bilayers contain charges on their surfaces. When present in an ionic environment, these charges of macromolecules' surface associate themselves with oppositely charged counter-ions in the salt solution. These counterions thus act like screen against the electrostatic field generated by other charged groups on the macromolecular surface (Linderström-Lang, 1924; Stigter and Dill, 1990; Tanford and Kirkwood, 1957). In such situations the effect of salt on the macromolecular activity is mainly dependent on its ionic strength. The effect of salt on protein's activity and stabilisation can be explained by two opposing processes described below:

Salting-in: It refers to the effect where at low salt concentrations, increasing the ionic strength of salt solution increases the protein solubility. The mechanism behind this effect can be explained by the Debye-Huckel theory. In brief, it states that there is an ionic atmosphere around each ion. This atmosphere comprises of a number of counter ions forming a cloud. Physically, the thickness of this ionic cloud is referred to the Debye length. As the ionic strength of the salt solution increases, the Debye length decreases. The Debye length is inversely proportional to the square root of the ionic strength. When proteins are exposed to

increasing salt concentrations, more ions bind to the proteins and increase their net charge. This in turn reduces the net neutral charges thereby leading to an increase in the solubility of proteins. Biochemical studies show that denaturation or destabilization of proteins and enzymes is caused by adding concentrated urea or guanidine. However, this theory doesn't hold good for salt solutions with higher concentrations.

Salting-out: This is commonly a protein purification method based on the solubility properties of protein. Using this method, proteins can be precipitated at high salt concentration or can be subjected to a series of low concentration to get it precipitated.

At very high salt concentrations it is seen that protein solubility decreases when the salt concentration is increased. It is seen that there is a linear negative correlation between the ionic strength and logarithm of protein solubility. The slope of the line relating these two variables is generally found to be constant for a particular salt and identified by Ks - the salting-out constant or the Setschenow constant. Hence to keep the biologically isolated enzymes and proteins functionally active, high concentrations of salts such as ammonium sulfate is used by biochemists.

Salting out is often used as a purification and separation method for biological macromolecules, typically proteins. It results from interfacial effects in a solution of very high anionic strength near protein surface by reducing the solubility of the protein. These interactions are largely responsible for governing the final conformation of the protein by folding it in such a way that most hydrophobic groups are buried and shielded from the polar solvents surrounding the cell.

The Stability in protein unfolding processes and mechanisms can be correlated with the change in free energy of the native conformation and denatured state upon addition of osmolytes to the aqueous buffer (Tanford, 1964).

Osmolytes with no net charge are known to be preferentially excluded from the vicinity of the protein surface which drives the protein to collapse into a more compact structure.

Likewise, salts should also be preferentially excluded from macromolecular surfaces (Onsager and Samaras, 1934). This ion exclusion is known to induce precipitation of the protein (salting-out), as well as aggregation of other macromolecules (Collins and Washabaugh, 1985).

Salt and organic osmolytes, both have the stabilising properties and can modulate protein stability. But in most cases it has been observed that organic osmolytes are used to stabilise protein against unfolding, although many organisms use salt to counteract the osmotic imbalance (Galinski, 1995). So, it can be said that depending upon the impact on protein is

aimed at, either of the salts or osmolytes may be used for protein stability.

Chapter 4 Membrane Proteins

Cellular membranes provide a physical barrier and isolate the cytosolic and extracellular compartments in a living organism by enclosing cells and cellular organelles by lipid bilayers. These lipid bilayers contain specialized proteins embedded in them, which occupy the full width of the bilayer and act as the mediator between the extracellular and intracellular environments. Proteins in general, are biological macromolecules that carry out a wide and varied range of crucial functions in a cell. Membrane proteins are of great significance and interest because of their ability to perform a multitude of tasks and their biologically important role in allowing cells to communicate with their surroundings.

4.1 Introduction

Membrane proteins play a vital role in controlling life processes and are the targets of most pharmaceutical drugs, they control cell adhesion to form tissues, they control important metabolic processes, including osmotic balance, energy production and transmission, and photosynthesis. Transmembrane proteins account for approximately 30% of all proteins and play an important role in almost all cellular processes. They act as sensors, catalysts, receptors (G-protein coupled receptors and growth factor receptors), transporters (solute transport) and channels (ion transport), energy convertors (respiration, ATP synthesis) and in cell signaling so on and are attached to or associated with the cell membrane. Proteins undergo folding, unfolding and refolding besides interacting with lipid membranes and DNA and with ligands that associate and dissociate. Osmolytes modulate these exchanges are modulated by osmolytes which are typically small and abundant molecules, through nonspecific and weak interactions. Hence the stabilization of these proteins is very important for maintaining a multitude of molecular pathways and thus crucial for the survival of the cell. Osmolytes which are compatible with cell metabolism even at very high concentrations are known as compatible solutes.

The interactions of the polypeptide chains of a protein with each other and with the surrounding medium determine the structure and stability of the protein. The study of soluble proteins is both simplified and complicated by the bulk aqueous phase. It is simpler to study a given protein in a way that a bulk aqueous phase is easier to comprehend than the anisotropic and inhomogeneous bilayer phase. At the same time, the fact that the aqueous phase provides restrictions and limitations on the structural motifs accessible to the polypeptide chain of the protein makes it complicated to study in its native form, while in the case of membrane

proteins, the lipid bilayer complicates the situation. It imposes thermodynamic and geometric constraints that limit the accessible structural domains. However, in protein folding studies, membrane proteins have the advantage over soluble proteins where the (un)folding events are interconnected in a complex way which can be disentangled to a remarkable extent in case of membrane proteins.

Very little is known about the structure, stability and folding of membrane proteins relative to their water-soluble counterparts. This is in part due to the technical problems associated with membrane proteins. The hydrophobic nature of this class of proteins makes their isolation and purification an extremely difficult task. Moreover, membrane proteins tend to be highly prone to irreversible denaturation and aggregation. Although, more than half of all proteins interact with membranes, their dependence on lipids renders them difficult to crystallize, and biophysicists have traditionally relied on indirect techniques to investigate their structural framework.

The role of hydrophobic interactions has a minimal and insignificant effect in the stabilization of membrane proteins unlike soluble proteins whose structure is mostly governed by the hydrophobic effect. The stabilization of membrane protein structure is mostly dominated by non-polar residues in a bilayer milieu. Inter-helix side chain hydrogen bonds have been identified in a number of membrane proteins, but their impacts on overall stability and folding were found to be not so overwhelming (Booth, 1997). In contrast, extensive van der Waals interactions play a major role in stabilizing the helical packing in the bilayer. Small residues such as glycine, alanine and serine, preferably appear at the helix-helix interface where they are often involved in specific interactions. Setting aside prosthetic groups and lipid interactions, helix packing is the dominant factor to provide specific helix-helix recognition and stabilization.

There are two broad categories of membrane proteins namely,

(i) Peripheral membrane proteins and

(ii) Integral membrane proteins.

Peripheral membrane proteins are found attached to the exterior of membranes and can serve as attachment proteins between cells or enzymes, etc., but not as transport proteins. Proteins are normally found on one side or the other of a membrane, and not on both sides. These proteins cannot simultaneously influence both sides of a membrane; they can play a role either in the interior of cells (or other membrane-enclosed compartments within cells), or on the exterior of cells.

Integral membrane proteins are the type of membranes proteins, which are integrated or

attached to the membrane. They are also known as transmembrane proteins. Many functions of the membrane are mediated by integral membrane proteins. The integral membrane proteins fall into two major classes that are characterized by their transmembrane structure, $\boldsymbol{\alpha}$ - helical and $\boldsymbol{\beta}$ - barrel transmembrane proteins. A large and important family of integral proteins consists of seven transmembrane - spanning α helices. More than one hundred fifty such seven helical membrane proteins have been identified. The protein that we used in this work is also a seven $\boldsymbol{\alpha}$ - helical integral membrane protein, called Bacteriorhodopsin, which is the topic of the next sub-chapter.

4.2 Bacteriorhodopsin

The class of integral proteins is most prominently represented by *Bacteriorhodopsin,* found in the photosynthetic bacterium, *Halobacterium salinarum.*

Halobacterium salinarum is a halophilic archea that thrives in high temperature, saturated saline conditions in brine pools that are exposed to bright sunlight. They form red tinged colonies in habitats such as the Dead Sea. They possess a set of four kinds of retinal proteins in their membrane namely, bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I and sensory rhodopsin II.

Bacteriorhodopsin is about 26kDa transmembrane protein that captures light energy and converts it into electrochemical proton gradient, which is further used in the production of ATP. Hence it is also known as a light driven proton pump. For its chemical similarity with rhodopsin, the visual pigment of the animal retina, and the fact that the material had been isolated from bacteria, the protein molecule was named Bacteriorhodopsin. It has been found from structural analysis that the photoactive retinal is embedded in seven closely packed alpha-helices (Grigorieff, 1996; Kimura *et al.,* 1997; Pebay-Peyroula, 1997; Essen *et al*., 1998; Luecke 1998), and builds a common structural motif among a large class of related G-protein coupled receptors (Helmreich, 1996; Baldwin, 1993; Hargrave, 1991). This $\boldsymbol{\alpha}$ - helical protein is arranged in an arc like structure.

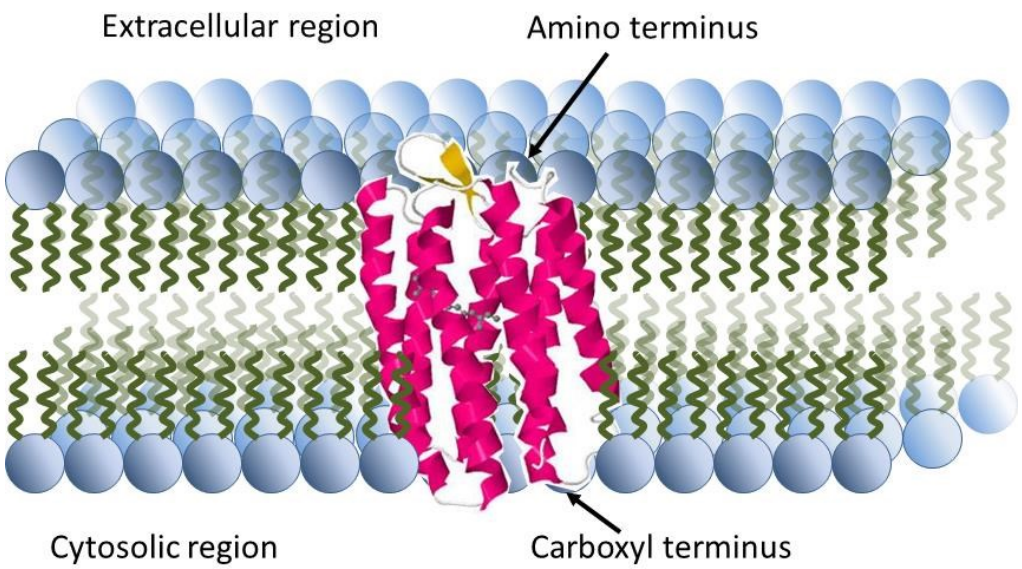

Figure 4-1: Bacteriorhodopsin in the membrane.

Overall Structure of the membrane protein Bacteriorhodopsin as deduced from electron diffraction analyses of two-dimensional crystals of the protein in the bacterial membrane integrated inside the lipid bilayer. The seven membranes spanning α helices from A-G are shown in ribbon structure. The retinal pigment (in grey) is covalently attached to lysine 216 in helix G. The approximate position of the protein in the phospholipid bilayer is indicated.

Bacteriorhodopsin has seven transmembrane helices joined by short interconnecting loops and naturally forms patches of two-dimensional crystals in the cell membrane, known as purple membrane. The helices are named from A to G and they surround the retinal molecule that forms a Schiff base (SB) with a conserved lysine residue on helix G. This retinal chromophore molecule absorbs light Figure 4-1. The biological activity of bacteriorhodopsin is dependent upon the photoisomerization of its retinal chromophore. Upon light activation, retinal is isomerised from all-*trans* to a 13-*cis* configuration, which is responsible for the conformational rearrangements in the protein Bacteriorhodopsin. As a result of this, proton is pumped from the cytosol outwardly across the cell membrane to the extracellular matrix. This generates a proton gradient across the membrane, which is then used to synthesize ATP.

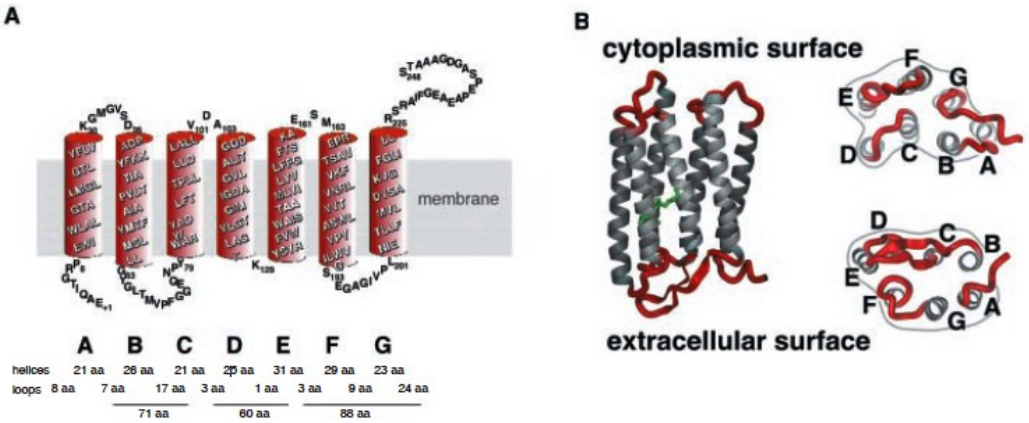

Figure 4-2: Model of the three-dimensional structure of BR.

(A) BR is a 248 amino acid membrane protein that consists of seven transmembrane α -helices, which are connected by loops. (B) Three-dimensional model and top and bottom view show spatial arrangement of the helices. Helices F and G are neighbouring helices A and B and thus can stabilize them. (Adapted from Oesterhelt *et al*., 2000).

Moreover, BR has become a paradigm for α-helical membrane proteins in general and for ion transporters in particular (Haupts *et al*., 1999; Oesterhelt, 1998; Lanyi, 1997; Luecke *et al*., 1999). Along with adjacent lipids, BR molecules assemble into trimers, which are packed into two-dimensional hexagonal lattices, the so-called purple membrane of *Halobacterium salinarum*. Bacteriorhodopsin is also involved in phototaxis (Bibikov, 1993, Bibikov, 1991) via the generation of membrane potential changes across the membrane, which is sensed by the transducer mcpT (Koch, 2005).

We chose Bacteriorhodopsin for our study because it is one of the best representatives of this class of membrane proteins and so far it is arguably the most studied transporter making it a model membrane protein for scientists. Bacteriorhodopsin shares many structural and functional characteristics of a wide variety of proteins. The presence of the retinal chromophore is one of such features that many proteins involved in cellular signal transduction share in common with Bacteriorhodopsin in a way that their activity is dependent upon the binding of a small ligand molecule. Similar features make bacteriorhodopsin an attractive candidate for studying membrane protein folding. Also, three-dimensional structure of bacteriorhodopsin is known to near-atomic resolution. Furthermore, it is interesting to observe the role of compatible solutes in prokaryotic stress management.

Chapter 5 Atomic Force Microscopy

In 1986, G. Binnig, C.F. Quate and C. Gerber invented the scanning probe microscope (SPM). This name originated from the fact that this is based on a local probe that is scanned onto a sample, allowing one to measure physical properties of materials on a small scale, by mean of a variety of different spectroscopic methods. In this microscope, a sensor tip carried by a flexible cantilever is used to touch, scan and characterize a surface. One of the established and important types of SPM is the Atomic force microscope (AFM).

Soon after its invention, atomic force microscopy (AFM) became popular as a tool for studying various samples, both living and non-living at nano level. It also emerged as an excellent instrument to investigate biological macromolecules and dynamic biological processes at different scales. It enables three dimensional surface imaging in real time at sub molecular resolution under various environmental conditions including aqueous solutions and physiological temperatures with minimal sample preparation. This was a significant breakthrough that allows biological macromolecules to be scanned in aqueous solution and makes it possible to image DNA, viruses, soluble proteins, membrane proteins crystals and other live biological samples. AFM is more than a surface-imaging tool by which force measurements can be performed to study the physical properties of the specimen, such as molecular interactions, surface hydrophobicity, surface charges, and as well as mechanical properties (Dufrêne, 2002). These measurements provide new insight into the structure-function relationships between the sample and the surface.

5.1 General Principles

Atomic force microscopy does not use lenses or an incident beam as used in other classical microscopy techniques, but instead it uses a sharp and pointed sensor or tip, at the end of a flexible cantilever to scan and sense the topography of a sample under investigation (Figure 5-1). This principle allows us to visualize nanoscale objects where conventional optics cannot resolve them due to the wave nature of light.

Atomic force microscopy (Binnig, 1986) is a powerful tool to observe, image and characterize membrane proteins in their physiological environment at the molecular level (Scheuring *et al*., 2003; Müller *et al*., 1998; Bryngelson *et al*., 1995, Frederix *et al*., 2003; Frederix *et al*., 2004). In the simplest mode of operation, the contact mode (discussed in section 5.3.1), the sample is scanned underneath the tip; the cantilever deflects up or down depending on the structure and topography of the underlying surface, tracking the surface.

Interactions between the AFM probe and the sample exert forces on the probe and thus cause the cantilever to deflect. The deflection is detected due to the changes in the position of the laser beam reflected from the end of the cantilever on a positionsensitive photodetector (Figure 5-1). The difference in voltage between the segments of the detector is linearly related to the deflection of the cantilever with a resolution down to 0.1 Å (Sarid 1994). Topographs of various biological samples can be obtained using different imaging modes of the AFM, from tissues and cells to single molecules (Le Grimellec *et al*. 1998; Schabert *et al*. 1995). The deflection of the cantilever can also be converted into force units by the use of Hook's law knowing the spring constant of the cantilever. Thus, forces at nano level acting between the probe and the sample can be quantified. This feature of the AFM probe evolved principally a new mode of the AFM operation, the so called force mode.

An AFM image is generated by recording the changes in force as the sample (or probe) is scanned in the *x* and *y* directions. The sample is mounted on a piezoelectric scanner, which ensures three dimensional positioning with high resolution. The force is monitored by attaching the probe to a flexible cantilever, which acts as a spring, and measuring the bending or deflection of the cantilever. The larger the cantilever deflection, the higher will be the force experienced by the probe. Most instruments use an optical method to measure the deflection of the cantilever with high resolution; a laser beam is focused on the free end of the cantilever, and the position of the reflected beam is detected by a position-sensitive detector (photodiode) (Dufrêne, 2002).

Because it is relatively simple to operate, and imaging does not require an external mean of contrast, the AFM has a vast range of imaging applications from single molecules to living cells. In addition, the AFM is a powerful method for manipulating single molecules, as cantilevers are being used to exert forces on individual molecules to probe their mechanical stability and elasticity. With ongoing developments in AFM studies, high resolution and high speed imaging of biological macromolecules can now be observed performing their physiological activities in real time. Thus, atomic force microscopy (AFM) has emerged as arguably the only technique that enables the real-time imaging of the surface of a living cell or biomolecule at sub-nanometer resolution, more than 1000 times better than the optical diffraction limit. Since AFM provides the advantage of directly observing living biological cells in their native environment, this technique has found many applications in pharmacology, biotechnology, microbiology, structural and molecular biology, genetics and other biology-related fields. In this chapter, different components and working modes of the AFM is discussed and illustrated.

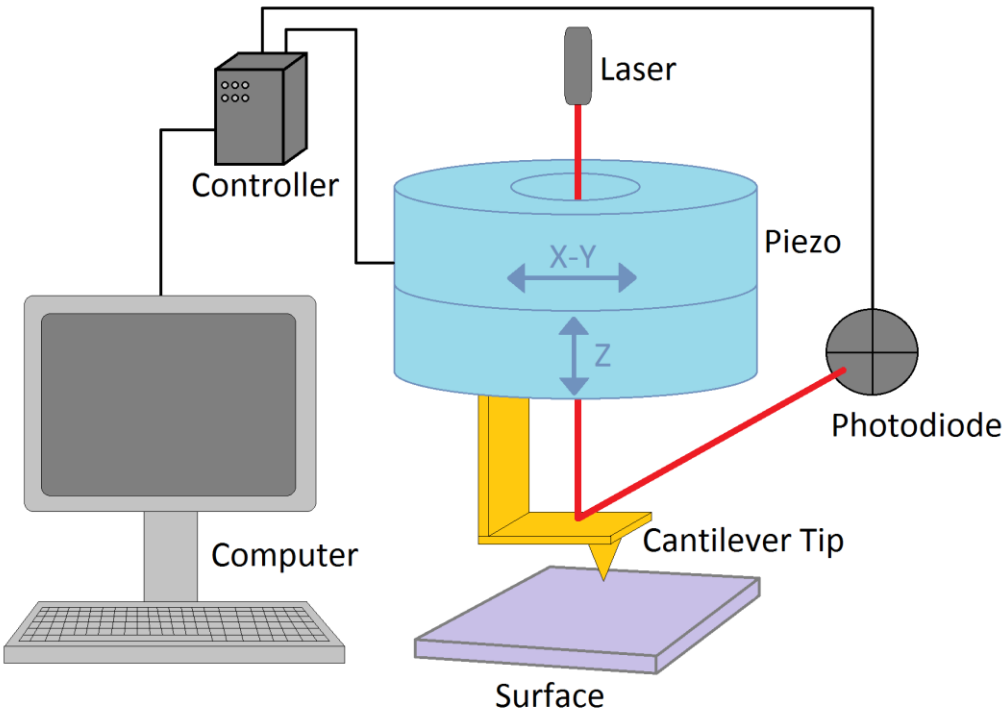

Figure 5-1: Schematic illustration of an AFM system.

A flexible cantilever with a tip at the end is rigidly connected with a xyz piezoelectric element. The optical lever consists of a laser diode beam that is focused on the back of the cantilever and bounces off reaching the photodetector.

5.2 The individual components of a force microscope

The two main components of the atomic force microscope are the tip attached to the cantilever and the piezo electric scanner. These two parts are discussed below in the following sub chapters.

5.2.1 The tip

The principal component of the Atomic Force Microscope is a pyramid-shaped stylus mounted on a flexible cantilever (Figure 5-2). One of the most important characteristic of the AFM probes is the tip size. In fact this parameter is dominating the final resolution of the microscope; a sharper tip will allow a higher lateral resolution. It determines the quality and nature of the images obtained by this technique.

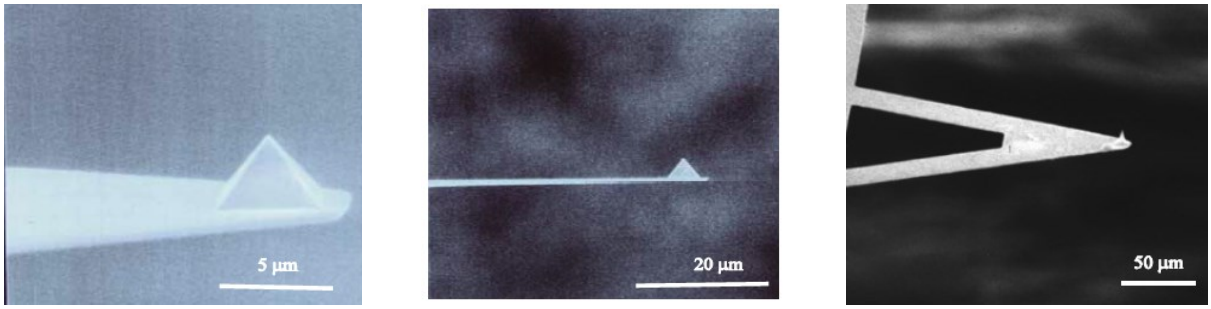

Figure 5-2: Images of the cantilevers taken by Scanning Electron Microscope (Pictures taken from Valle, 2003).

The sharpness of the tip can be well described by two parameters. One parameter being the tip curvature radius, which is the real radius of the tip in the approximation that it is spherical and determines the size of the details that the AFM will be able to resolve. Another parameter is the aspect ratio or opening angle of the tip which measures the opening angle of the cone representing the full tip, and determines how deep the tip will penetrate between two adjacent structures on the surface. Because of their flexibility, the tips are resistant to damage from tip crashes, while their slenderness permits the imaging of sharp recesses in surface topography.

5.2.2 The Piezoelectric scanner

The AFM scanners are made from piezoelectric material, which expands or contracts proportionally to an applied voltage. The scanner itself is constructed by stacking three independent piezoelectric crystals, each of them being responsible for movements on one axis x, y or z. These crystals create a voltage if pressure is applied, or in reverse, can create a pressure by expanding or contracting if a voltage is applied. Using the contraction and expansion of the crystal, the configuration in a scanner allows the controlled movement on the order of a fraction of a nanometer.

The piezoelectric scanner can move very precisely with good reproducibility for small displacements, but for displacements more than 70% of the full-scale displacement the piezoelectric response is non-linear (hysteresis). The commercially available AFMs featuring “closed loop” feedback systems independently monitor the scanner movements and correct its motion for hysteresis. The scanner configuration consists of a hollow tube with x and y-axis controlled by two segments of the piezoelectric crystal, and one segment for z-axis.

5.3 Imaging modes

Atomic force microscope (AFM) can simultaneously image several interactions between the sample, the surface and the tip. The manner in which the interactions can be used to obtain an

image of the sample is generally termed as a mode.

One of the most common and earliest used imaging modes is the constant force mode (contact mode), sometimes referred as the height mode, which displays the changes in the sample height during scanning. This mode gives calibrated height information about the sample surface. Alternatively, small changes in cantilever deflection that signal the feedback loop to make the height adjustments can also be monitored by this technique; this imaging mode is called deflection mode. In addition to faster scanning, the cantilever can oscillate at high frequency as well. When the tip encounters elevation-changes due to difference in topography on the sample surface the amplitude of the oscillation changes. The feedback system then responds to keep the amplitude of cantilever oscillation at constant level. This mode of operation reduces the lateral forces that can push the sample around.

While scanning tip interacts with the surface only for short time intervals at the bottom of its oscillation turning point. Several parameters could be used by the feedback of the system to obtain the topography of the surface, i.e. amplitude of oscillation, frequency, and phase shift (Garcia and Perez, 2002). This mode exhibits low lateral shear forces enabling non-destructive imaging of fragile biological objects in fluid such as living cells (Putman *et al,*. 1994), moving DNA and DNA-enzyme complexes (Kasas *et al*., 1997).

5.3.1 Contact (constant force) mode

The first and for a long time the most extensive way of operating the atomic force microscope for collecting topographic images was the contact mode. In the constant force mode of AFM, the constant force, between the tip and the sample, which is preset at a minimum value, is held constant by an electronic feedback loop. Whenever the cantilever begins to deflect upward (or downward), indicating that the tip is encountering an obstacle (or a trough) on the surface, the sample is retracted (or raised) to cancel the deflection of the cantilever, thereby keeping the tip-sample force constant. The surface topography is obtained by displaying the advance and withdrawal of the sample required to prevent the cantilever. The image obtained in this way is really an equiforce surface. It is evident how such a kind of image may be strongly influenced by the local tip-sample force interaction.

Another way to image the sample's surface in contact mode is the "variable deflection imaging" or "constant height mode". In this case the deflection of the cantilever is allowed to change while scanning. The deflection of the lever is recorded as topography. The almost complete absence of feedback regulation allows to the microscope, when operated in this way, to scan faster. On the other hand the data obtained are of difficult interpretation because

the measured contours do not represent an equiforce surface.

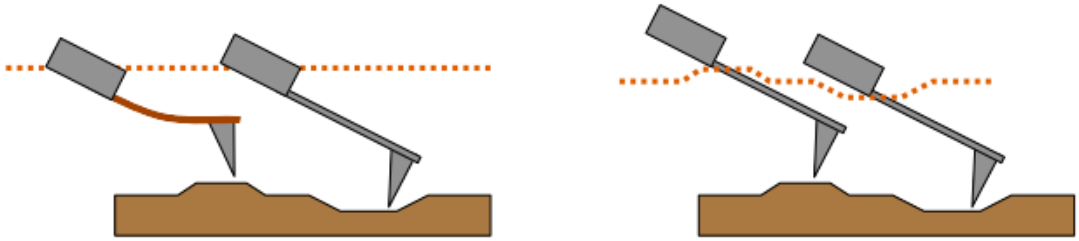

Figure 5-3: Scanning in contact mode.

On the left side a scan at constant height is shown, resulting in a deflection image. The cantilever is deflected upwards (shown in red) because of the surface constitution. The height is constant (dashed line). On the right a scan at constant force is shown, resulting in a height image. The deflection of the cantilever is kept constant while the height is continuously adjusted (dashed line).

5.3.2 Tapping (oscillation) mode

In early 1990s, D.J. Keller, Q. Zhong and C.A.J. Putman independently proposed the tapping mode. In the tapping mode, the cantilever tip does not stay in contact with the sample continuously. This mode uses the oscillation of the cantilever to scan the sample where the cantilever vibrates close to its resonance frequency. Upon approaching the sample on the surface, the tip briefly touches the bottom of each swing, resulting in a decrease in oscillating amplitude. The feedback loop keeps this decrease at a preset value and a topographic image of the sample surface can be obtained. In this mode, the cantilever is externally driven by forces of acoustic, magnetic, or electric nature such that the tip undergoes vertical sinusoidal oscillations with amplitudes of a few nanometers and with frequencies close to resonant frequency of the cantilever. Low lateral shear forces are exhibited in this mode and has this enabled non-destructive imaging of fragile biological objects in fluid for e.g. living cells (Putman *et al*. 1994), moving DNA and DNA-enzyme complexes (Kasas *et al.* 1997).

The continuous oscillation of the cantilever tip allows for a much more accurate height adjustment, leading to a very sensitive scanning method.

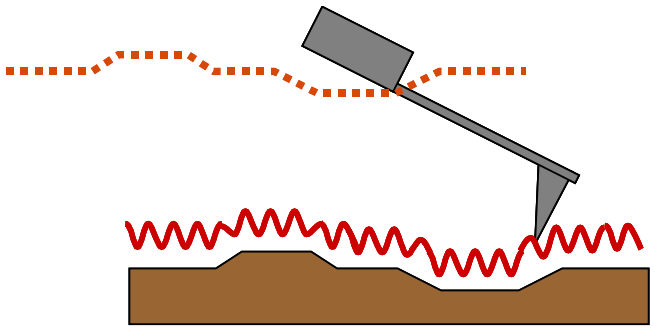

Figure 5-4: Scanning in taping mode.

There are significant disparities observed in the oscillation of cantilevers in fluid than of it in air or vacuum. This is because while scanning the sample topography the cantilever motion drags the surrounding liquid which leads to an increase of the effective mass and a corresponding decrease of the resonant frequency. Another account for this difference is also the cantilever oscillation is inharmonic and asymmetric when the quality factor is low, in contrast with a tapping mode AFM in air, where the cantilever is approximately sinusoidal and symmetric. In this mode of operation, usually the tip touches the sample just at the very end of its oscillation, reducing in this way at the minimum the lateral stimulation on the sample. This is the main reason of the large scale use of the Tapping Mode-AFM for imaging soft and sensitive biological samples. This mode requires, to strongly fix the samples to the surface limiting the number of observable biomacromolecules. The heterogeneity of the molecules does not permit to use one fixed protocol to bind them onto the surface. Even more important, the characteristic softness of the biological materials does allow one to predict the effects of the strong binding on the structure and function of the different molecules. Working in tapping mode, the samples can be simply deposited onto the surface, using the weakest possible immobilization, preserving their structure and function. Further more the contact time of the tip with the sample is faster then the relaxation time of the most of biomaterials preventing any deformation. But one limitation of measuring in air is that the biological samples do not get their physiological environment which renders them functionally inactive. From the technical point of view, to operate an AFM in tapping mode it is necessary to generate inside the imaging cell the oscillation of the cantilever. Acoustic and magnetic excitation modes have been developed to excite the cantilever-tip ensemble. In the acoustic excitation mode a piezoelectric actuator is attached below the substrate containing the cantilever-tip ensemble (Anselmetti, 1994). When the oscillating voltage is applied to the actuator, it generates a vibration in the actuator which in turn results in oscillation of the

cantilever. This oscillating mode is quite frequently used in measuring samples in air as well as in liquid, as it also excites the liquid.

Han and co-workers in 1996, have directly excited the tip of microcantilever by applying an oscillating magnetic field to the magnetised microcantilever (Han *et al.,* 1996).

5.3.3 Imaging biological samples with Atomic force microscopy

The mechanism of image capturing by AFM is very different from those that of optical and electron microscope. A spatial resolution of optical and electron microscope is an inherent property of the instrument and depends ultimately on the design and principles of operation of the microscope. In contrast, the spatial resolution of the AFM depends as much on the characteristic and nature of the sample as on the inherent properties of the instrument (Bustamante *et al*., 1996).

The AFM introduced the first revolution, allowing one to work in liquid, the natural condition for all biomolecules. Living cells as well as their constituents were successfully imaged with very good results. The only remaining problem was how to avoid the lateral stimulation of the biomolecules on the surface when imaging in contact mode. This problem was partly overcome by the use of the tapping mode AFM. In the last decade many advances in living science were achieved by AFM in particular concerning the nucleic acids, the imaging of proteins together with their dynamic and static properties. For instance, it is always difficult to handle and crystallize membrane proteins, specially derive them and make then form a three dimensional structure. However, they can form two dimensional crystals in presence of appropriate lipid medium. These two dimensional crystals of membrane proteins can be studied by AFM, both in tapping and in contact modes, allowing the high resolution of some structural details of cells and molecules which were previously not possible (Muller *et al.,* 1997, Muller *et al.,* 1999). The Bacteriorhodopsin sample used in this study is also in the form of two dimensional native crystals.

Many factors contribute to an AFM image of biological structures in addition to the topography of the sample surface. They include the size and shape of the tip and its sharpness, properties of the feedback loop, and the mechanical and chemical properties of the sample and imaging environment. It is possible to image rigid and atomically flat surfaces at sub nanometer resolution using a relatively blunt tip because of the hardness of the surface.

On the contrary, soft samples tend to conform to the harder tip surface and tip-sample interactions take place over a larger area (Valle, 2003).

5.3.4 Imaging proteins with Atomic force microscopy

Imaging proteins successfully in their physiological environment while retaining their biological activities has always been a concern for different research groups working in the related field around the globe. Many groups like that of Ando, Scheuring etc. have come up with various techniques to record real time activities of functionally active protein. Several attempts were done, some of which were successful and some of them were critically received by the respective scientific community (Radmacher *et al*., 1994; Thomson *et al*., 1996). It is noteworthy to mention here that in order to successfully investigate the dynamic characters of a protein by AFM; the protein has to comply with the following criteria:

(i) It must have a measurable conformational diversity in its structure at microscopic resolution (1nm) range, and

(ii) For high speed imaging, the time scales of such dynamic changes must be in the order of few seconds.

Coping up the imaging speed with the speed of biological processes is a limiting factor for the scanning speeds of the available microscopes. Presently, there are many attempts to improve the time resolution of the atomic force microscopes, they are mainly dealing with very fast electronic feedbacks, new mechanical design and new probes (Viani, 1999) and some groups like that of Toshio Ando and Simon Scheuring have successfully imaged protein and recorded their activities using high speed and high resolution AFM imaging.

Besides topography and surface imaging, force spectroscopy has been powerful and successful in unraveling different structures in proteins, RNA and other polymers. Although, extraction of submolecular information from AFM topography for isolated globular proteins is a very difficult task. Achieving atomic resolution on soft biological samples is difficult when compared to attaining the same on solid-state flat materials or two-dimensional crystals (Figure 5-5). On soft samples and surfaces, deformities lead to a strong and pronounced indentation of the sample by the tip. This effect has led to the prediction that the lateral resolution of such a sample would be limited to several nanometers.

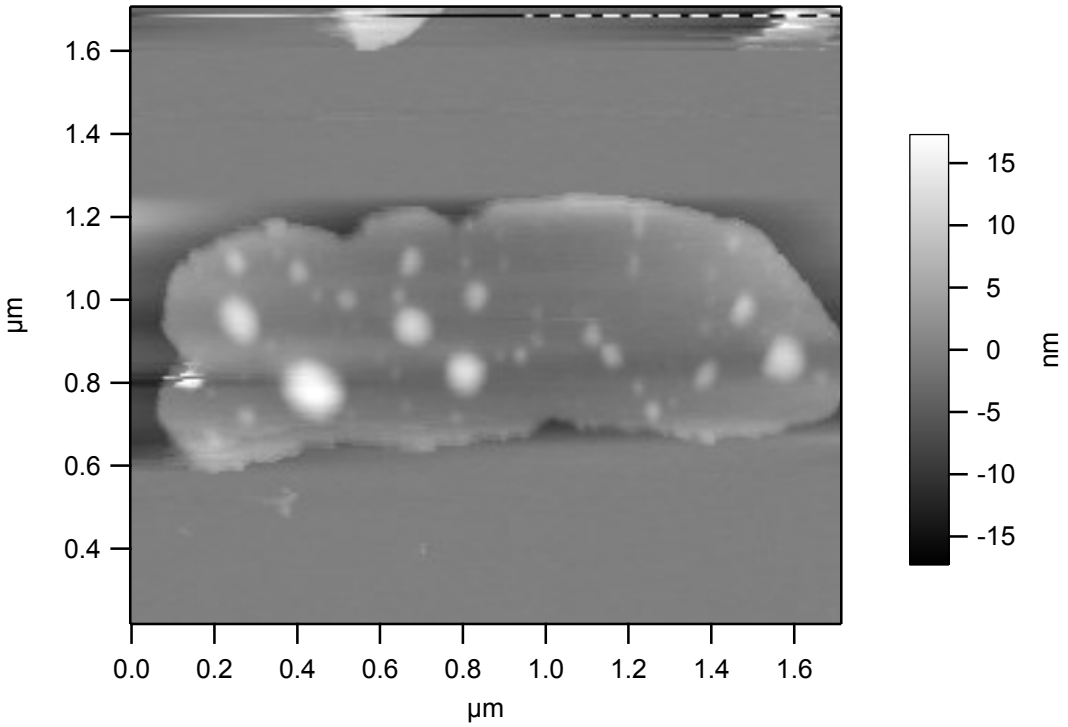

Figure 5-5: AFM image of native 2-D Bacteriorhodopsin patches.

Once we succeeded with the imaging of our protein sample, we used the single molecule force spectroscopic technique to further study their behaviour and dynamics. Single molecule methods provide a broad range of biological properties. By imaging or by force spectroscopy, single molecule methods often open a myriad of property options to investigate. Forces, elasticity, torsion, adhesion, mechanical strength and stability and motion of molecules are currently determinable with these techniques.

Chapter 6 Single Molecule Force Spectroscopy

Biomolecules such as proteins, lipids, and nucleic acids among others form the basic building blocks of life. Structurally, these biomolecules are macromolecules formed by aggregation of multiple molecules (subunits) held together by weak intermolecular forces. These intermolecular forces are non-covalent interactions (a chemical bond which does not involve the sharing pair of electrons) and can be classified under ionic interactions, Van der Waal's interactions, Hydrogen bonds and hydrophobic interactions. The study of such forces at molecular levels is of utmost importance in structural biology: not only to understand how the macromolecules acquire the structures but also to investigate how any alterations to these structures affect their function.

The advantage of single molecule techniques over ensemble techniques is that the intricate molecular events like change in conformation of a particular molecule, polymer elasticity, mechanical resistance of chemical bonds, protein unfolding etc. can be investigated at atomic resolution using these techniques. Single molecule techniques also get an edge over ensemble measurements because they do not have the complexities and problems associated with population averaging, which is inevitable in the case of ensemble techniques. The transient states and rarely occurring events which would, in the case of ensemble measurements otherwise be obscured by employing averaging methods can be resolved by super resolution atomic force spectroscopy taking care that events recorded are not artifactual.

AFM based single molecule force spectroscopy has been very successful in characterizing mechanical (un)folding of individual membrane proteins under the influence of externally exerted mechanical force. Multiple inter- and intramolecular forces working within a single protein molecule contribute to the mechanical resistance of the molecule.

Single Molecule Force Spectroscopy has already been applied to study the dynamics and stepwise unfolding of different water-soluble (Marszalek *et al.* 1999; Rief *et al.* 1997; Schwaiger *et al.* 2004) and transmembrane proteins (Oesterhelt, 2000; Cisneros *et al*. 2005; Janovjak *et al.* 2004; Kedrov *et al*. 2004). It has been shown that the topology and arrangement of the secondary structure elements are responsible for the mechanical stability of the protein. Other factors like direction of the pulling (Dietz *et al*. 2006; Lu *et al*. 1998), type of electrolytes (Park *et al*. 2007), and binding of ligand (Kedrov *et al*. 2005) to the protein affect the mechanical properties of the protein.

In typical pulling experiments, force is applied as a means to selectively the steps in a biochemical reaction cycle that involves motion. Detailed analysis of the force-extension relationship of each individual protein molecule (Fernandez and Li, 2004) by analysing the rupture force of molecular bonds, or force spectra provides a measure of bond energies, lifetimes and also extraction of detailed equilibrium thermodynamic parameter and reconstructing free energy landscapes (Hummer and Szabo, 2005; Jarzynski, 1997).

In our study, we exploited the ability and accuracy of single molecule force spectroscopy to perform an image scan of the adsorbed Bacteriorhodopsin molecule on mica surface which is mounted on a piezo electric actuator and find the location of an individual Bacteriorhodopsin molecule, which was then extracted with the same AFM tip and the unbinding forces and the force spectrum of extraction of the protein molecule from the membrane is recorded and analysed (Oesterhelt et.al, 2000). Piezoelectric elements perform continuous movements in z-axis increasing or decreasing the distance between the sample and the AFM probe. When the microscopic tip of the AFM cantilever and the sample surface are brought together and then separated, one or more molecules can attach to the probe by absorption. A single molecule tethered between the tip and a sample responds to the extension by generating a restoring force that causes the cantilever to bend. The obtained data could be displayed as force curves which are plots of the cantilever deflection as a function of sample position along the z-axis (towards and away the AFM probe). Knowing elastic properties of the AFM cantilever one could directly measure the force applied to the molecule as a function of sample position (Hook's law).

6.1 Force- extension curve: measurements of elasticity and force

An AFM force-extension curve is a plot of the tip-sample interaction forces *versus* the tip-sample distance. For such plot, the tip of the cantilever is lowered towards the sample surface and is retracted to its starting point. When the tip reaches the surface of the sample, the cantilever is pressed onto the sample surface with a predetermined constant force. The cantilever deflection is noted by a position-sensitive split photodiode detector.

The cantilever deflection resulted due to inter-atomic interactions are converted into force by using Hook's law:

$$F = -k_c d, \qquad \textbf{Equation 6-1}$$

where F is the force acting on the cantilever, k_c is the spring constant of the cantilever and d is its deflection, and is the measure of the force acting on the stretched molecule or its part. The tip is then retracted from the surface. With the attachment of tip and sample, the forces

between the atoms of both the surfaces begin to interact, causing the cantilever to bend toward the sample surface as the tip is retracted.

As depicted in the Figure 6-1 shows how an uncorrected force-extension (distance) curve looks like.

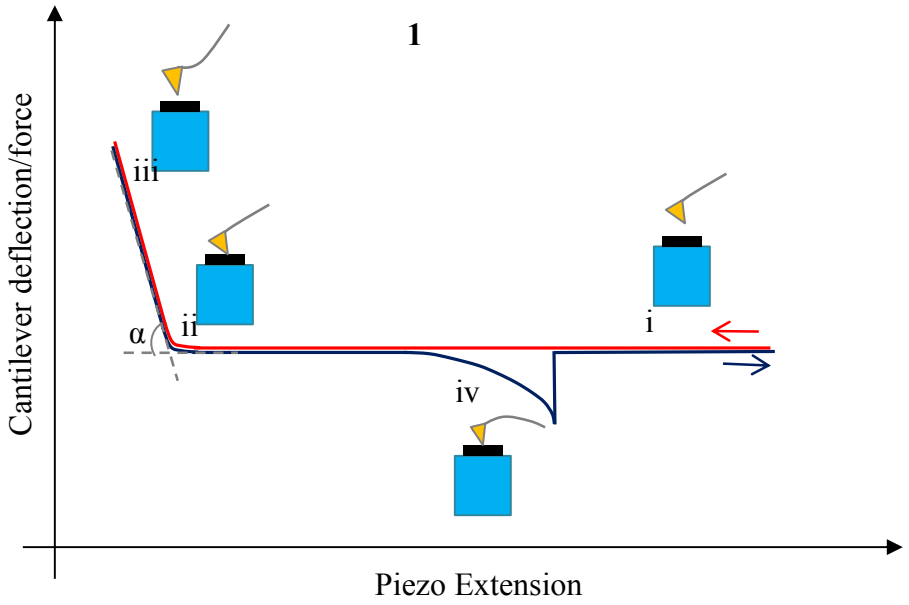

Figure 6-1: Polymer stretching by AFM cantilever.

Force induced stretching curve of a small molecule: Raw data: uncorrected force curve. (Adapted from Baltrukovich,2008)

i) – iv) represents all the steps of approach-retraction cycle.

(i) The tip is away from the surface and is slowly approaching: the deflection at this stage stays constant and the approach curve at this point is horizontally parallel to the x- axis (piezo extension).

(ii) Cantilever extends and at this stage it reaches the surface and is in direct contact with the surface causing bending of the cantilever by the force of the piezo. In this position attractive Van der Waals forces dominates in absence of any long-range interactions.

(iii) The deflection increases and at maximal deflection point (preset force acting upon the cantilever) the piezo electric scanner retracts.

(iv) The deflection of the cantilever decreases slowly (shown in retraction, blue curve). Mostly this retraction part of the force curve does not resemble the approach curve and exhibits hysteresis due to the polymer molecule attached to the tip. The downwards deflection of the cantilever starts only when the polymer is fully stretched to its maximum length and reaches its peak. (Israelachvili, 1991).

Raw Force-curves need to be corrected because of the deflection caused during pulling the

sample. The experimental situation is not ideal and there are various factors affecting the accuracy of the measurements like hysteresis, cantilever deflection, snap in or pull off. The Figure 6-2 shows a typical corrected force-curve after taking into account and correcting these factors.

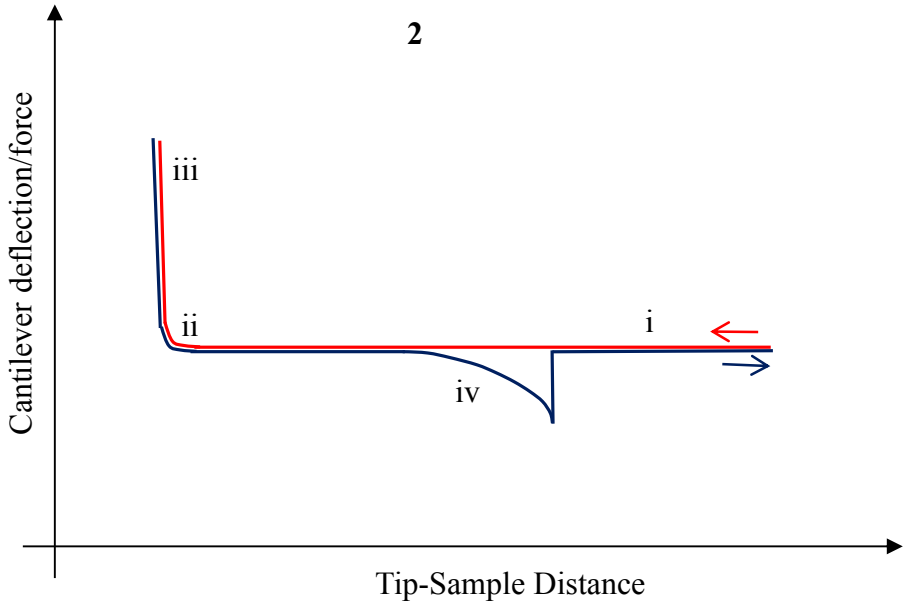

Figure 6-2: Corrected force curve.

The interpretation of the force curves relies almost entirely on the force laws which describe force as a function of tip-sample distance and not as a function of the piezo displacement (Israelachvili 1991). Thus, to be useful, the force curves must be transformed into the plots of force as a function of tip-sample distance (Figure 6-3). Tip-sample distance, $\mathbf{d_{ts}}$, is calculated from the piezo movement, $\mathbf{z_p}$, and metric cantilever deflection, $\mathbf{\Delta x}$, (Figure 6-3) by the following equation, (Baltrukovich, 2008)

$$d_{ts} = z_p - \Delta x, \qquad \textbf{Equation 6-2}$$

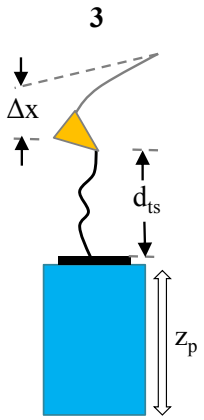

Figure 6-3: Schematic representation of how tip-sample distance is calculated.

A typical force versus extension curve on the membrane protein Bacteriorhodopsin generated by an AFM is shown in Figure 6-3. A complete force-extension curve provides information on the structure, folding and unfolding processes and also intermediates formed during protein (un)folding, refolding processes. A typical force curve on a surface without any sample adsorbed into it usually looks like as displayed in Figure 6-4. There is no interaction between the tip and the surface. The red line represents the trace track and the blue the retrace of the cantilever movement. While the cantilever approaches the surface slowly and it's still away from the surface (1), the deflection of the cantilever does not change and stays constant. This condition yields the force curve that is parallel to the x-axis. Upon reaching the surface (2), the cantilever bends by the force of the piezo. The deflection signal rises more (3) and reaches the maximum force already set before the experiment. This is the point (4) where piezo starts to retract the cantilever. The deflection of the cantilever decreases slowly (5) until it is away from the surface (6). The cantilever now goes away from the surface giving us the retrace curve (7).

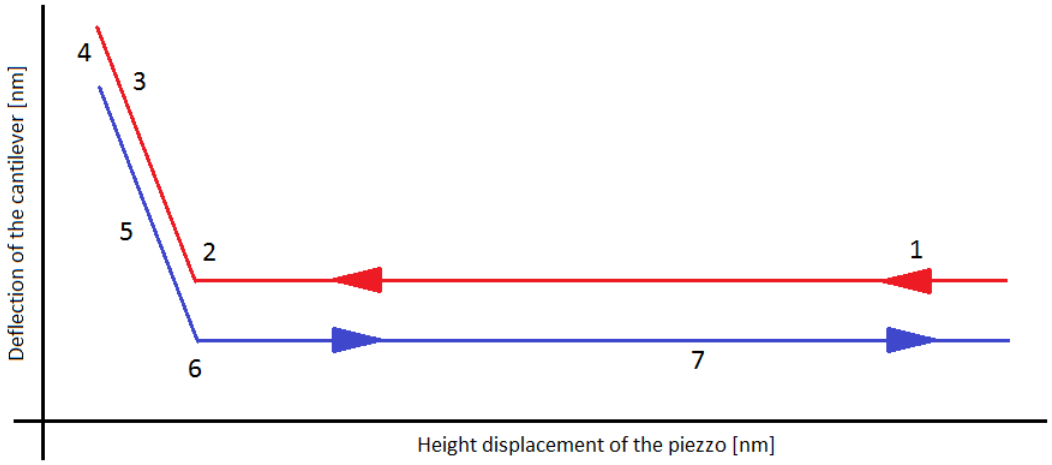

Figure 6-4: Force curve of a naked surface.

While measuring small molecules attached or adsorbed to the surface, a different kind of force curve is observed in which the sample attaches to the tip of the cantilever Figure 6-5. Here, the trace (in red) stays the same while the retrace (in blue) shows a downward peak (6) due to direct contact and attachment to the molecule. When the piezo retracts from the surface, the flexible cantilever bends towards the surface, as it is observed when a spring is elongated. The downward deflection continues to increase until the force is high enough for the molecule to either detach from the surface or from the tip. In both the cases the cantilever jumps back into its neutral position and continues to be retracted from the surface.

Figure 6-5: Force curve of a small molecule detaching at an early point.

In most cases, force-extension curves, also known as force-distance curves display a non-linear pattern indicative of an entropic spring, which is well described by the worm like chain model (WLC), which is explained in detail in later sections. Between the unfolding events, the polymer behaves like a non-linear elastic spring. In our experiments, the unfolding force curves are fitted by WLC model to obtain the persistence length and peak force of the stretched region of the polypeptide chain.

When the polymer sample is a membrane protein, with helices integrated inside the membrane and joined by interconnecting loops protruding out of the membrane, pulling experiment will result in the cantilever deflection downwards until the force reaches the maximum to extract one or more helices out of the membrane, in which case the cantilever will snap back into its neutral position. The deflection is then converted into force and resulting peaks are correlated to the unfolded helices of the protein Figure 6-6. This provides information about the constitution of the protein inside the membrane, the force required to unfold the protein indicating its general stability and influence of different environmental

condition on it (Roychoudhury *et al.,* 2012).

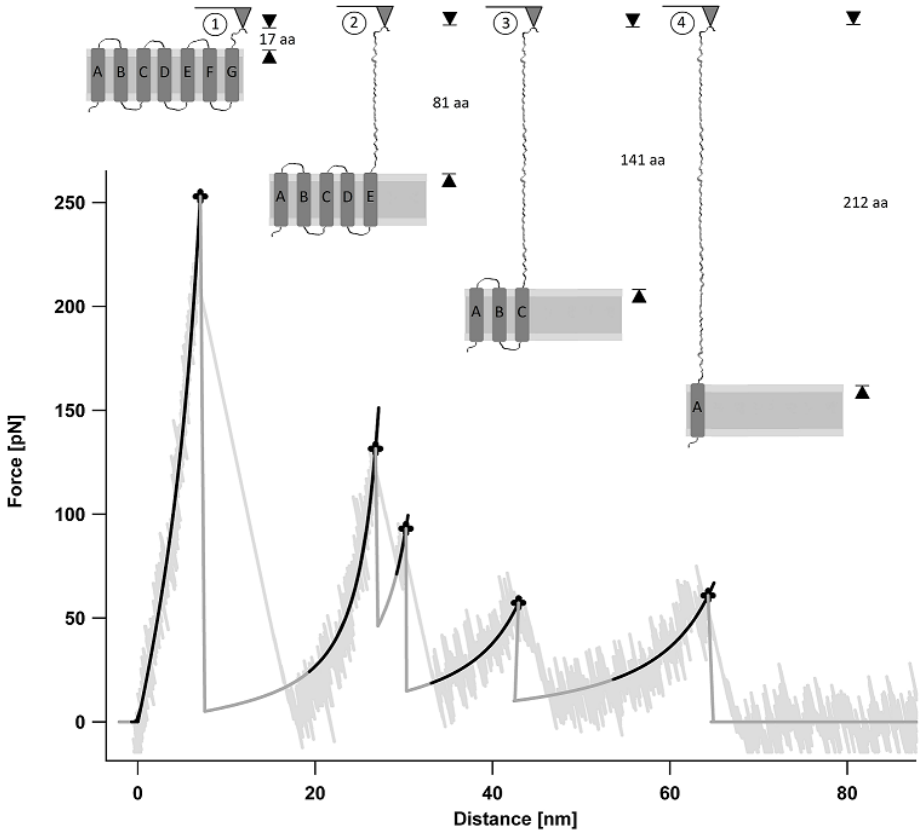

Figure 6-6: Bacteriorhodopsin unfolding force curve.

Unfolding of bacteriorhodopsin and the corresponding force curve Bacteriorhodopsin has seven α- helices inside the membrane (A-G), connected by loops. Each peak of the force curves resembles one inner loop connecting two incorporated domains being pulled out.

The preparation of sample and its attachment to the surface plays a crucial role in studying biological macromolecules by AFM. The solutions and buffers need to be extremely clean and pure. The attachment of the sample to the surface is one of the common requirements for sample preparation to avoid undesired motions of the sample. And also, too firm an attachment along considerable portion of the molecular surface may lead to undesirable denaturing effects and should be avoided. Hence in our experiments, it was desired and necessary to allow the molecule to get only adsorbed and not fixed to the surface while taking care that the scanning probe may slightly shift the adsorbed molecule (Müller *et al*, 1997).

6.2 Technical requirements: probe design and calibration

In an AFM based single molecule force spectroscopic measurement, two kinds of data can be recorded while mechanically stretching the protein, these are force and extension. Proteins can be considered as springs that generate a restoring force when mechanically stretched. The force-sensitivity is limited by the stiffness of the cantilever and the laser power. The

extension is the measure of the distance between the anchoring points at which the two ends of the polypeptide chain are attached.

An appropriately designed AFM probe is necessary for good quality and informative images and also for accurate measurements of the local forces and interactions. Conventional AFM cantilevers are mostly made up of silicon or silicon nitride (Albrecht *et al*., 1990). The sharpness of the tip plays a very important role in determining the atomic resolution of the image scanned and hence needs to be considered before choosing the appropriate tip depending on the nature of the sample (Engel *et al*. 1997). Most widely used tips are pyramidal in shape with radius ranging from 30nm to 50nm (Albrecht *et al*. 1990).

In force spectroscopic experiments, force is generally calculated from the bending or deflection of the cantilever with known spring constant. The stiffness of the cantilever depends upon its shape and material properties. Usually the numerical value of stiffness ranges from $10^1 – 10^5$ pN/nm. For obtaining high precision force data, each cantilever needs to be properly calibrated prior to use and also after the measurements.

In single molecule data recording, noise plays an important factor. Circumventing the noise of various sources and forms is of utmost concern. The sources of noise include the thermal noise of the cantilever, mechanical vibrations of the parts of the AFM, besides the electrical and optical contributions of the corresponding components in the system. The dominant and significant contributor of noise are thermal fluctuations of the cantilever motion, the bandwidth (the frequency range over which the signal is recorded, i.e. a slow pulling with limited bandwidth will give better signal-to-noise ratio, the noise being proportional to the square root of the bandwidth), and the distribution of intensity of the laser beam. The minimum detectable force is determined by the thermal vibration. Taking into account only the thermal noise, the smallest force, $F_{min,}$ which can be measured in an AFM based force spectroscopic experiment neglecting noise from the detection system and other sources is given by (Sarid, 1994),

$$F_{min} = \sqrt{\frac{4k_B TBk_c}{w_o Q}}, \qquad \textbf{Equation 6-3}$$

where $\mathbf{k_B}$ is the Boltzmann constant (1.38Å ~ 10^{-23}k J/K), **T** is the temperature in Kelvin, **B** is the measurement of bandwidth, $\mathbf{w_0}$ is the angular resonance frequency in vacuum, $\mathbf{k_c}$ is the spring constant and **Q** is the quality (Q-) factor. The Q-factor describes viscous damping of the cantilever oscillation. Smaller cantilevers with higher resonance frequencies can detect smaller forces than large cantilevers with the same spring constant (Sarid, 1994; Viani *et al.,* 1999).

In order to precisely and quantitatively measure the local interactive forces recorded by an AFM, it is mandatory to properly calibrate the cantilever and know the bending stiffness (or spring constant) of the cantilever as accurately as possible. There are several methods to measure the spring constant of the cantilever.

The spring constant of the cantilever k_c is usually estimated by its manufacturer but due to fabrication heterogeneity this estimate can be quite gross, even a 10 per cent deviation from the stated estimate is not unusual which is very crucial for force measurements at sub molecular level. For this reason, several methods have been developed to arrive at a more accurate estimate. The most commonly used calibration methods (Cumpson *et al*., 2004) are,

(i) through comparison with a reference cantilever of known stiffness,

(ii) through calibration using thermal vibrations,

(iii) by the method of added particle masses, or

(iv) by combining measurements of the resonant frequency with the cantilever physical dimensions and material properties.

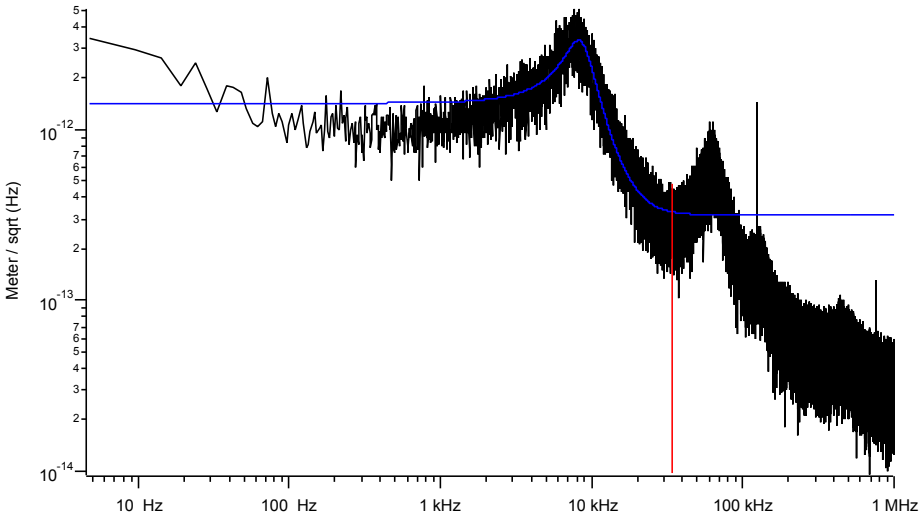

Figure 6-7: Determination of spring constant using thermal tune method.

The most commonly adapted convenient method is the thermal tune method (Butt and Jaschke 1995; Hutter and Bechhoefer 1993) which we also exploited for determining the spring constant of the cantilever used in our measurements. Hence, here it is described in details. The spring constant k_c is given by,

$$k_c = \frac{E w_c t_c^3}{4 L_c^3}, \qquad \textbf{Equation 6-4}$$

where, E, w_c, t_c, and L_c are the Young's modulus, the width, the thickness, and the length of the cantilever, respectively.

The resonance frequency of a fundamental mode of a rectangular cantilever in vacuum is estimated as,

$$w_0 \cong \frac{t_c}{L_c^2}\sqrt{\frac{E}{\rho_c}}, \qquad \textbf{Equation 6-5}$$

where ρ_c is the density of the lever's material.

Applying the equipartition theorem to an AFM cantilever considered as a simple harmonic oscillator with one degree of freedom with higher vibrational modes being neglected, results in the following equation for the cantilever's stiffness (Hutter and Bechhoefer 1993),

$$k_c = \frac{k_b T}{(\Delta x^2)}. \qquad \textbf{Equation 6-6}$$

While calibrating, the cantilever is positioned far away from the surfaces, and the thermal vibration signals are recorded. From the above equation it is clear that the spring constant of the AFM probe can be determined by the stored thermal energy and mean-square cantilever deflection, ($\mathbf{\Delta}x^2$). The power spectrum of cantilever's thermal fluctuations, P_{therm}, calculated by numerically integrating the frequency domain resulting signal and equals the mean square of the fluctuations in the time series data (Hutter and Bechhoefer 1993), spring constant estimation is given as,

$$k_c = \frac{k_B T}{P_{therm}}. \qquad \textbf{Equation 6-7}$$

Power spectrum of the cantilever in air reveals an intensive resonant peak of the fundamental vibration mode. The power spectra show a Lorentzian shape and the resonant peak is fitted by Lorentzian function and the area under the fit provides $\{\mathbf{\Delta}X^2\}$ value. This is a convenient and fairly simple way to calibrate a cantilever. This method does not include the noise from other sources into the calculation and also ignores fluid damping. This would lead to an underestimation of the measured stiffness. Some sources claim the method can yield up to 20% of the error in the calibration (Walters *et al*., 1996). Other sources of error come from the fact that a cantilever is not an ideal one dimensional oscillator and also that noise from the environmental disturbance and instruments is always included in the recordings of the signal.

6.3 Polymer stretching under externally applied force: elastic model of protein

The bases of polymer elasticity theory were postulated around 60 years ago (Guth and Mark, 1934) when the remarkably unusual elastic properties of polymer materials were explained for the first time. With the introduction and eventual popularisation of Single Molecule Force Spectroscopy over the past few years the investigation and understanding of mechanical properties of individual polymer molecules, biomolecules like DNA, polysaccharides, lipids,

proteins and peptides (Roychoudhury *et al.* 2012; Janshoff *et al.* 2000) at nano level has noticeably advanced.

A protein consists of one or more chains of amino acids and macroscopically can be considered as a mechanically elastic polymer subject to thermal fluctuations. Modeling the protein as a polymer is useful because certain aspects of polymer theory can be applied to proteins and used to predict their behavior (Smith *et al.* 1996).

Two types of restoring forces (Austin *et al*. 1997; Treloar 1975) act while stretching a long, flexible elastic polymer, like protein, which are:

(i) Entropic elastic forces originated from the reduction in the number of conformations prevail at small displacements.

(ii) Enthalpically driven elastic effects like rupture of hydrogen bonds, salt bridges, conformational changes, bond dissociation (Janshoff et al., 2000) etc. resulted due to the enthalpic tension and forces at the large extensions of the polymer backbone.

The elastic nature of a polypeptide chain can be described using two models from statistical mechanics namely: the freely jointed chain and the wormlike chain (WLC) model (Janshoff *et al*. 2000). They are often used to quantitatively describe the force - distance (F- D) relationships during polymer stretching.

6.3.1 The freely jointed model

The freely jointed chain model is the simplest model used in polymer extension studies. In this model, the polymer is considered to be made up of rigid segments with the length l_k, called Kuhn lengths, joined at the ends and the segments are known as Kuhn segments. These segments are connected through flexible joints and have fixed length, straight and very rigid and unstretchable and are direct measure of stiffness of the polypeptide chain (Smith *et al.*1992). They can take any orientation and are free to rotate in any direction.

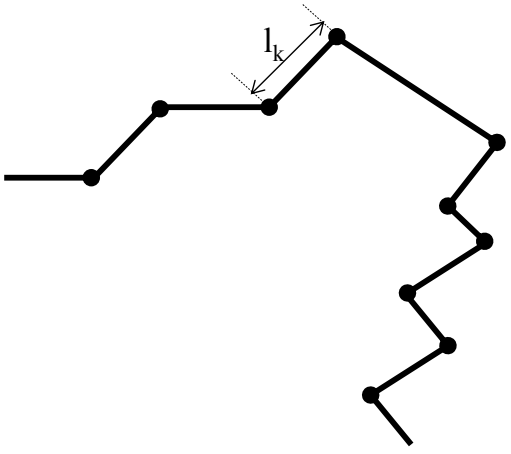

Figure 6-8: Freely jointed model.

When a force is applied, the alignment of segments tends to take place in the direction of the force. According to this model, fluctuations may take place only at the flexible joints of the rigid segments which can be arbitrarily close to each other and are assumed to act independent of each other. The neighbouring bonds being uncorrelated is the fundamental assumption behind this model. An extension of the polymer chain, x, depends on the applied force, F, in the following manner,

$$F(x) = L\left[coth\left(\frac{Fl_k}{k_BT}\right) - \frac{k_BT}{Fl_k}\right] = L\,\boldsymbol{L}\left(\frac{Fl_k}{k_BT}\right), \qquad \textbf{Equation 6-8}$$

where **L** is the Langevin function and L is the contour length of the polymer, which is defined as $L = nl_k$, l_k is the Kuhn length, k_B is the Boltzmann constant and T is the absolute temperature

6.3.2 The worm like chain model

Most biopolymers like protein, DNA etc. are semi flexible which means that they are rigid over length scales much larger than the size of a monomer unit. These polymers are described by the worm-like chain model as unstretchable, continuously flexible chains with homogenous spring of constant bending elasticity (Bustamante *et al*., 1994). This model was first proposed by Kraty and Porod and sometimes also known as the Kratky-Porod model.

In this model depicted in Figure 6-9, the polymer molecule is represented as an irregular curved filament, a worm like chain, which is linear within the persistence length, l_p, a parameter that indicates the stiffness of the molecule. This is the parameter of our interest in the study of understanding the impact of compatible solutes in membrane protein unfolding. According to the worm like chain (WLC) model the direction of the curvature at any point is random and the contour length is fixed. In polymer physics, contour length can be described as the length of a polymer at its maximum extension under fully stretched condition. The Force-distance relationship can be expressed as

$$F(x) = \frac{k_BT}{l_p}\left[\frac{1}{4}\left(1 - \frac{x}{L}\right)^{-2} + \frac{x}{L} - \frac{1}{4}\right]. \qquad \textbf{Equation 6-9}$$

Here F(x) is the applied force, x is the extension of polymer chain, k_B is the Boltzmann constant, l_p is the persistence length and L is the contour length.

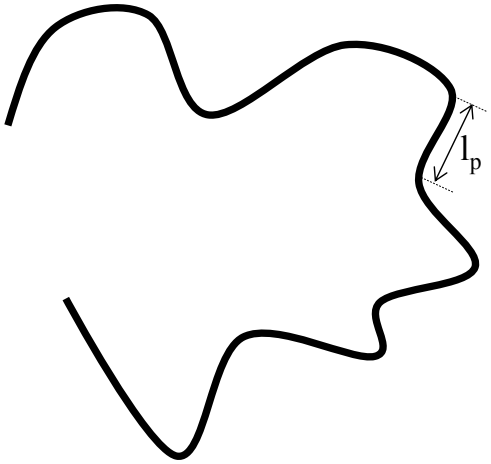

Figure 6-9: Worm Like Chain model (WLC).

This model has been successfully used in describing the biopolymers like protein and DNA (Roychoudhury *et al.* 2012, Oesterhelt *et al*. 2000, Bustamante *et al.* 1994).

In conclusion, as this project is focused on identifying the effects of compatible solutes on the mechanical unfolding properties of the membrane protein Bacteriorhodopsin, forces obtained in a mechanical unfolding experiment are fitted to a worm-like chain model only to identify true unfolding events in a pulling trace, by verifying that the contour length increment obtained is comparable to the contour length of the protein under study.

Chapter 7 Materials, Methods and Experimental Set-up

7.1 Introduction

The sample preparation step is very crucial for single molecule experiments in order to obtain good quality and reliable data. Utmost care needs to be taken in preparing a proper surface to adsorb the sample, buffers, protein sample and solutions of different osmolytes at different concentrations.

7.2 Surface preparation and treatment for adsorption of the sample

Scanning probe microscopes and in particular the atomic force microscope, enables performing experiments of both imaging and force spectroscopy, with a sample adsorbed at a surface and immersed in an appropriate buffer. It is understood that a protein fixed onto a surface, adsorbed maintains an exact or nearly the same activity that it would have in the bulk solution at physiological conditions.

To achieve stable, reliable AFM measurements and to avoid sample movements while scanning it is important to obtain flat supporting surfaces for sample adsorption. To prepare sample support, normal glass slides were taken. Disks of around 10 mm diameter were punched from a thin Teflon foil (thickness ≈ 0.3 mm, commercially available) and glued to glass slide with instant glue (UHU Plus, Sofortfest). Muscovite mica (Mica New York Corp., USA) was punched into disks of 5 mm diameter and glued onto the teflon disk with a water-insoluble, two component epoxy glue (UHU Plus, Sofortfest). Prior to experiments the mica surface of the support was cleaved with a glued tape. The freshly cleaved surface was imaged with an AFM at a scan area of 20×20 μm^2 under buffer conditions to check its flatness. Mica, besides the normal advantage of being atomically flat over long distances and easy to prepare by cleaving, is strongly hydrophilic, a property which in our case is very good for our sample.

A critical step toward observing a macromolecule at work onto a surface, as it is the case of an AFM experiment, is the procedure used to deposit it onto the substrate surface. A very strong binding could in fact somehow limit the movements of the protein, leading to its inactivation; with a too weak binding, the proteins could be impossible to be imaged even in the gentlest possible scanning conditions, because the probe would move them all around the surface. A judicious way is to bind the protein very weakly to the surface. For the purpose of attaining good imaging conditions the protein molecules are laterally packed on to the surface; in such a condition if the conformational changes, which the protein is supposed to

undergo, are not constrained by the lateral packing, it will maintain the activity and, at the same time, it will not be displaced by the scanning tip because it is part of an extended two dimensional layer.

7.3 Buffers and osmolyte solutions

The natural surroundings of protein(s) in the interior of cells are an environment where some conditions like pH, temperature and ionic strength are kept relatively constant. To keep the pH constant at physiological levels *in vitro*, a buffer solution is commonly used. All buffer solutions were made with fresh nanopure water and chemical reagents with the purity of an analytical grade from Sigma/Merck (Germany). For our measurements we used Tris+KCl buffer. KCl (Potassium chloride) is the salting agent and Tris (tris(hydroxymethyl)aminomethane), is the buffering agent for slightly basic conditions (pH 7.8).

To study the stabilizing effects of osmolytes, several concentrations of all three osmolytes (ectoine, betaine and taurine) were used during the experiment. All three osmolytes were tested under several concentrations (1mM, 10mM, 100mM and 1M) in a solution of Tris+KCl buffer to study their stabilising effect and understand the physical or chemical interactions between the solute molecules and the protein. Ectoine was received as a gift from Bitop Gmbh, Witten and betaine and taurine were purchased from Sigma-Aldrich. All of these were 99.9% pure.

7.4 Protein sample

2D native crystals of the membrane protein bacteriorhodopsin were received as gifts. 2D crystallization supports the native environment of membrane proteins and provides flat protein-enriched membrane patches, which is beneficial for Single Molecule Force Spectroscopic techniques. The preparation of the sample is rather easy. Sample concentration must be carefully chosen to avoid overcrowded distribution and vice versa. On one hand, a high concentration of proteins may lead to aggregation while on the other hand, few proteins will be adsorbed if a low concentration of proteins is used, producing insufficient unfolding events.

The protein sample is diluted in 300mM KCl and 20mM Tris at pH 7.8 (standard buffer). For studying the concentration dependency of unfolding events different concentration of osmolyte solutions were prepared by adding the corresponding amount of solutes in the Tris + KCl buffer. Around 10 µl of protein solution in buffer was incubated for 15 minutes to assure proper adsorption of the sample onto the surface. After the deposition, which has

always taken place at room temperature, the sample was rinsed intensely to remove the loosely bound proteins with the standard buffer. This buffer is also used during imaging and force spectroscopy.

7.5 Setting up the AFM

Before starting the measurements it is important to arrange the set-up for accurate measurements. The cantilever is chosen and checked under a microscope for bends and cracks or presence of any other potential mechanical problems. Buffer solutions were prepared in advance and filtered. The cleaned cantilever holder (Figure 7-1) was then placed on the work bench and the chosen cantilever was put in it and fixed. The cantilever holder is made up of plastic with the transparent glass place like prism and a designated place to hold the cantilever allowing the laser beam and the reflected light to pass through the glass.

Figure 7-1: Cantilever holder inside a clean petri dish.

For a successful experiment, the cantilever holder needs to be very clean without the presence of dust particles or broken pieces of previously used cantilevers. If any contaminants remain, they can interfere with the tip or the protein solution giving rise to artifacts during measurements. The cleanliness of the holder is also crucial as it allows the required reflection from the cantilever to be precisely sensed and read by the photodetector. Grease or dust on the surface of the fluid cell may create diffraction and introduce errors in the measurements. For cleaning, the holder is handled very carefully with hand gloves on and is washed five times with ultra-pure spectroscopic grade ethanol (99.9% purity, Uvasol, Germany) and nanopure milliQ (18.2 MΩ) water each, alternatively. Care is taken that it does not fall off the hand or touch any unclean surface. The holder is then dried by using nitrogen flow and the

cantilever is inserted for the experiment.

7.6 The Experimental Set-up

The effects of osmolytes on mechanical stability of proteins were studied by using the combination of Atomic force microscopy and single molecule force spectroscopy.

The measurements for our experiments were performed using a commercial Asylum Research MFP 3D AFM instrument. The cantilevers used in our experiments were Olympus OMCL TR400 PSA silicon nitride cantilevers with a given spring constant of around 20 pN/nm. The commercial AFM was upgraded with two personal computers (PCs) equipped with 64 bit data acquisition electronics. For data capture, the deflection from the photodetector and z -position signal were adjusted.

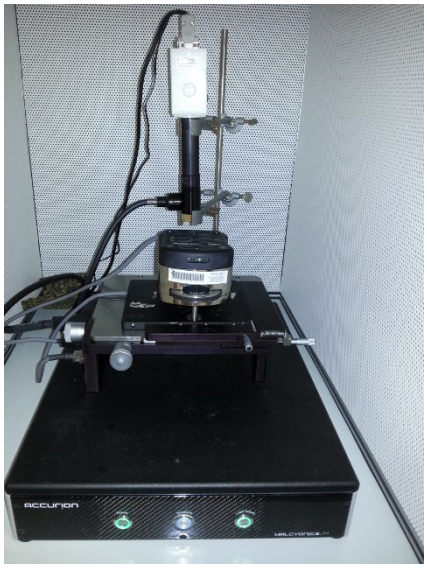

Figure 7-2: AFM set up (Asylum).

Once the AFM is ready to get started, the sample solution in the buffer is placed on the sample bench of AFM. The spring constants of cantilevers were calibrated in buffer using thermal fluctuation analysis, already discussed earlier.

The tested buffers were 20mM Tris + 300mM KCl (standard buffer) and standard buffer with different protecting osmolytes. Due to the weakening effect of osmolytes on the binding affinity of protein to both cantilever and substrate, the pick-up efficiency was typically very low, making these experiments quite challenging.

At first the sample is scanned and a nice patch of protein is selected for performing mechanical pulling experiments on it Figure 5-4. The procedure for stretching proteins, as shown in Figure 7-3 is as follows: the tip is pressed against the mica surface at forces of

several piconewtons, which allows the protein to stick to the tip by non-specific interactions. Then, the cantilever is moved away at a constant speed and the protein chain begins to extend. Force curves were recorded at a retraction speed of 400 nm/s.

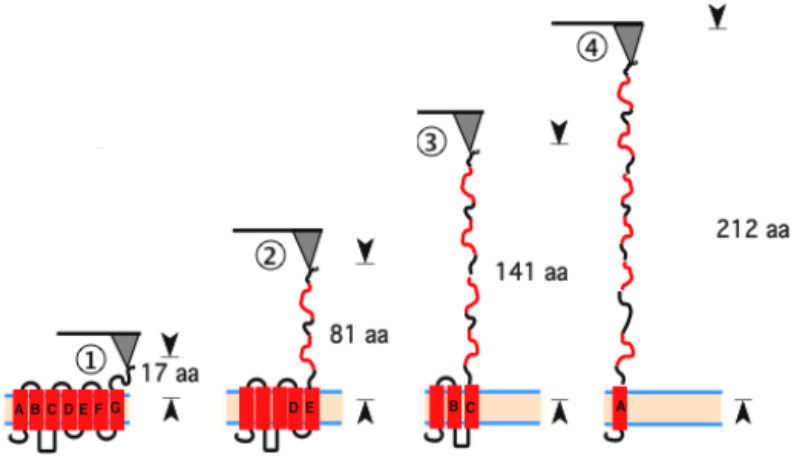

Figure 7-3: The model showing how the lengths of unfolded parts of a polypeptide chain can be measured. This model explains the peaks in the force spectra as the sequential extraction and unfolding of a single Bacteriorhodopsin molecule. If the force is applied on the COOH-terminus, helices F and G will be pulled out of the membrane and unfold. Upon further retraction, the unfolded chain will be stretched and a force will be applied on helices D and E until they are extracted from the membrane. Thus, peak 2 denotes unfolding of helices D and E and peak 3 indicates unfolding of helices B and C. Peak 4 shows extraction of the last remaining helix A (Adapted from Oesterhelt *et al.*, 2000).

The tension in the polypeptide chain is increased until it is high enough to extract a pair of helices or domain out of the membrane and unfolds it. This rising force is constantly monitored by the deflection of the cantilever registered by the photodetector. When a pair of helices, in the case of bacteriorhodopsin is unfolded, the tension is released and it also most goes back to zero, relaxing the cantilever. This also prevents another loop of helix to get unfolded simultaneously. This cycle of tension-relaxation continues until the full length of the protein molecule is stretched and extracted out of the membrane. This is how single and individual unfolding events can be observed at real time while the experiment is being executed.

A single experiment involves obtaining many force-extension curves containing particular unfolding events whose unfolding forces could be used as representative values of the unfolding force of the protein for each testing condition. Data corresponding to the characteristics of the trace such as number of samples, piezo sensitivity, photodetector sensitivity as well as cantilever deflection and extension are stored in the computer on line and then they can be analysed after the experiment is finished. The system has a screen that

shows an instant "force-extension" curve for each trace that allows monitoring the quality of the data being taken. Several options present in the software allow real time viewing of the various parameters like speed, contact time, force etc.

7.7 Data analysis

The final outcome of mechanical pulling experiments of protein is the common called force – extension or force – distance (F - D) curves. In order to extract and obtain information from these data, they need to be processed and analysed before they are finally interpreted. The data recorded during the experimental procedure and collected thereafter are stored as voltage values or binary values, directly from the analog-to-digital converter; one set of data from the cantilever output that will be converted into force values, and the other from the position of the scanner, that will be converted into extension values. The deflection values from the cantilever are converted into displacement by multiplying the output value by the photosensitivity of the photo quadrant detector: $x = \sigma, v$, x being the displacement of the cantilever (modeled as an ideal spring), σ, the photosensitivity constant and v, the deflection data, in volts, from the Analog-to-digital-convertor (ADC) system. The output from the cantilever is then transformed into force units by multiplying off-line, the deflection values, by the spring constant of the cantilever, expressed in pN/nm.

A single molecule experiment aims at testing one molecule at a time and in unspecific attachment experiments, the likelihood that a single molecule would attach to the tip is very low. Also, there is a possibility of more than one to several molecules to be able to attach to the tip of the AFM cantilever. So, to be sure that only a single molecule has been stretched, a very characteristic pattern of force-extension curve is collected, where each peak corresponds to a pairwise unfolding event of the membrane protein bacteriorhodopsin being pulled out of the membrane. Because the helices are structurally the same, unfolding forces are expected to be similar and peaks be equally spaced. This characteristic pattern is also called the "mechanical fingerprint" of the protein. The use of multiple copies of similar and nicely superimposing force curves are collected to be analysed, for a better statistics and also to unequivocally identify the unfolding events from any other non-specific surface-cantilever interactions. The data analysis procedure starts with the selection of traces that exhibit the typical 4 peak force curves of unfolding events of a single bacteriorhodopsin molecule, no diffraction pattern and a clear baseline after the detachment of the protein from the tip.

The data analysis was performed using the custom software IgorPro 6.1 (Wavemetrics, USA). IgorPro being a custom software made it easier for us to analyze the force curves

using self-written programs on Igor that does an automatic peak search and fits the rising part of the force curves ahead of each peak with the worm like chain (WLC) model (as described previously). This model considers the protein chain as a continuous flexible rod subject to thermal fluctuation and allows the protein to be characterized in terms of the total length (contour length, **L**) and its persistence length ($\mathbf{l_p}$). From this we obtain the length of the stretched amino acid chain and thereby the number of amino acids as well as its persistence length, which may depend on the surrounding conditions. From the number of amino acids obtained from each fit, counting back from the C-terminus, it can be calculated which part of the protein is left in the membrane as an intermediate. The first peak in a force curve can be attributed to a direct interaction between the tip and the surface and also includes the unfolding of the first two helices. As the cantilever separates from the surface, the protein begins to stretch and bends the cantilever. This reveals that helices G and F, D and E, and B and C, respectively, mostly unfold (Muller *et al*, 2002; Oesterhelt *et al*., 2000) sometimes showing less stable intermediates where only one helix is extracted out of the membrane. The seventh helix, A, is then extracted from the membrane in a last step (Oesterhelt *et al*., 2000).

A sudden drop in the force curve after a peak indicates the extraction of a pair of helices of the membrane protein bacteriorhodopsin out of the membrane. The other peaks similarly show the unfolding events of remaining helices eventually and the last drop in the force curve corresponds to the extract of the full protein out of the membrane and subsequent detachment of the protein from the tip.

The parameters of interest in our study are the unfolding force and the persistence length of the unfolded polypeptide chain. The measured unfolding forces reveal specific inter and intra molecular interactions that stabilise the protein structure and can be taken as a measure for the protein stability in the membrane against denaturation due to external stress like temperature or ionic strength as well as various additional solutes. The persistence length is a measure of the flexibility of the chain. In other words, persistence length of an unfolded amino acid chain is a measure for its tendency to form a compact coil in solution which supports (re)folding of the protein. Shorter persistence lengths correspond to a higher tendency to coil up while longer ones indicate a more extended conformation.

7.8 The technical problems of force spectroscopic measurements and measures to handle those

One of the most important and crucial concern in force spectroscopic experiments is to be able to control and lock the deflection of the cantilever to the user-defined set-point so as to

set the proper pulling force. Thermal drift of the cantilever yields lower actual values of the pulling force compared to user-defined ones. In theory, the resting position of the cantilever is assumed to remain the same (zero force position). Bending with the ambient temperature changes and due to the laser heating is a big disadvantage of commercial silicon nitride cantilevers (Radmacher *et al*. 1994), which were also used here in force-clamp measurements.

However, drift of the cantilever results in a shift of zero force position, which cannot be tracked during the measurements. Prior to the start of each experiment, every cantilever was calibrated and checked for its sensitivity to thermal fluctuations in the system and also measured again after the completion of the experiment. When the buffer and osmolyte solutions dried up while measurements were running it was refilled using micropipettes and the AFM was allowed to equilibrate for at least 30 mins to get rid of the drift.

It is not very easy to collect "enough" force curves to be used for analysis, mostly because the tip-sample attachment is through non – specific interaction. Hence, only a few "good" force-extension curves can be obtained from each experiment. The challenges that arise are typically, attachment of more than one protein molecules to the tip of the cantilever which represents similar peak pattern but with higher unfolding forces. Another commonly encountered problem in pulling experiments is that is because of unspecific interact between tip and sample, so it is possible, in many instances that the position where the tip contacts the protein is not known or also could be that the tip attaches somewhere in the middle of the polypeptide chain and pick up the molecule from there and not in the terminus. This way, the data will show unfolding events. So, to minimize the probability of such errors and collect only the proper force curves, only the traces with typical four peaks were taken into consideration as representative of the protein under study.

Chapter 8 Results

Compatible solutes increase the stability of proteins and have positive effects on binding affinity etc. It has also been demonstrated that these molecules have use in several other biological and biotechnological applications such as in Polymerase Chain Reaction (PCR), in health drinks, in medicines to state a few. Among these various applications and uses, both in basic research and commercial purposes, the stabilising effects of these molecules on proteins are of greater interest because it's well known that they stabilise the protein but the mechanism of action of these solutes is still not very clear and well understood.

The aim of this work was to study the impact of compatible solutes on membrane protein (un)folding, how these solutes affect the mechanical stability of the membrane protein Bacteriorhodopsin and subsequently facilitating the process of (re)folding. To achieve this, different concentrations of different compatible solutes were chosen for the experiments based on previous knowledge and the hypothesis formed. After setting up the instrument and adsorbing the sample on a mica surface, as described in Section 6.1, AFM measurements were done. At first a topography image of the sample was taken followed by mechanical pulling of single proteins by extracting them out of the membrane.

To understand the stabilising effects of the compatible solutes ectoine, betaine and taurine particularly on membrane proteins, we analysed their effects on the unfolding force and persistence length of the membrane protein Bacteriorhodopsin. Both the parameters were extracted from all recorded force curves by fitting with the WLC model to them and were histogrammed for representation.

The results of the concentration dependency study of ectoine, betaine and taurine are discussed below. The peak forces and persistence lengths data were also displayed as scattered plots in addition to histograms to get accurate and more information out of these data for each intermediate peaks. We studied and compared the results obtained from different concentrations of the same compatible solute as well as the disparity in influence of among different compatible solutes.

In addition to carrying out experiments on concentration series, we also analysed the data for individual unfolding intermediate peaks of each osmolytes at all the concentrations used and compared them together and also with no osmolyte condition to see more in detail if the effect of osmolytes is on full length of the protein altogether or if they have different effects on single intermediate peaks as well.

Taking previous works and knowledge about the compatible solutes and their impact on

living cells and biological macromolecules, we started our first set of measurements in presence of 1M concentration of each compatible solute.

In this chapter, the findings of the force spectroscopic measurement data are presented. During mechanical unfolding of membrane proteins using single molecule force spectroscopy, force is applied from external source which induces the pairwise unfolding of the structural elements of the protein BR (Kedrov *et.al*, 2007). Since, the interaction between the tip and the sample is non-specific; there were force curves recorded with various lengths. Of these, only the force curves which showed full length unfolding of the membrane protein Bacteriorhodopsin (Oesterhelt *et. al*, 2000) were taken for further analysis.

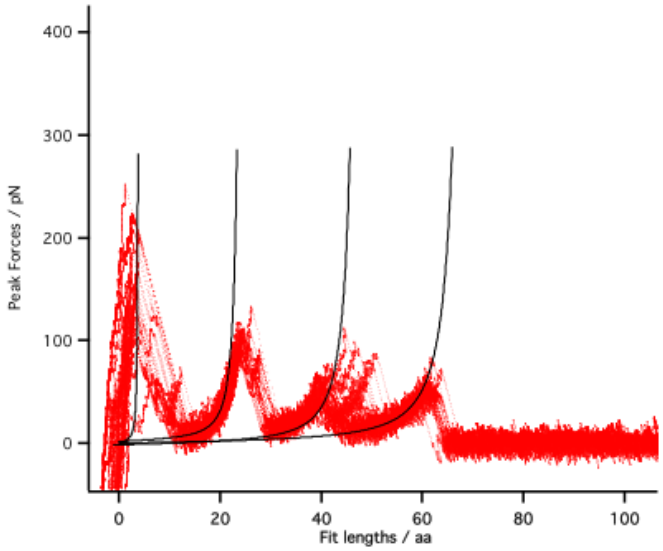

Figure 8-1: Superimposed Bacteriorhodopsin force curves.

Force-Distance spectra of Bacteriorhodopsin unfolding showing typical four peak pattern (in red) with WLC fits (in black).

A careful collection and selection of force curves for histogramming were done by overlaying the nice curves. The force-extension curves of Bacteriorhodopsin exhibiting typical four peaks were collected and superimposed Figure 8-1 to find a pattern of similar curves. The curves were fitted with the WLC model and analysed. This analysis generated histograms for each experimental condition.

8.1 Increase in unfolding forces and reduction in persistence lengths in presence of compatible solutes

As described earlier, in a single force curve of BR unfolding, there are four main peaks. Of the four main peaks, each peak represents a pair of α-helices, pulled out of the membrane.

In the histograms, the peak force and persistence lengths are plotted against the number of

counts recorded in each experimental condition. By definition, peak force is the force at which an unfolding event takes place and a peak is detected. And persistence length of a polymer molecule is defined as the parameter that quantifies the stiffness of the polymer. Due to the possibility of shorter fit ranges yielding inaccurate fits, we fitted only the peaks with specific and fairly longer fit ranges to avoid the errors resulting because of inaccurate fits.
In the displayed histograms, the peaks of all selected force curves, with specific fit ranges, chi square values and force ranges were plotted to control the quality of the fits. Using the software Igor, hence the peaks with the desired qualities were taken in for generating the histograms. Initially, an average force and persistence lengths of the unfolding of all loops and helices were plotted and not of individual unfolding events to get an overview of how the unfolding forces and persistence length of the full polypeptide chain is affected under the varying experimental conditions.

8.1.1 Change in peak force

From our first set of experiments, we first histogrammed the unfolding forces recorded while extracting Bacteriorhodopsin molecule out of the membrane. Unfolding experiments on Bacteriorhodopsin were first done only in the buffer, without adding any compatible solutes. As mentioned earlier, compatible solutes are called so because they are compatible with cellular metabolism and other physiological activities even when they are accumulated in the cells at molar concentration. Hence, we started our experiments with 1M ectoine followed by 1M betaine and 1M taurine.
As we can see in the displayed histogram in Figure 8-2, in absence of any compatible solute, the force histogram shows a mean unfolding force of around 59pN, whereas in presence of the different compatible solutes the unfolding forces have been increased by different magnitude in case of different concentrations. The results found were as expected from the previous knowledge that osmolytes stabilise the protein structure. Here also in case of the membrane protein Bacteriorhodopsin, it can be seen that the presence of osmolytes in the environment result in a mild to significant rise in peak forces. The increased unfolding forces thus recorded reveal the localization of intra- and intermolecular interactions that stabilise the protein structure. The shift to higher forces in presence of different compatible solutes can be attributed to the stabilisation of the membrane protein bacteriorhodopsin by these molecules resulting in the requirement of higher forces to extract the protein out of the membrane. It was also quite interesting to notice that although the molecules under study are all compatible solutes, and they all result in increased peak forces but the magnitude of the increase in peak

forces varies considerably.

A) B)

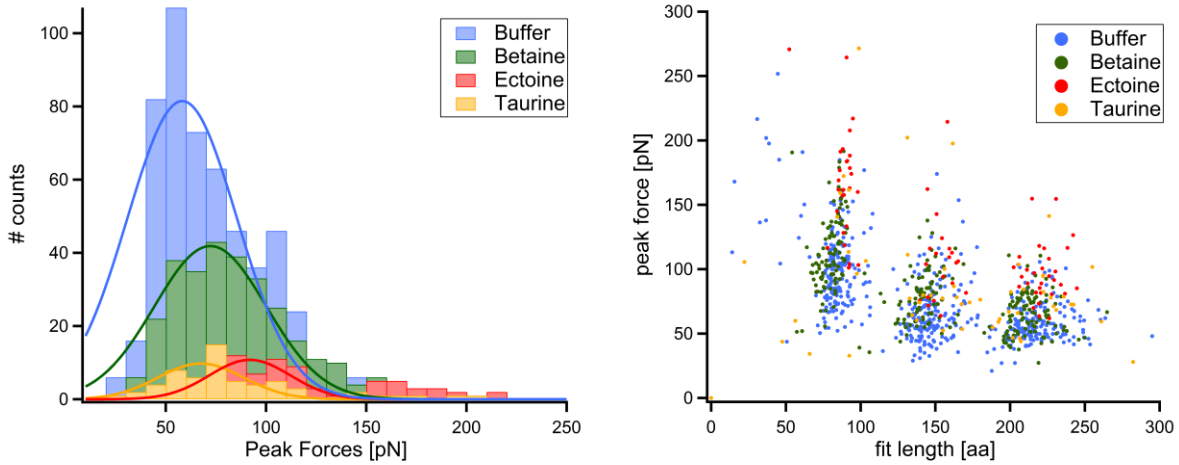

Figure 8-2: Histogram showing the dependency of unfolding forces of BR.

A) Unfolding forces obtained from single molecule force spectroscopic experiments on Bacteriorhodopsin are histogrammed here. Here, the unfolding forces in pN are plotted against the number of counts of data recorded.

B) Scattered plots showing the effect of 1M concentration of osmolytes on peak force for each unfolding peak of a single BR extraction out of the membrane.

Betaine and taurine showing the least increase in peak forces in comparison with the no compatible solute condition and ectoine almost double the peak force indicating more pronounced effects on membrane protein stabilisation. Upon fitting the histograms with Gaussian distribution it was found that in case of 1 M each of the osmolytes ectoine, betaine and taurine mean unfolding forces were 92 pN, 72 pN and 67 pN respectively.

Individual unfolding peaks were then analysed using self-written programs to get a measure of forces for the four main peaks of each unfolding event. These results shown in the form of histograms were of all the unfolding peaks taken into account. Two-dimensional scattered diagrams were then plotted as function of peak forces *versus* fit lengths which revealed the changes in unfolding forces, for each intermediate peak. In Figure 8-2 B) we can see three distinct distributions and one unclear. The first one is very scattered and unclear with less data, which indicates that the first unfolding peaks are not clear enough to get a good quality and reliable fit. This is because of the unspecific attachment of the tip to the terminus while the protein is pulled out of the membrane. This peak has the highest unfolding force, which ranges from around 150 pN to about 250 pN. The four distributions indicate the unfolding forces required for pairwise extraction of helices G-F, E-D, C-B and A of the seven alpha helical membrane protein Bacteriorhodopsin respectively. We can notice here that all three osmolytes stabilise the unfolding intermediates in a similar fashion, just in case of betaine there is almost no change in the peak forces for the last peak as compared to peak forces of

last unfolding peak without any osmolyte. The probable causes that of all three osmolytes betaine has more data counts than ectoine and taurine are discussed in chapter 9.

8.1.2 Change in persistence length

The persistence length of BR was affected by the presence of these compatible solutes quite significantly. A reduction in the value of persistence length, from minor to profound ones was recorded in presence of 1 M concentration of each osmolyte. In this case, taurine has the most pronounced effect by reducing the persistence length of the unfolded polypeptide chain to almost half while ectoine has a moderate effect and betaine almost a negligible one.

A) **B)**

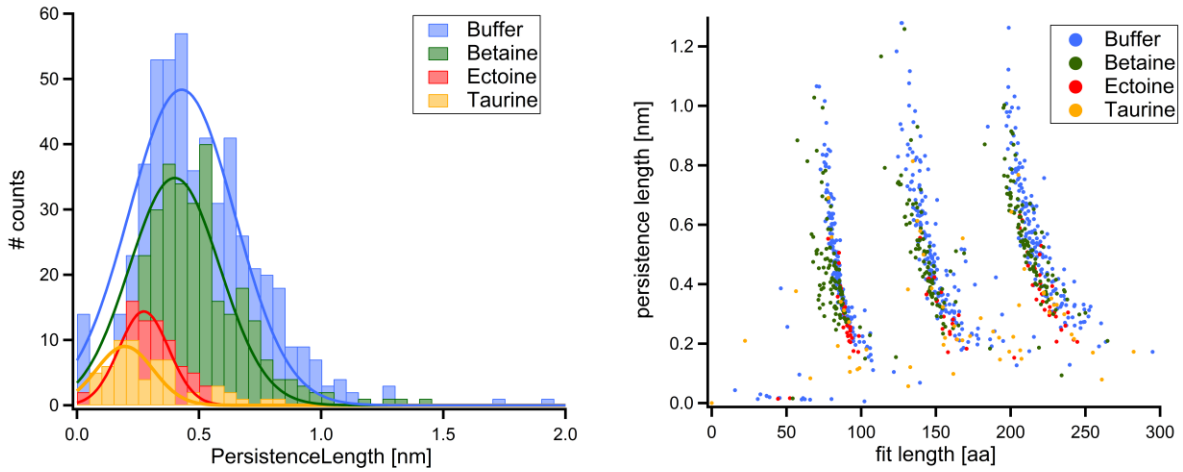

Figure 8-3: Histogram depicting the dependency of persistence length of BR.

A) Unfolding forces obtained from single molecule force spectroscopic experiments on Bacteriorhodopsin is histogrammed here. Here, the persistence lengths in nm are plotted against the number of counts of data recorded.

B) Scattered plots showing the effect of 1M concentration of osmolytes on persistence length for each unfolding peak of a single BR extraction out of the membrane.

In absence of the osmolytes the persistence lengths was found to be 0.44 nm, and in presence of 1 M ectoine it got reduced to 0.28 nm while 1 M betaine and 1 M taurine reduced it to 0.4 nm and 0.2 nm respectively as seen in Figure 8-3 A. This gives us an idea how the whole length of the stretched polypeptide chain is affected by different osmolytes at a high concentration of molar level.

As in the case of the 2D scattered plots of peak forces, similarly we plotted scattered diagrams to see the effect of osmolytes on persistence lengths of the each individual unfolding event. In Figure 8-3 B), betaine has least effect while taurine and ectoine show more significant effects exhibiting a pronounced reduction in persistence lengths. But again, what is visible in these average scattered plots of each unfolding event of all selected force

data gives us the idea that the whole polypeptide chain is affected and stabilised by the osmolytes in a similar way. All the helices behave similarly and exhibit similar unfolding pattern. A decrease in persistence length is observed in case of all intermediate peaks. We also see a very or almost missing first distribution in Figure 8-3 B) which is due to the filtering out bad fits to the first peak of the curves because of unspecific interaction between the tip and the sample.

These significant findings and observations using 1 M concentration of each of the compatible solute lead us to go for a concentration dependent study with lower concentrations of the osmolytes to see how and at which concentration these solutes start influencing unfolding forces and persistence lengths of the membrane protein bacteriorhodopsin.

8.2 Dependence of unfolding forces on different osmolytes and their varying concentrations

From the initial experiments with 1 M concentration of each of the compatible solutes, it was observed that all three compatible solutes resulted in an increase in peak forces required in unfolding of the protein. But the magnitude of increase in the forces was not the same and there was a significant difference in stabilising effects of each compatible solute. So, it was interesting to see where did the effect starts to occur and at what scale. Hence, we decided to go on with further measurements at different concentrations of the compatible solutes. We did force measurements at 1 mM, 10 mM, 100 mM and 1 M concentration of ectoine, betaine and taurine to see where and at which concentration osmolytes start influencing unfolding forces and persistence lengths of the membrane protein Bacteriorhodopsin. In this section, the results of concentration dependency of unfolding forces are presented.

8.2.1 In presence of Ectoine

After my experiments with 1M ectoine, it was found that this compatible solute, also working as osmoprotectant has a significant effect on membrane protein stability. Hence, I performed concentration dependent experiments and the results are represented in the form of the histogram below (Figure 8-4).

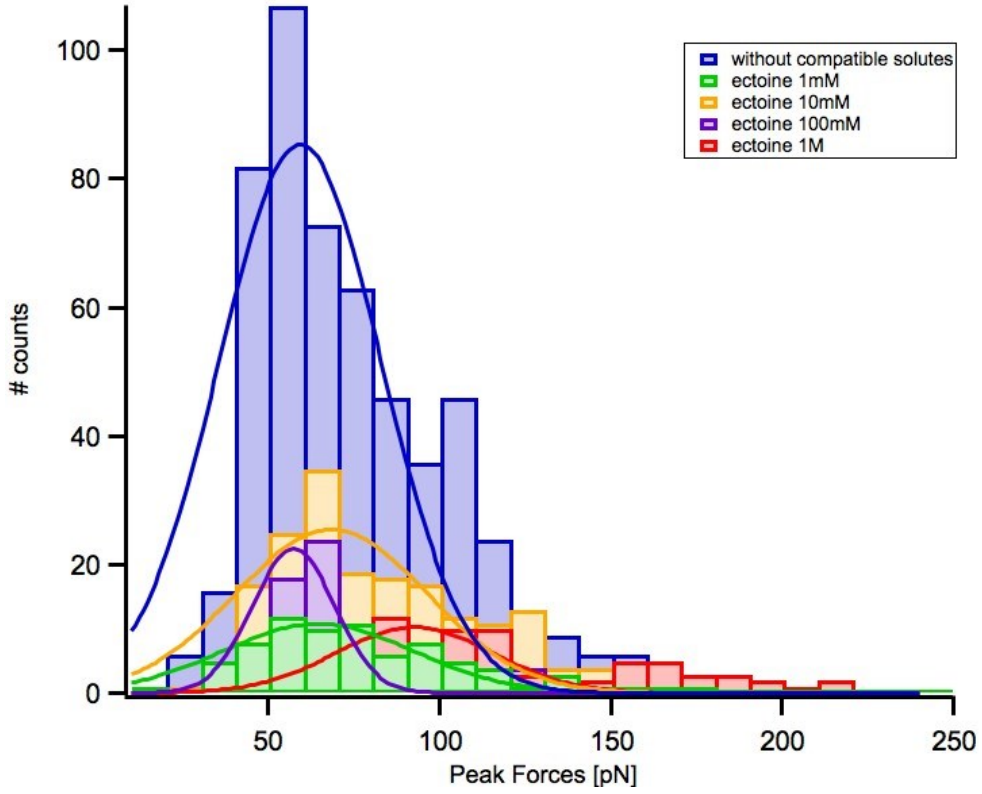

Figure 8-4: Histogram showing the dependency of unfolding forces of BR on changing concentrations of ectoine.

Similar number of experiments were carried out for each condition (approximately two weeks were devoted to measurements of each condition) to collect a considerable number of force curves for the analysis. These histograms give us the idea of changes in peak forces upon addition of ectoine in the environment at different concentrations. Also we can observe the reduction in the number of data counts for each condition as compared to the only buffer conditions. We can see that the increase in force is not linearly dependent on the increase in concentration of ectoine. While 1M concentration shows maximum increase in peak forces, the least increase is observed in the case on 100mM concentration. These observations indicate that the effect of an osmolyte on membrane protein stabilisation is not local or specific. They act differently at different concentrations.

8.2.2 In presence of Betaine

Similar as in the case of ectoine, histograms were plotted for different concentrations of betaine ranging from 1mM to 1M as a function of data counts *versus* peak forces (Figure 8-5). In this case, we can interestingly observe that there is a nearly linear dependency of increasing force with the increase in concentration.

The histograms are fitted with gauss distribution and the mean value of the Gaussian fits is taken to be the average force for each conditions.

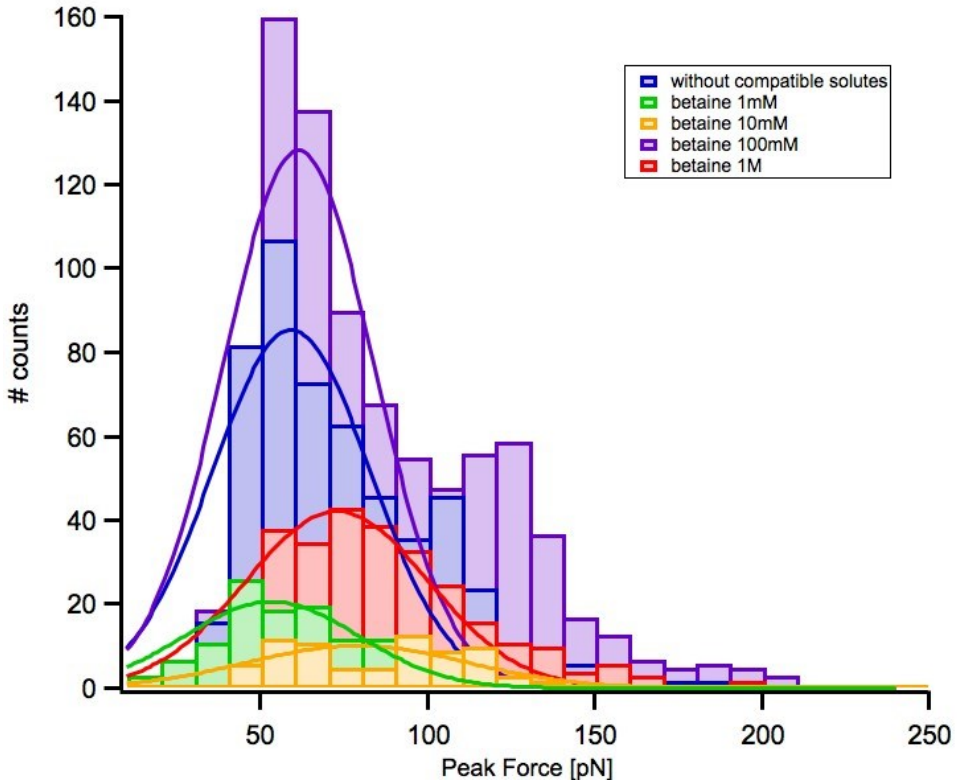

Figure 8-5: Histogram showing the dependency of unfolding forces of BR on changing concentrations of betaine.

8.2.3 In presence of Taurine

The overlay of histograms displayed below exhibits the dependency pattern (Figure 8-6) of unfolding forces on various concentrations of taurine. It has been noticed that in case of taurine, there is a very significant reduction in the recorded data count. The counts were so low for 1mM taurine (only 8 fairly good quality curves could be recorded) that a proper and accurate histogram could not be plotted because of the dispersed and broad distribution of the data and a very high noise level. In this case, surprisingly the low concentration of 10mM exhibits highest increase in peak force. Of all three osmolytes used, the unfolding experiments in presence of taurine yielded the least number of proper full length unfolding force curves.

The potential causes of these effects are discussed in Chapter 1.

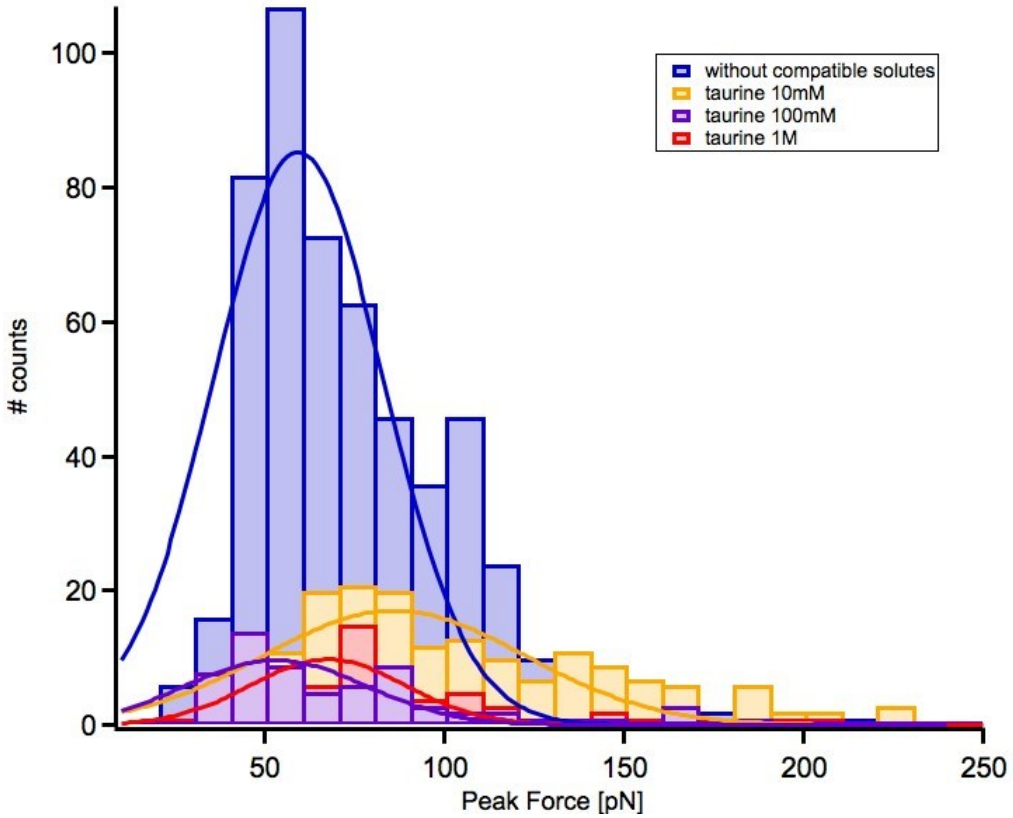

Figure 8-6: Histogram showing the dependency of unfolding forces of BR on changing concentrations of taurine.

8.3 Comparing unfolding force at different concentrations of ectoine, betaine and taurine

Figure 8-7 represents the impact of increasing concentration on peak forces of BR. There is a slight increase in overall unfolding forces in case of taurine; betaine shows an almost linear increase in forces while ectoine has the highest effect on unfolding forces resulting in nearly double the value at 1 M ectoine than no osmolyte condition. There is a slight dip in the forces at 100 mM concentration, mainly in case of ectoine and taurine.

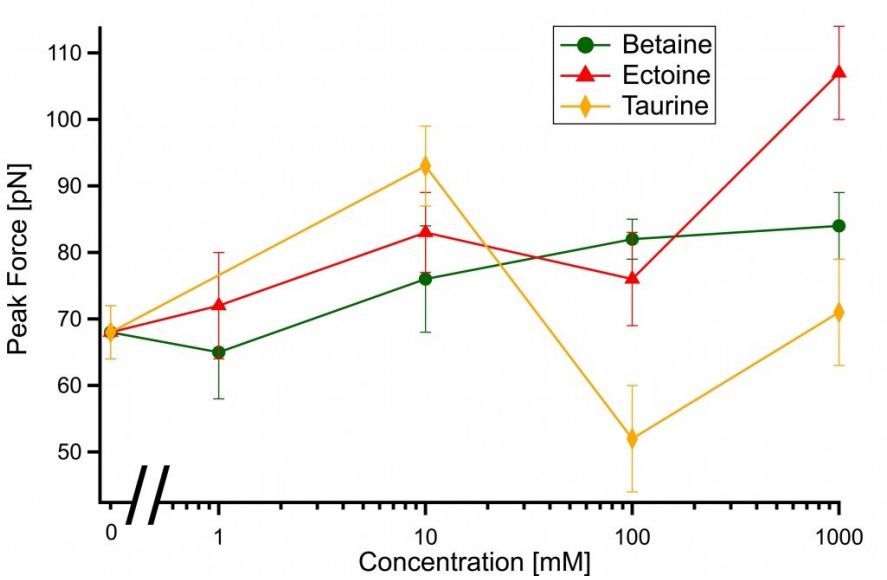

Figure 8-7: Impact of concentration changes on unfolding force.

It is also noticeable in the graph that ectoine and taurine follows a similar course. This graph shows how in four different concentrations and at no osmolyte condition each of the osmolytes behaves with respect to protein stabilisation. The peak force value plotted against each concentration is the sum of all four peaks of one single unfolding force curve for all good quality curves. We also plotted same graph with the values derived from fitting the histograms where an average of all four peaks of each force was taken to get the mean value to be plotted on the graph (Data not shown here). This was done to be sure of the distribution and its error and if taking an average of four peaks of each curve or plotting separate histograms for each curve and then adding these four yields similar or same result. We could see that the outcome of these two graphs was similar and Gaussian model is nearly most appropriate model for fitting these data.

In case of 1mM taurine, there is no value plotted in the graph because of too less data available to make a good and statistically reliable distribution and fit. It is observed that ectoine and taurine follow a similar pattern. This graph helps to get the information out of it more easily with a comparison of all three osmolytes at various concentrations within one framework.

8.4 Dependence of the persistence length on different osmolytes and concentrations

The persistence length of the stretched polymer was determined, compared and studied for different experimental conditions of ectoine, betaine and taurine. In this section, the changes in persistence length of BR upon addition of compatible solutes at 1mM, 10mM, 100mM and

1M concentrations of these three osmolytes under study are presented. A reduction in persistence lengths is noted in presence of all osmolyte.

8.4.1 In presence of Ectoine

In Figure 8-8, the changes in persistence length in presence of ectoine at various concentrations is shown. Ectoine being used in cosmetics, sunscreen lotion etc. makes it an interesting molecule to study its effect on membrane proteins. The graph presented below is a function on number of data counts *versus* persistence length of the polymer. Here also the dependence of increase or decrease in persistence length of BR is quite non-linear on the concentration changes of ectoine. Although we can see a shift in all the histograms of all concentrations of ectoine towards left hand side of the histogram depicting the data without any osmolyte in it. This indicates that under the influence of ectoine the polypeptide chain gets a reduced persistence length but the reduction is very specific for definite concentrations. This suggests that the tendency of the membrane protein BR to collapse into a more compact conformation by specifically excluding the ectoine molecules from the protein vicinity increases upon addition of ectoine but there is no local or specific binding site.

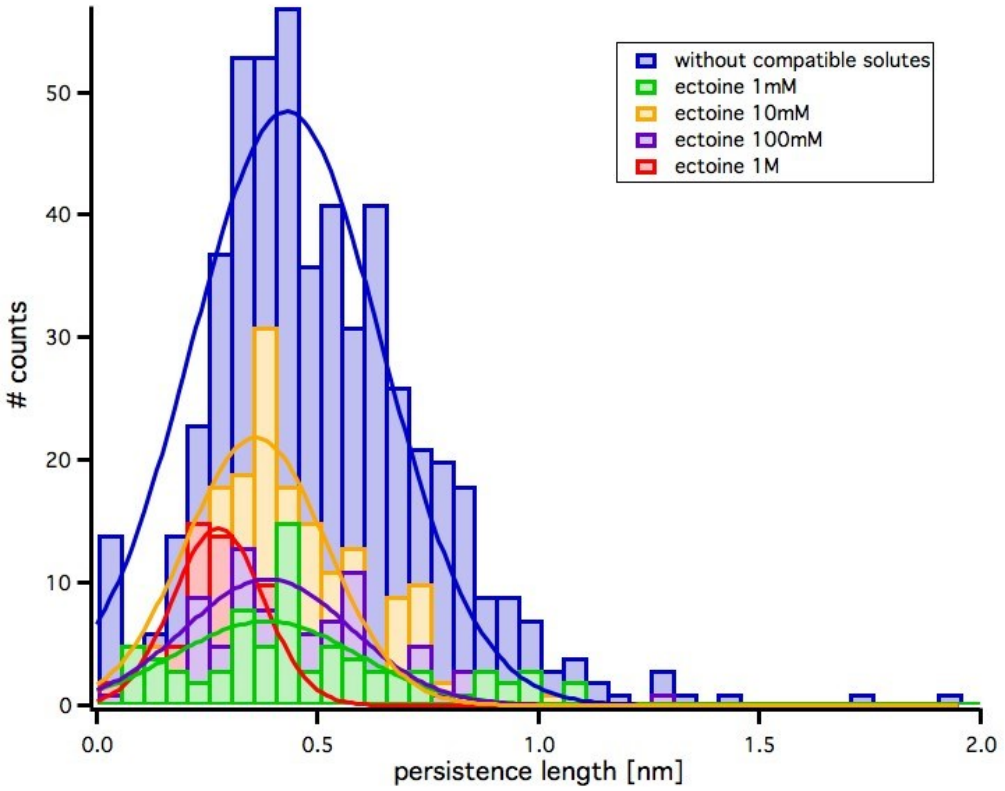

Figure 8-8: Histogram showing the dependency of persistence lengths of BR on changing concentrations of ectoine.

8.4.2 In presence of Betaine

It is synthesized from choline in both liver and kidney (Garcia-Perez and Burg, 1991) and is also used as a component of some drugs used again hepatic diseases. Betaine/GABA transporter (BGT1) is the osmoregulated betaine transporter (Yamauchi, *et al*., 1992). In addition to transcription, BGT1 is also regulated by insertion of plasma membrane (Kempson and Montrose, 2004).This makes it very interesting to study the impact of betaine and its varying concentrations on membrane proteins.

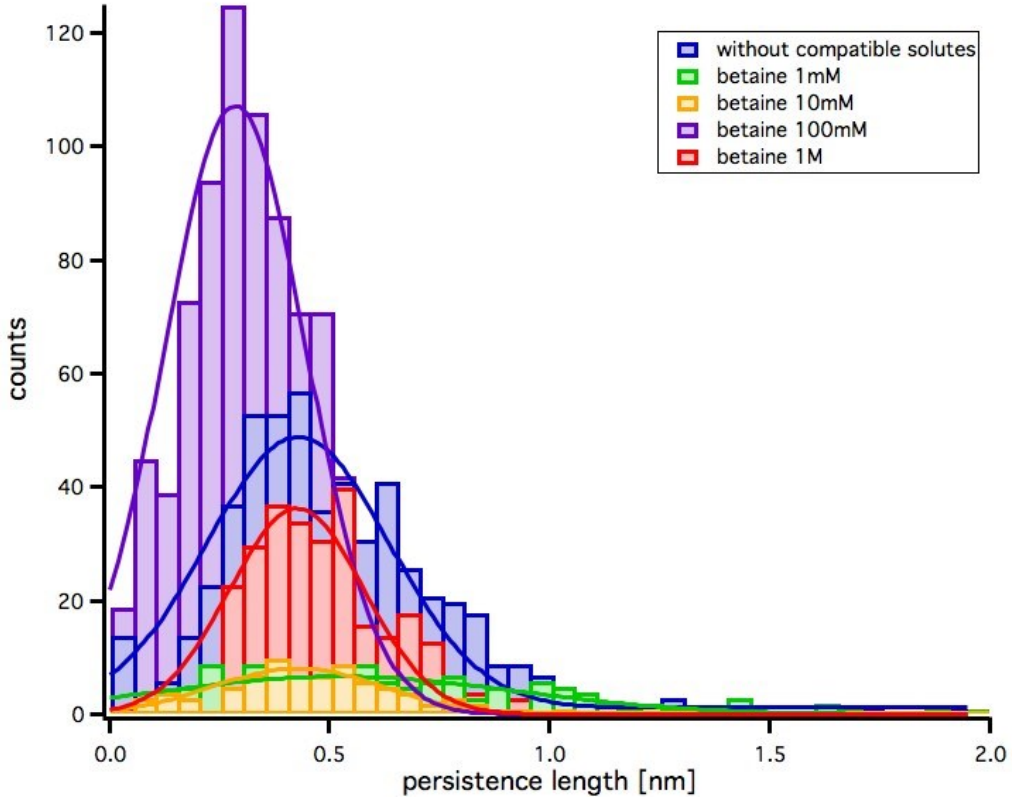

Figure 8-9: Histogram showing the dependency of persistence lengths of BR on changing concentrations of betaine.

In Figure 8-9, it can be observed that betaine reduces the persistence length of BR but the degree of reduction is not very high. Also, the number of data count is not very low in this case; sometimes it's even higher than no osmolyte condition (in case of 100mM). The maximum decrease in persistence length is also seen in the case of 100mM betaine. 1M concentration had comparatively milder effect on the persistence length. It is interesting to see that highest effect is seen in moderate concentrations (10mM and 100mM) betaine and the least and highest concentrations (1mM and 1M) show the minimum reduction in persistence length. This suggests that betaine has a particular effect on membrane protein

structure which is not directly proportional to its concentration in the medium.

8.4.3 In presence of Taurine

Taurine is mainly synthesized in liver from cysteine. It is circulated in general to the whole body and the bulk portion is taken up by kidney and is excreted. Hence it becomes very interesting and important to study the effects of changing concentration of taurine on membrane proteins.

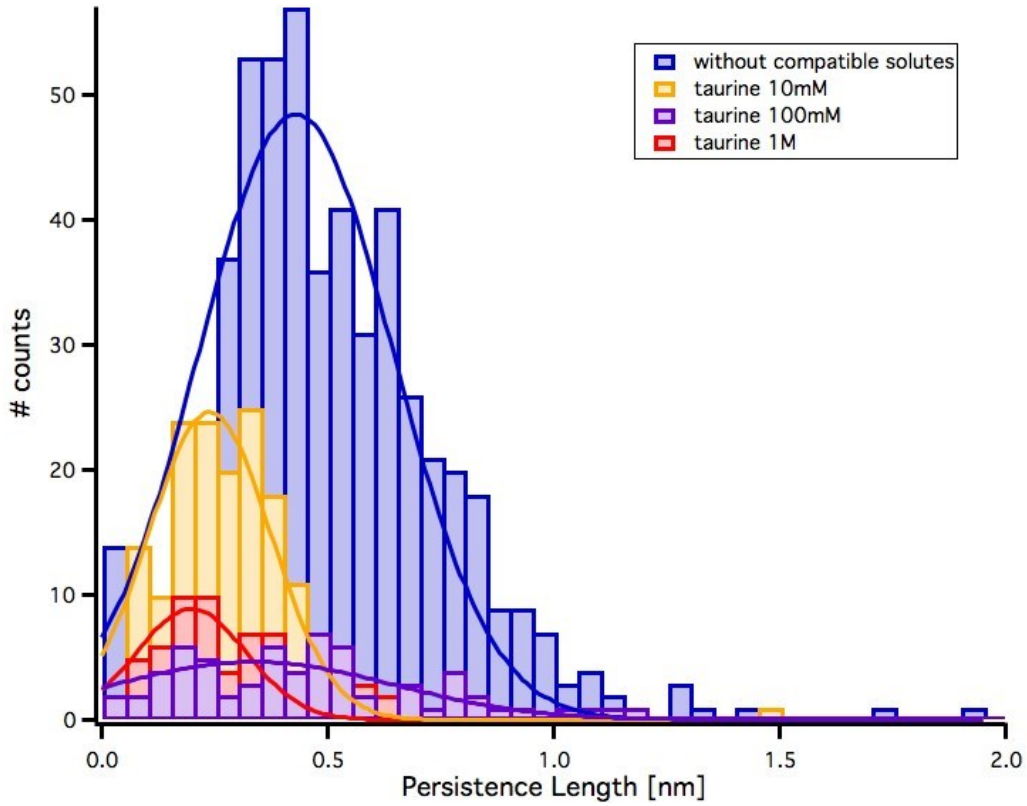

Figure 8-10: Histogram showing the dependency of persistence length of BR on changing concentrations of taurine.

This is because membrane proteins act as transporters and carriers through which taurine may pass and counteract the destabilising effects of urea in the kidney. A significant reduction in the numbers of force curves recorded has been observed in presence of taurine as well apart from ectoine. As seen in Figure 8-10, the membrane protein BR exhibits a decrease on persistence length with the increasing concentration of taurine. Hence, it is noticeable that there is a concentration dependent decrease in persistence length with the increase in concentration of taurine which was not really the same way in case of other two osmolytes, ectoine and betaine.

8.5 Comparing persistence lengths changes of BR under the influence of different concentration of ectoine, betaine and taurine

The analysis and graphs with histograms plotted in the previous section provided the information on how different concentrations of the same osmolyte affect the unfolding properties of membrane protein Bacteriorhodopsin. In Figure 8-11, a comparison among all osmolytes at all concentrations studied is plotted. This gives an overview of how persistence length of bacteriorhodopsin is influenced by different osmolytes at various concentrations.

It can be observed from the graph that the influence of concentration in persistence length change is also mostly non-linear and very specific for the particular concentrations. But in case of taurine, it is noticeable that there is a concentration dependent decrease in persistence lengths with the increase in concentration (Figure 8-11).

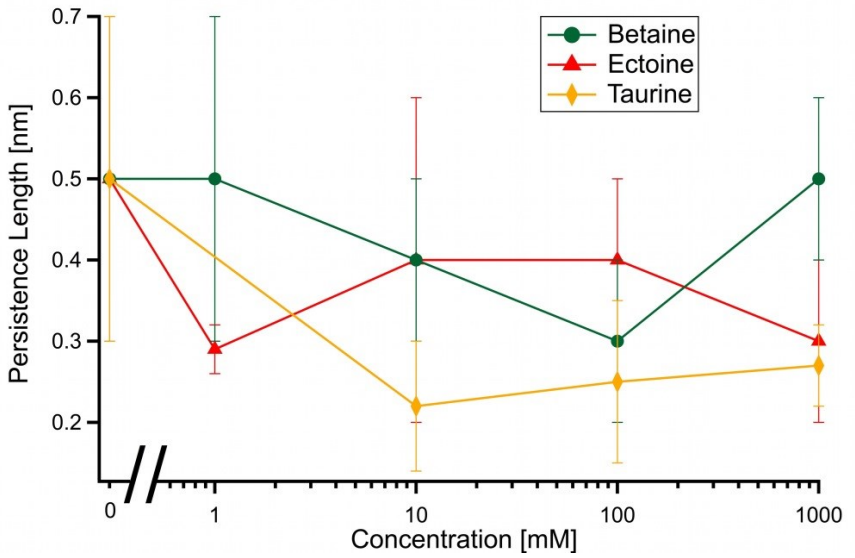

Figure 8-11:Impact of different osmolytes on persistence length of Bacteriorhodopsin. The error bars here represent the quality of the Gaussian fits used to fit the histograms.

8.6 Summary

This shortening of persistence lengths resulting into a more compact structure has also been observed in case of soluble proteins (Janshoff *et al*., 2000, Oberdörfer *et al*., 2003).But there is a particular pattern of it observed mainly in the case of ectoine and taurine at several conditions although the magnitude of their effect varies.

We also observed that the number of counts of recorded data went down in case of the osmolytes mainly for ectoine and taurine. These histograms hence suggest that different osmolytes have different modes of action elucidating different degrees of effects on membrane protein stability. The increase in unfolding forces and the reduction in persistence

length indicate the stabilisation of membrane proteins against unfolding and the tendency of the polypeptide to adopt a more coiled structure and globule conformation in presence of osmolytes. For better understanding and getting an overview of all three osmolytes at different concentration and also for the no osmolyte conditions for study the above graph, Figure 8-11 was plotted.

8.7 Dependence of the single unfolding peaks on concentration

This section displays the results of the analysis done to see the impact of compatible solutes on unfolding intermediates. In addition to carrying out experiments on concentration series, we also analysed the data for individual unfolding intermediate peaks of each force curve under different experimental conditions and compared them together to see more in detail if the effect of osmolytes is on the full length of the polypeptide chain or they have different effects on single intermediate peaks as well.

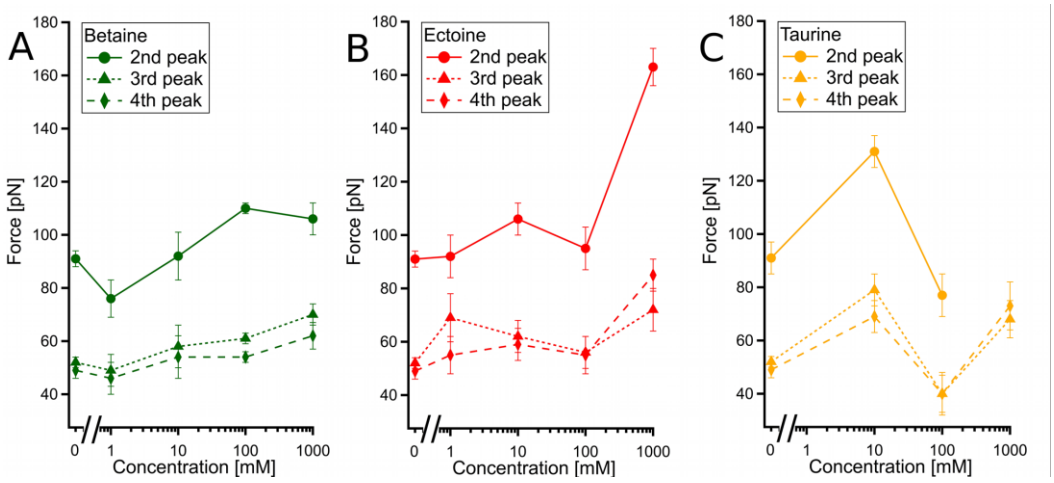

Figure 8-12: Dependency of single peak unfolding force on compatible solute concentration.

The analysis for observing the effects of concentration changes on single intermediate steps involved in unfolding of BR was performed for measuring the changes in the unfolding force and the persistence length for each peak. Due to the unspecific interaction between tip and surface we did not have enough data to analyze the first unfolding step. So the first peak is not shown. In Figure 8-12 we present the forces for the second, third and fourth peak for the measurements with different osmolytes. In general we can observe and confirm that the second peak always has a higher unfolding force than the third and fourth one. For betaine we can observe an increase in the unfolding force for all three peaks dependent on the increase in concentration. For ectoine we see a nonlinear dependency of the unfolding force on the

concentration but the course for all peaks is the same. For taurine also we see similar effects but it shows higher fluctuations. Due to less data counts available for analysis, the second peak at 1M taurine could not be analysed and displayed.

The dependency of the persistence length on the varying osmolyte concentration is shown in Figure 8-13. The first detection is that for the buffer solution in absence of any osmolyte (what stands for 0 M of osmolytes). The persistence length decreases for each sequenced unfolding step as a result of addition of the osmolytes.

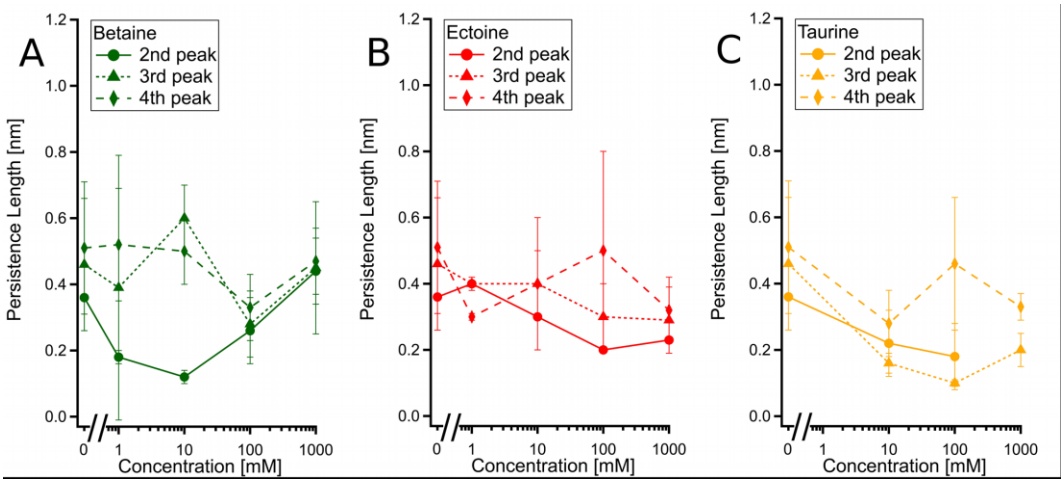

Figure 8-13: Dependency of single peak persistence length on compatible solute concentration.

Furthermore we can conclude from our observation that under the influence of betaine the effect on the different peaks is non-linear. For example for the second peak we have a reduction of the persistence length for 1mM and 10mM but then the persistence length decreases with higher concentrations. For ectoine we see a reduction of the persistence length for the second and third unfolding step with an increasing concentration. The fourth peak shows a non-linear dependency. Taurine shows for all peaks a decrease in persistence length with increasing concentration. This course is similar to that of the osmolyte concentration dependent measurements of (Oberdörfer *et al*., 2003) on soluble proteins.

Assigning error values to the analysed data was not straight forward and could not be directly taken from the experimental noise recorded. This is because of the thermodynamic influence on the dissociation. The unfolding force does not have a distinct value but a broad distribution. Therefore Monte Carlo simulations of the dissociations curves were performed to determine the standard deviation of the force histogram without any experimental error. The principal simulations carried out and results obtained from these are presented in the next section.

8.8 Error calculation for the forces derived from simulation

The dissociation force of an unfolding event is not solely dependent on the statistics. Thermal fluctuations also have an effect on this which results in a distribution of unfolding forces dependent on both stretching behavior of the polymer chain as well as the thermal influence. Hence an increase in temperature results in a broadened histogram and higher unfolding forces can be obtained. An asymmetric distribution is seen because of such stretching behaviour. The probability of unfolding steps occurring at lower forces is higher than at higher forces.

Figure 8-14 shows the histogram derived from dissociation forces obtained from simulated force unfolding events. The worm like chain model that explains the stretching behaviour of the polypeptide chain, thermal noise for adding fluctuations and a harmonic oscillator binding potential were included in the simulated curves.

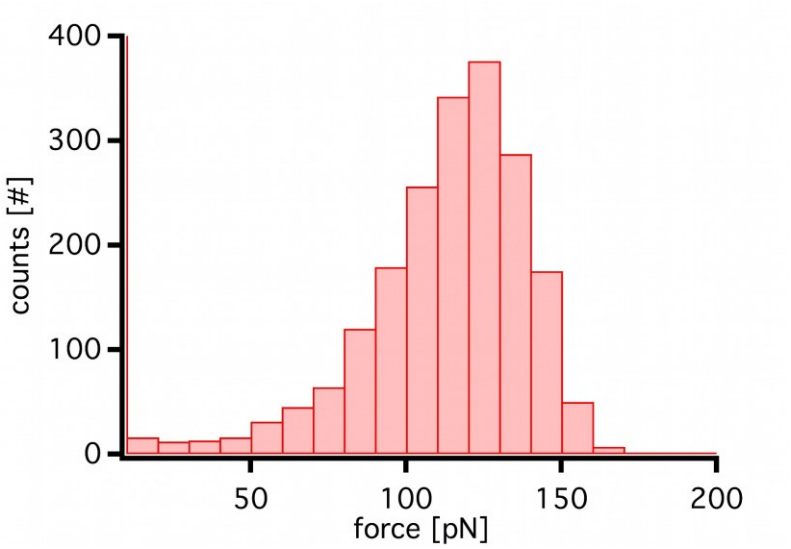

Figure 8-14: Histogram of 1000 simulated dissociation forces.

Hence, it is important to take this factor into account while analysing the experimentally recorded data in the form of force curves. So, we tried to evaluate what is the experimental error for the data recorded when the parameter which we want to analyse and determine is not just influenced by statistical errors. Another issue to consider was the reduced number of data counts recorded from the experiments with different concentrations of osmolytes because osmolytes lower the binding probability of the tip of the AFM cantilever to the polypeptide molecule. Each concentration of the three osmolytes used in this study yielded different number of data counts which were used for further analysis. Thus, for the simulated force curves also different groups were made which had as many counts as obtained from

experimental conditions and histogrammed to exactly simulate the analysis conditions. Simulated curves were grouped into 200, 100, 50, 25 and 12 counts.

Here, the results of highest counts and lowest counts for the experimental conditions are shown. In Figure 8-15, the influence of noise can be observed but the pattern of distribution are similar for all 10 histograms of each condition. To each of these histograms, a Gaussian fit was applied with keeping the parameter y0 constant at zero.

In Figure 8-16, the mean force (x0) is displayed and the error for it derived from the Gaussian fit. For the calculation of the mean force and its error, we calculated the arithmetic mean and its standard deviation:

$$\bar{F} = \sqrt{\frac{\sum_{i=0}^{N} F}{N}}, \qquad \textbf{Equation 8-1}$$

$$\sigma_F = \sqrt{\frac{1}{N-1}\sum_{i=0}^{N}(F-\bar{F})^2}. \qquad \textbf{Equation 8-2}$$

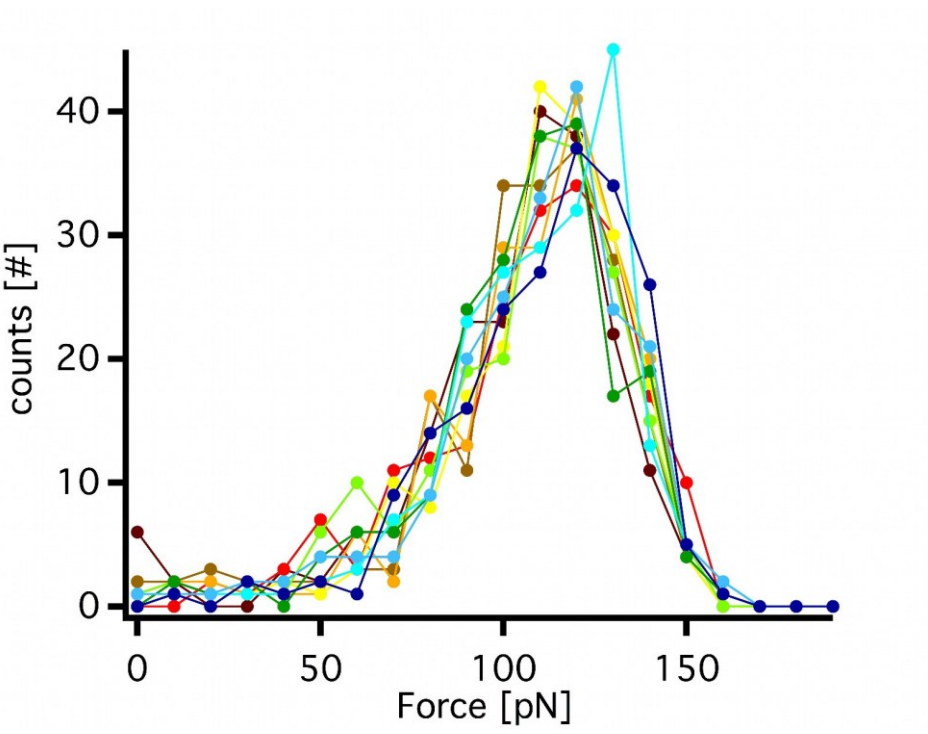

Figure 8-15: Overlay of 10 histograms of simulated forces containing 200 counts.

Each colour belongs to one data set containing 200 counts, but simulated with the same parameters.

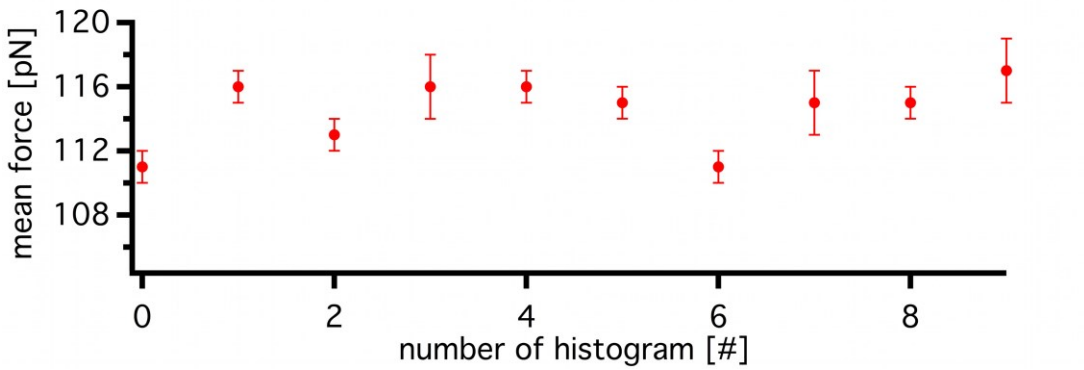

Figure 8-16: Mean force of the histograms shown in Figure 8-14, derived from a Gaussian fit, and its standard deviation.

So we derived for the histogram containing 200 counts a mean force of 115 pN and a standard deviation of 2 pN.

Because the procedure is the same for all counts we show now just the result for the histogram with the lowest number of counts, namely 12 counts. Based on the low number we decided to use here not just 10 histograms but 20, Figure 8-17. Although the histograms do not contain many data points the distribution is not uniformly and flat. When we look at, Figure 8-18, it can be seen that the first histogram could not be fitted properly while the rest of the histograms generated good fits.

Applying the same procedure for calculating the error histograms containing the least number of count of 12 (as found in case of taurine) a mean force of 113 pN and a standard deviation of 9 pN was obtained.

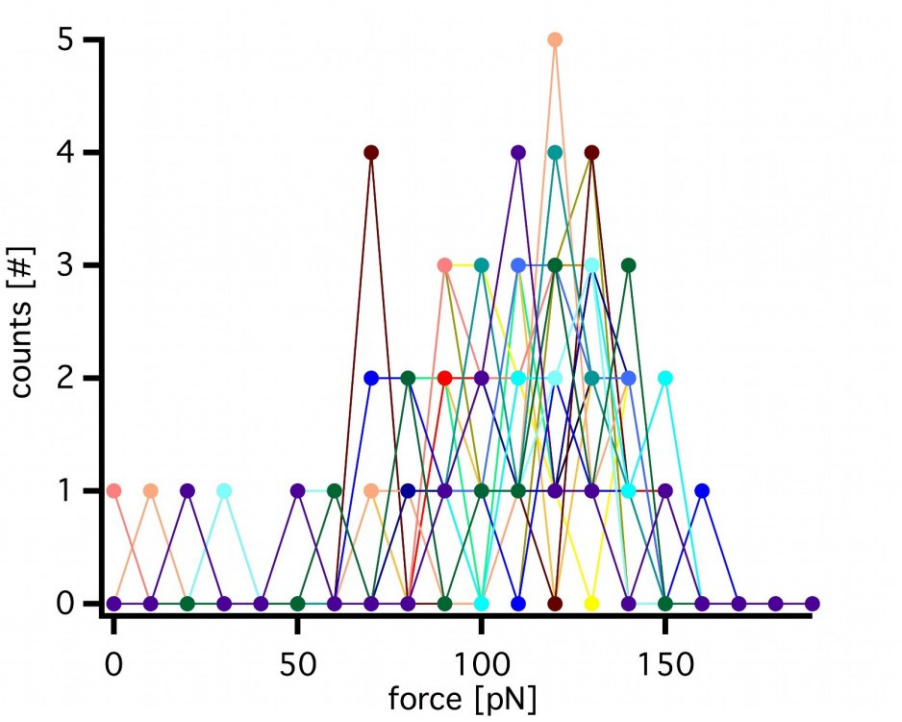

Figure 8-17: Overlay of 20 histograms of simulated forces containing 12 counts.

Each colour corresponds to one data set of 12 counts (same simulation parameters).

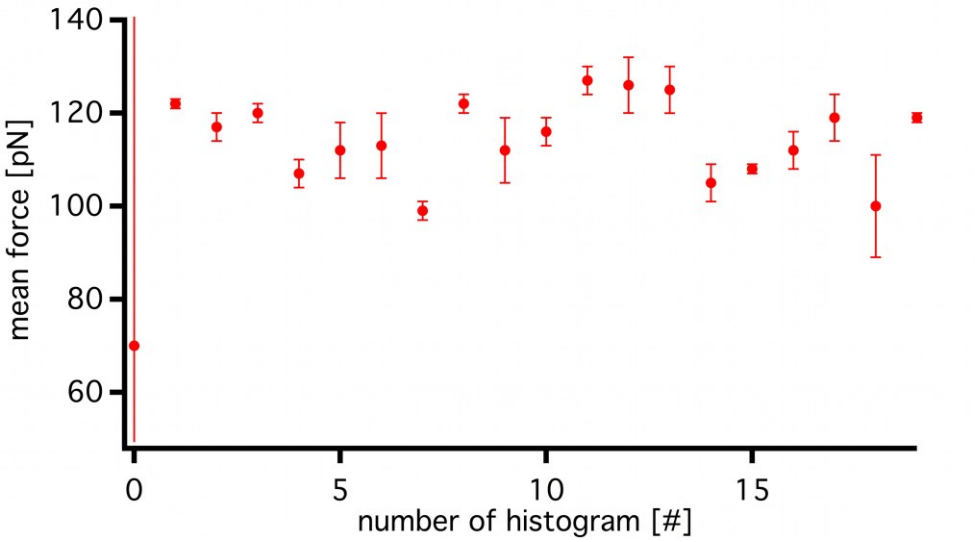

Figure 8-18: Mean force of the histograms shown in Figure 8-14, derived from a Gaussian fit, and its standard deviation.

To summarize these results and put them together, Figure 8-19 was plotted which describes the dependency of the standard deviation of the unfolding force on the number of data counts.

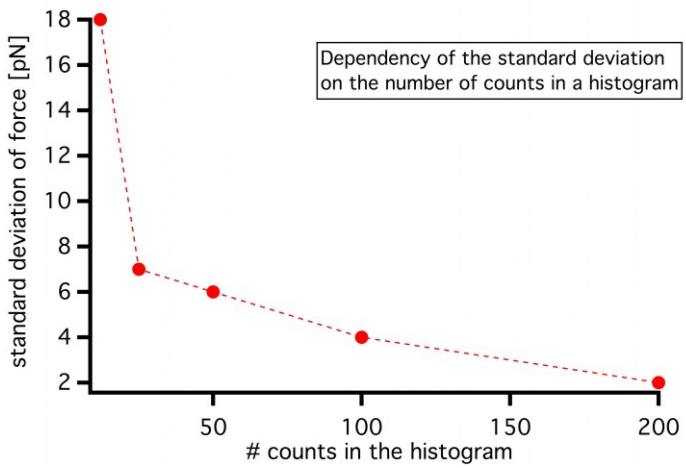

Figure 8-19: Graph of the standard deviations dependent on the number of counts in a histogram.

From this graph we deduced the standard deviation values for the mean forces which are dependent on the number of counts present in each of the results obtained from various experimental conditions.

counts	standard deviation of force [pN]
12 – 15	9
16 – 20	8
21 – 30	7
31 – 66	6
67 – 88	5
89 – 113	4
114 – 165	3
166 - 200	2
> 200	2

These standard deviations of the Gaussian fit are used in the single peak analysis and the sum of all four peaks, as the error bars for different values of peak forces and persistence lengths.

Chapter 9 Discussion and Conclusion

It is known that compatible solutes from different molecular classes ranging from amino acids and their derivatives, sugars, polyols etc. induce the formation of a stabilising hydration shell around proteins (Arakawa and Timasheff,1985; Bolen and Baskakov, 2001). The effect of osmolytes on protein stabilisation can either be mild, moderate or strong, depending upon the kind of solute molecule present in the surrounding and whether a protein interacts more with the water molecule in the aqueous buffer or with the added compatible solutes.

Previous studies on organic osmolytes have illustrated that the stabilising properties of these molecules correlate with the preferential exclusion of these solutes from the vicinity of the polypeptide. This results into accumulation of water molecules present in aqueous buffer and formation of a denser water shell (preferential hydration) around the unfolded polypeptide chain (Xie and Timasheff., 1997; Xie and Timasheff., 1997). The compatible solutes raise the Gibbs free energy of the unfolded state of the protein by interacting unfavourably with the unfolded state and expel the solute molecules from near the protein surface (Bolen D. W., 2001). This is due to a net repulsive interaction between the compatible solutes and the protein. The rise in free energy of the unfolded state consequently favours the stabilisation of folded conformation in the native state (Bolen D. W., 2001).

In this study of stabilising effect of the three different compatible solutes ectoine, betaine and taurine on the membrane protein Bacteriorhodopsin using three different compatible solutes ectoine, betaine and taurine the results obtained suggest that compatible solutes act as a stabilising agent on membrane proteins. This work was carried out using Atomic force spectroscopy on single molecules of the sample making it novel and different compared to other ensemble experiments. Bacteriorhodopsin represents one of the most detailed and extensively studied membrane proteins making it ideal for the study of various life processes. Hence it was suitable to be taken up for our study involving compatible solutes, which are known to stabilise protein structures and support folding but up to now it is not shown for membrane proteins with single molecule techniques. Also little is known about the complex mechanism involved in this. The purpose of this work was to understand whether biological effects on the cellular level induced by an increase or decrease in the solute level, respectively, may be explained by the direct interaction of the compatible solutes with the membrane proteins under investigation. Therefore, we focused in this study on the mechanical properties and changes in force and persistence length during unfolding of the protein that result from applying external stress in presence of compatible solutes ectoine,

betaine and taurine at various concentrations.

Mechanical protein unfolding experiments were conducted using a commercial AFM at constant pulling speed. Force spectroscopic results obtained from these experiments are a spectrum of traces, usually called force - extension or force - distance (F-D) curves exhibiting unfolding forces as a function of the pulling coordinate. All collected force curves with typical four major unfolding events from the recorded force spectrum were selected for analysis and extracting desired information. Each of the selected unfolding events was fitted using a worm like chain model fitting algorithm which is described in Chapter 6.

In general, higher unfolding forces indicate that a protein is mechanically more stable and it is more too difficult to extract out of the membrane while the decrease in the value of persistence length indicates a tendency of the polypeptide to form a more compact structure.

9.1 Effects of compatible solutes on the stability of Bacteriorhodhopsin

Here, the effects of several protecting osmolytes at different concentrations were examined on Bacteriorhodopsin. The results of the force spectroscopic studies of protein folding dynamics revealed significant effects of these compatible solutes on unfolding properties of Bacteriorhodopsin, a reduction in persistence length and an increase in the unfolding forces of the protein varying with different concentrations of compatible solutes. Hence it was deduced that the solutes tested have positive effect on stabilization of Bacteriorhodopsin. This indicates that these osmolytes may play an important role in mechanical stability of membrane proteins in addition to globular proteins (Oberdörfer *et al*., 2003) against externally induced unfolding stress. These conclusions were derived from the understanding and interpretation of the experimentally obtained results for the parameters of unfolding forces and persistence lengths. The inference of these results are discussed below.

In a Bacteriorhodopsin unfolding force curve, each peak of the major four peaks represents the unfolding event of a pair of helices pulled out of the membrane and gives the mechanical force required to unfold a single molecule of the protein. Some intermediate events in the form of small peaks are also observed which indicate the occurrence dynamic intermediate conformations in the unfolding pathway of the protein.

The results presented in Chapter 8 show that higher forces are required to extract and unfold a single molecule of Bacteriorhodopsin in presence of the compatible solutes as compared to the condition where only aqueous buffer is present.

Significant changes in unfolding forces in the case of the membrane protein

Bacteriorhodopsin under the influence of varying concentrations of osmolytes were observed (Figure 8-7). There was an increase in the peak force for each condition although the impact varied from mild to pronounced but almost non-linear increase was observed. Only in case of betaine, the increase of unfolding forces with osmolyte concentration was found to be nearly linear. But other than that in most cases the forces did not increase particularly with increasing concentrations (1 mM, 10 mM, 100 mM and 1 M) which makes this result very interesting as previous similar work on the effects of osmolytes on fibronectin III showed no influence on unfolding forces. This increase in force may also be understood on the basis of the different shapes of the free energy landscapes along the unfolding pathways. When unfolding a beta barrel structure as a Fibronectin III domain, the breakage of the interaction between the first and last beta strand, which are oriented in an antiparallel manner, is sufficient to induce the unfolding of the whole domain. Due to the antiparallel orientation, all hydrogen bonds between the strands are loaded simultaneously and a small shift of approximately 2Å induced by the applied force leads to the breakage of all bonds at once. Such a small shift only leads to a very small change in the protein surface. In contrast the unwinding of an alpha helix is followed by a major surface increase, and thus it experiences a stronger stabilising effect by the osmolyte-induced hydration shell (Yu *et al*., 2007; Politi, and Harries, 2010).

The observed decrease in the persistence length of the stretched amino acid chain directly shows the stronger tendency of the entropic coil to collapse into a dense conformation which profoundly increases the protein folding rate. As seen in Figure 8-11, the heterogeneity in the behavior of protein with regard to changes in the persistence length can be explained by the different degree of unfavourable interaction between the protein and the solute molecules. At specific concentrations, these effects can vary from mild to severe depending on the enhancement of hydrophobic collapse of the membrane protein Bacteriorhodopsin by the compatible solutes.

As observed in our concentration dependent study, the effect of various concentrations on the persistence length of Bacteriorhodopsin is different in the analysis where we take all peaks into account. All osmolytes at different concentrations reduce the persistence length of the protein, which implies that the tendency of the molecule to acquire a more compact conformation is different depending upon the concentration. These molecules act very specifically at particular concentrations and their preferential interaction with water and with protein is different at different concentrations.

This suggests a general tendency of the osmolytes to render the unfolded state of protein into

a more collapsed structure than that of aqueous buffer. The molecular origin of such effects and changes still remain fairly elusive making it difficult to suggest a univocal general mechanism of action of these osmolytes on protein stabilization. From our experimental results and observations the mechanism of how compatible solutes stabilise membrane proteins can be understood based on the model of preferential exclusion (Kurz, 2008).

9.2 Mechanism of osmolyte action in stabilising membrane protein Bacteriorhodopsin: preferential exclusion model

In general, osmolytes are widely known for their stabilising properties on globular proteins (Oberdörfer *et al*., 2003, Lippert *et al*. 1992, Lentzen *et al*., 2006) and lipid bilayers (Harishchandra *et al*., 2010). In accordance with the preferential exclusion model of osmolyte action, the results of our experiments show a stabilisation of the membrane protein structure in the presence of osmolytes.

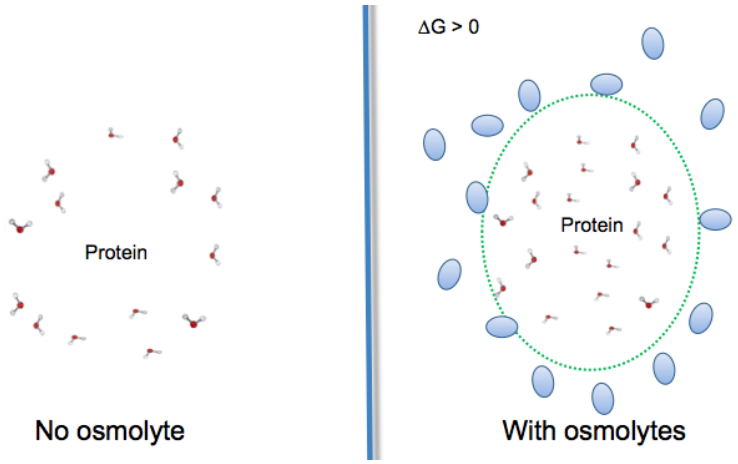

Figure 9-1: Schematic depiction of membrane protein BR preferential hydration in the presence osmolytes.

Osmolytes are excluded from the protein surface due to steric repulsions from water (blue spheres represent osmolyte molecules, green dotted lines depicts hydration layer and red and white structures are water molecules). Preferential exclusion of osmolytes from the vicinity of protein surface results in enrichment of water in the solvation shell due to the unfavourable interactions occurring between protecting osmolytes and Bacteriorhodopsin surface. Rise in free energy ΔG is seen in presence of osmolytes.

From the decrease in persistence length it can be said that the polypeptide folds into a dense conformation by expelling the solute molecules from the exposed protein surface area. Due to the rise in the chemical potential of the stretched protein, the protein prefers to reduce the solvent exposed surface by adopting a more condensed structure. This shortening of persistence lengths resulting into a more compact structure has also been observed in case of soluble proteins (Janshoff *et al*., 2000, Oberdörfer *et al*., 2003). As observed in our concentration dependent study, the effect of various concentrations on the persistence length

of Bacteriorhodopsin is different in the analysis where we take all peaks into account. All osmolytes at different concentrations reduce the persistence length of the protein, which implies that the tendency of the molecule to acquire a more compact conformation is different depending upon the concentration. These molecules act very specifically at particular concentrations and their preferential interaction with water and with protein is different at different concentrations.

In case of the single peak analysis for the different concentrations, each osmolyte shows a similar pattern in the forces and persistence lengths for each unfolding peak (Figure 8-12 and Figure 8-13) which indicates again that there is no specific binding on the protein and the osmolytes do not affect only a specific part of the protein. An almost similar increase in force required to extract the intermediate peaks suggests that no specific hydrogen bonds are responsible for the increase in force. Furthermore we could observe a similar pattern for the forces and persistence lengths for each peak for ectoine and taurine dependent on the concentrations.

For these both osmolytes we got also a remarkably reduction in the binding probability of the tip to the terminus of the protein and therefore less unfolding events within the same amount of force curves. This leads to the assumption that ectoine and taurine act in a similar way on the membrane protein Bacteriorhodopsin in contrast to betaine and also it establishes that not all protecting osmolytes have similar effects on protein.

Hence, we conclude that the stability of the membrane protein BR in general is enhanced by the organic osmolytes by raising the peak force and shortening the persistence length. The increase in unfolding forces can also be attributed to be a measure of the stability of the protein in the membrane against denaturation caused by various external stresses such as high salinity, change in surrounding temperature, addition of solutes etc. These results obtained indicating disparities in concentration dependency thus suggest that different osmolytes have different and specific effects on stability of BR. This implies that the membrane protein is strongly affected by compatible solutes when it comes to extracting it out of the membrane. A profound increase in the peak forces in presence of osmolytes could be attributed to the formation of a dense and more structured water shell around the exposed part of BR.

At point 1 in Figure 9-2, it is assumed that upon addition of the solute molecules, the interaction between the transmembrane part of the first helix of Bacteriorhodopsin and water, and the same region of BR and osmolytes govern the rearrangement of water and osmolyte molecules around the stretched or exposed polypeptide in the bulk solution.

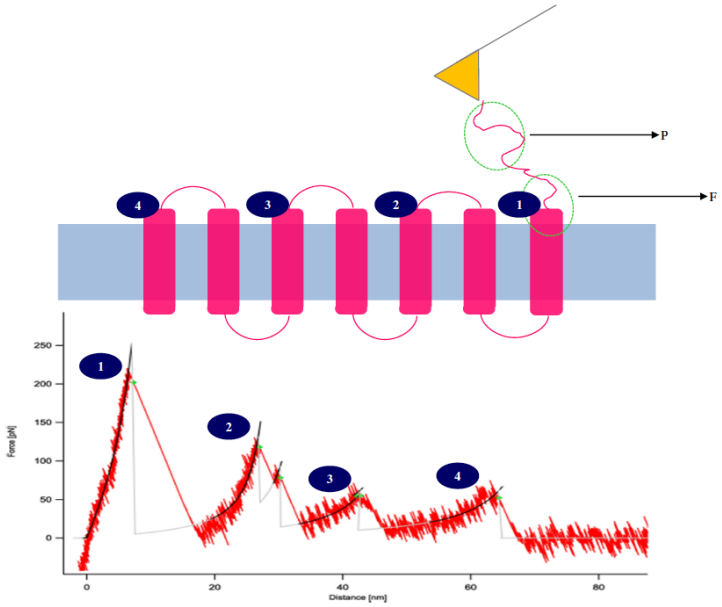

Figure 9-2: Schematic representation of Bacteriorhodopsin unfolding in presence of osmolytes.

This scheme depicts the pairwise extraction of individual BR molecules out of the membrane. When a force is applied on the terminus of the protein, helices F and G are pulled out. The increase of unfolding forces in presence of osmolytes is resultant of the formation of a denser hydration shell around that area. Here, F if the force acting upon the protein to pull it out of the membrane and the green dotted circle in the cartoon resembles the part where a restructured water shell might have formed. P is the part, which can give a measure of persistence lengths or the ability of the protein to collapse into a more coiled structure.

As shown in Figure 9-2, unfolding of a membrane protein requires the extraction of its helices out of the membrane into an aqueous environment. Because of the positive interaction between these solute molecules with no net charges and water, these molecules dissolve well in aqueous surrounding available for it. This in turn makes the protein to collapse into a more compact coil and renders it more stable. Our results showing a decrease in persistence length in presence of osmolytes supports the enhanced stability of membrane protein in presence of these compatible solutes. Also, during the unfolding of protein, the system becomes enthalpically unfavourable due to breaking of bonds in presence of osmolytes. This enhances a greater structuring of the hydration shell around the unfolded protein and results in the thermodynamic stability of the protein.

9.3 Dynamic behavior in presence of osmolyte and the free energy change due to increased concentration conditions

According to the preferential exclusion model, the chemical nature of the exposed protein surface to the surrounding is a very important factor in determining the interactions between different molecules in the environment. Similarly in our case, the expulsion of osmolytes is strongly driven by solvophobic effect which results in preferential hydration (Bolen D. W.,

2001). The degree of exclusion depends upon the affinity of the between the particular osmolytes and the protein at various concentrations which is exhibited in the result obtained from our experiments. It can be said that the results of this study support and illustrate the model that osmolytes interact unfavourably with the unfolded state of the protein which results in the expulsion of the solute molecules which are at proximity to the protein surface. The stabilisation is more significant in case of Bacteriorhodopsin because of its alpha helical structure uncoiling of which results in a major increase in surface area causing a stronger stabilisation.

This behaviour and interaction between the protein and osmolytes and water is very dynamic and leads to the stabilisation of the protein by forcing it to acquire and adopt a more compact structure. This is caused due to the steric repulsion perturbation of the surface free energy of water by osmolytes.

Steric exclusion of osmolytes from protein surface first proposed by Kauzmann (Kauzmann, 1974) with respect to preferential hydration attributes the exclusion of molecules from the protein surface on the bulkiness of the molecules of osmolytes and water. Osmolytes being larger in size cannot further penetrate the shell formed around the polypeptide which can be accessible to the water molecules that are smaller in size. This leads to excess of water around stretched protein surface forming a dense water shell separating from solvent by an interface. This mechanism is thermodynamically termed as preferential hydration (Arakawa and Timasheff, 1985; Bhat and Timasheff, 1992). Changes in the surface tension thus leads to the changes in the concentration of osmolytes in surrounding (Gibbs, 1878) which is manifested by varying degree of effects these osmolytes have on membrane protein stability at different concentrations.

The unfolding of BR exposes it to the surrounding environment of aqueous buffer and osmolytes and increases the protein surface area and also leads to changes in the free energy.

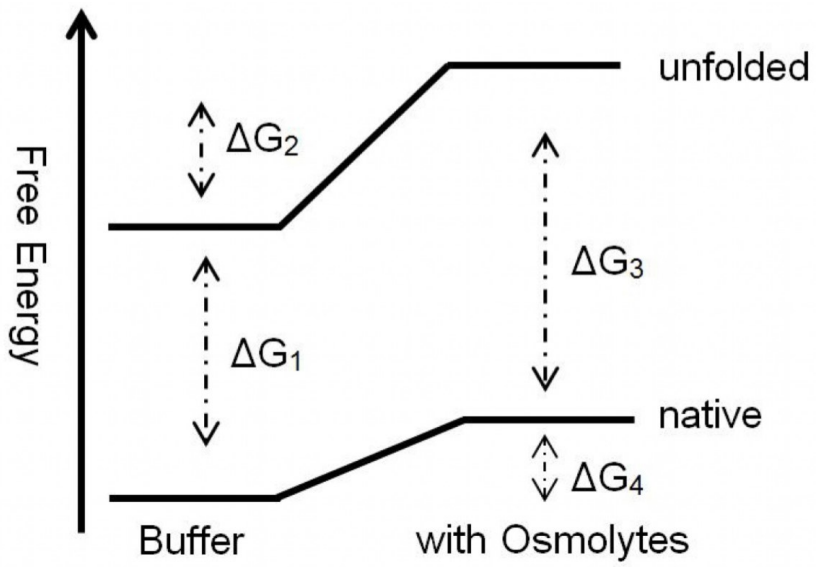

Figure 9-3: Schematic representation of protein stabilisation induced by thermodynamic effects of osmolyte action.

The diagram depicts the increase in free energy of both native and unfolded state due to unfavourable interaction of solutes with protein. ΔG1 and ΔG3 are the Gibbs free energy difference between the native and unfolded state of protein in buffer and in presence of osmolyte, respectively. ΔG2 and ΔG4 are the rise in Gibbs free energy of the unfolded state ensemble and native state ensemble. (Adapted from Baskakov *et al*., 1998 and Street *et al*., 2006).

Osmolytes and proteins do not display a favourable interaction. These solutes dissolve well in water at high concentrations and exhibit different preferences to interact with the protein compared to water (Timasheff, 1998). If water and the solute have the same tendency to interact with the protein, the free hydration energy is the same in presence and absence of the solute. If the solute preferentially is excluded (Figure 9-1) from the protein, its state is similar to that in pure water. They act by raising the free energy of both native and unfolded ensemble states, but the increase in Gibbs free energy is stronger in case of the unfolded state because of the larger exposed surface area (Figure 9-3). The increased Gibbs free energy difference between the folded and unfolded state of the polypeptide chain results in the increase in unfolding free energy barrier, which is manifested by the increase in measured unfolding forces. This results in the greater stability of the protein in the osmolyte solution than in aqueous buffer. The stability of Bacteriorhodopsin observed in our study hence can be explained by this theory. The exclusion of the solute from the protein creates a concentration gradient of the osmolyte at the protein surface. An unfolded peptide chain will be driven to a more compact coil to minimize its surface area and thus the loss of entropy, which is caused by the concentration gradient of the osmolyte. By that it supports the formation of a molten globule and protein folding.

The same effect also accounts for the stabilisation of proteins against unfolding since this is

always associated with an increase of the protein surface. Unfolding of a membrane protein requires the extraction of the peptide chain out of the membrane into the aqueous surroundings. The increased free energy difference between the folded and unfolded conformation induced by the osmolyte leads to an increase of the unfolding free energy barrier, which is manifested in the increased measured unfolding forces.

9.4 Conclusion

Protecting osmolytes are known to have a general stabilising effect on cytoplasmatic proteins [13, 29, 30] and lipid bilayers [31]. But the mechanism by which they induce conformational changes, facilitate protein folding rendering it more stable is still under investigation (Baskakov *et al.* 1998; Kumar *et al.* 1999; Bolen 2001; Ratnaparkhi *et al.* 2001; Zou *et al.* 2002; Auton *et al.* 2005; Ignatova *et al.* 2006; Street *et al.* 2006; Beck *et al.* 2007; Loo *et al.* 2007; Street *et al.* 2009; Venkatesu *et al.* 2009). Most of the related studies have been concentrated on the thermodynamic principles involving ensemble experiments to investigate the magnitude and impacts of osmolyte accumulation at various concentrations and their effect on protein folding dynamics and stabilisation (Tokuriki, *et al*., 2004; Sasahara *et al*., 2003). Very few single molecule techniques have been so far been employed in such studies.

In this study, single molecule force spectroscopic data that reveal the stabilising effects of such osmolytes on membrane proteins have been presented. From unfolding experiments on the seven helical membrane protein Bacteriorhodopsin (BR) we obtained the forces required to unfold the protein and its intermediates as well as the persistence length of the unfolded amino acid chains. In the presence of the compatible solutes ectoine, betaine and taurine a strong stabilising effect on BR was observed. The forces required to unfold Bacteriorhodopsin were found to have increased. This result reveals a stabilising effect of compatible solutes, which is likely to be valid for alpha helical membrane proteins in general. In addition a decrease in the persistence length of the unfolded amino acid strands was observed, which indicates an increased tendency to coil up in accordance with the presented data. This results in a more collapsed structure of the polypeptide molecule in presence of compatible solutes than in water, which in turn supports the (re)folding of the protein.

The results of this work support the preferential exclusion model in two ways. On one hand, compatible solutes support folding of Bacteriorhodopsin, on the other hand, it helps Bacteriorhodopsin to withstand unfolding stress. The observed decrease in persistence length of the stretched amino acid chain directly shows the stronger tendency of the entropic coil to collapse into a dense conformation which strongly increases the protein folding rate.

It is also important to mention here that for ectoine and taurine, we got also a remarkable reduction in the binding probability of the tip to the terminus of the protein and therefore less unfolding events within the same amount of force curves. This indicates that in presence of these solutes the unspecific interactions between the tip and the sample goes down considerably. This also leads to the assumption that ectoine and taurine act in a similar way on the membrane protein Bacteriorhodopsin in contrast to betaine.

With our concentration dependent measurements on the membrane protein Bacteriorhodopsin we could show that it is affected by the compatible solutes in the expected way: the protein is more folded into the membrane to reduce the contact of the protein surface to the compatible solutes and the binding probability is reduced. Furthermore we observed that each unfolding peak is affected in the same way so that we can assume for the mechanism of the interaction of the osmolytes and Bacteriorhodopsin that there is no specific binding to the protein. These molecules act very specifically at particular concentrations and their preferential interaction with water and with protein is different at different concentrations.

Besides we saw a similar way of interaction of ectoine and taurine with the protein. Hence, we conclude that the stability of the membrane protein BR in general is enhanced by the organic osmolytes. The overall increase in force indicates that the osmolytes may act as protectant for cell surfaces to withstand the external stress while the decrease in persistence length indicates a facilitated refolding process. Because of the increasing clinical relevance of osmolytes in understanding and curing human diseases, we hope that our results and findings will help and lead to a further investigation of osmolytes due to the importance of these solutes in human organs and the important role of membrane proteins as transporters and drug targets.

Chapter 10 Outlook and Perspectives

Protein folding is a dynamic and reversible process. It has been shown that protein folding equilibrium is pushed by osmolytes towards the natively folded state by increasing the Gibbs free enegry of the unfolded state (Street *et al*., 2006). Osmolytes shift the folding equilibrium and enhance protein folding by affecting the polypeptide chain though hydrophobic effects.

They can influence the structure, dynamic and conformation of the proteins which can prove to be very important in studies involving protein misfolding. The non intrusive and non toxic nature of these organic osmolytes with the cellular mechanism are set to provide new and alternative measures to understand and design curing aaproaches for diseases caused by alterations in protein folding pathways (Tanaka *et al*., 2004). The regulation of cellular activity and function is largely dependent on the cosolute and their concentrations.

The results obtained from this study establishing the impacts of compatible solutes on the stabilisation of the membrane protein Bacteriorhodopsin also will help in understanding prokaryotic stress management.

The photoactive protein Bacteriorhodopsin is used here as a model system for studying membrane protein folding because of its popularity as the model membrane protein in various other studies. It's well known structure-function dynamics helps us to study the changes this protein undergoes in presence of compatible solutes used in our study. Making this protein as the model and observing the effect of compatible solutes in we plan to venture further in protein folding and stabilisation studies on medically relative membrane proteins. From the medical point of view, membrane proteins have their special importance for drug delivery, as transporters, and misfolding studies to name a few.

Hence, the future experiments and applications of this work directly focuses on the study of medically relevant membrane proteins. In the study described in this thesis, we observed a pronounced effect of compatible solutes on membrane protein stability. These interesting results act as the precursor of our future work involving similar stabilisation of membrane proteins using compatible solutes. But unlike Bacteriorhodopsin, most of the integral membrane proteins need to be incorporated inside the cell membrane to retain its biological activity. In the harsh external environment, out of the membrane most of the membrane proteins lose their biological function. Being physiologically inactive they become unstable and denature. This particular nature of membrane proteins make them very complex and difficult to study and investigate. But this study establishing stabilising property of compatible solutes on membrane proteins address the issue of better and more stable ways of

handling membrane proteins.

An extension of this work aims at leading investigation of several other phenomena like preferential exclusion or interaction, concentration dependency, and disparity among the impacts of different osmolytes, as well as the statistical and simulation works which were done to validate the authenticity of the results obtained. This study involving single molecule force spectroscopic technique is also significant because no denaturant that could counteract with compatible solutes used was required to reach the transition state. Force spectroscopy is especially suited to investigate the surface effects making it an ideal tool to study effects of osmolytes on membrane proteins where the information about the crucial parameter like the activation barrier from unfolding kinetics of single protein molecule can be obtained.

With the interesting results obtained from this work, more studies need to be carried out to demonstrate and further illustrate the impacts of osmolytes. These can be achieved by understanding the basis of osmolyte–macromolecule (membrane proteins in our case) interactions in aqueous environments.

The findings and understanding from this work can also be used for refolding kinetics of protein folding. Such studies of stabilising properties of osmolytes can give a unifying mode of action resulting in better and effective insight into the therapeutic approaches for neurodegenerative diseases caused by protein misfolding as well as in case of gastroenterological and hepatic diseases. The distinct and specific effects among different osmolytes at various concentrations may eventually reshape the already existing theories of how the stabilising properties vary within different concentrations of same osmolyte as well as the disparity among different osmolytes in the case of protein folding and also other macromolecular processes. This will not only allow a better explanation of the role of osmolytes in protein stabilisation based on osmolyte-macromolecule interactions in a stressed surroundings typical for cellular environment but also in resolving the effects and the behaviour of such solute molecule involved in pathological conditions. The far reaching outcome of this work will also help in further formulation and testing of theories of osmolyte action and their impact on regulation of protein structure and function.

Presentations at Scientific Conferences

Oral talks

1. **Stabilization of Membrane Proteins using Compatible Solutes** at 1st Biostruct Master Class, Heinrich Heine University, Düsseldorf, Germany; **6th -9th September 2010**
2. **Effect of Compatible Solutes on Membrane Protein Stability: A Single Molecule Study** at Linz Winter School; Linz, Austria; 1st – 7th February 2011,
3. **Observing the protecting effect of Organic Osmolytes in Action at the Single Molecule Level** at 2nd BioStruct Master Class "Macromolecular Interactions", HHU, Düsseldorf, Germany; 12th **-15th September 2011**
4. **Stabilization of Membrane Proteins by Compatible Solutes: Single Molecule Force Spectroscopic Study** in 11th **Greta Pifat-Mrzljak International School of Biophysics, Primosten, Croatia; 30th September – 9th** October 2012

Posters

1. **Impact of Compatible Solutes on Mechanical Properties of Bacteriorhodopsin: An Atomic Force Microscopic Study** at 54th Annual Biophysical Meeting, San Francisco, USA; 19th -24th February 2010
2. **Stabilization of Membrane Proteins using Compatible Solutes at 1st BioStruct master class, Heinrich Heine University, Düsseldorf, Germany; 6th -9th September 2010**
3. **Stabilization of Membrane Proteins using Compatible Solutes at 9th International Symposium on Scanning Probe microscopy & Optical Tweezers in Life Sciences, Berlin, Germany; 6th -7th October, 2010**
4. **Mechanical Stabilization of Membrane Proteins with Compatible Solutes** at 55th Annual biophysical meeting, Baltimore, Maryland, USA; 5th -9th March 2011
5. **Light Induced Structural Changes in Bacteriorhodopsin and Sensory Rhodopsin II: A Single Molecule Comparative Study** at IVth International Meeting on AFM in Life Sciences and Medicine**, Institute Curie, Paris, France; 23rd -27th August, 2011**
6. **Reconstitution of membrane proteins with a defined orientation into a tethered lipid membrane in 2nd Biostruct master class "Macromolecular Interactions", Heinrich Heine University, Düsseldorf; 12th-15th September 2011**
7. **Stabilizing of membrane proteins by compatible solutes: single molecule force spectroscopic study** in Focus on Microscopy 2012 (25th International Conference on 3D Image Processing in Microscopy, 24th International Conference on Confocal Microscopy), Singapore, 1st – 4th April 2012
8. **Stabilization of Membrane Proteins by Compatible Solutes: Single Molecule Force Spectroscopic Study** in 11th **Greta Pifat-Mrzljak International School of Biophysics, Primosten, Croatia; 30th September – 9th** October 2012

List of Enclosures

1. **Studying Bacterial Membrane Proteins with Single-Molecule Atomic Force Spectroscopy**
   Roychoudhury, A and Oesterhelt, F., Microscopy and Analysis (2011) *25*, 15-17.

2. **Effect of the Compatible Solute Ectoine on the Stability of the Membrane Proteins**
   Roychoudhury, A., Häussinger, D., Oesterhelt, F. Protein Pept. Lett. (2012) *19*, 791-794.

3. **Concentration dependent Membrane Protein Stability under the Influence of Different Compatible Solutes Studied by Single Molecule Force Spectroscopy**
   Roychoudhury, A., Bieker, A., Häussinger, D., and F. Oesterhelt, Biol. Chem.
   (Under revision)

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Studying Bacterial Membrane Proteins with Single-Molecule Atomic Force Spectroscopy

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BIOGRAPHY

Filipp Oesterhelt received his PhD from the Ludwig-Maximilians University Munich, Germany, for the development and application of single-molecule force spectroscopy and its application to membrane proteins. In 2003, he was appointed assistant professor and now holds a lectureship and group leader position in the Institute for Physical Biology at Heinrich Heine University. He is now working in the field of single-molecule biophysics, with a focus on force spectroscopy, force microscopy and single molecule fluorescence.

ABSTRACT

Atomic force microscope-based single-molecule force spectroscopy has made it possible to probe the mechanical stability and unfolding pathways of membrane proteins as well as the free-energy landscape of inter- and intramolecular interactions. Single-molecule force spectroscopy probes the mechanical properties of individual molecules by exerting mechanical forces and measures the induced conformational changes. Forces applied to a single protein acts as a denaturant leading to unfolding of its three dimensional structure thereby revealing its unfolding pathways. This technique is of great interest especially in the field of membrane proteins, which can be investigated under physiological conditions and in their native membrane environment. Examples illustrated here are that of bacteriorhodopsin and sensoryrhodopsin II.

KEYWORDS

atomic force microscopy, force spectroscopy, bacteriorhodopsin, sensoryrhodopsin, free energy landscape, protein unfolding

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INTRODUCTION

The atomic force microscope (AFM), invented in the year 1986, has for the first time enabled the addressing and manipulation of individual biomolecules, triggering also the development of a variety of scanning probe microscopes and single-molecule techniques. Apart from its ability to image biological surfaces with sub-nanometre resolution, AFM also enables the manipulation of matter at the nanoscale. The exceptionally high spatial resolution of the AFM and its high sensitivity to mechanical forces allow for the imaging of transmembrane proteins and membrane associated proteins in their natural environment and enables force measurements on the individual molecule [1, 2, 3]. AFM-based single-molecule force spectroscopy is specially suited to measure their mechanical response, revealing inter- and intramolecular interactions [4]. Thus it provides a powerful tool to characterize the structure-function relationships of native membrane proteins [5].

Force Spectroscopy

The atomic force microscope takes advantage of a very sharp needle-like tip with a radius of 2-10 nm mounted on an elastic cantilever, to address the single molecule to which forces are to be applied. The cantilever can be moved by a piezo element, which allows for performing very accurate tip movements with sub nanometre resolution. To measure the force acting on the tip, a laser beam is focused onto the cantilever while the position of the reflected beam is measured via a four segmented photodiode. The position of the reflex is a measure for the deflection of the cantilever, which is proportional to the applied force (Figure 1).

A plot of measured forces versus the distance between the tip and the surface is called a force-versus-distance (F-D) curve (Figure 2). When pulling on the terminus of a membrane protein, the deflection of the cantilever will increase until the force is high enough to unfold a part of the protein. This allows the cantilever to relax, resulting in a sudden decrease of the applied force. Stretching the unfolded part allows one to determine its contour length which is a direct measure of the number of unfolded amino acids. This indicates which part of the protein is left in the membrane and reveals stable unfolding intermediates [6, 7].

Calculating from the force curves how long a force can be applied to a part of the protein until it unfolds, reveals the force dependent dissociation rates. From these the shape of the free-energy landscape of the intra- and inter-

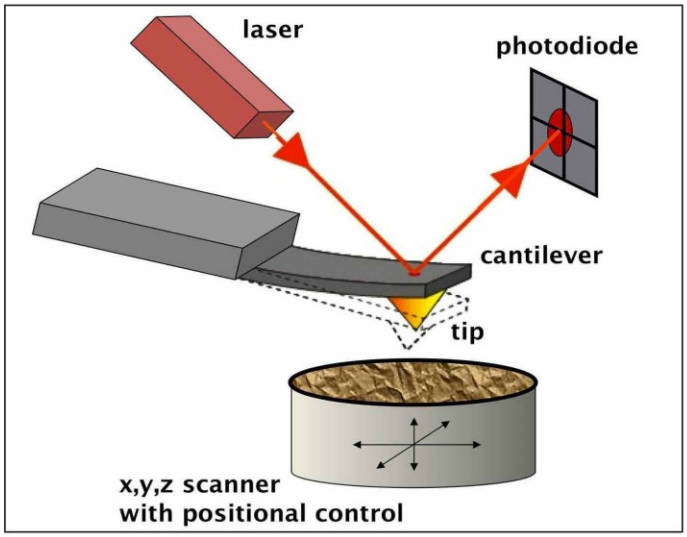

Figure 1:
Basic principle of atomic force microscope-based force spectroscopy. See text for details.

molecular interactions can be determined.

Controlled Unfolding of Membrane Proteins

Bacteriorhodopsin is a seven-α-helical transmembrane protein belonging to the family of microbial rhodopsins. In *Haloarchaea*, bacteriorhodopsin is a light-activated proton pump which performs a second type of photosynthesis. Applying the method of force measurements to individual bacteriorhodopsin molecules, revealed a very detailed map of its unfolding pathways and the stability of the different unfolding intermediates (Figure 2).

MATERIALS AND METHODS

Sample Preparation

Native purple membrane patches from *Halobacterium salinarum* were adsorbed onto freshly cleaved mica surfaces.

Atomic Force Microscopy

The patches were imaged in contact mode with an Asylum Research MFP 3D atomic force microscope using an Olympus TR400 PSA cantilever.

Attachment of the terminus to the tip was obtained via non-specific adsorption. To perform unfolding experiments on the membrane proteins the tip was positioned on a selected patch and subsequent force measurements were taken.

RESULTS

The force-distance curves obtained (Figure 3a) show a series of unfolding events. The curves show four main peaks, each reflecting the unfolding of a pair of transmembrane helices.

When stretching the terminus, a force is applied to the end of helix G (Figure 3 a, b, part 1). After unfolding of helices G and F, the force is acting at the end of helix E (Figure 3 a, b, part 2). The same is valid for the pairs of helices E and D as well as C and B leaving only helix A in the membrane (Figure 3 a, b, part 4).

These observations support the two-stage model of membrane protein folding in which alpha helices insert into the membrane as stable units and then assemble into the functional protein. However, in between several intermediate unfolding events can be recognized, revealing additional stabilizing interactions within the molecule. This allows studying the different unfolding pathways that a membrane protein may take [8-10]. In addition, upon relaxing the unfolded molecule, also the refolding of the stretched molecule can be observed, giving insights into the folding free energy barriers [11]. In general, the answer of membrane proteins to the change of external factors like pH, temperature or ion concentrations can be studied in detail [8, 9].

Revealing Conformational Changes by Differential Force Spectroscopy

Sensory rhodopsin II (SRII) is another seven-α-helical transmembrane protein that mediates a photophobic response of *Halobacterium salinarum* and *Natronomonas pharaonis* to avoid photo-oxidative damage in bright light. Upon

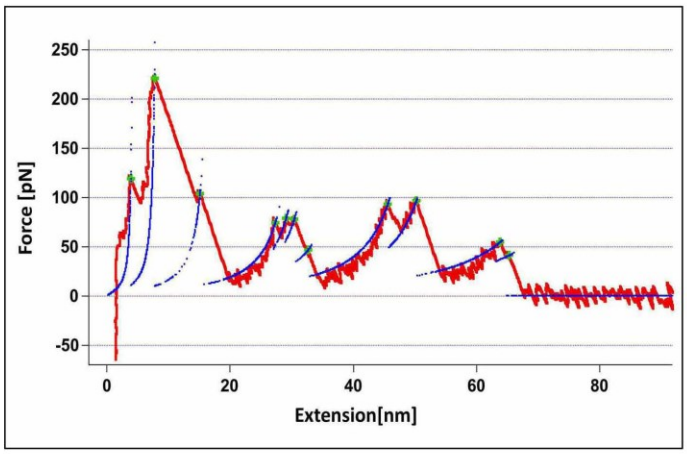

Figure 2:
Force-elongation curve of bacteriorhodopsin after analysis by a self-written algorithm. The blue curves show WLC (worm-like chain) fits to the stretching of the unfolded part of the protein which reveal the number of amino acids stretched. The green crosses indicate the localization and the force at which the unfolding events occurred.

light activation the receptor undergoes a conformational change that activates a tightly bound transducer molecule (HtrII), which in turn, by a chain of homologous reactions, transmits the signal to the chemotactic eubacterial two-component system [12, 13] .

The technique of single-molecule force spectroscopy was applied to transducer binding studies to SRII as well as light activation. Using this technique, intramolecular interactions within SRII of *Natronomonas pharaonis* (NpSRII) upon NpHtrII binding were localized and quantified.

Applying single molecule force measurements, stabilizing interactions of NpSRII in presence and in absence of its transducer were detected. It was shown, that transducer binding stabilized the extracellular half of the transmembrane helix G where specific hydrogen bonds are formed between helix G and the TM2 of the transducer, as well as the center of helix F where it interacts with the central water cluster (Figure 4a) [14].

The observed local stabilisations were in accordance with the results obtained with other techniques like crystallography, electron paramagnetic resonance spectroscopy and fluorescence resonance energy transfer. The specific interactions found might reflect a mechanism by which the receptor transducer interaction could have evolved without compromising the photocycle of microbial rhodopsins. Single-molecule force spectroscopy was also employed to study the changes in stability upon light activation of NpSRII in presence and absence of its transducer.

The experiments revealed that successive light activation in the presence of the transducer leads to destabilization of the central region of helix F due to breakage of the

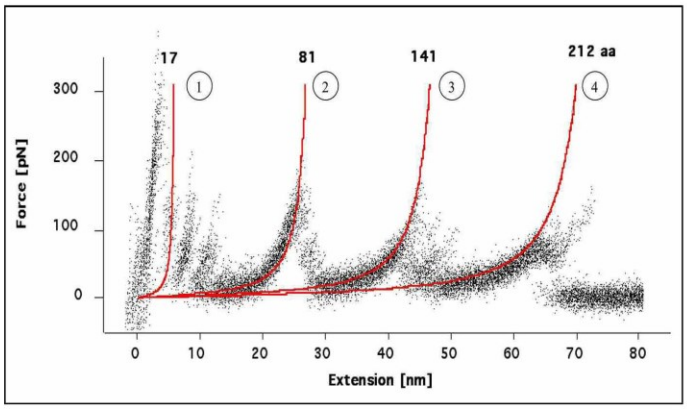

Figure 3a:
Several force curves of bacteriorhodopsin were superimposed showing four main intermediates. WLC (worm-like chain) fit curves indicating the force-elongation characteristic of an individual amino acid strand are shown in red, numbers above give the number of stretched amino acids.

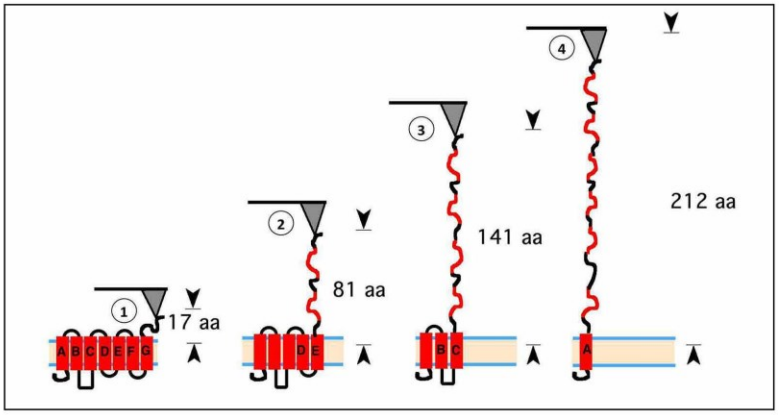

Figure 3b:
By counting back the number of calculated amino acids, the localization of stabilizing interactions are revealed. The peaks in the force spectra are explained by this model as the sequential extraction and unfolding of a single bacteriorhodopsin molecule. If a force is applied on the C-terminus, helices G and F get pulled out of the membrane and unfold. Upon further retraction, the unfolded chain gets stretched and a force is applied on helices E and D until they are extracted from the membrane. The same holds true for helices C and B. These unfolding events are reflected by peak 2 and peak 3 for helices E-D and C-B respectively. Peak 4 shows extraction of the last remaining helix A from the membrane.

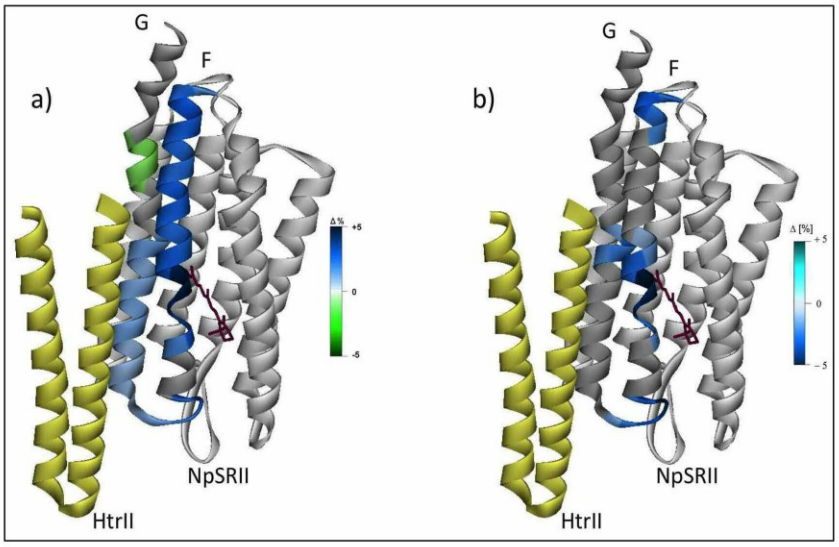

Figure 4:
Stabilising effect of transducer HtrII binding and light activation onto NpSRII.
(a) Transducer binding stabilizes the extracellular half of helix G due to specific hydrogen bridges and leads to a stabilization of helix F.
(b) Subsequent light activation destabilizes the center of helix F, indicating breakage of hydrogen bonds with the central water cluster.

hydrogen bonds between the amino acids Thr204 and Asp201 with the water cluster (Figure 4b).

This elucidates the complex structure-function relationship of the NpSRII-HtrII complex and helps us to further understand the mechanism of signal transduction.

CONCLUSIONS

The examples given above show that force spectroscopy is a powerful new technique, which is able to give detailed insight into the dynamic structure-function relationships of proteins and other biological molecules. With its ability to measure forces down to the piconewton range it provides a tool for routinely characterizing mechanical properties of individual biomolecules.

Single-molecule force spectroscopy is capable of detecting individual unfolding pathways of single membrane proteins under physiologically relevant conditions. In this way it can localise the interactions that stabilize the protein structure.

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Effect of the Compatible Solute Ectoine on the Stability of the Membrane Proteins

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Abstract: Mechanical single molecule techniques offer exciting possibilities for investigating protein folding and stability in native environments at sub-nanometer resolutions. Compatible solutes show osmotic activity which even at molar concentrations do not interfere with cell metabolism. They are known to protect proteins against external stress like temperature, high salt concentrations and dehydrating conditions. We studied the impact of the compatible solute ectoine (1M) on membrane proteins by analyzing the mechanical properties of Bacteriorhodopsin (BR) in its presence and absence by single molecule force spectroscopy. The unfolding experiments on BR revealed that ectoine decreases the persistence length of its polypeptide chain thereby increasing its tendency to coil up. In addition, we found higher unfolding forces indicating strengthening of those intra molecular interactions which are crucial for stability. This shows that force spectroscopy is well suited to study the effect of compatible solutes to stabilize membrane proteins against unfolding. In addition, it may lead to a better understanding of their detailed mechanism of action.

Keywords: Atomic force microscopy, membrane proteins, force spectroscopy, compatible solutes, ectoine.

INTRODUCTION

Microorganisms produce and accumulate compatible solutes in the cytoplasm aiming for protecting themselves from environmental stress. "Compatible solutes" generally have a low molecular weight, mostly either uncharged or zwitterionic organic molecules including polyols, sugars, amino acids and their derivatives. These types of solutes help maintaining osmotic balance without interfering with the essential cellular processes. Since they have relatively little effect on the cytosolic ionic strength, no special adaptation of the molecular pathways is required even at molar concentrations [1, 2].

Ectoine is a heterocyclic amino acid (Fig. **1**) that serves as a protective substance in many bacterial cells [3, 4, 5]. It allows microorganisms to resist extreme living conditions like drastic temperature variations [2, 6, 7], high salinity [8] and osmotic shocks [9, 10, 11]. Hence it is also known as an osmoprotectant, which is obtained from halophototrophic bacteria. These effects are also based on the direct stabilisation of cytoplasmatic proteins against denaturation [12, 13, 14]. However little is known about the stabilising effects of ectoine on membrane proteins.

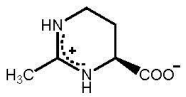

Figure 1. Chemical structure of Ectoine

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Transmembrane proteins account for approximately 30% of all proteins and play an important role in almost all cellular processes. They act as sensors, catalysts, receptors, transporters and channels and perform essential vital functions. Hence the stabilization of these proteins is very important for maintaining a multitude of molecular pathways and thus crucial for the survival of the cell. Here we present experimental results that reveal the stabilising effects of Ectoine against denaturation of a membrane protein. We investigated this effect of ectoine on Bacteriorhodopsin, by performing mechanical unfolding experiments.

Atomic force microscopy [15] is a powerful tool to observe, image and characterize membrane proteins in their physiological environment at the molecular level [16, 17, 18, 19, 20]. Single molecule force spectroscopy offers novel ways to investigate the stabilisation of membrane proteins. It provides detailed insights into inter and intra molecular interactions and enables us to go beyond the ensemble average [21, 22, 23].

Force spectroscopy applies a force to single molecules while observing the extraction of the protein from the membrane. By contacting the protein with the AFM tip, the protein terminus can be attached to the tip by unspecific or specific (e.g. thiol) interactions [21]. While pulling back the tip we apply an increasing force to the terminus and detect the protein unfolding by measuring length change of its unfolded part, being stretched between the tip and the support revealing (un)folding intermediates.

For investigation BR purple membrane patches [24, 25] were adsorbed onto a freshly cleaved mica surface. Unfolding experiments were done on single proteins after selecting a patch by imaging and subsequent positioning of the tip above a selected patch (as previously described) [21, 26].

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The measurements were performed using an Asylum Research AFM instrument and Olympus OMCL TR400 silicon nitride cantilevers with a spring constant of 20 pN/nm. The buffer used was 300mM KCl and 20mM Tris at pH 7.8 with 1M Ectoine and without Ectoine, respectively. Force curves were recorded at a retraction speed of 400 nm/s.

The force curves were analyzed using self-written software that does an automatic peak search and fits the rising part of the force curves before each peak with the worm like chain model,

$$F = K_B T/p\,(1/4(1-x/L_0)^{-2} - 1/4 + x/L_0),$$

which describes the stretching of an unfolded polypeptide chain. Here F is the applied force, x is the end to end distance, K_B is the Boltzmann constant, p is the persistence length and L_0 is the contour length. From this we obtain the length of the stretched amino acid chain and thereby the number of amino acids as well as its persistence length, which may depend on the surrounding conditions. From the number of amino acids obtained from each fit, counting back from the C-terminus, it can be calculated which part of the protein is left in the membrane as an intermediate. This reveals that helices G and F, D and E, and B and C, respectively, mostly unfold pairwise [21] sometimes showing less stable intermediates where only one helix is extracted out of the membrane. The seventh helix, A, is then extracted from the membrane in a last step (Fig. **2**).

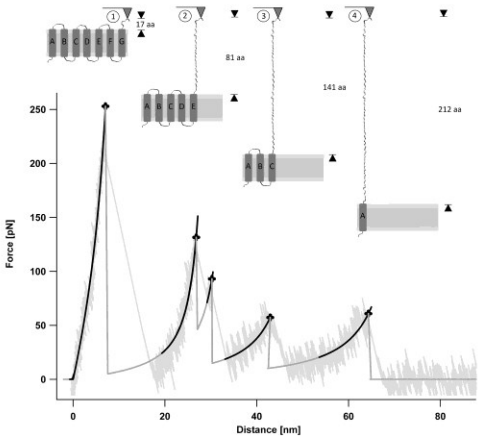

Figure 2. A typical Force – elongation curve on a single native Bacteriorhodopsin stretched by AFM. The black crosses indicate the localization and the force at which the unfolding took place. The black curves show a Worm Like Chain fit (WLC) to the unfolded part of the protein, revealing the number of amino acids of the stretched chain. The little sketches show the AFM tip attached to the terminus of BR. The seven transmembrane helices are depicted in grey and denoted from A to G. The sketches 1 to 4 indicate the main intermediate steps of the unfolding of BR that reveal pair wise unfolding.

The parameters of interest in our study are the unfolding force and the persistence length of the unfolded polypeptide chain. The measured unfolding forces reveal specific inter and intra molecular interactions that stabilise the protein structure and can be taken as a measure for the protein stability in the membrane against denaturation due to external stress like temperature or ionic strength as well as various additional solutes. On the other hand the persistence length of an unfolded amino acid chain is a measure for its tendency to form a compact coil in solution which supports (re)folding of the protein. Shorter persistence lengths correspond to a higher tendency to coil up while longer ones indicate a more extended conformation.

To understand the stabilising effects of ectoine on membrane proteins, we analysed its effect on the unfolding force and persistence length. Both parameters were extracted from all recorded force curves were histogramed as shown in (Fig. **3**). In absence of ectoine the force histogram shows a mean unfolding force of around 59pN (Fig. **3a**), whereas in presence of ectoine a pronounced increase of the unfolding force to 92pN and 164pN, respectively was found. Also the persistence length is affected by the presence of Ectoine (Fig. **3b**). In the absence of ectoine the it was found to be about 0.44nm whereas upon addition of Ectoine the persistence length was significantly decreased down to 0.28nm.

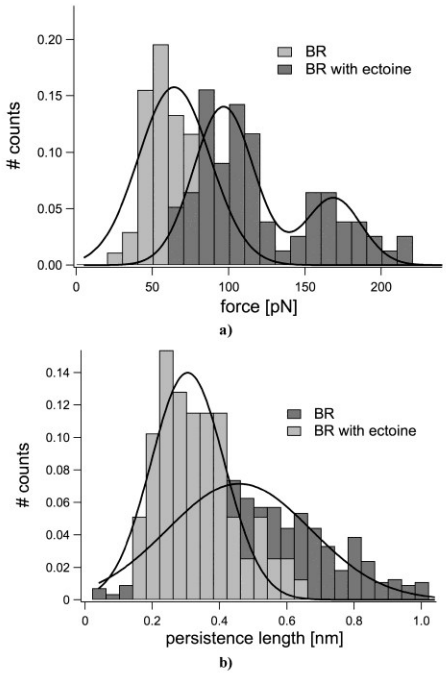

Figure 3. a) Histograms showing the dependency of unfolding forces of BR on Ectoine. **b**) Histograms showing the dependency of persistence length of BR on Ectoine. The second peak appearing in the presence of ectoine may be ascribed to the miscellaneous stabilising effects of ectoine on the different unfolding intermediates. However, this is beyond the scope of this paper and will be discussed elsewhere.

It is known that compatible solutes from different molecular classes like amino acids and their derivatives, sugars or polyols, induce the formation of a stabilising water shell around soluble proteins [12, 27].

The results presented here suggest that compatible solutes may also act as a stabilising agent on membrane proteins. The mechanism of how compatible solutes stabilise proteins can be understood based on the model of preferential exclusion [32].

These solutes dissolve well in water at high concentrations and exhibit different preferences to interact with the protein compared to water [28]. If water and the solute have the same tendency to interact with the protein, the free hydration energy ΔG_h is the same in presence and absence of the solute. If the solute preferentially is excluded from the protein, its state is similar to that in pure water. However, the exclusion of the solute from the protein creates a concentration gradient of the osmolyte at the protein surface. This leads to an increased ΔG_h and thus a stabilization of the folded conformation. An unfolded peptide chain will be driven to a more compact coil to minimize its surface area and thus the loss of entropy, which is caused by the concentration gradient of the osmolyte. By that it supports the formation of a molten globule and protein folding.

Our results support this model in two ways. On one hand ectoine supports folding of BR, on the other hand, is helps BR to withstand unfolding stress. The observed decrease in persistence length of the stretched amino acid chain directly shows the stronger tendency of the entropic coil to collapse into a dense conformation which strongly increases the protein folding rate. This is a general effect, which can be observed also in the case of soluble proteins [13].

The same effect also accounts for the stabilisation of proteins against unfolding since this is always associated with an increase of the protein surface. Unfolding of a membrane protein requires the extraction of the peptide chain out of the membrane into the aqueous surroundings. The increased free energy difference between the folded and unfolded conformation induced by the osmolyte leads to an increase of the unfolding free energy barrier, which is manifested in the increased measured unfolding forces.

Interestingly, as described by others, ectoine had shown no effect on the unfolding forces of globular fibronectin III domains [13]. This may be understood on the basis of the different shapes of the free energy landscapes along the unfolding pathways. When unfolding a beta barrel structure as a Fibronectin III domain, the breakage of the interaction between the first and last beta strand, which are oriented in an antiparallel manner, is sufficient to induce the unfolding of the whole domain. Due to the antiparallel orientation, all hydrogen bonds between the strands are loaded simultaneously and a small shift of approx. 2Å induced by the applied force leads to the breakage of all bonds at once. Such a small shift only leads to a very small change in the protein surface. In contrast the unwinding of an alpha helix is followed by a major surface increase, and thus it experiences a stronger stabilising effect by the osmolyte-induced hydration shell [33-35].

CONCLUSION

Ectoine is known to have a general stabilising effect on cytoplasmatic proteins [13, 29, 30] and lipid bilayers [31]. Here we presented force spectroscopic data that reveal also its stabilising effect on membrane proteins. From unfolding experiments on the seven helical membrane protein Bacteriorhodopsin (BR) we obtained the forces required to unfold the protein and its intermediates as well as the persistence length of the unfolded amino acid chains. In the presence of the compatible solute ectoine a strong stabilising effect on BR was observed. The forces required to unfold BR were found being increased. This result may reveal a stabilising effect of ectoine, which is likely to be valid for alpha helical membrane proteins in general. In addition a decrease in the persistence lengths of the unfolded amino acid strands was observed, which indicates an increased tendency to coil up in accordance with shown data. This leads to a more compact structure of the unfolded amino acid chain, which supports the (re)folding of the protein.

We conclude that the stabilisation of BR by ectoine may provide a general protecting mechanism. While the enhancement of protein stability might be important to maintain cellular processes, ectoine especially may act as a protectant for cell surfaces against external stress. We expect the results also to be of high interest for medical research due to the importance of osmolytes in human organs and the important role of membrane proteins as drug targets.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

None declared.

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Concentration dependent Membrane Protein Stability under the Influence of Different Compatible Solutes Studied by Single Molecule Force Spectroscopy

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Running title: Membrane protein stabilisation by compatible solutes.

Abstract:

Compatible solutes are small, uncharged, zwitter ionic, osmotically active molecules produced and accumulated by microorganisms inside their cell to counteract different kinds of environmental stress. They enhance protein stability without interfering with the metabolic pathways even at molar concentrations. In this paper, we report the stabilizing effects of compatible solutes, ectoine, betaine and taurine on membrane protein Bacteriorhodopsin at different concentrations. Using Atomic Force Microscopy based Force Spectroscopy their impact was quantified by measuring the forces required to extract the protein from the membrane and the change in the persistence lengths of the unfolded polypeptide chain.

Increasing unfolding forces were observed indicating the strengthening of intra-molecular interactions, which are vital for protein stability. The decrease in persistence lengths was recorded showing increasing tendencies of the polypeptide strand to coil up. Interestingly, it was revealed that these molecules have different stabilizing effects on protein unfolding at different concentrations. These information are aimed to achieve a better understanding of the interaction between the unfolded protein and the solutes. The results show that the unfolding of single proteins provides in depth and crucial information about their structure-dynamic relationship at sub-nanometer scale, which are aimed at providing detailed insight into various life processes.

Keywords: osmolytes; protein unfolding; membrane proteins; atomic force microscopy; single molecule force spectroscopy; compatible solutes.

Compatible solutes are small, low molecular weight, naturally occurring compounds that are known to stabilise proteins against external stresses. They are zwitter ionic with no net charge, and belongs to different categories e.g. amino acids, their derivatives, methylamines, polyols etc. They are accumulated inside the cells when the cell is subjected to adverse conditions like extreme temperature conditions, pressure, chemical denaturation, and presence of high salt concentration and other solutes in the environment they live in. They are known as compatible solutes because of their compatibility with that they bolism and do not interfere with cellular composition and function even when they are accumulated in the cell at molar concentrations (Yancey et al., 1982; Burg, 1995). These as well as tioned and other external stresses create a harsh condition for the cellular macromolecules, threatening the loss of their biological properties and functions (Pace, 1975; Xie et al., 1994; Privalov et al., 1993). Hence, many compatible solutes are accumulated and used by the cells to counter-act such denaturing effects by stabilising the intracellular macromolecules such as proteins.

A number of experimental studies involving ensemble measurements have shown that accumulation or addition of compatible solutes have reduced and prevented structural destabilisation and helped restore the stability of the protein (Knapp et al., 1999; Lee et al., 1981; Lamosa et al., 2003; Foord et al., 1998; Yancey et al., 1982).

These molecules have gained much focus recently for their interesting role in stabilising proteins against denaturing forces. Hence, they are also known as osmoprotectants or protecting osmolytes.

Experimental works done by the groups of Galinski, Timasheff and Yancey show that compatible solutes like ectoine increase the melting temperature Tm of the proteins thereby rendering it thermodynamically

more stable that help the proteins to retain their folded native conformations.

The stabilising osmolytes exert a force that causes the protein to co-operatively fold into a native-like functional conformation from an unstructured conformation in aqueous environment (Burg, 2000; Schellman, 2002; Murphy et al., 1992; Baskakov et al., 1998). Several studies have demonstrated that the stabilising property of naturally occurring osmolytes correlate with the preferential exclusion of these osmolytes from the vicinity of unfolded or denatured protein which result in the formation of a denser and more structured water shell (preferential hydration) around the stretched protein (Xie et al., 1997a; Xie et al., 1997b, Yu et al., 2007).

This expulsion of solutes from the protein surface corresponds to the increase in the chemical potential of the protein (Lee et al., 1981; Knapp et al., 1999; Janshoff et al., 2000).

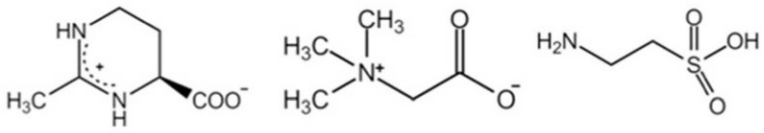
The protein backbone is osmophobic in nature, so when the concentration or number of compatible solute molecules increase in the solvent accessible area of the protein, the protein acquires a more compact conformation by hiding the backbone into the core of folded proteins in order to reduce its exposure to the added solutes (Bolen, 2001; Yancey, 2005; Baskakov et al., 1998). This raises the enthalpy of the system and as a result the unfolded state of the protein will be more destabilised due to its greater solvent exposed area in turn resulting in stabilisation of the folded conformation.

Apart from bacterial cells, mammalian cells are also known to contain a considerable amount of organic osmolytes. Renal medullary cells of mammals contain the highest concentration organic osmolytes like betaine, taurine, sorbitol, inositiol etc. to counter-balance the effects of very high concentration of NaCl and urea during urine concentration (Somero, 1986).

In our studies, we used three different compatible solutes namely, ectoine, betaine and taurine and studied their effect on the mechanical stabilisation of the membrane protein Bacteriorhodopsin using unfolding experiments carried out by Atomic force microscopy based force spectroscopy.

Ectoine is a heterocyclic amino acid accumulated in bacterial cells that maintains osmotic balance and serves as protective agent for cells against hostile conditions. It is most commonly derived from the halophototrophic bacteria *Halomonas elongata* and is accumulated inside the cell to help the cell survive temperature variations, differences in osmotic pressure, draught, high salinity and other environmental shocks.

Betaine is another organic osmolyte that is known more for its role in osmo-regulation. Mammalian cells can accumulate betaine in the cytoplasm (Burg et al., 2007). It is mostly synthesized from choline in mammalian liver and kidney. It is a methyl donor and known to be used as medicine, as a PCR rate enhancer, tissue protection etc. (Timasheff, 1992).



Ectoine Betaine Taurine

Figure 1. Chemical structure of compatible solutes used in the study. All of these three compatible solutes belong to the class of amino acids and their derivatives.

Taurine is present in meat, fish, milk and an essential nutrient for mammalian growth particularly for neurological development. It helps in regulating the level of water and mineral salts in the blood. Sodium- and chloride-dependent

taurine transporter TauT is known to carry out the osmoregulation during hypertonic condition. Hypertonictiy increases the transcription of TauT. Taurine is accumulated in mammalian liver cells (Häussinger, 1998).

Proteins are extraordinarily complex bio macromolecules that perform fundamental functions for the viability of the cell. Understanding the dynamics of protein folding, the mechanism and pathways they take up to acquire their native conformation is very important. Their complexity lies not only in their static conformation but also in their structural dynamics, which are critical for biological functions. Of all the proteins, transmembrane proteins constitute about 30% population and perform a key role as transporters, receptors, sensors, ion channels, catalysts and so on. But at the same time the proteins belonging to this class are less stable and are difficult to handle. Hence, stabilisation of membrane proteins is of great significance for carrying out various cellular activities.

Bacteriorhodopsin is a transmembrane protein that belongs to the family of microbial rhodopsins and is found in the cellular membrane of the archaeon *Halobacterium salinarum*. It folds into a seven alpha helical topology joined by short interconnecting loops. It contains a covalently bound retinal molecule, which absorbs light thereby contributing in process of photosynthesis by the cell (Pedersen 1995).

The atomic force microscope (AFM), invented in the year 1986, has for the first time enabled the addressing and manipulation of individual biomolecules, triggering also the development of a variety of scanning probe microscopes and single-molecule techniques. Apart from its ability to image biological surfaces with sub-nanometer resolution, AFM also enables the manipulation of matter at the nanoscale. The AFM-based single molecule force spectroscopy is specially

suited to measure the mechanical response of proteins, revealing inter- and intramolecular interactions. It exploits the advantage of using a sharp needle like tip attached to the flexible cantilever to unspecifically attach and manipulate the terminus of a single protein.

Protecting effects of compatible solutes on soluble proteins have been studied to a fairly good extent, the studies on membrane proteins have not been concentrated upon so far. Intrigued by this fact and also that the natural stabilisation mechanisms of protein is quite fascinating, yet little understood, we employed single molecule force spectroscopic techniques to understand the mechanical properties of the membrane proteins and how they are stabilised by using natural osmolytes.

Here, in this paper, we report the findings of single molecule unfolding experiments of the membrane protein Bacteriorhodopsin (BR) and the influence of the compatible solutes ectoine, betaine and taurine on the mechanical stabilisation of it in presence of these osmolytes at various concentrations. The effects are represented by the change or difference in unfolding forces of individual BR molecule and their persistence lengths. Stability of intermediates is also observed and reported in these experiments.

Results

Increase of unfolding forces and reduction in persistence lengths

The organic osmolyte ectoine is known to stabilise membrane protein Bacteriorhodopsin (BR) (Roychoudhury et al., 2012). In this study, force curves were fitted with the Worm Like Chain (WLC) model (Figure 2) and histogrammed by taking into account all the four peaks of each selected force curves. The parameters like unfolding forces and persistence length of the stretched polymer were compared and studied for different experimental conditions. The measured unfolding forces reveal the localization of intra- and intermolecular interactions that stabilise the protein structure.

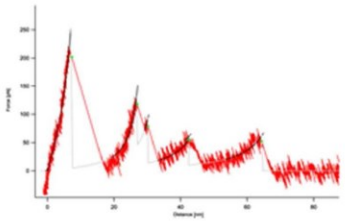


Figure 2. A force-extension curve representing extraction of a single BR molecule out of the membrane. The green crosses indicate the localization and the force at which the unfolding took place. The black curves show a Worm like Chain fit (WLC) to the unfolded part of the protein, revealing the number of amino acids of the stretched chain. Each of the four peaks correspond to defined chain length.

These forces can be attributed to be a measure of the stability of the protein in the membrane against denaturation caused by various external stresses such as high salinity, change in surrounding temperature, addition of solutes etc. Figure 3 A) and B) display the results obtained from the unfolding experiments with 1M concentration of all three osmolytes, ectoine, betaine and taurine compared to that of without any osmolyte on peak forces and persistence lengths respectively.

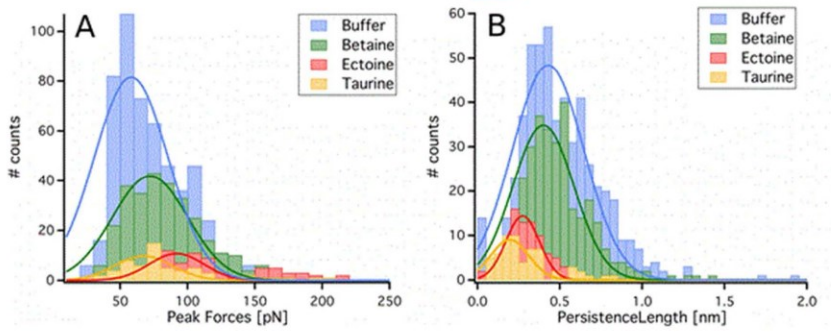


Figure 3. A) Histograms showing the dependency of unfolding forces of BR on different osmolytes (1M). B) Histograms showing the dependency of persistence length of BR on different osmolytes (1M).

In Figure 3A) it can be seen that, as expected from the already known information that osmolytes stabilise the protein structure, here also in case of

membrane proteins it can be observed that the peak forces rise significantly high in presence of osmolytes. Although, all three osmolytes result in higher unfolding forces, the degree in the raise of forces is different. In case of peak forces taurine and betaine show the lesser effect and ectoine has the most pronounced effect by almost doubling the dissociation forces in its presence. In absence of any osmolyte, unfolding force was found to be 59pN, while in case of 1M each of ectoine, betaine and taurine it is 92 pN, 72pN and 67pN respectively. On the other hand, as seen in Figure 3B), a reduction in persistence lengths is noted in presence of osmolytes. In this case, taurine has the most pronounced effect by reducing the persistence length of the unfolded polypeptide chain to almost half while ectoine has a moderate effect and betaine almost a negligible one. In absence of the osmolytes the persistence lengths was found to be 0.44 nm, and in presence of 1M ectoine it got reduced to 0.28nm while 1M betaine and 1M taurine reduced it to 0.4nm and 0.2 nm respectively. This gives us the idea how the whole length of the stretched polypeptide chain is affected by different osmolytes at a high concentration of molar level.

We also observed that the number of counts of recorded data went down in case of the osmolytes. These histograms hence suggested that different osmolytes have different modes of action elucidating different degrees of effects on membrane protein stability. The increase in unfolding forces and the reduction in persistence length indicate the stabilisation of membrane proteins against unfolding and tendency of the polypeptide to adopt a more coiled structure and globule conformation in presence of osmolytes.

These significant findings and observations using 1M concentration of each of the osmolytes lead us to go for a concentration dependent study with lower concentrations of the osmolytes to see where and at which concentration

osmolytes start influencing unfolding forces and persistence lengths of the membrane protein bacteriorhodopsin.

Influence of concentration changes on unfolding forces and persistence lengths

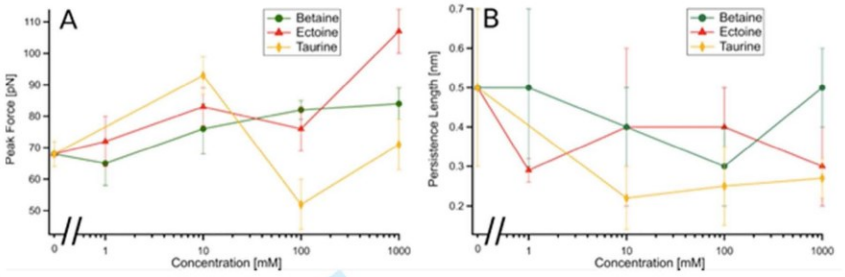


Figure 4. A) Influence of concentration changes on unfolding forces B) Influence of concentration changes on persistence lengths

Figure 4A) and B) depict the impact of increasing concentration on peak forces and persistence lengths of BR. There is a slight increase in overall unfolding forces in case of taurine; betaine shows an almost linear increase in forces while ectoine has the highest effect on unfolding forces resulting in almost double the value at 1M ectoine than no osmolyte condition. There is a slight dip in the forces at 100mM concentration, mainly in case of ectoine and taurine (Figure 4A). The influence of concentration change in persistence length change is also mostly non-linear and very specific for the particular concentrations. But in case of taurine, it is noticeable that there is a concentration dependent decrease in persistence lengths with the increase in concentration (Figure 4B). These results obtained thus indicate that different osmolytes have different and specific effects on stability of BR.

Impact of compatible solutes on unfolding intermediates

So far the results shown were of all the unfolding peaks taken into account. We then analysed individual unfolding peaks to get a measure of forces and persistence

lengths for the four main peaks of each unfolding event. Two-dimensional scattered diagrams were plotted as function of peak forces *versus* fit lengths and persistence lengths *versus* fit lengths, which revealed the changes in unfolding forces, and persistence lengths for each main peak.

In Figure 5A) we can see there are three distinct distributions. The first one is very scattered and unclear with less data, which indicates that the first unfolding peaks are not clear enough to for a good quality and reliable fit. This is because of the unspecific attachment of the tip to the terminus while the protein is pulled out of the membrane. This peak has the highest unfolding force, which ranges from around 150pN to about 250pN. The four distributions indicate the unfolding forces required for pairwise extraction of helices G-F, E-D, C-B and A of the protein. We can notice here that all three osmolytes stabilise the unfolding intermediates in a similar fashion, just in case of betaine there is almost no change in peak forces for the last peak as compared to peak force of last unfolding peak without any osmolyte.

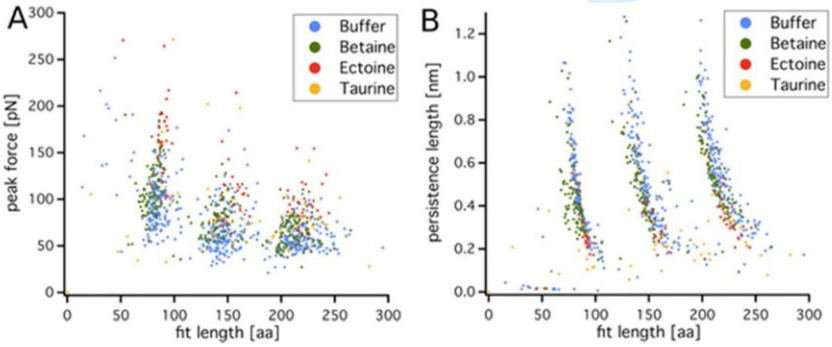


Figure 5. A) Scattered plots showing the effect of 1M concentration of osmolytes on peak forces for each unfolding peak of a single BR extraction out of the membrane B) Scattered plots showing the effect of 1M concentration of osmolytes on peak forces for each unfolding peak of a single BR extraction out of the membrane

Similarly, we plotted scattered diagrams to see the effect of osmolytes on persistence lengths of the each individual unfolding event. In Figure 4b), betaine has least effect while taurine and ectoine show more significant effects exhibiting a pronounced reduction in persistence lengths. But again, what is visible in these average scattered plots of each unfolding event of all selected force data gives us the idea that the whole polypeptide chain is affected and stabilised by the osmolytes in a similar way.

In addition carrying out experiments on concentration series, we also analysed the data for individual unfolding intermediate peaks of each osmolytes and compared them together to see more in detail if the effect of osmloytes is on the full length of the protein altogether or they have different effects on single intermediate peaks as well.

The analysis for observing the effects of concentration changes on single intermediate steps involved in unfolding of BR was performed for measuring the changes in the unfolding force and the persistence length for each peak. Due to the unspecific interaction between tip and

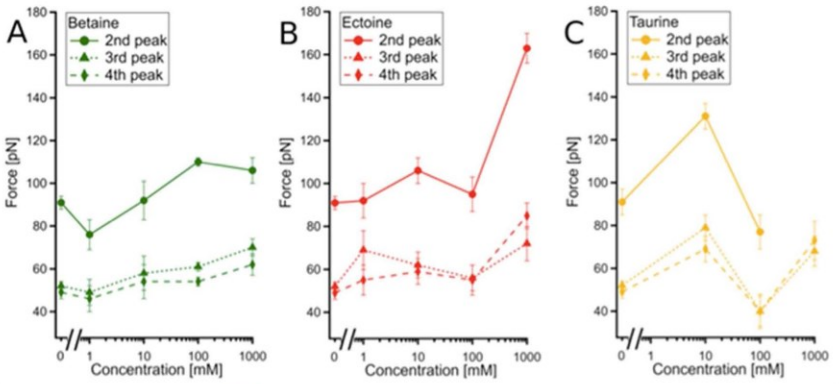


Figure 6. Dependency of single peak unfolding force on osmolyte concentration.

surface we did not have enough data to analyse the first unfolding step. So the first peak is not shown. In Figure 6 we present the forces for the second, third and fourth peak for the measurements with different osmolytes. In general we can observe and confirm that the second peak always has a higher unfolding force than the third and fourth one. For betaine we can observe an increase in the unfolding force for all three peaks dependent on the increase in concentration. For ectoine we see a non-linear dependency of the unfolding force on the concentration but the course is for all peaks the same. For taurine counts the same but it shows higher fluctuations. Due to less data available, we could not analyse the second peak at 1M for taurine.

The dependency of the persistence length on the varying osmolyte concentration is shown in Figure 7. The first detection is that for the buffer solution in absence of any osmolyte (what stands for 0M of osmolytes) the persistence length shows for all osmolytes a decrease for each sequenced unfolding step. Furthermore we can conclude from our observation that under the influence of betaine the effect on the different peaks is non-linear. For example for the second peak we have a

reduction of the persistence length for 1mM and 10mM but then the persistence length decreases with higher concentrations. For ectoine we see a reduction of the persistence length for the second and third unfolding step with an increasing concentration. The fourth peak shows a non-linear dependency. Taurine shows for all peaks a decrease in persistence length with increasing concentration. This course is similar to that of the osmolyte concentration dependent measurements of (Oberdörfer et al., 2003) on globular proteins. So we can conclude for the single peak analysis that the osmolytes betaine and ectoine affect the protein in a non-linear way which goes in accordance with the result of the single peak force analysis.

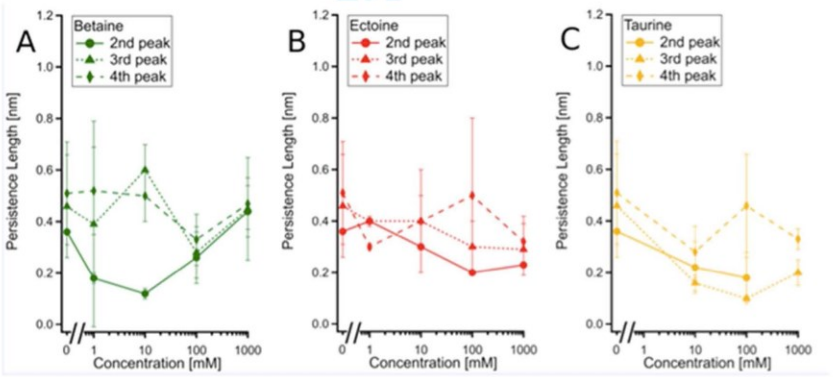


Figure 7. Dependency of single peak persistence length on osmolyte concentration.

Discussion:

In this study, the stabilisation of the membrane protein Bacteriorhodopsin upon addition of compatible solutes ectoine, betaine and taurine at various concentrations was investigated by applying atomic force microscopy based single molecule force spectroscopy.

Bacteriorhodopsin represents one of the most extensively studied and one of the best known membrane proteins making it ideal for the study of various life processes. Hence it was suitable to be taken up for our study involving compatible solutes, which are known to stabilise protein structures and support folding but up to now it is not shown for membrane proteins with single molecule techniques. Also little is known about the complex mechanism involved in this. The purpose of this work was to understand whether biological effects on the cellular level induced by an increase or decrease in the solute level, respectively, may be explained by the direct interaction of the compatible solutes with the membrane proteins under investigation. Therefore, we focused in this study on the mechanical properties and changes in force and persistence length during unfolding of the protein that result from applying external stress in presence of compatible solutes ectoine, betaine and taurine. In general, higher unfolding forces indicate that a protein is mechanically more stable and is difficult to extract out of the membrane while the decrease in the value of persistence length indicate a tendency of a polypeptide to form a more compact structure.

The results of the force spectroscopic studies of protein folding dynamics revealed significant effects of these compatible solutes on unfolding properties of Bacteriorhodopsin, a reduction in persistence length and an increase in the unfolding forces of the protein varying with different concentrations of compatible solutes.

This suggests that compatible solutes also act as stablising agent for membrane proteins in addition to globular proteins (Oberdörfer et al., 2003). The principal cause behind this stabilisation by rendering the protein into a more compact conformation can be attributed to the unfavourable interaction between the polypeptide backbone and the compatible

solutes present in the aqueous environment. This goes in accordance with the mechanism of preferential exclusion for osmolyte action (Kurz, 2008). Compatible solutes dissolve well in water, even at very high molar concentration and leads to the exclusion of the solute molecules from protein vicinity in the aqueous environment (Timasheff, 1992).

Protein stability could be governed by water structure

In general, osmolytes are widely known for their stabilising properties on globular proteins (Oberdörfer et al., 2003, Lippert et al. 1992, Lentzen et al., 2006) and lipid bilayers (Harishchandra et al., 2010). In case of globular proteins, previous studies showed that there were no significant changes in the unbinding force of the FN-III domains under the influence of compatible solute ectoine (Oberdörfer et al., 2003). But we observed significant changes in unfolding forces in case of the membrane protein Bacteriorhodopsin under similar conditions (Figure. 4A). This implies that the membrane protein is strongly affected by compatible solutes when it comes to extracting it out of the membrane. A profound increase in the peak forces in presence of osmolytes could be attributed to the formation of a dense and more structured water shell around the exposed part of BR (Figure 8). At point 1 in Figure 8, it is assumed that upon addition of the solute molecules, the interaction between the transmembrane part of the first helix of BR and water, and the same region of BR and osmolytes govern the rearrangement of water and osmolyte molecules around the stretched or exposed polypeptide in the bulk solution.

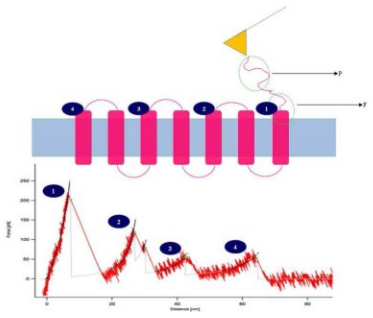


Figure 8. Schematic representation of Bacteriorhodopsin unfolding in presence of osmolytes.

This scheme depicts the pairwise extraction of individual BR molecules out of the membrane. When a force is applied on the terminus of the protein, helices F and G are pulled out. The increase of unfolding forces in presence of osmolytes are resultant of the formation of a denser hydration shell around that area.
Here, F if the force acting upon the protein to pull it out oft he membrane and the green dotted circle in the cartoon resembles the part where a restructured water shell might have formed. P is the part, which can give a measure of persistence lengths or the ability of the protein to collapse into a more coiled structure.

As shown in Figure 8, unfolding of a membrane protein requires the extraction of its helices out of the membrane into the aqueous environment. Because of the positive interaction between these solute molecules with no net charges and water, these molecules dissolve well in aqueous solutions even at very high concentrations and are eventually preferentially excluded from the protein vicinity. A denser and more compact water structure is formed upon addition of solutes, which causes a dehydration of the protein surface making less aqueous surrounding available for it. This in turn makes the protein to collapse into a more compact coil and renders it more stable. Our results showing a decrease in persistence length in presence of osmolytes supports the enhanced stability of membrane protein in presence of these compatible solutes. Also, during the unfolding of protein, the system becomes enthalpically unfavourable due to breaking of bonds in presence of osmolytes. This enhances greater structuring of hydration shell around the unfolded protein and results in the thermodynamic stability of the protein.

The unfolding of BR exposes it to the surrounding environment of aqueous buffer and osmolytes and increases the

protein surface area and also leads to changes in the free energy.

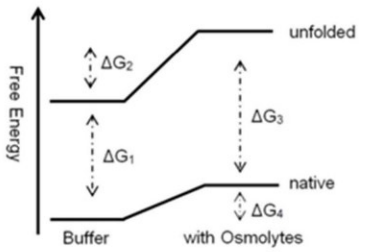


Figure 9. Schematic representation of protein stabilisation induced by thermodynamic effects of osmolyte action.

The diagram depicts the increase in free energy of both native and unfolded state due to unfavourable interaction of solutes with protein. ΔG1 and ΔG3 are the Gibbs free energy difference between the native and unfolded state of protein in buffer and in presence of osmolyte, respectively. ΔG2 and ΔG4 are the rise in Gibbs free energy of the unfolded state ensemble and native state ensemble (adapted from adapted from Baskakov et al., 1998b and Street et al., 2006).

Osmolytes and proteins do not have a favourable interaction. They act by rising the free energy of both native and unfolded ensemble states, but the increase in Gibbs free energy is stronger in case of the unfolded state because of the larger exposed surface area (Figure 9). The increased Gibbs free energy difference between the folded and unfolded state of the polypeptide chain results in the increase in unfolding free energy barrier, which is manifested by the increase in measured unfolding forces. This results in the greater stability of the protein in the osmolyte solution than in aqueous buffer. The stability of BR observed in our study hence can be explained by this theory.

Mechanism of osmolyte action in stabilising membrane protein Bacteriorhodopsin: preferential exclusion model

In accordance with the preferential exclusion model of osmolyte action, the results of our experiments show a significant stabilisation of the membrane protein structure in the presence of osmolytes. They also establish the fact that different osmolytes have different stabilising effects on unfolding forces and persistence lengths. On one hand, osmolytes facilitate (re)folding of BR, and on the other hand supports it in withstanding the unfolding stress. Our

experimental results show a decrease in the persistence lengths of the stretched amino acid chain of Bacteriorhodopsin, which indicates that under the influence of osmolytes there is a stronger tendency of the entropic coil to collapse and fold into a dense conformation by expelling the solute molecules from the exposed protein surface area. Due to the rise in the chemical potential of the stretched protein, the protein prefers to reduce the solvent exposed surface by adopting a more condensed structure. This shortening of persistence lengths resulting into a more compact structure has also been observed in case of soluble proteins (Janshoff et al., 2000, Oberdörfer et al., 2003).

As observed in our concentration dependent study, the effect of various concentrations on the persistence length of Bacteriorhodopsin is different in the analysis where we take all peaks into account. All osmolytes at different concentrations reduce the persistence length of the protein, which implies that the tendency of the molecule to acquire a more compact conformation is different depending upon the concentration. But there is a particular pattern of it observed mainly in the case of ectoine and taurine at several conditions although the magnitude of their effect varies. These molecules act very specifically at particular concentrations and their preferential interaction with water and with protein is different at different concentrations. A significant reduction in the numbers of force curves recorded has been observed in presence of ectoine as well as taurine. This indicates that in presence of these solutes the unspecific interactions between the tip and the sample goes down considerably.

An almost similar increase in force required to extract the intermediate peaks suggests that no specific hydrogen bonds are responsible the increase in force.

In case of the single peak analysis for the different concentrations, each osmolyte shows a similar pattern in the forces and persistence lengths for each unfolding

peak (Figure 6 and 7) which indicates again that there is no specific binding on the protein and the osmolytes do not affect only a specific part of the protein. Furthermore we could observe a similar pattern for the forces and persistence lengths for each peak for ectoine and taurine dependent on the concentrations. For these both osmolytes we got also a remarkably reduction in the binding probability of the tip to the terminus of the protein and therefore less unfolding events within the same amount of force curves. This leads to the assumption that ectoine and taurine act in a similar way on the membrane protein Bacteriorhodopsin in contrast to betaine.

With our concentration dependent measurements on the membrane protein Bacteriorhosopsin we could show that it is affected by the compatible solutes in the expected way (more folded into the membrane to reduce the contact surface to the compatible solutes and reduction in the binding probability). Furthermore we observed that each unfolding peak is affected in the same way so that we can assume for the mechanism of the interaction of the osmolytes and Bacteriorhodopsin that there is no specific binding to the protein. Besides we saw a similar way of interaction of ectoine and taurine with the protein. Hence, we conclude that the stability of the membrane protein BR in general is enhanced by the organic osmolytes. The overall increase in force indicates that the osmolytes may act as protectant for cell surfaces to withstand the external stress while the decrease in persistence length indicates a facilitated refolding process.

Because of the increasing clinical relevance of osmolytes in understanding and curing human diseases, we hope that our results and findings will help and lead to a further investigation of osmolytes and their role in drug development with respect to the important role of membrane proteins as transporters and drug targets.

Materials and methods:

Sample Preparation- Native purple membrane patches* from *Halobacterium salinarum* were dissolved in solution buffer. The sample of Bacteriorhodopsin diluted in buffer, was then adsorbed onto freshly cleaved mica surfaces (Müller, 1997).

The effect of all the three compatible solutes on mechanical unfolding of membrane protein were studied under several concentrations (1mM, 10mM, 100mM and 1M) in a solution 20mM Tris + 300mM KCl buffer in nanopure water (R > 18 M Ω cm) at physiological pH of 7.8. The sample containing the purple membranes was diluted such that the solution is not too dense and the adsorption time was chosen to be around 15 minutes to ensure high coverage, but no overlapping of membrane patches. After 15 minutes incubation time, the sample was washed with the same buffer for three times to wash away the unadsorbed protein and the sample was then immersed in the standard buffer with or without the compatible solutes at above mentioned concentrations for concentration dependent unfolding experiments. We carried out the same number of pulling experiments for each condition.

Ectoine was provided by BiTop, Witten, Germany and betaine and taurine were purchased from Sigma Aldrich, Germany. All of these had analytical grade purity of 99.9%.

Atomic Force Microscopy and Single Molecule Force Spectroscopy- The membrane patches were imaged using Atomic Force Microscope from MFP 3D, Asylum Research (Santa Barbara, CA) instrument and Olympus OMCL TR400 silicon nitride cantilevers. Imaging was performed in fluid using contact mode with the same cantilever like in the force spectroscopy measurements.

After imaging mechanical unfolding experiments were carried out on single proteins by selecting a patch and thereafter

directing the tip of the cantilever on top of the selected patch.

The attachment of the terminus to the tip was obtained via non-specific adsorption by pressing the cantilever onto the sample. Force curves were recorded at a retraction speed of 400nm/s. The stiffness of the cantilever exhibited by the measure of the spring constant was calculated for each unloaded cantilever by positioning it away from the surface and taking the mean of thermal vibration signal and applying the equipartition theorem (Hutter et al., 1993). The spring constant calculated by this method was measured to be around 20pN/nm.

Data analysis - Force versus extension curves were recorded in repeated pull and release cycle using self-written procedures implemented in the Asylum AFM software. Selected force *versus* extension curves were corrected for virtual deflection of the cantilever, displacement of the cantilever, tip-surface conversion and other factors using the graph correction options in IGOR. Afterwards the curves were analysed by fitting each force peak to the Worm Like Chain (WLC) model with a monomer length of 3.6Å in IGOR using self-written procedures. The WLC model gives the simple approximate force extension relationship of Bacteriorhopsin unfolding, given by:

$F = KB\,T/p\,(1/4(1-x/L0)-2 - 1/4 + x/L0)$.

Here F is the applied force, x is the end to end distance, KB is the Boltzmann constant, p is the persistence length and L0 is the contour length, i.e. the length of the completely stretched polymeric chain. The WLC model was used to fit the force and persistence length in the protein unfolding force curve although our system is a complicated one and this model is a simple model based on assumptions, which do not fully comply in accordance with the requirements of our system. But the WLC has been often found to have given reliable results of studies involving force spectroscopic techniques for stretching polypeptide molecules.

The recorded force curves showed the typical characteristic pattern of four main peaks (Figure 2) (Oesterhelt et al., 2000). In these force curves, each main peak represents the extraction of an alpha helical pair of the protein out of the membrane, which is typical for various seven transmembrane helical proteins of the rhodopsins family (Sharma et al., 2006; Klare et al., 2008; Oberbarnscheidt, 2009). Additionally, force curves reveal further side peaks representing unfolding intermediates revealing different unfolding pathways (Figure 2) that differ depending on the experimental condition (Kedrov et al., 2007; Cisneros et al., 2008; Müller et al., 2002; Janovjak et al., 2006).

From the number of amino acids obtained from each fit, counting back from the C-terminus, it can be calculated which part of the protein is left in the membrane as an intermediate.

Persistence length (P) is derived from fitting the curvature of each peak and it is a measure of elasticity or bending rigidity of the protein. The unfolding force (F) is the measure of mechanical force required to extract the protein out of the membrane. It is derived by fitting the highest point of each peak before it shows a rupture resulting in the drop of unfolding force. Localization of stabilising intramolecular interactions reveal that helices G and F, D and E, and B and C, respectively, mostly unfold pair wise, sometimes showing less stable intermediates where only one helix is extracted out of the membrane. The seventh helix, A, is then extracted from the membrane in the last step.

Error calculation - Because of the thermodynamic influence on the dissociation the unfolding force does not have a distinct value but a broad distribution. Therefore we performed Monte Carlo simulations of the dissociations curves to determine the standard deviation of the force histogram without any experimental error. After analyzing the simulated curves, we made histograms with as many curves that were

taken for plotting the histograms from the experimentally recorded data. This we made ten times and fitted a Gaussian distribution to them, which we used to calculate the mean force and its standard deviation. We used this standard deviation as the error of the force in the graph showing unfolding force for each intermediate peak dependent on concentration (Figure 6 and Figure 7).

For the error of the persistence length of the single peak analysis we used the standard deviation value of the Gaussian fit to the persistence length histogram.

In Figure 5A) and B) we took into account all peaks and used the Gaussian error propagation law assuming that the errors of the single peaks are uncorrelated.

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*Native purple membrane patches of Bacteriorhodopsin were a gift from the group of Prof. Dr. Norbert Hampp, Department of Chemistry, University of Marburg, 35032 Marburg, Germany.

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Figure legends:

Figure 1. Chemical structure of compatible solutes used in the study.

All of these three compatible solutes fall under the class of amino acids and their derivatives.

Figure 2. A force-extension curve representing extraction of a single BR molecule out of the membrane. The green crosses indicate the localization and the force at which the unfolding took place. The black curves show a Worm like Chain fit (WLC) to the unfolded part of the protein, revealing the number of amino acids of the stretched chain. Each of the four peaks correspond to defined chain length.

Figure 3. A) Histograms showing the dependency of unfolding forces of BR on different osmolyte (1M).

B) Histograms showing the dependency of persistence length of BR on different osmolyte (1M).

Figure 4. A) Influence of concentration changes on unfolding forces.

B) Influence of concentration changes on persistence lengths.

Figure 5. A) Scattered plots showing the effect of 1M concentration of osmolytes on peak forces for each unfolding peak of a single BR extraction out of the membrane.

B) Scattered plots showing the effect of 1M concentration of osmolytes on peak forces for each unfolding peak of a single BR extraction out of the membrane.

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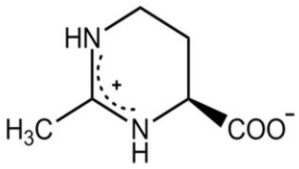

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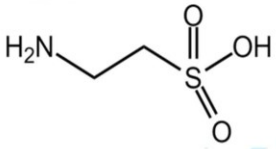

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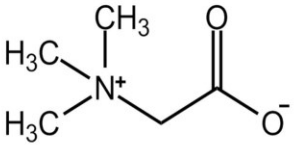

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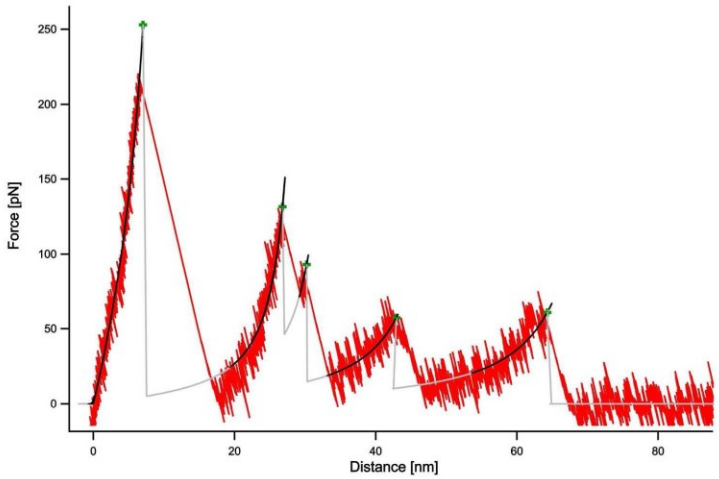
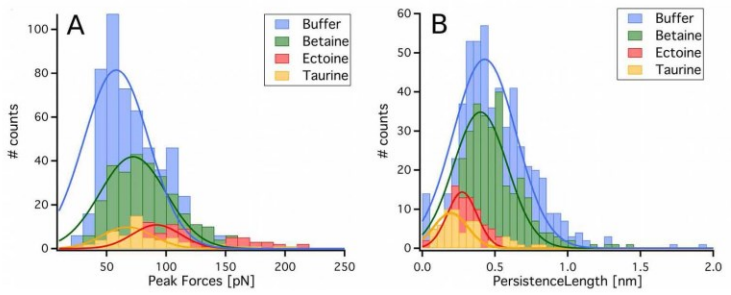
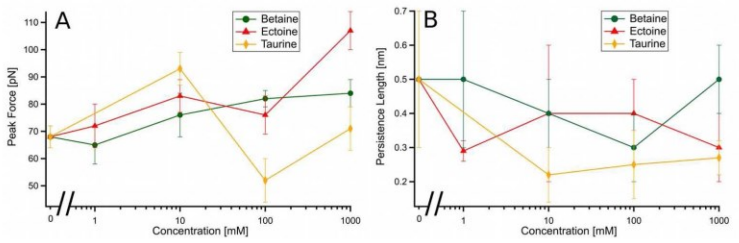


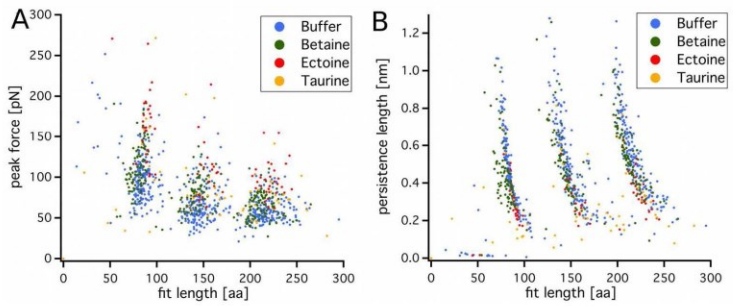
210x137mm (300 x 300 DPI)



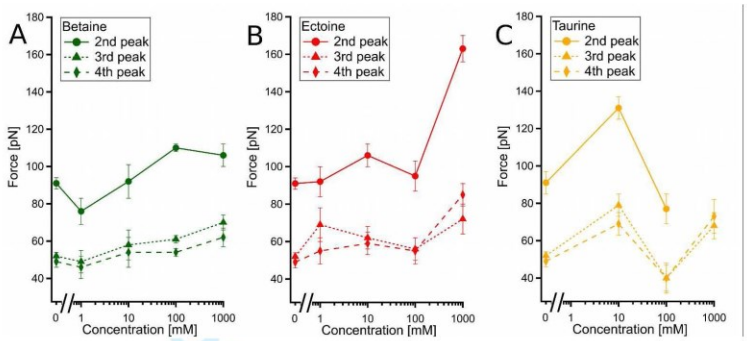
82x32mm (600 x 600 DPI)

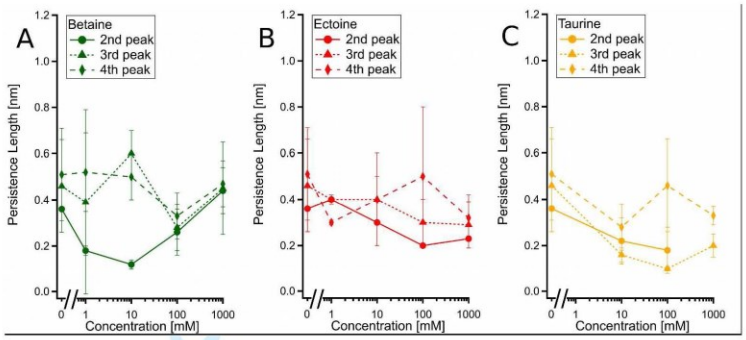


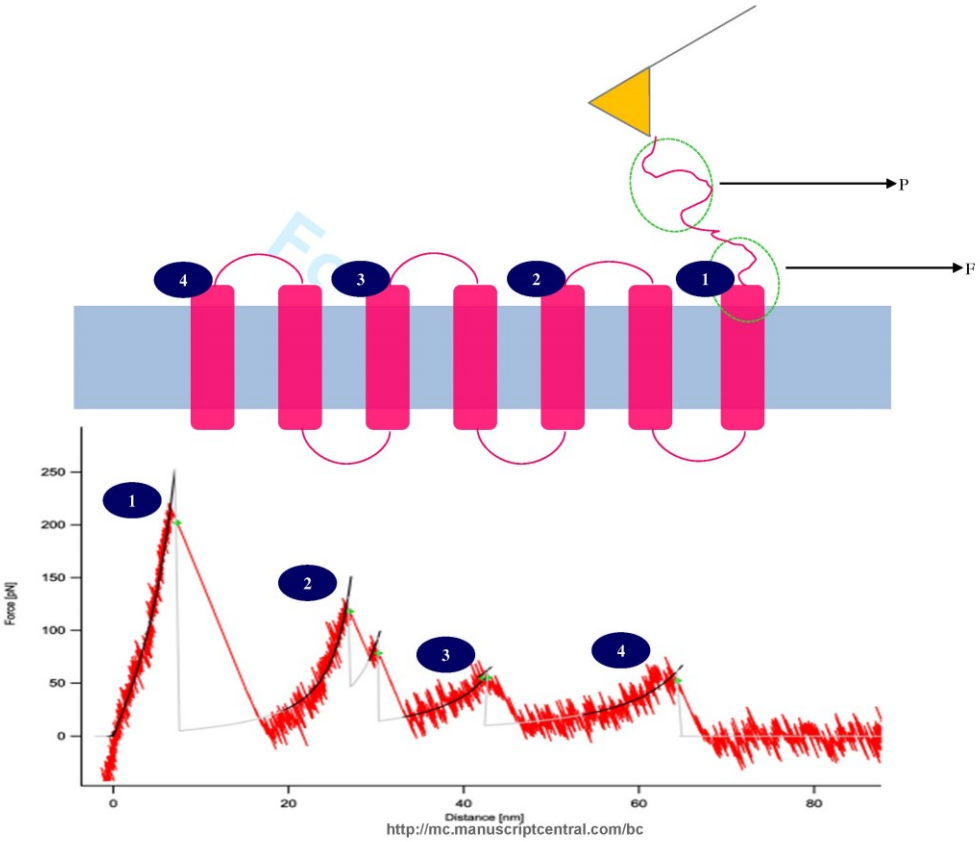
67x21mm (600 x 600 DPI)

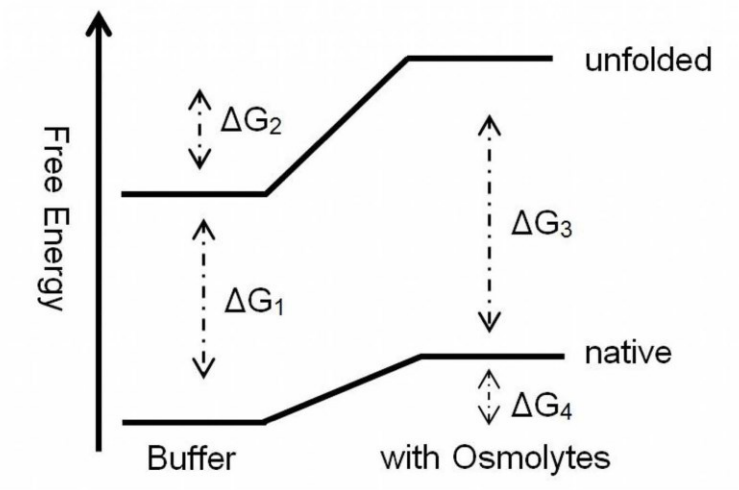


85x35mm (600 x 600 DPI)









53x35mm (600 x 600 DPI)