Characterizing the Functional and Folding Mechanism of β-barrel Transmembrane Proteins Using Atomic Force Microscope

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To my Parents
Summary:
Single-molecule force spectroscopy (SMFS) is a unique approach to study the mechanical unfolding of proteins. SMFS unfolding experiments yield insight into how interactions stabilize a protein and guide its unfolding and refolding pathways. In contrast to various water-soluble proteins whose unfolding and refolding patterns have been characterized, only α-helical membrane proteins have been probed by SMFS. It was shown that α-helical membrane proteins unfold via many intermediates; this differs from the two-state unfolding process usually observed in water-soluble proteins. In membrane proteins, upon mechanically pulling the peptide end of the protein, single and grouped α-helices and polypeptide loops unfold in steps until the entire protein is unfolded. Whether the α-helices and loops unfold individually or cooperatively to form an unfolding intermediate depends on the interactions established within the membrane protein and the membrane. Each unfolding event relates to an unfolding intermediate with the sequence of these intermediates defining the unfolding pathway of the protein. β-barrel-forming membrane proteins are the second major group of membrane proteins and have not yet been studied by SMFS. To fill this void this study was designed to characterize interactions, unfolding, and refolding of the β-barrel forming outer-membrane protein G (OmpG).

Folding of transmembrane proteins, despite the important part these proteins play in every biological process in a cell, is studied in only a few examples. Of those only a handful were β-stranded membrane proteins (Tamm et al., 2004; Kleinschmidt et al., 2006). Current models describe that transmembrane β-barrels fold into the lipid membrane via two major steps. First the unfolded polypeptide interacts with the lipid surface where it then folds and inserts into the membrane (Kleinschmidt et al., 2006; Huysmans et al., 2010). Conventionally, thermal or chemical denaturation is used to study folding of membrane proteins. In most cases membrane proteins were solubilized in detergent or exposed to urea to be studied, conditions that are not compatible with In vivo conditions. This suggests that the folding pathways described so far may not be a realistic representation of such pathways in nature.

SMFS represents a unique approach to study the unfolding and refolding of membrane proteins into the lipid membrane (Kedrov et al., 2006; Kessler et al., 2006). Using SMFS makes it possible to study unfolding and refolding of membrane proteins in their native
physiological environment with controlled pH, electrolyte, temperature, and most importantly in the absence of any chemical denaturant or detergent.

In this thesis, SMFS was utilized to unfold and refold OmpG in E coli lipid extract. Bulk unfolding experiments suggested that OmpG unfolds and folds reversibly and much faster than α-helical proteins (Conlan et al., 2000). The folding process is thought to be a coupled two-state membrane partition-folding reaction. To the contrary, the mechanical unfolding of OmpG consisted of many sequential unfolding intermediates. Our SMFS refolding experiments showed that a partially unfolded OmpG molecule also refolds via several sequential steps. The predominant refolding steps are defined by individual β-hairpins that could later assemble the transmembrane β-barrel of OmpG. In conclusion, the most probable unfolding and refolding pathways of OmpG as a membrane β-barrel protein go through the β-hairpins as the structural segments or unfolding-refolding intermediates and the process is a multi step one rather than the simple two state process.

We also used SMFS to study the physical interactions that switch the functional state and gating of OmpG. The structural changes that gate OmpG have been previously described by X-ray crystallography (Yildiz et al., 2006). They showed when the pH changes from neutral to acidic the flexible extracellular loop L6 folds into the pore and closes the OmpG pore. Here, SMFS was used to structurally localize and quantify the interactions that are associated with the pH-dependent closure. At an acidic pH, a pH-dependent interaction at loop L6 was detected. This interaction changed the unfolding of loop L6 and β-strands 11 and 12, which connect loop L6. All other interactions detected within OmpG were found to be unaffected by changes in pH. These results provide a quantitative and mechanistic explanation of how pH-dependent interactions change the folding of a peptide loop to gate the transmembrane pore. It has also been shown how the stability of OmpG is optimized so that pH changes modify only those interactions necessary to gate the transmembrane pore and there are no global changes in protein conformation or mechanical properties.

In the next step of interactions study, dynamic SMFS (DFS) was applied to quantify the parameters characterizing the energy barriers in energy landscape for unfolding of the OmpG. Some of these parameters are: free energy of activation and distance of the transition state from the folded state. The pH-dependent functional switching of OmpG directs the protein along different regions at the unfolding energy landscape. The two functional states of OmpG
sequential folding take the same unfolding pathway as β-hairpins I–IV. After the initial unfolding events, the unfolding pathways diverge. In the open state, the unfolding of β-hairpin V in one step precedes the unfolding of β-hairpin VI. In the closed state, β-hairpin V and β-strand S11 with a part of extracellular loop L6 unfold cooperatively, and subsequently β-strand S12 unfolds with the remaining loop L6. These two unfolding pathways in the open and closed states join again in the last unfolding step of β-hairpin VII. Also, the conformational change from the open to the closed state witnesses a difference in $Xu$ and $\kappa$ in the energy landscape that translates to rigidified extracellular loop L6 at the gating area. Thus, a change in the conformational state of OmpG not only bifurcates its unfolding pathways but also tunes its mechanical properties for optimum function.
# Table of Contents

## INTRODUCTION

1. **THE FIRST UNIT OF LIFE STARTED WITH MEMBRANE** 1  
1.1 **CELL MEMBRANE STRUCTURE** 2  
1.2 **MEMBRANE PROTEINS** 3  
1.2.1 **α**-HELICAL MEMBRANE PROTEINS 5  
1.2.2 **β**-BARREL MEMBRANE PROTEINS 5  
1.3 **MEMBRANE PROTEINS FOLDING** 12  
1.3.1 Models for **α**-HELICAL MEMBRANE PROTEIN FOLDING 13  
1.3.2 Models for **β**-BARREL MEMBRANE PROTEIN FOLDING 15  
1.4 **GATING STUDY OF MEMBRANE PROTEINS** 18  
1.5. Atomic Force Microscopy 19

## ATOMIC FORCE MICROSCOPY

2. **1. HISTORY** 19  
2.1 **PRINCIPLE** 19  
2.1.3 The Cantilever 20  
2.1.4 AFM Modes 23  
2.2 **SINGLE-MOLECULE FORCE SPECTROSCOPY** 25  
2.2.1 Dynamic Force Spectroscopy, (Dynamic SMFS) 27  
2.3 **WHAT IS THE ADVANTAGE OF USING ATOMIC FORCE MICROSCOPY IN MEMBRANE PROTEIN STUDIES?** 29

## FOLDING MECHANISM OF OMPG

3. **1. UNFOLDING PATTERN: ONE β-HAIRPIN AFTER THE OTHER** 31  
3.1 **OUTER MEMBRANE PROTEIN G (OmpG)** 31  
3.1.2 Mechanical Unfolding Pathways of the Membrane β-Barrel Protein OmpG 33  
3.1.3 Material and methods: 34  
3.1.4 Results and Discussion 41  
3.2 **REFOLDING PATTERN: ONE B-HAIRPIN AFTER THE OTHER** 48  
3.2.1 Exploring Refolding Pathways and Kinetics of the Membrane β-Barrel Protein OmpG 48  
3.2.2 Experimental procedures 49  
3.2.3 Results 50  
3.2.4 Discussion 52

## INTERACTION STUDIES

4.1.2 **Introduction** 59  
4.1.2 Experimental procedures: 61  
4.1.3 Results and Discussion: 62  
4.2 **DUAL ENERGY LANDSCAPE: THE FUNCTIONAL STATE OF THE OUTER MEMBRANE β-BARREL PROTEIN OmpG MOLDS ITS UNFOLDING ENERGY LANDSCAPE** 67  
4.2.2 Experimental Procedures 71  
4.2.3 Results and discussion 74  
4.2.3.1 Functional state of OmpG directs its unfolding route 74
4.2.3.2 Quantifying the Unfolding Energy Barriers of OmpG in the Closed and Open Conformations 75
4.2.3.3 Transition State Distances of Unfolding Energy Barriers 77
4.2.3.4 Activation Free Energy of B-Strands and B-Hairpins 79
4.2.3.5 Mechanical Properties of OmpG 83
4.2.3.6 Mapping the Unfolding Energy Landscapes of OmpG in the Open and Closed States 85
4.2.4 Conclusion: 86

Outlook 89

5.1 Introduction 89
5.2 Interaction Study and Unfolding Energy Landscape 90
5.3 Membrane Protein Folding 92

References: 96

Abbreviations 110

Symbols 111

Publications: 113

Acknowledgment: 114

Declaration: 115
INTRODUCTION

1.1 THE FIRST UNIT OF LIFE STARTED WITH MEMBRANE

Our ancestors, not knowing anything about molecular biology, cells and genes, called the common features amongst all the living beings as “life”. They marveled at life, struggled to discover, define and describe it. Based on all the discoveries in the past century, now we know that all living beings are made of cells, and that these units of living matter are enclosed by membranes. So, simply, we can consider a cell as a small balloon filled with all chemicals necessary for sustaining life and the balloon itself is the membrane. Accordingly, individual cells team up to form tissues, which can be structured to form organs that are capable of performing complex functions and cooperate among other organs to sustain an organism (Albers et al., 2000).

The most valid theory of the formation of the first membrane is the following: About two billion years ago, an early prebiotic environment existed in which very versatile simple molecules formed. These reacted into more complex molecules and eventually developed into complicated catalytic and self-replicating systems. These very first systems had to compete for available resources in the primordial pond. In this environment, resources were limited and shared among all competing pre-cellular self-replicating systems. The development of the plasma membrane provided a strong selective advantage in that resources could be restricted from competitors. They also could gather nutrients from the environment and retain their own within the system. Moreover, with the plasma membrane, the effective concentrations of substrates were adjusted to be able to change the catalytic rates of the enzymes (Van Meer et. al., 2008).

Thus, in this way, the plasma membrane promoted evolution of the first cell. Some of the characteristics of these first membranes were: being dynamic, thin, self-healing, and having insulating boundaries. These boundaries have the same characteristics as the lipid bilayer. Lipids comprise a collection of molecules that are insoluble in water,
while being soluble in fat and organic solvents. The lipid composition of membranes highly depends on cell type, organelle, and organism (Rothman and Lenard, 1977).

1.2.1 CELL MEMBRANE STRUCTURE
Membranes are one of the most critical components of the cell. They enclose the cell and act as a selective barrier to let nutrients enter the cell and retain its own synthesized products while excreting waste materials. Membranes also define cell boundaries and shape. They separate the organelles like the Golgi apparatus and mitochondrion inside the eukaryotic cell. More importantly, the ion gradient across mitochondria membranes is used for producing ATP, which is the consumable source of energy inside the cell.

The basic structure of membranes is provided by the lipid bilayer, which can be easily seen by electron microscopy (EM). Phospholipids are the most abundant lipids in the cell membrane. However cholesterol and glycolipids can also be found in large amount in different cell membranes. Phosphoglycerides and Sphingomyelin are considered the most important phospholipids in the cell membrane. Phospholipids spontaneously form bilayers in aqueous environment because of their structure, amphiphilic nature, and the fact that bilayers are energetically the most favorable arrangement in aqueous solution (Rothman and Lenard, 1977).

A lipid bilayer has at least two distinct regions, which are chemically distinguishable: The interfacial space (interface) and the hydrophobic core (Chamberlain et al., 2004). The interfacial region is about 15 Å wide and is chemically very heterogeneous. It also has functional groups that participate in hydrogen bonding and ionic interactions (Kang et al., 2011; Wiener et al., 1991). The hydrophobic core, which is about 30 Å wide, has a very low dielectric constant\(^1\) that enhances certain molecular interactions. For instance, the energy calculated for backbone hydrogen bonds of proteins in the bilayer is five times higher than that in water. This difference in energy of the hydrogen bonds suggests that the cost of dissolving the backbone into the hydrophobic core would be compensated by the enhanced stability of secondary structure formation in the lipid bilayer. Overall, membrane proteins form their secondary structures easier and faster in lipid bilayers than in other environments (Kang et al., 2011; Bowie, 2011).

Lipids are not equally distributed within the two leaflets of a cell membrane, which

\(^1\) The dielectric constant of the lipid bilayer hydrophobic core is \(~2\).
results in asymmetry (Rothman and Lenard, 1977). The best example for this asymmetry is the plasma membrane of eukaryotic cells, where phosphatidylserine (PS) and phosphatidylethanolamine (PE) are found more in the cytoplasmic interface and phosphatidylcholine (PC) and sphingomyelin (SM) are primarily enriched in the exoplasmic interface. Glycolipids are exclusively located on the outer interface of the cell membrane (Zaczkowski, 1993). This asymmetry is very important for the function of membranes, since some proteins specifically bind to certain lipid headgroups (Steinberg, 2008). For example, PS lipids are negatively charged so enrichment of PS lipids in the cytosolic interface causes a charge difference between the two leaflets of the cell membrane. This difference is helpful in determining the transmembrane topology of membrane proteins based on the "positive-inside" rule (von Heijne, 1986). Moreover, cholesterol and sphingolipids may transiently associate to form lipid rafts in the outer leaflet that hosts some particular proteins. These specialized domains of different compositions in the plasma membrane of animal cells may have special roles in biological processes such as endocytosis (Kahya et al., 2004).

The lipid bilayer is a two-dimensional fluid, and the fluidity of the bilayer is dependent on its composition (Singer and Nicolson, 1972; Chiu et al., 1995). This fluidity makes the cell membrane a dynamic structure where molecules are able to move within or throughout it almost freely. This was the model considered for the cell membrane in 1972 by Singer and Nicolson. Since then there have been numerous studies on membranes and membrane proteins directing us to the point where we now know that the cell membrane is more mosaic than fluid (Engelman, 2005). In a mosaic bilayer, proteins have confined or directed movement within the membrane.

1.3 MEMBRANE PROTEINS
Although the lipid bilayer is the scaffold of the membrane, proteins carry on most of the functions. Being on the border of the cells, they are the major gates for nutrient uptake and waste disposal. Localization at the boundaries of the cell and between cellular compartments has made membrane proteins the key player in communications and signal transductions. Photosynthesis, cell-cell adhesion, transport of ions and small molecules, osmotic pressure maintenance and cellular organization are some examples of roles of membrane proteins. On the other hand, the fact that about 30% of all the open reading frames in the genome of all cells encode membrane proteins emphasizes the importance of these proteins (Wallin and Heijne, 1998). Despite the importance of membrane proteins, our knowledge about their
structure and function is very poor, as only less than 1% of protein data bank (PDB) contains data about membrane proteins (White, 2004).

Two structural motifs have been observed for secondary structure of membrane proteins inside the bilayer: transmembrane α-helical bundles and β-barrel structures (Figure 1.1) (Bowie, 2005). α-helical membrane proteins are predominant in cell membranes and β-barrel membrane proteins are found mainly in the outer membrane of gram-negative bacteria, chloroplasts, and mitochondria (White and Wimley, 1999). Some bacteria use β-barrel membrane proteins as toxins (Olson & Gouaux, 2003).

Figure 1.1 Membrane proteins with focus on the secondary structural motifs: The two most common architectures of membrane proteins: a) Voltage dependent anion channel, VDAC, from human mitochondria, is an example of β-barrel proteins. Most of the β-barrel proteins are porins in the outer membrane of gram negative bacteria, mitochondria and chloroplasts (Damaghi et al., 2011). b) Light driven proton pump Bacteriorhodopsin, bR, from H. salinarium, an example of α-helical proteins, contains seven transmembrane helices (Lanyi, 2004).

The general structural features of membrane proteins are very similar to soluble proteins. The interior amino acids are mostly nonpolar (Rees et al., 1989; Adamian et al., 2002). Hydrogen bonding interactions stabilize membrane proteins structure (Call et al., 2004; Senses et al., 2004). Like soluble proteins, the α-helices and β-sheets make internal hydrogen bonds with the same strength. Although for soluble proteins, water is a very competitive solvent for hydrogen bonding; in contrast, the
hydrophobic core of lipid membranes prepares an ideal environment for hydrogen bond formation with no competition. Thus, the backbone hydrogen bonds may be much stronger in membrane proteins than in soluble ones (Bowie, 2011).

There are other characteristics of membrane proteins that we can consider as general themes for both α-helical and β-barrel groups, namely, the localization of polar amino acids such as arginine and lysine at the N-terminus and the localization of amphipatic residues such as tryptophan and tyrosine at the bilayer, which forms the aromatic belt region that may serve as an anchor for proteins in the membrane (Von Heijne, 1986; Schiffer et al., 1992; Kang et al., 2011).

1.3.1 α-helical membrane proteins

Transmembrane (TM) α-helices are rich in non-polar amino acids such as alanine, valine, leucine, and isoleucine. They generally form ~ 20 amino acid (aa) long segments that pass the entire cell membrane (Von Heijne, 1986; Popot and engelman, 2000). Transmembrane α-helices are not always straight or rod-shaped structures. Some of them are kinked or distorted (Fu et al., 2000). Polar and charged residues are located in the outer membrane loops and domains. The role of these extramembrane segments can provide additional stability, protein folding assistance, and even environmental sensors (Yernool et al., 2004).

Single and bundled transmembrane α-helices are held together by electrostatic and van der Waals interactions (Torres et al., 2003). The TM helices adapt packing angles around 20° with a preference for left-handed helices, compared to right-handed helices with a packing angle about -35° of water-soluble proteins (Bowie, 1997).

1.3.2 β-Barrel membrane proteins

As an evolutionary view, gram-negative bacteria are the ancestors of mitochondria and chloroplasts; therefore they all have two surrounding lipid bilayers (Sagan, 1967). While the lipid composition of their lipid bilayer has changed significantly over their evolutionary course, the protein composition remained partly the same. Almost all proteins found in the outer membranes of gram-negative bacteria, mitochondria and chloroplasts are β-barrel proteins such that 2-3% of the whole genome in gram-negative bacteria encodes β-barrel membrane proteins (Wimley, 2003; Walther et al., 2009).

Usually, in β-barrel membrane proteins, β-strands of eight to eleven residues with a tilt of 25-45° spans the membrane bilayer in an anti-parallel manner (Wimley, 2003).
The smallest membrane β-barrel known to date has eight trans-membrane strands (Sansom and Kerr, 1995), while the largest, PapC, has 24 strands (Fairman et al., 2011).

1.3.2.1 β-barrel membrane proteins of gram-negative bacteria

As mentioned before, gram-negative bacteria have two distinct layers of membrane: the outer membrane and the inner membrane. Between these two membranes there is an aqueous compartment called the periplasm that contains soluble proteins. The peptidoglycan cell wall of the bacteria is also located inside the periplasm.

The two membrane layers are asymmetric in both lipid composition and proteins. The inner membrane is composed of phospholipids and contains integral α-helical proteins and peripheral lipoproteins and is in charge of energy-driven transport processes. The outer membrane is an asymmetric bilayer itself, which is composed of phospholipids in the inner leaflet (70-80% phosphatidyl ethanol amine and 20-30% phosphatidyl glycerol and cardiolipin) and lipo-polysaccharides (LPS) in the outer leaflet. It also contains β-barrel proteins and lipoproteins. The β-barrel proteins make pores in the outer membrane and allow nutrients and solutes to enter the organism and waste products to exit.

Almost all β-barrel proteins of gram-negative bacteria are located in the outer membrane and are known as Outer Membrane Proteins (OMPs). The number of strands in the barrel in bacteria ranges from eight to twenty four. All bacterial OMPs have an even number of strands. OMPs play many versatile roles in bacteria and we may categorize them in six distinct groups: (i) general porins such as OmpA, OmpF, OmpC, and OmpG, (ii) passive transporters such as ScrY and LamB, (iii) active transporters such as FhuA, Fec A, and Fep A, (iv) enzymes such as the phospholipase ompLA, and the protease OmpT, (v) defensive proteins such as OmpX, (vi) and structural transmembrane proteins such as OmpA [table 1.1] (Tamm et al., 2004; Walther et al., 2009; Fairman et al., 2011).

Synthesis of outer membrane proteins and the other integral membrane proteins in gram-negative bacteria is carried out in the cytoplasm. All integral proteins have a signal sequence at the N-terminal that guides them to the Sec complex, which is a secretory machine in the inner membrane (von Heijne, 1990). Nevertheless, the delivery mechanism for α-helical and β-barrel proteins to the Sec complex is different (Figure 1.2).
### Summary of all known structures of bacterial β-barrel membrane proteins:

<table>
<thead>
<tr>
<th>No. of strands</th>
<th>Protein name</th>
<th>Organism</th>
<th>Function</th>
<th>PDB ID</th>
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<tr>
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<td>Adhesin/invasion</td>
<td>3QRA, 3QRC</td>
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<tr>
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<td>Adhesin</td>
<td>1P4T</td>
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<td>Escherichia coli</td>
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<td>Klebsiella pneumoniae</td>
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<td>Adhesin/invasin/vasin</td>
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<td>Putative channel</td>
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<td>Pseudomonas aeruginosa</td>
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<td>Plasminogen activator</td>
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<td></td>
<td>Gdpa</td>
<td>Rhodobacter capsulatus</td>
<td>Porin</td>
<td>2POR</td>
</tr>
<tr>
<td></td>
<td>Gdpa</td>
<td>Rhodopseudomonas blastic</td>
<td>Porin</td>
<td>1H6S, 1PRN, 2PRN</td>
</tr>
</tbody>
</table>
Table 1.1 List of β-barrel membrane proteins in bacteria that have been identified to date.

In contrary to the inner membrane that is translocated cotranslationally, outer membrane proteins are directed to the Sec complex post-translationally. As soon as the OMPs are synthesized and emerged from the ribosomes, a group of proteins called trigger factors (TF) are attached to the signal sequence and prevent the cotranslational secretion of OMPs (Hoffmann et al., 2010). Then SecB, which is a chaperon and a member of the Sec machinery, binds to the OMPs and directs them to the Sec complex in the inner membrane (Bechtluft et al., 2010). Consequently they are transported into the periplasm by the SecYEG complex, which is a Translocon complex in the inner membrane (von Heijne, 2011; Hagan et al., 2011). When these proteins are in the periplasm, they are supported by chaperons such as Skp and SurA and delivered to the Bam complex in the outer membrane. Although translocation of the inner membrane proteins is carried out using the energy produced in ribosomes during protein synthesis, the translocation of OMPs requires extra energy that is supplied by SecA, an ATPase (Zimmer et al., 2008).

Translocation of β-barrel proteins through the inner membrane is much faster than other proteins, since β-barrel proteins have lower hydrophobicity, and they are translocated post-translationally (Duong et al., 1998). When β-barrel proteins are
being translocated, they are unfolded therefore they have a very high propensity to aggregate. For this reason chaperons are utilized in the periplasm to prevent aggregation and misfolding of these proteins. There is no ATP outside the inner membrane, so the procedure must be carried out without any energy consumption. SurA is a major periplasmic chaperon that transports the bulk mass of OMPs to the Bam complex, and it has been shown that in the absence of SurA this responsibility is shouldered by Skp and DegP (Sklar et al., 2007). Skp and DegP also take care of the peptides that were not able to enter the major pathway of SurA (Sklar et al., 2007). One interesting fact about SurA is the structural similarity of its core domain to the C-terminal of trigger factor (TF), and the fact that both can recognize unfolded peptides and prevent their misfolding or aggregation (Bitto et al., 2002).

The Bam complex is the last stop for the OMPs before entering the outer membrane. It is composed of five major components, BamA, BamB, BamC, BamD, and BamE. BamA and BamD are the two most essential components of the complex (Malinverni et al 2011; Malinverni et al., 2006). BamA is an integral β-barrel protein with a massive periplasmic domain; on the other hand, BamD is a lipoprotein with unknown structure. However, it is predicted that BamD has five or six tetratricopeptide repeats and as it has been recently discovered, these tetratricotides have a key role in protein-protein interactions. It has recently been shown that BamD mediates BamA’s interactions with BamC and BamE (Kim et al., 2007). BamA is the central player in OMPs folding and assembly that does most of the job with the help of polypeptide transport-associated domains (POTRA), which are the periplasmic appendages of BamA. An unfolded peptide interacts with the POTRA domains by β-strands augmentation (Hagan et al., 2011). β-strands augmentation is a process, in which, one or more β-strands of an unfolded peptide interacts with one or more of the POTRA domains β-sheets (von Heijne, 2011). It has been suggested that the binding of the unfolded peptide to the POTRA domain will initiate the formation of β-strands by providing parts of the entropic cost of β-strands formation; or consecutive strands could be brought together to form β-hairpins or match the first and last β-strands of the barrel to facilitate the cylinder formation of the barrel. This β-strands augmentation is very compatible with the hairpin-wise mechanical unfolding and refolding patterns of β-barrel proteins, in this case OmpG and VDAC (Damaghi et al., 2011; Gatzeva-Topalova et al., 2008).
Figure 1.2 OMP biogenesis and assembly in Gram-negative bacteria: All proteins whose destination is one of the two membranes of gram-negative bacteria are synthesized in ribosomes. Inner membrane proteins are co-translationally transferred to the Sec machinery and then inserted into the inner membrane. OMPs and also periplasmic proteins get translocated posttranslationally. β-barrel proteins of the outer membrane are transited through the periplasm with the help of chaperones (majorly SurA) in an unfolded conformation. Then they get folded in the BAM complex and inserted to the outer membrane. The Bam complex has five different components, in which Bam A and BamD are the two essential ones. BamA is a β-barrel protein itself with a periplasmic appendage. The five green circles in the figure that are attached to the BamA barrel are named POTRA domains. BamD is a lipoprotein with unknown structure, however, it is predicted to have five or six tetratricopeptide repeats and these repeats usually mediate protein-protein interactions. Recently it has been shown that BamD plays a major role in keeping the total stability of the complex by facilitating the protein–protein interactions among the other components of the BAM complex (Anwari et al., 2010; Gatos et al., 2008).
In short words, the β-barrel assembly in the Bam complex is a multi-step process. These steps are binding to the unfolded peptide, folding and then inserting the peptide into the outer membrane. BamA and BamD serve in folding and insertion. BamB, -C, and -E presumably may modulate the activity of other proteins as well as some regulatory roles (Hagan et al., 2011).

### 1.3.2.2 Mitochondria and Chloroplast β-barrel membrane proteins

In gram-negative bacteria membrane proteins are synthesized in the cytoplasm and transferred to the membranes; while mitochondria and chloroplasts import 90-99% of their proteins from the cell cytosol in the opposite direction of bacteria. Nonetheless, outer membrane biogenesis of mitochondria and chloroplasts resembles very much that of bacteria (Zeth, 2010).

In mitochondria, nascent polypeptides of OMPs, after being synthesized in cytosolic ribosomes, are imported through the outer membrane into the inner membrane space (IMS) via the TOM complex (Endo et al., 2010). In the IMS, the nascent polypeptide is escorted by TIM complex to the sorting and assembly machinery (SAM) complex in which it is folded and inserted into the outer membrane of mitochondria (Paschen et al., 2005).

In chloroplasts, like mitochondria, the nascent polypeptide after being synthesized in the cytosol is transported to the inner membrane space via the translocon of the outer chloroplast (TOC) membrane complex. Seemingly TOC plays the same role in chloroplast as TOM does in mitochondria. However, there is some doubt about the insertion mechanism of proteins into the outer membrane of chloroplasts. Toc75-V, which is an essential β-barrel protein in the outer membrane of chloroplasts, might be...
involved in the folding and insertion of the proteins into the outer membrane, the same role as SAM complex plays in mitochondria (Figure 1.3) (Schleiff et al., 2005). In comparison to the bacterial and mitochondrial outer membrane proteins, much less is known about the chloroplasts OM proteins (Figure 1.3).

1.4 MEMBRANE PROTEINS FOLDING

In 1961, Anfinsen showed the spontaneous formation of disulfide bonds in ribonuclease A (RNaseA) and proper folding of proteins after being unfolded by denaturant (Anfinsen and Haber, 1961). The importance of this finding was the fact that the hidden code of protein folding is in its amino acid sequence. Since then, the question has been how a chain of amino acids folds properly and so accurately to acquire the conformation of the native and biologically active protein from its denatured state. Besides, this has been a goal of bioinformatic studies to predict the three dimensional structure of a protein from its primary sequence.

Thereafter in 1968, Levinthal proposed the idea that it is impossible for a protein to fold into its native state by randomly checking all of the pathways. The Levinthal paradox expresses that if a polypeptide were to fold by random search of all possible intermediate conformations, the folding process of one small protein would take longer than the age of the universe (Levinthal, 1968). Hence, his suggestion for the folding process was a set of defined pathways that a protein would follow under kinetic control.

The ‘Ensemble view’ of protein folding has partly solved the Levinthal’s paradox. In the 1990s, Ken A. Dill introduced an intriguing theory describing protein folding and dynamics in the concept of energy landscape and folding funnels (Dill, 1985; Dill, 1997; Dill, 1999). On a three dimensional energy landscape, each point correlates the free energy of a conformation to its entropy. Since the folded native state is the most stable conformation, the free energy is minimized during folding such that the final structure corresponds to the deepest valley of the funnel-shaped energy landscape (Figure 1.4).

In addition, the energy landscape of the protein folding can provide a framework to connect the thermodynamics and kinetics of the folding process. Although some models such as the hydrophobic collapse, the framework model and the nucleation-condensation have been proposed to describe folding of globular proteins, there is no universal agreement on any one of these models (Daggett and Fersht, 2003a; Daggett and Fersht, 2003b).
Figure 1.4 From the Levinthal hypothesis to pathways to the funnel shaped energy landscape: a) Levinthal argued that randomly finding the native folding conformation for a protein resembles finding a needle in a haystack. b) Levinthal’s argument led to the arising of the folding intermediates and folding pathways. c) The new ensemble model puts more emphasis on multiple folding routes and much less on specific pathways. These figures are adopted from the exceptional review of Prof. Ken A. Dill (Dill and Chan, 1997).

1.4.1 Models for α-helical membrane protein folding

In 1989, Jacobs and White proposed their thermodynamic model for protein folding. This model is called the three-step model and is based on the so-called helical hairpin insertion model previously proposed by Engelmen and Steitz. This model is built on thermodynamic measurements of small hydrophobic peptides partitioning on the membrane (Engelman and Steitz, 1981; Jacobs and White, 1989). The three steps of this thermodynamic model for protein folding are: interfacial partitioning, interfacial folding, and insertion. Meanwhile Popot and Engelman proposed the ‘two-stage hypothesis’ for the folding mechanism of α-helical proteins (Popot et al., 1987; Popot and Engelman, 1990). In their model the process of membrane protein folding happens in two simple stages: insertion and folding (Figure 1.5). In the first step, the transmembrane segments, after insertion into the lipid bilayer, form independent stable α-helices across the membrane lipid bilayer. In the second step, the tertiary and quaternary structure of the protein is completed inside the bilayer by establishing interactions between helices and the helices and the membrane. This was one of the primary and simplified models of a very elaborate mechanism.

Later, Popot et al., while studying ligand binding on Bacteriorhodopsin (BR) discovered the third stage of the folding process (Popot et al., 1987). They showed that during the third stage, higher order structures or conformations facilitate partitioning of regions other than the helix, such as coil regions, loops, or prosthetic groups into the lipid membrane. Consequently, these additional polypeptide
structures, via adopting their native conformation, may pull the secondary structures of the protein and helices into the native functional structure. In 1999, White and Wimley, using synthetic peptides and small proteins in systematic biophysical measurements, proposed a four-step thermodynamic model for membrane protein folding mechanism (White and Wimley, 1999). In this model, the four steps include: membrane partitioning, helix formation, insertion into the membrane, and association (Figure 1.5). The other important implications of this model are: (i) contrary to the two and three step models, in this model the folding and membrane association of helices can happen either in the interface or the aqueous environment, and the final process even can be a combination of them, (ii) in this model all the folding processes are spontaneous, and after each step there is a decrease in free energy of the whole system, which includes the polypeptide, solvent, and lipid bilayer, and (iii) this free energy minimization is because of the hydrophobic effect (burying polar groups and especially the peptide bond), and this hydrophobic effect is the driving force that also maintains the secondary structure in the folded protein (White and Wimley, 1999).

After proposing this model, White and Wimley attempted to measure transfer energy of individual amino acids from water to the lipid bilayer models such as carbon tetrachloride, octanol, or 1-palmitoyl 2-oleoyl phosphatidylcholine lipids to make a hydrophobicity scale that describes propensity of different amino acids in membrane insertion and membrane protein folding (White and Wimley, 1996; Wimley et al., 1999).

This hydrophobic scale beautifully explains the energy contribution of each single residue of 20 different amino acids in a polypeptide integrating into the lipid bilayer. For example, isoleucine residues have the highest hydrophobicity ($\Delta G = -0.6$ kcal/mol) and aspartates have the lowest ($\Delta G = +3.5$ kcal/mol). As another example, transferring a glycine residue, which is more favorable than aspartate, costs free energy ($\Delta G = 1.25$ kcal/mol). Let’s clarify it with a different example:

transferring a polypeptide with 20 polyalanine residues costs a $\Delta G$ of 10 kcal/mol, which suggests the segment is not hydrophobic enough to partition into the model membrane spontaneously. But if we replace five of the alanines with five leucines, the insertion will become favorable with a $\Delta G$ of -1.25 kcal/mol (Liu et al., 1996a). These

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2 The hydrophobic effect here is described as burying polar groups and specially peptide bonds inside the polypeptide.
measurements have been performed on individual transmembrane α-helices (20 amino acids makes a helix in the membrane) and the interesting result is that the range of energy transfer for all different combinations varies between 10 to 20 kcal/mol, which is a typical stabilization energy for small soluble proteins, and indicates the reverse process of polar groups burying energy release.

In 1982, Engelman and Steitz proposed that the helical pair or helix hairpin could also play a role as folding elements (Steitz and Engelman, 1981). This was because of the existence of hydrophobically unfavorable helices due to having polar and charged residues coupled with the highly favorable ones. Therefore they proposed that the high hydrophobicity of neighboring helices could compensate for partitioning of those polar or charged residues and consequently the two helices or helical hairpins can be inserted as one folding unit. Later on in vitro and in vivo studies proved this hypothesis (Kuhn, 1987; Hessa et al., 2005b). Molecular dynamic simulation studies on helical pairs also validated them as a folding unit (Khutorsky, 2003).

The real scenario for α-helical protein folding in the cell is much more complicated and involves numerous molecular complexes and machineries. Briefly, the nascent chains coming out of the ribosomes is directly inserted into the membrane translocon, in which the proteins are folded, assembled, and released into the lipid membrane (Kedrov et al., 2011). After the process is completed, the ribosome-translocon complex leaves the folded protein inside the membrane.

Von Heijne and colleagues through an elegant work discovered most of the insertion codes and mechanisms happening in the translocon (Hessa et al., 2005a; Hessa et al., 2005b). They substitute different amino acids of a polypeptide segment to measure the free energy of partitioning for each of the 20 amino acids. Their results showed that the insertion probability not only depends on the residual composition of the peptide, but also depends on the position of each residue in the polypeptide segments.

1.4.2 Models for β-barrel membrane protein folding

β-barrel membrane proteins differ from α-helical proteins in primary sequence code, secondary structure elements, and the lipid environment. The membrane spanning sequences of β-barrel membrane proteins have less hydrophobicity than α-helical membrane proteins. Accordingly, it makes sense to expect membrane insertion, assembly, and folding mechanisms to be different for these two different groups of membrane proteins. Recent discoveries in fact proved this difference (Tamm et al.,
Generally, β-barrel membrane proteins feature tight turns on the periplasmic side and large, flexible loops on the extra cellular side of the lipid membrane. The lipid bilayer face of the barrels is mostly composed of hydrophobic residues, and the residues facing the lumen of the barrel are polar. Therefore, like α-helical membrane proteins, the general distribution of residues in β-barrel membrane proteins is the opposite of soluble proteins. There are many glycines and aromatic residues such as Trp and Tyr in the transmembrane strands of β-barrel membrane proteins. These residues are normally located in two rings contacting the lipid bilayer interfaces at both ends of the barrel (Tamm et al., 2004). Loops have the broadest range of sequence variability and carry most of the functional characteristics of the β-barrel membrane proteins.

The first model for β-barrel membrane proteins proposed by Tamm and colleagues after kinetics studies on OmpA describes a simple two step process with some intermediates. Using time-resolved Trp fluorescence quenching (TDFQ)\(^3\), and design of OmpA mutants with one Trp at the beginning of each outer loop of all four β-hairpins, they measured the membrane translocation rate of β-hairpins. They found that all four β-hairpins of OmpA crossed the membrane in the same time course. Therefore, they concluded that all four β-hairpins of OmpA insert and fold into the lipid membrane in a concerted manner. In conjunction with kinetics study results, these data led them to the idea that the insertion and folding of the barrel happens simultaneously in β-barrel membrane proteins (Tamm et al., 2001). This mechanism contrasted with the two-stage mechanism proposed for α-helical membrane proteins that suggested the separate folding and insertion of α-helices into the membrane (Popot and Engelman, 1990; Tamm et al., 2001).

However, as mentioned above, according to distinct amino acid frequencies for α-helices and β-sheets, it would be reasonable to expect different rules to govern the insertion of α-helical and β-barrel membrane proteins. Furthermore, α-helices and β-barrel membrane proteins utilize different ‘snorkeling’\(^4\) for amino acids such as lysine or arginine (Chamberlain et al., 2004a; Chamberlain et al., 2004b).

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\(^3\) The time-resolved Trp fluorescence quenching technique enables a direct observation of tryptophan residues translocation across the lipid bilayer. Briefly, Trp fluorescence quenchers are placed in the membrane at different depths, and the time course of the Trp residues passing through these zones of quenchers are followed.

\(^4\) ‘snorkel’ means allowing the amino acid residues to locate its polar moiety in the aqueous/head group region while burying the aliphatic part of the side chain in the hydrophobic site of the lipid bilayer.
Figure 1.5 Membrane protein folding models. **Top-left:** The two-stage model that later developed to the three-stage model that accounts for folding of extra membrane domains, interactions with cofactors and oligomerization (Popot and Engelman, 1990). **Bottom-left:** Four-stage model based on thermodynamics and kinetic studies (Wimley and White, 1999). **Top-right:** Simultaneous insertion and folding process proposed by Hang and Tamm (Tamm et al., 2004). **Bottom-right:** Tilted insertion model (Huysmans et al., 2010).
Recently, a study on PagP, which is a monomeric eight-stranded β-barrel membrane protein in E. coli and has a role in the bacterial stress response mechanism, elaborated the two-stage model for β-barrel membrane proteins (Huysmans et al., 2010). It has been shown that the folding of the PagP β-barrel goes through a transition state, which is a formation of an extracellular half β-barrel in the membrane interface. Based on this finding, a new model known as tilted insertion model was proposed. A similar tilted insertion mechanism has been proposed by simulation studies on OmpA (figure 1.5) (Bond and Sansom, 2006).

1.5. GATING STUDY OF MEMBRANE PROTEINS

Recently, while looking for more suitable proteins as a main channel of biosensors constructions, outer membrane proteins attracts interests due to their robust structure against harsh environment as well as their simple structure for genetic engineering. However, the main challenge has been the mechanism of their pore gating to be able to control it to the favourable state. There have been a lot of different studies focused on Omps gating mechanism; nevertheless, the details of theses proteins gating mechanism remain to be explained more accurately (Todt et al., 1992).

Some of the transmembrane pores formed by Omps in E. coli are pH gated and low pH, which is toxic for the microrganism, induces the closing of the channels of, for example, OmpC, OmpF, LamB and PhoE (Conlan et al., 2000). Which conformational changes and what interactions cause channel closure has been different and debated. In 1999, AFM it has been shown for the first time that the large extracellular loops of OmpF collapses onto the channel entrance and closes it at low pH (Muller and Engel, 1999). This finding supported the theory that conformational changes of the flexible loops could control gating of Omp channels. Later on, more experiments on LamB, an E. coli β-barrel maltoporin that is specific for maltooligosaccharides uptake, corroborated the flexible loop controlled gating model. LamB proteins, which are lacking the major extracellular loops L4 and L6, failed to close at acidic pH (Andersen et al., 2002). However, these insights need to be further substantiated. Furthermore, it remains to be shown whether conformational changes of flexible extracellular loops are a common gating mechanism of Omps (Todt et al., 1992).
Chapter 2

ATOMIC FORCE MICROSCOPY

2.1 ATOMIC FORCE MICROSCOPE

2.1.1 History
Scanning probe microscopy (SPM) started when in the early 1980s Binnig and Rohrer invented the scanning tunneling microscope (STM) at IBM research in Zurich, Switzerland (Binnig et al., 1982). They won the Nobel Prize in Physics in 1986 for this fabulous innovation. In the same year Binnig together with Quate and Gerber built a new SPM method that could image both conducting and non-conducting samples. In this SPM method, the interaction between a very sharp tip and the sample surface is exploited to make topographic images. The name of this newborn SPM is Atomic Force Microscopy (AFM), which also is known as Scanning Force Microscopy (SFM). The AFM is a very high-resolution type of SPMs with practically exhibited resolution of less than a nanometer (Binnig et al., 1986).

2.1.2 Principle
Simplicity is central to AFM. The main parts of the AFM are: a cantilever with a sharp tip, a piezoelectric transducer, a head in which a laser diode and a quadrant photodetector are located, and a computer for control and visualization (Figure 2.1). For working with biological samples, a glass block (also known as a fluid cell) is required to mount the cantilever and keep the buffer fresh.

The sample is placed on freshly cleaved mica, properly cleaned gold, or glass glued onto a piece of Teflon with a steel disc underneath, which will be mounted on top of the piezoelectric transducer. The piezoelectric transducer is the motor of the machine. The ceramic elements of the piezoelectric actuator is voltage driven and can move with sub-nanometer accuracy in the $x$, $y$ and $z$ directions.

While doing imaging or force spectroscopy, the cantilever senses the interactions between the tip and the surface. Forces produced by these interactions deflect the cantilever. Bending the cantilever changes the angle of an aligned laser beam and
consequently the position of the laser spot on the position-sensitive photo detector (PSPD). The red light-emitting laser diode ($\lambda = 625$ nm) and a four quadrant PSPD are located inside of the AFM head (Meyer et al., 1988). The laser beam that is focused onto the end of the cantilever is reflected onto a mirror before going to the PSPD. The mirror is also placed inside the head and can be used for laser alignment. The position alteration of the laser spot on the PSPD results in a change of the deflection signal. The deflection signal is kept constant with the help of a controller that is fed into a feedback loop to control the piezoelectric actuator movement. Then the cantilever deflection is amplified by an optical beam deflection detection system and PSPD provides a voltage signal proportional to the cantilever deflection. This voltage signal will be converted into absolute deflection in nanometer scale, which can be subsequently converted into force (N) using Hook’s law.

![Figure 2.1 Setup of an AFM instrument: Different parts and the working mechanism of an AFM are shown schematically. A laser beam is manually adjusted on the cantilever when setting up to obtain a maximum sum signal on the photodiode. Scanning the sample deflects the cantilever and results in conversion of the voltage signal to force (pN) or deflection (nm) by the software. The output signal shown in the figure is for contact mode.](image)

2.1.3 The Cantilever

The cantilever can be described as the figurative finger of an AFM, which with a tip

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5 This is actually the mechanism in contact mode AFM that will be described in more detail in the following part.
mounted at its end is able to touch the sample surface. Cantilevers are micro-fabricated from silicon, silicon-nitride or diamond. Lithographic photo-masking, dry and wet etching and vapor deposition are some of the techniques used to fabricate cantilevers. Cantilevers are either triangular (V shaped), or rectangular (| shaped) and are 100-200 µm long and 0.5-2 µm thick. They are coated with a thin layer of gold and aluminum for beam deflection instruments, and with ferromagnetic materials for magnetic measurements (http://www.veeco.com).

The V shaped cantilevers are mostly used for imaging because they exhibit less torsional motion or twisting than rectangular cantilevers while scanning. On the other hand, rectangular cantilevers are mostly used for frictional measurements such as protein unfolding studies because of their massive degree of rotational freedom that makes them sensitive to lateral forces too.

Normally, cantilevers have silicon or silicon-nitride tips. Silicon-nitride tipped cantilevers are most commonly used in force spectroscopy studies. A silicon-nitride tip is covered by a non-conducting layer of native oxide and usually has a conical or pyramidal shape. Dimensions of the tips range from 2-100 µm in height and 2-60 nm in radius. However, tips can be coated with a thin layer of different precious metals such as gold, silver, and platinum for special goals. Moreover, tips can be modified by adding desired functional groups chemically (i.e. chemical modification) for some special interaction studies.

Regardless of the shape, cantilevers can be considered as a Hookean spring. Thus, the applied or experienced force, F, would be proportional to the vertical deflection, \( \Delta x \), of the cantilever and be measurable by Hook’s law:

\[
F = -\kappa \Delta x
\]

Where \( \kappa \) is the cantilever spring constant and \( \Delta x \) is its vertical displacement. The cantilever spring constant depends on its shape and material. For different applications, cantilevers with different spring constants are used. For example, for contact mode imaging of biological samples, it is better to use cantilevers with spring constants ranging from 60-100 pN/nm while for force spectroscopy softer cantilevers would be more suitable. Moreover, the resolution of an AFM is limited by the thermal noise of the cantilever, which is determined by its spring constant.
Chapter 2

There are some other characteristics such as resonance frequency, quality factor, and measurement bandwidth to be considered for proper cantilever selection in particular applications (Viani et al., 1999).

### 2.1.3.1 Cantilever calibration:

As mentioned above, the resolution of an AFM is directly dependent on thermal noise of the cantilever and its spring constant. Furthermore, to convert the deflection of the cantilever to the force in SMFS experiments, we need to determine the spring constant of the cantilever quite accurately.

**Cantilever spring constant calibration, added mass method:**

The added mass model for calculating the force constant of cantilevers, $\kappa$, for the first time introduced by Meyer et al. is as follows:

$$ k = \frac{Ewt^3}{4l^3} \quad (2.2) $$

Where $w$, $l$, and $t$ stand for the cantilever’s width, length, and thickness, respectively (Meyer et al., 2003). $E$ is the Young’s modulus of the material. $w$ and $l$ can be measured by the scanning electron microscope, and $t$ can be accurately measured from the resonance frequency of the cantilever using this equation.

$$ t = \frac{2\sqrt{12\pi}}{1.875^2} \sqrt{\frac{\rho}{E}} f_r l^2 \quad (2.3) $$

Where silicon $\rho$ is 2330 kg/m$^3$ and $E$ is 1.69 x 10$^{11}$ N/m$^2$ (Meyer et al., 2003).

Since the tips’ coatings and manufacturer variability could potentially affect the force constant, instead of determining the resonance frequency of the cantilever, changes in the resonance frequency are measured. This is possible by using the added mass method (Cleveland et al., 1993). In this method the cantilever is considered as a single harmonic oscillator and its resonance frequency can be measured by:

$$ \nu_0 = \frac{1}{2\pi} \sqrt{\frac{\kappa}{m}} \quad (2.4) $$

Where $m$ is the mass of the cantilever and $\kappa$ is the cantilever force constant. The resonance frequency of the cantilever is measured after the mass was added and
the force constant of the cantilever is measured by the following equation:

\[
\kappa = 4\pi^2 \frac{m^*}{\nu_1^2 - \nu_0^2}
\]  

(2.5)

Usually, a small tungsten sphere is used as added mass. An optical microscope is used to measure its diameter and calculate its volume. Knowing tungsten’s density, one can calculate the mass.

**Cantilever spring constant calibration, Thermal Fluctuation Analysis:**

This method of calibration is the most convenient and often used for measuring the spring constant. In this model the cantilever is considered as a single harmonic oscillator, and the thermal motion of the first elastic mode is correlated to its thermal energy

\[
\frac{1}{2} k_B T = \frac{1}{2} \kappa \langle x^2 \rangle
\]

(2.6)

The power spectral density (PSD) of the cantilever’s thermal fluctuations has a peak at the cantilever's resonance frequency (Figure 2.2). The area below the PSD curve is equal to \( \langle x^2 \rangle \).

In our lab (and for all experiments within this thesis), the thermal fluctuations method is used for force constant calibration. Practically, the cantilever is retracted from the surface for several microns and the resonance frequency is monitored for three to five seconds. Then, the computer, from the thermal oscillation of the cantilever, calculates the PSD of the cantilever. The PSD shows a peak at the cantilever's resonance frequency. This calibration has been applied before and after all the SMFS experiments.

**2.1.4 AFM modes**

**Contact or dc mode**

In contact mode AFM, the cantilever's tip scans the sample surface while having soft physical contacts with it. The changes in the cantilever deflection during scanning are recorded to make topographic images of the sample. During raster-scanning, a low and non-destructive force of <100 pN is normally applied. A feedback loop maintains
the interaction force while imaging; meaning if the cantilever bends because of the sample roughness, then the feedback loop will adjust the position of the piezoelectric to the original set point.

In contact mode, data is generated in either constant-height or constant-force mode. In constant-height mode, the scanner’s height is kept constant during scanning and the spatial difference of the cantilever deflection is used to generate topographic images. A few examples for applications of this mode are in immobilized samples such as membrane proteins, real-time imaging of dynamic surfaces, and atomic scale images of very flat surfaces. In constant-force mode, the applied force on the sample stays constant by moving the scanner up and down while scanning the surface.

![AFM modes diagram](image)

**Figure 2.2 AFM modes:** a) In contact mode, the cantilever is quasi-static and the system gets the topography of the surface by maintaining the cantilever deflection at the original set point. b) In tapping mode, the cantilever is oscillated at a constant frequency and amplitude. Oscillation amplitude works as the set point and feedback, which should be constant at all times. Therefore, if a decrease in amplitude is detected it is compensated by retraction of the piezo-electric actuator. Additionally, the mechanical properties of the sample are obtained from the phase shift of drive signal and cantilever oscillation (green and blue sine waves on top of part b).

**TappingMode (TM) AFM**

Tapping mode or dynamic mode AFM imaging was developed at the beginning of the 1990s to overcome the problem of destroying soft samples and also to minimize the lateral forces while using contact mode AFM (Karrasch et al., 1993; Hansma et al., 1994). In tapping mode, the cantilever oscillates acoustically, and the tip touches the surface once in any oscillation cycle. Amplitude-modulated tapping mode is the most used tapping mode technique in biology. In this mode, the cantilever gets excited close to its resonance frequency, and then a PSPD detects the RMS amplitude of the oscillation. The problem with tapping mode images is the low resolution compared to contact mode. Also, the height information acquired in tapping mode is not as accurate as in contact mode. However, it is a good choice for imaging of soft and
fragile samples (Fritz et al., 1995; Guthold et al., 1999; Muller et al., 1999).

2.2 SINGLE-MOLECULE FORCE SPECTROSCOPY

AFM based SMFS has made it possible to measure pico-Newton forces at the single molecule level and at Angstrom resolution (Clausen-Schaumann et al., 2000; Fisher et al., 2000). It has given fundamental insights into the molecular basis of some biological events such as molecular interactions and recognition (Hinterdorfer et al., 1996; Lee et al., 1994), protein folding and unfolding (Kedrov et al., 1998; Rief et al., 2004), DNA mechanics (Rief et al., 1999), as well as cell adhesion and mechanics (Krieg et al., 2010).

Generally, in an SMFS experiment, individual molecules are pulled apart under force to study their structure, dynamics, interactions and mechanical properties (Cui and Bustamante, 2000; Merkel et al., 1999; Oesterhelt et al., 2000). Particularly in AFM based SMFS, the target molecule is attached to the tip of the cantilever by pushing the tip into the molecule and pulling it apart by retracting the cantilever away at a certain velocity (Binnig et al., 1986). This pulling exerts mechanical stress on the protein that eventually unfolds the molecule. The unfolding of the molecule is sensed by the cantilever deflection and results in so-called force-distance (F-D) curves.

F-D curves are obtained from scoring the cantilever deflection (d) as a function of vertical displacement of the piezoelectric (z) which yields a raw ‘voltage-displacement’ curve that finally is converted into a ‘force-distant’ curve using two conversions; i) conversion of voltage to cantilever deflection using the sensitivity of the AFM detector that is the slope of the retraction curve in contact region (Figure 2.3), and ii) conversion of the cantilever deflection to force by using Hooke’s law.

In a typical F-D curve, the distance (D) between distinct peaks represents unfolding of a folded structure inside the macromolecule e.g. protein or DNA and force (F) is a measure of the force required to break the interactions and unfold that structure. Therefore, both the amplitude of the force peak and the distance from one force peak to the next one specifies an unfolding event. The collection of all these unfolding events (peaks) in one F-D curve makes the unfolding pattern of the molecule that is very specific like a ‘fingerprint’ for that molecule. These mechanical ‘fingerprints’ differ in versatile situations and conditions that give different conformations to a molecule. Consequently, different fingerprints reveal different populations of unfolding events and unfolding patterns that can represent new interactions and/or new conformations in the molecule.
Chapter 2

Figure 2.3 A typical force-distance curve: Schematic representation of an F-D curve showing steps of the interactions between the tip and the surface during the vertical movement of the piezoelectric. The slope of the region between (C) and (D) is used to determine the deflection of the cantilever (nm) with the known spring constant.

SMFS has been used to characterize the mechanical properties of many biomolecules such as titin (Marszalek et al., 1999; Rief et al., 1997; Rief et al., 1998), FN-III domains of tenascin (Oberhauser et al., 1998), FN-III (of fibronectin) (Oberhauser et al., 2002), polysaccharides (Marszalek et al., 2002), how strong transmembrane α-helices integrate in lipid bilayers (Ganchev et al., 2004), viscoelastic properties of a single polysaccharide (Kawakami et al., 2004), the molecular spring made of Ig and fibronectin type III (FN-III) domains – myomesin (Schoenauer et al., 2005), the nanospring behavior of multidomain protein ankyrin (Lee et al., 2006), the ubiquitous collagen (Gutsmann et al., 2004), spider silk (Oroudjev et al., 2002), and even a non-mechanical protein barnase (Best et al., 2001). It has also been used for determining misfolding of single proteins (Oberhauser et al., 1999), the unfolding kinetics of ubiquitin (Schlierf et al., 2004), mapping the energy landscape of GFP (Dietz and Rief, 2004), energy landscape roughness study of proteins (Nevo et al., 2005), folding pathway of fast-folding Ig domain (Schwaiger et al., 2005), and protein molecules (Janovjak et al., 2005). It even has been applied to determine protein structure using a method termed ‘mechanical triangulation’ (Dietz and Rief, 2006) and many other surveys, which needs a book to count. Amongst all of these applications, the most important study has been done on ubiquitin refolding by Fernandez and colleagues (Fernandez and Li, 2004). Their work initiated the application of AFM in protein refolding studies.
SMFS has been becoming a powerful technique to study membrane proteins, and it has been used in many studies such as unraveling the mechanical stability of proteins and their structural segments inside the protein (Oesterhelt et al., Kedrov et al., 2006), unfolding pathways of different proteins and their relative likelihood (Bertz and Rief, 2008), and ligand and inhibitor binding studies (Cao et al., 2007). SMFS has also been applied to study the refolding mechanism and misfolding of α-helical membrane proteins (Kedrov et al., 2006).

2.2.1 Dynamic Force Spectroscopy, (Dynamic SMFS)

AFM-SMFS unfolding experiments measure the force that breaks the interactions stabilizing a protein’s structural segments. It was previously mentioned that when a molecule is unfolded by a cantilever withdrawal in AFM, it would result in F-D curves. F-D curves define the force and the length of structural elements of the unfolded protein and reveal the protein’s mechanical unfolding intermediates and subsequently its mechanical unfolding pathway.

To understand the forced mechanical unfolding event in an F-D curve, a classical two state model is considered. An energy profile diagram of a two-state reaction is shown in Figure 2.4. It consists of a low-energy folded state, which is stable, and a high energy unfolded state, as well as a transitional state. The transition state separates the folded and unfolded states and needs to be overcome in the unfolding procedure (Evans and Ritchie, 1997). The unfolding rate, \( k_u \), can be calculated by this equation:

\[
k_u = \left( \frac{1}{t_D} \right) \exp \left( - \frac{\Delta G_u^*}{k_B T} \right)
\]

\[
\Delta G_u^* = G^* - G_n
\]

where \( G^* \) is the energy of the transition state and \( t_n \) is the relaxation time of the transition state.

In equilibrium conditions, molecules continuously bond and unbind under zero force. When exposed to an external force, these reacting molecules reduce the ratio of bound to free components (unbound ones). On the other hand, at infinite dilution, which is the case in single-molecule experiments, an isolated single bond exists far from equilibrium and only has non-zero strength on time scales shorter than its lifetime. Therefore, the bond will dissociate under any force loaded for adequate periods of time. Thus, when the bond is loaded at a high rate, it would experience shorter
lifetime, and when loaded at a low rates it experiences a longer lifetime. In other words, higher loading rates lead to higher detachment forces, and lower loading rates lead to lower breaking forces. It also means slow pulling velocities result in long lifetimes but low rupture forces, and high pulling speed reveal high rupture forces but short lifetimes (Evans and Ritchie, 1997; Evans, 1998; Evans, 2001).

Figure 2.4 Conceptual unfolding energy barrier tilted by an externally applied force according to the Bell-Evans theory: a) The potential along the reaction coordinate (vector of externally applied force) in the absence of force (black curve) exhibits one energy barrier that separates the folded state from the unfolded state. A folded protein is confined in a potential well by the energy barrier, $\Delta G^*_u$, that unfolds with an unfolding rate of $k_u$. $ts^*$ is the transition state and $x_u$ is a distance of transition state from the folded state. Application of an external force, $F$, changes the thermal likelihood of reaching the top of the energy barrier. It is assumed that although for a sharp barrier the distance, $x_u$, of the energy barrier relative to the folded state is not changed, the thermally averaged projection of the energy profile along the pulling direction is tilted by the mechanical energy $-(F \cos \theta)x$ (red line). This tilt decreases the energy barrier. At low loading rates, the thermal contribution to overcome the energy barrier is higher, and therefore, the mechanical energy required to overcome the barrier is smaller. b) With increasing loading rates, the mechanical force increases owing to a reduced lifetime of the folded state (Evans, 2000).

Applying an external force, $F$, with the angle of, $\theta$, relative to the microscopic reaction coordinate, $x$, adds a mechanical potential, $-(F \cos \theta)x$, which tilts the energy landscape, $E(x)$, along this coordinate and will lower the energy barrier, $G^*$, at the transition state (Figure 2.4).

In short, in a reaction under force, the force lowers the energy barrier in the energy landscape (Figure 2.4). Even though this force can change the position and the shape of the barrier, it cannot change the shape and the location of the transition state for a sharp energy barrier (Evans 1998).
2.3 What is the advantage of using atomic force microscopy in membrane protein studies?

AFM is the only technique that does not require any crystallization, freezing or staining of the samples. Indeed, AFM images the topography of the sample down to the sub-nanometer resolution in a physiological buffer environment and a temperature close to native conditions. Outstanding signal-to-noise ratio is another advantage of AFM that provides very useful information that cannot be achieved by other techniques. Conventionally, protein stability is studied using heat or denaturants like urea, which place proteins in conformations that are far from their native conformation. Furthermore, it is very difficult to carry out this technique on membrane proteins because of the lipid bilayer and their biphasic environment. In contrast, SMFS unfolds membrane proteins from their folded to a stretched unfolded conformation that lacks almost all secondary structures. Another advantage of SMFS is the ability of this method to provide rare data on single molecule unfolding intermediates that are missed in ensemble studies due to averaging of the interactions.
Chapter 3

FOLDING MECHANISM OF OmpG

3.1 UNFOLDING PATTERN: ONE β-HAIRPIN AFTER THE OTHER

3.1.1 Outer Membrane Protein G (OmpG)

The outer membrane of gram-negative bacteria is a selective permeable barrier that controls exchange of solutes and nutrients such as sugars, nucleotides, and amino acids across the membrane (Benz et al, 1978; Nikaido, 2003). This selective permeability is because of the existence of channel-forming integral membrane proteins known as outer-membrane porins that function as molecular filters and facilitate uptake of substances from the environment to the periplasm. All outer-membrane proteins known to date are transmembrane β-barrels proteins with 4-12 β-hairpins, and all outer membrane porins are β-barrels with 16 or 18 strands constructing a pore in the membrane (yildiz et al., 2006). Porins conform to a common pattern where three monomers associate into a trimer, which is their oligomeric state in 2D and 3D crystals and their functional state in lipid membranes (Jap, 1989; Sass et al, 1989), (Benz et al, 1978). The other β-barrel membrane proteins don’t have this pattern.

In E. coli, sugar uptake is normally carried on by LamB (Szmelcman et al, 1976) and ScrY (Schmid et al, 1991) as main porins. However, in mutants where LamB is either nonfunctional or deleted, the sugar uptake is managed by OmpG (Misra and Benson, 1989). The OmpG gene encodes a polypeptide with 301 amino acids, of which 21 amino acids at its N-terminus is a signal sequence and is cleaved after reaching to the

---

6 β-hairpin is an antiparallel pair of β-strands in β-barrel proteins.
outer membrane. Lack of long hydrophobic stretches and cysteine residues (Fajardo et al, 1998) are some special features of porins, which are present in OmpG too. It also contains a C-terminal phenylalanine, which can play a role in membrane insertion (Struyve et al, 1991).

Figure 3.1 Equipotential surfaces of OmpG with bound detergent molecules: Views of the open conformation, pH 7.5, in two different views inside the membrane (a, b) and top view from the periplasmic side (c). (d) Top view from the periplasmic side of the closed conformation, pH 5.6. LDAO detergent molecules (green molecules in a, b, and c). The B250A° 2 hydrophobic crystal contact surface between adjacent OmpG molecules is bare (b). In the closed conformation, low-pH, OmpG binds one molecule of OG and one glucose (yellow molecules in the pore), and 13 OG molecules on its outer hydrophobic surface (d). Surface areas comprising negative charges at neutral pH colored as red, positively charged areas are colored blue. Hydrophobic areas without charge are colourless. This figure is adopted from the brilliant work of Yildiz and colleagues on the crystal structure of OmpG (Yildiz et al., 2006).
Circular dichroism studies of OmpG in detergent demonstrated that, as other outer-membrane porins, OmpG consists of almost entirely of β-sheets with very little helix content (5 amino acids in loop 4 making a small helix). In the same study using proteoliposome-swelling assays, it has been shown that OmpG is a nonselective channel for mono-, di- and trisaccharides, with an unusual pore size (2 nm in diameter) (Fajardo et al, 1998). Cryo-electron microscopy of two-dimensional (2D) crystal proved the monomeric state of the barrel, with no evidence of oligomerization. Secondary structure predictions of OmpG estimated 16 β-strands (Fajardo et al, 1998), but later predictions (Conlan et al, 2000; Behlau et al, 2001) proposed 14 β-strands, which fit better with the projection maps.

Another study on chemical cross-linking and two-dimensional electrophoresis for the first time showed that OmpG is a monomer (Fajardo et al, 1998), in contrary to the other porins known at that time that were trimers (Nikaido, 2003).

Recently, the structure of OmpG from E. coli was solved in the open and closed state (Yildiz, et al., 2006). They showed that OmpG comprises fourteen β-strands that form a transmembrane β-barrel pore (Subbarao, et al., 2006; Yildiz, et al., 2006). Six short polypeptide turns on the periplasmic side and seven longer loops (L1-L7) on the extracellular side connect individual β-strands. OmpG is thought to be gated by its largest extracellular loop L6, which in a pH-dependent manner controls the flux of small molecules through the pore (Conlan et al., 2000). At acidic pH < 6 loop L6 folds into the pore constricting the channel entrance. Being able to withstand rather harsh environmental conditions, OmpG forms a robust pore, which makes it suitable for applications as a biosensor.

3.1.2 Mechanical Unfolding Pathways of the Membrane β-Barrel Protein OmpG

Single-molecule force spectroscopy (SMFS) is a unique approach to study the mechanical unfolding of proteins (Borgia et al., 2008; Kedrov et al., 2007). Such forced unfolding experiments yield insight into how interactions stabilize a protein and guide its unfolding pathways.

Previous SMFS work has probed the mechanical stability of water-soluble proteins composed of α-helices and β-strands. A prominent example of unfolding of a β-barrel structure is that of the green fluorescent protein (GFP) (Diez, et al., 2004), the stability of which plays a major role for its application as a marker in modern fluorescence microscopy. In contrast to the variety of water-soluble proteins
Chapter 3

characterized, only α-helical membrane proteins have been probed by SMFS. It was found that α-helical membrane proteins unfold via many intermediates, which is different to the mostly two-state unfolding process of water-soluble proteins. Upon mechanically pulling the peptide end of a membrane protein, single and grouped α–helices and polypeptide loops unfold in steps until the entire protein has unfolded. Whether the α-helices and loops unfold individually or cooperatively to form an unfolding intermediate depends on the interactions established within the membrane protein and with the environment (Kedrov et al., 2007).

Each of these unfolding events creates an unfolding intermediate with the sequence of intermediates describing the unfolding pathway taken. However, so far, β-barrel-forming membrane proteins have not been characterized by SMFS. For these reasons, we have characterized the interactions and unfolding of the β-barrel-forming outer-membrane protein OmpG from Escherichia coli by AFM based SMFS.

### 3.1.3 Material and methods:

**Protein expression and purification**

For 2D crystallization, the OmpG gene fragment without its signal sequence (amino acids 22–301) was cloned into the pET26b plasmid vector, resulting in an additional methionine at the N-terminus, and expressed in *E. coli* strain BL21 (DE3)-C41 grown in TB medium. The protein formed inclusion bodies, which were collected by low-speed centrifugation after breaking the cells in a cell disruptor (Constant Systems). The pellet was washed in buffer (25 mM Tris–HCl, pH 8) containing 1 M urea and 1% Triton-X 100, and the inclusion bodies were dissolved in 8 M urea in the same buffer. The solubilized protein was loaded onto an anion exchange column, and unfolded OmpG was eluted by a NaCl step gradient. Refolding of OmpG was achieved by dilution in 1% (wt/vol) *n*-octy-β-D-glucopyranoside (OG), at a final urea concentration of 3 M. Refolding was monitored by SDS–PAGE, taking advantage of the difference in apparent masses of refolded (28 kDa) and unfolded (36 kDa) OmpG (Conlan et al., 2000) Subsequent ion-exchange chromatography removed small amounts of remaining, unfolded or partially refolded OmpG and served to exchange the buffer. An additional gel filtration step on a Superdex-200 16/60 column was not critical for crystallization. The protein was concentrated to ~50 mg/ml by ultrafiltration (Centricon) or dialysis against buffer containing 20% PEG-35000 in dialysis tubes with a 12000 Da cutoff. Buffer was exchanged either by ultrafiltration
or dialysis. The desired final protein concentration was adjusted by adding an appropriate amount of buffer to the concentrated protein stock. The final yield was 20–30 mg of refolded OmpG per litre of culture. Protein purity was greater than 95%, as estimated by SDS–PAGE and Coomassie staining. Selenomethionine-substituted OmpG was expressed similarly in BL21 (DE3)-C41 cells in M9 minimal medium, suppressing methionine biosynthesis by a five-fold increase in the concentration of the amino acids Leu, Ile, Lys, Phe, Thr and Val. Precultures were grown overnight in LB medium, which was removed by gentle centrifugation prior to inoculation. Expression and purification was performed as described for the underivatized protein.

2D crystallization

2D crystals of refolded OmpG were obtained by detergent dialysis (Kuhlbrandt et al., 1993) in the presence of E. coli polar lipids (Avanti), essentially as described (Behlau et al., 2001; Hiller et al, 2005). At lipid/protein ratios (LPR) between 0.25 to 1.5 (wt/wt), reconstituted OmpG formed 2D lattices in tubular vesicles that were ~150 nm wide and up to 1 µm long. At lower LPR 2D crystals were extensive membrane sheets measuring several µm across. Electron micrographs of tubular 2D crystals washed with 4% unbuffered trehalose as a cryo-protectant were recorded at a specimen temperature of ~ 4 K in a JEOL 3000 SFF electron microscope. Crystallographic image processing (Crowther et al, 1996) yielded projection amplitudes and phases to ~ 8Å resolution, from which projection maps were calculated.

Support Preparation for single-molecule force spectroscopy

AFM measurements do not need any staining, labeling or fixation of the sample. For imaging and SMFS experiments we need to design special support to immobilize the sample (Figure 3.2). Another prerequisite for an AFM measurement is an atomically flat surface. Materials commonly used for this purpose are mica, graphite, gold and glass. For imaging and SMFS measurements of OmpG, mica is used as the support (Müller et al., 1997).

For support preparation, 8 mm diameter magnetic steel discs (Agar Scientific, Stansted, UK) were treated with 37% hydrochloric acid for ~5 mins, This step is done for surface activation, and washed intensively with de-ionized water. Then Teflon discs were punched in 10 mm diameter circles and glued onto the cleaned steel discs with instant glue Loctite 770 (Koenig, Dietikon, Switzerland).
An approximately 0.2-0.3 mm thick slice of muscovite mica (Shree G. R. Exports, Kolkata, India) was punched onto the disc of 5 mm diameter. Then, the upper layer of the mica disc was cleaved using a tape and this side glued onto the Teflon disc using water-insoluble, twin epoxy glue (Konrad Electronic, Hischau, Germany). The last layer of mica should be cleaved just before adsorbing the sample to give an atomically flat surface.

**Single-molecule force spectroscopy and imaging**

2 µl of the OmpG sample was adsorbed onto freshly cleaved mica covered by 28 µl of adsorption buffer (pH 7; 25 mm tris (hydroxymethyl) aminomethane–HCl, 25 mm MgCl₂, 300 mm NaCl). After 30 mins, the sample was washed by pipeting the same buffer used for adsorption at least 6 times to remove loosely bound and unbound membrane patches.

After this, the membranes were localized by AFM in the same buffer solution at room temperature (Möller et al., 2000; Müller et al., 1999c). For SMFS, the AFM cantilever tip (60 mm long Biolever, Olympus) was pushed onto the OmpG membrane with the application of 500–750 pN of force for 500 ms. In approximately 0.01% of all cases the OmpG terminus attached non-specifically to the silicon nitride AFM tip. The AFM tip was then retracted at 600 nms⁻¹ to induce unfolding. All experiments were performed in the same buffer for adsorption at room temperature (24 ± 1°C).

Before and after each experiment, the spring constant of each cantilever (0.03 Nm⁻¹) was estimated from its thermal noise by using the equipartition theorem (Butt et al., 1995). An F–D curve recorded the forces required to overcome the interaction strengths that stabilized the unfolding intermediates of the membrane protein. The F–D spectra recorded from OmpG sample being either densely packed or crystallized two-dimensionally showed no difference in pattern.
Selection of force-distance (F-D) curves

In SMFS experiments the AFM tip can attach to any of the exposed parts of the proteins such as loops, turns, and anywhere inside the polypeptide thus leading to F-D curves of various lengths particularly short curves. Besides simultaneous pickup of multiple proteins with AFM tip, creates higher forces and steep slopes to the peaks. Covering all the interactions happening inside the protein necessitates selecting full-length curves.

A suitable criterion that used to distinguish curves of OmpG molecules as well as the other membrane proteins studied in our group is the overall length of the force curve (Müller et al., 2002b; Oesterhelt et al., 2000). Using the worm like chain model and the secondary structure of the molecule, the length of the unfolded stretched molecule can be easily calculated (Oesterhelt et al., 2000). The length of the unfolded polypeptide chain depends on the amino acid residue at the N-terminal end of OmpG molecule at where the cantilever tip attaches. For analysis, we selected only F–D curves that were sufficiently long to ensure that OmpG was unfolded from its terminus. The fully stretched OmpG peptide (281 amino acids) is approximately 84 nm long. Thus, we selected F–D curves that were >70 nm long (Sapra et al., 2009).

![Figure 3.3 Mechanical unfolding of the β-barrel membrane protein OmpG: a) Structural representation of a single OmpG molecule nonspecifically attached to the tip of an AFM cantilever. An increase in theDistance between the tip and the membrane establishes a force that induces unfolding of OmpG. b) Force–distance (F–D) curves recorded during the unfolding show certain force peaks (marked by arrows) that correspond to the unfolding intermediates of OmpG. Occasionally, F–D curves lacked force peaks (marked by ellipses); this indicates alternating unfolding pathways.](image)

Analysis of F-D curves

Force-Distance curves recorded during an SMFS experiments of OmpG sample were
imported and analyzed in Igor Pro (Wavemetrics Inc, USA). Raw data displays force as a function of vertical displacement, $z$, of the piezoelectric actuator. However, the end-to-end distance of the stretched protein is smaller than $z$ because of the deflection of the cantilever, $\Delta x$, which can be several nm for cantilevers normally used in our experiment of pulling OmpG ($\kappa = 25$-$30$ pN/nm) (Figure 3.4). To compensate this $z$ value for cantilever deflection, the force was plotted versus the distance between the tip and the sample surface instead of piezo movement, $z$. That distance is called tip-sample separation ($tss$):

$$tss = Z - \Delta x$$

(3.1)

All F-D curves of mechanical unfolding of OmpG having overall lengths of 70-75 nm and almost similar unfolding patterns have been selected and aligned manually using Igor Pro (Wavemetrics Inc., Oregon, USA). The adhesion peak which occurred at a tip-to-membrane separation of $\sim 5$ nm has been taken as the reference. Then we assigned events in the force-distance spectra to the secondary structural elements of OmpG structure using the worm like chain (WLC) model.

*Models used to describe polymer behavior under force*

A polymer is willing to acquire non-structured random coil configurations in solution, to get the maximum conformational entropy. Applying an external force reduces this conformational entropy because of the alignment of the molecule along the direction of applied force. Assuming the simplest case with unaffected backbone bonds and work is just done against pure entropic restoring forces, the polymer molecule behaves like an ideal spring that its spring constant only depends on the length of the monomers, the Kuhn length ($Kl$), and contour length ($Lc$) of the polymer (Janshoff et al., 2000).

In an SMFS experiment the cantilever of the AFM that stretches up and unfolds the molecule applies such external forces on the molecule in the direction of pulling. As
mentioned above this mechanical stretching results in reduced conformational entropy. When a mechanical stretching deforms intra-molecular interaction within a polymer, a change in enthalpy will also happen. Using the entropic and enthalpic character of restoring forces makes it possible to describe mechanical properties and behavior of the polymer under tension and accordingly makes it possible to model the streching process of the polymer and get fitting procedure.

Two fitting procedure based on two decent models are routinely used. Freely jointed chain (FJC)\(^7\) and the worm-like chain (WLC) model are these two models (Figure 3.5) that are developed to predict such restoring forces. The FJC model can explain the structure or lack of structure and mechanical properties of unfolded macromolecules such as proteins and DNA (Bustamante et al., 1994; Smith et al., 1992; Kellermayer et al., 1997; Rief et al., 1997a). As we see in Figure 3.5 in a freely jointed chain, short segments are connected by swivels and presents an extreme case of freely moving rods. All the segments behave as a very flexible slender rod, which in their extreme flexibility it would behave like a worm-like chain. Actually, in absence of external force the worm-like chain and freely jointed chain are the same; nevertheless, they behave differently under an external force\(^8\) (Bustamante et al., 1994). FJC model is applied for polysaccharides and some synthetic polymers. However, the model we use in our lab is WLC model, which is more realistic and will be described as following.

Wormlike Chain Model

In the worm like chain model, the polymer is assumed as a continuous, elastic and freely moving in all dimensions chain (Figure 3.5) (Kratky and Porod, 1949). The WLC model can describe accurately each discrete segment between force peaks in an F-D curve. Taking into account the entropic and enthalpic contributions of the polymer the WLC model describes the force-extension relationship in pN accuracy (Bustamante et al., 1994; Rief et al., 1997). Using the contour length of the chain, \(L_c\), and its persistence length, \(l_p\), the WLC model relates extension of the stretched chain, \(x\), to the force, \(F\), (Bustamante et al., 1994) in the following equation:

\[^7\] The FJC model can be used to understand the mechanics of proteins that have segmental flexibility, e.g., motor proteins (Howard, 2001).

\[^8\] Under zero force, \(2l_p = s\), in which \(l_p\) is the persistence length of the worm-like chain and \(s\) is the length of the freely jointed chain’s segment. The equivalent of this segment length, \(\delta\), in a worm-like chain is called the Kuhn length (Howard, 2001).
where $l_p$ is the persistence length of the polymer, which is the length of filament over which thermal bending becomes appreciable and describes the rigidity of polymer. However, polymers cannot be stretched beyond their contour length.

\[
F(x) = \frac{k_B T}{l_p} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right) + \frac{x}{L_c} - \frac{1}{4} \right]
\]

(3.1)

Figure 3.5 Freely jointed chain (FJC) and worm-like chain (WLC): The FJC models a chain with discretely rigid segments that are connected to each other by swivels. Dashed circles in FJC denote these swivels between the segments with the length of $s$, which freely revolve in all directions. Under zero force WLC and FJC are similar. However, there are some small differences between them under an external force (Howard, 2001).

Using the WLC model every single event (peaks) in an F-D curve was fitted. We used the persistence length of 0.4 nm and a monomer length of 0.36 nm for aminoacids as of the polypeptide chain (Rief et al., 1997a). Then after, using the contour length acquired from the worm like chain fitting, the length of stretched polypeptide was calculated in the number of amino acids. These fits describe the length of stretched and unfolded parts of the protein assigned to each peak. Comparing these fits to the secondly structure of the protein allows us to identify mechanical unfolding barriers or the mechanical unfolding intermediates of the protein (Müller et al., 2002b).

If an unfolding barrier is located on the other side of the membrane (the side adsorbed to the mica) or within the lipid bilayer, the bilayer exclude a part of the polypeptide chain that results in an apparent shorter length. To compensate for this screening the bilayer thickness of $\sim 4$ nm, i.e., 11 amino acids, was added to the calculated contour

\[^9 l_p \text{ is } \sim 50 \text{ nm for a DNA molecule, and } \sim 0.4 \text{ nm for a polypeptide chain, which is almost the size of a single amino acid.}\]
length when locating the unfolding barrier on secondary structure (Müller et al., 2002b).

### 3.1.4 Results and Discussion

In our SMFS experiments, OmpG reconstituted in E. coli lipid membranes were first imaged by AFM. The AFM tip was then pushed onto the OmpG surface to facilitate the nonspecific attachment of the N or C terminus. Withdrawal of the AFM tip stretched the terminus and induced unfolding of OmpG. Force–distance (F–D) curves recorded the interactions that occurred upon unfolding of a single OmpG molecule (Figure 3.3b). We analyzed only F–D curves that correspond to the fully stretched length (>70 nm) of an unfolded OmpG polypeptide (281 amino acids). This selection criterion ensured that OmpG was mechanically unfolded by stretching one of its termini. Individual F–D curves showed a series of force peaks that varied in occurrence (Figure 3.3b). Every force peak of an F–D curve reflects an interaction that has been established by an unfolding intermediate, with all of the intermediates together describing the unfolding pathway of an OmpG molecule (Figure 3.6a). The superimposition of all of the F–D curves showed a clear pattern of predominant force peaks (Figure 3.6b).

Then we determined that this unfolding pattern belongs to pulling from which end (N terminus or C terminus) of OmpG molecules (Figure 3.8). To do so, we designed a mutant with a deletion in one loop closer to N terminous. Based on this design the only change should happen in the position of one last peak if pulling from the N terminal or the last six peaks should shift if pulling from C terminal (Figure 3.8a).

In the mutant OmpGΔ220-228 amino acids 220-228 of the extracellular loop L6 were deleted (ΔL). The SDS gel proved this deletion in the mutant OmpGΔ220-228 running at slightly different molecular weights (figure 3.8). The superimposition of F-D curves obtained on pulling OmpGΔ220-228 showed the existence of force peaks at positions similar to those of wild-type OmpG except for the last peak, which shifted from amino acid position 248 in wild-type OmpG to 239 in OmpGΔ220-228. This shift of 9 amino acids suggests that OmpG was mechanically unfolded by pulling its N-terminal.
Figure 3.6 Unfolding intermediates and pathways of OmpG: a) The F–D curve obtained from the unfolding of a single OmpG molecule. The colored lines are wormlike-chain model (WLC) fits to individual force peaks. The numbers denote the contour lengths (amino acids, aa) of the unfolded polypeptide chain obtained from the WLC fits. b) Superimposition of F–D curves (n=99) shows the reproducibility of the OmpG unfolding. The colored lines are WLC fits, as shown in (a). c) Histogram showing the contour lengths obtained upon fitting of all the force peaks from every F–D curve analyzed (n=136). The tertiary structure cartoons show the predominant mechanical unfolding pathway of OmpG, that is, b hairpins unfold individually.
Histogram of force peak positions (in amino acids) of wild-type (black) and mutant (red) OmpG showed the shift in the force peak by a distance corresponding to the deletion length in L6 loop (Figure 3.8c). This was a good proof of pulling from N terminus. Considering that the N-terminus comprising eight amino acids comparing to the one amino acid long C-terminus, makes it reasonable that the probability of non-specific attachment to the AFM tip to the N-terminus is higher than the C-terminus.

Data analysis:
Each force peak was fitted by using the worm like chain model (WLC) as described before to reveal the lengths of the unfolded polypeptide stretches (Figure 3.6b, c). The length determination of the unfolded polypeptide stretches allowed us to assign the structural segment of unfolded segment to the secondary structure of the protein and consequently describe the unfolding intermediates of OmpG (Figure 3.6c, 3.7).

Two antiparallel β-strands forming one β-hairpin created each unfolding structural
segment or unfolding intermediate. Although the heterogeneous population of the F–D curves indicated multiple unfolding pathways coexist, the common pattern from superimposition of the curves showed that the mechanical unfolding barriers always happen at the beginning of the β-hairpins.

Figure 3.8 OmpG unfolding occurred upon mechanically pulling its N-terminus: a) The secondary structure model of OmpG shows N- and C-termini being located on the periplasmic surface. The N-terminus comprises 8 amino acids compared to the 1 amino acid long C-terminus. Thus, the probability
to non-specifically attach the AFM tip to the N-terminus is higher than attaching the C-terminus. In the mutant OmpGΔ220-228 amino acids 220-228 of the extracellular loop L6 were deleted (ΔL). The SDS gel shows wild-type OmpG and mutant OmpGΔ220-228 running at slightly different molecular weights. b) The superimposition of F-D curves obtained on pulling OmpGΔ220-228 shows the existence of force peaks at positions similar to those of wild-type OmpG except for the last peak, which shifted from amino acid position 248 in wild-type OmpG to 239 in OmpGΔ220-228. This shift of 9 amino acids suggests that OmpG was mechanically unfolded by pulling its N-terminus. c) Histogram of force peak positions (in amino acids) of wild-type (black) and mutant (red) OmpG shows the shift in the force peak by a distance corresponding to the deletion length in L6 loop.

Whereas some individual unfolding pathways described by some F–D curves showed that every β-hairpin unfolded individually, other F–D curves showed two adjacent β-hairpins unfolded collectively or some times three β-hairpins unfolded together. With fourteen β-strands forming seven β-hairpins, OmpG showed different structural segments as multiple of β-hairpins that unfolded individually. This results in seven discrete unfolding intermediates for OmpG. The probability of the curves with every β-hairpin unfolded individually and having seven major peaks was 31%, the one with a pair of β-hairpin unfolded cooperatively was also 31%, that two pairs of β-hairpins unfolded cooperatively was 23%, and the three pairs of β-hairpins together unfolded was 8% (Table 3.1). In 7% of all cases, the F–D curves showed full-length unfolding but only three force peaks. We assumed that, in this case, more than two β-hairpins unfolded cooperatively.

<table>
<thead>
<tr>
<th>Unfolding pathways</th>
<th>Probability (%)</th>
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<tbody>
<tr>
<td>7 Unfolding intermediates (force peaks)</td>
<td>31</td>
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<tr>
<td>6 Unfolding intermediates (force peaks)</td>
<td>31</td>
</tr>
<tr>
<td>5 Unfolding intermediates (force peaks)</td>
<td>23</td>
</tr>
<tr>
<td>4 Unfolding intermediates (force peaks)</td>
<td>8</td>
</tr>
<tr>
<td>3 Unfolding intermediates (force peaks)</td>
<td>7</td>
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Table 3.1F–D curves recorded upon unfolding single OmpG molecules showed up to seven force peaks (Figure 3.3b, 3.6a). Each force peak occurred at specific positions of the F-D spectra (Figure 3.6b). Every WLC fit of such force peak structurally located the unfolding intermediate that established a sufficiently high interaction against mechanical pulling. Overcoming this interaction induced the unfolding of a structural segment that contributed to the unfolding intermediates. Further retracting the AFM tip stretched the unfolded peptide until the forthcoming unfolding intermediate was detected. It is found that each of the seven structural segments associated with the unfolding of OmpG were β-hairpins. However, some F-D curves showed only six instead of seven unfolding force peaks (Figure 3.3b). Since all six unfolding force peaks were at identical positions as in F-D curves showing seven peaks, we must assume that in this case two β-hairpins unfolded in one step. Similarly it occurred that some F-D curves showed five and four instead of seven unfolding force peaks.

Again the remaining force peaks were at identical positions as they were in F-D curves showing seven peaks. In case of five force peaks we assumed that two times two β-hairpins unfolded cooperatively and three β-hairpins unfolded individually. In case of four force peaks we assumed that three times two β-hairpins unfolded cooperatively and that one β-hairpin unfolded individually. We have analyzed the F-D curves (n=136) that showed seven, six, five, four and three unfolding peaks and calculated their occurrence probability.

Bulk unfolding experiments suggested that OmpG unfolds and folds reversibly.
(Conlan and Bayley 2003). The folding process is thought to be a coupled two-state membrane partition–folding reaction (Tamm et al., 2004). This contrasts with our results showing that OmpG unfolds via many sequential unfolding intermediates. Denaturation of β-barrel membrane proteins in the presence of urea or detergent suggests a surprisingly low stability (<10 kcal mol⁻¹) (Tamm et al., 2004), similar to that of GFP (>10–25 kcal mol⁻¹) (Ishii et al., 2007). Our results showed that unfolding of single β-strands of OmpG requires forces of approximately 150–250 pN. These forces, reflecting the interaction strengths stabilizing the β-strands, are much higher than those (100–150 pN) required to unfold single α–helices from membrane proteins (Kedrov et al., 2007) and than those (ca. 100 pN) required to unfold the entire β-barrel protein GFP (Diez et al., 2004) at similar conditions.

Thus, our experiments suggest that OmpG as a β-barrel membrane protein is unusually mechanically stable protein. The discrepancies in the OmpG stability determined by SMFS and by conventional denaturation methods may be because of the different experimental conditions.

In our SMFS experiments, OmpG was embedded in the native E. coli lipids and investigated in buffer solution at room temperature. Conventional unfolding experiments with thermal or chemical denaturants induce very different unfolding scenarios for, in most cases, solubilized OmpG.

In agreement with our observations, it was found that the β–barrel formed by α–hemolysin is far more stable in a lipid bilayer than in detergent (Kang et al., 2005). SMFS showed that the majority of the unfolding structural segments formed by OmpG are established by individual β–hairpins. This stepwise unfolding behavior of a transmembrane β–barrel protein is very different from the almost spontaneous force-induced unfolding of the water-soluble β–barrel protein GFP (Tamm et al., 2004). When water-soluble proteins are unfolded, the exposure of their hydrophobic core to the hydrophilic aqueous solution is one of the driving forces leading to destabilization. Thus, application of a force to initiate unfolding is often sufficient to complete the unfolding of water-soluble proteins (Borgia et al., 2008). By contrast, the anisotropic environment of the lipid bilayer contributes to the structural stability of an embedded membrane protein so that forces must be repetitively applied to a sequence of unfolding intermediates until the entire protein has been unfolded. Whether the mechanical unfolding of transmembrane β–barrel proteins is reversible is
a question that we will answer in next part of this chapter.

As we saw each β-hairpin of the OmpG β-barrel can unfold individually, or cooperatively with an adjacent β-hairpin. This yields a variety of diverse unfolding structural segments, intermediates, and pathways that is not observed in the mechanical unfolding of water-soluble β-barrel proteins. This could be because of the different environment of membrane proteins.

When α-helical transmembrane proteins were unfolded by SMFS, it was observed that their α-helices could unfold individually or together with adjacent α-helices (Kedrov et al., 2007). The probability for transmembrane α-helices to unfold individually or cooperatively was shown to depend on environmental conditions such as temperature, pulling speed, mutations, and the electrolyte (Kedrov et al., 2007). It remains to be investigated under which conditions β-hairpins may alter their propensity to unfold individually or cooperatively. The search for conditions under which β-hairpins cluster and form larger unfolding intermediates may provide insight into the mechanisms leading to the assembly of β-sheet-like aggregates such as those that occur in neurodegenerative diseases.
Chapter 3

3.2 REFOLDING PATTERN: ONE β-HAIRPIN AFTER THE OTHER

3.2.1. Exploring Refolding Pathways and Kinetics of the Membrane β-Barrel Protein OmpG

Introduction
The increasing number of discovered genes doesn’t concert with lack of knowledge about their functional and three-dimensional structures and folding mechanism. Studies on integral membrane proteins folding mechanism have been largely excluded of the folding investigations because they are much more difficult to work with. This mainly arises from the difficulties in mimicking the anisotropic environment of the lipid membrane, in which specific interactions shapes to give a precise folding to the inserted hydrophobic polypeptides. Accurate folding of proteins is in a high priority of importance for the cell and organism and any changes such as environmental changes or point mutations that cause instability in the structure of the protein that may lead to unfolding or misfolding of it, will cause diseases. So from the biological and biomedical points of view, the main questions to be answered are:
1. How the nascent polypeptides find their proper folding and functional 3-D structure?
2. Why different forms of folding conformations exist for the same protein and how do they forms from the same sequence?
3. What kind of interactions between and within biological macromolecules determines their final structure, and functions?
Accordingly, determining forces that stabilize membrane proteins secondary structural elements (folding intermediates) is of high importance. In addition membrane insertion mechanism of polypeptides and their folding into the final functional structure inside the membrane is one of the primary approaches that would help to answer these questions.
It has been recently showed that an AFM based SMFS technique can be exploited to
unravel some ambiguity of α-helical membrane proteins (Kedrov et al., 2004). This novel approach allowed single membrane proteins to be monitored during their folding into the membrane. OmpG has been chosen for this study as a representative of β-barrel membrane proteins for several reasons: i) it is a monomer β-barrel membrane protein with no disulfide bond ii) it has 14 β-strands, comprising 7 β-hairpins, with almost no α-helix iii) nobody has yet reported the folding mechanism of β-barrel membrane proteins using AFM-SMFS technique or any other mechanical unfolding approaches such as magnetic tweezers or optical tweezers. The structure and function of β-barrel membrane proteins, especially OmpG, has been described in previous sections and will be elaborated in the introduction of chapter 4.

3.2.2 Experimental procedures

AFM set-up
The experiments on OmpG refolding were performed using a NanoScope IIIa AFM equipped with the PicoForce module. The piezoelectric actuator was equipped with a Z-sensor and a closed-loop feedback that ensured vertical positioning of the sample stage with accuracy of ~1 nm. The software Igor Pro (Wavemetrics, Lake Oswego, OR) was used to control the AFM in the folding experiments. While the first PC controlled the piezo position, the second PC recorded the force spectroscopy data at rates up to 45,000 data points per second (Kedrov et al., 2004).

Refolding experiments
Sample preparation and primary AFM measurements are described in previous section of Chapter 3. Folding experiments were performed in 300 mM NaCl, 25 mM MgCl₂, 25 mM tris, and pH 7.0. Membranes were localized by AFM in the same buffer solution at room temperature. For SMFS, the AFM tip (60 mm long cantilever, Biolever, Olympus) was pushed onto OmpG to apply forces of approximately 500–750 pN for about 500 ms, which in approximately 0.1% of all cases attached to the N terminus of OmpG. Then the AFM tip was retracted at 600 nms⁻¹ to induce unfolding. Unfolding and extraction of the last β-hairpin VII occurred at pulling distances greater than 65 nm (force peak at 248 aa), thus we stopped initial unfolding at pulling distances of 65 nm by approaching back the cantilever and and letting the polypeptide relax in proximity (ca. 5 nm) to the membrane surface. Thereafter, OmpG was unfolded completely. Before and after each experiment, the spring constant of each
cantilever (ca. 0.03 Nm$^{-1}$) was estimated from its thermal noise using the equipartition theorem.

**Data analysis**

For analysis we selected only refolding experiments of which the F–D curves have both initial unfolding and refolding events (figure 3.13) and the curves showing the accurate movement of piezo as it designed at the software. These F–D curves were fitted using the WLC model as described. The folding rate ($k_{\text{fold}}$) for every refolded β-hairpin was acquired from single-exponential fit (continuous lines) of folding extent:

$$y=y_0+A\exp(-t\cdot k_{\text{fold}})$$

where, $y$ is the folding probability at certain time, $A$ is the folding extent after $t$ time (efficiency), and $k_{\text{fold}}$ is the folding rate of the structural element.

**3.2.3 Results**

**Refolding Pathways of the Transmembrane β-Barrel Protein OmpG**

Despite of their enormous relevance being virtually involved in many cell biological processes, the folding of only few transmembrane proteins has been studied. From these only a handful β-stranded membrane proteins was characterized (Tamm et al., 2004; Kleinschmidt et al., 2006). Current models describe that transmembrane β-barrels fold into the lipid membrane via two major steps.

![Figure 3.9](image_url) Experimental schemata of the mechanism of the SMFS technique to study refolding of the β-barrel membrane protein OmpG into the lipid membrane.
First the unfolded polypeptide interacts with the lipid surface where it folds, tilts and inserts into the membrane (Kleinschmidt et al., 2006; Huysmans et al., 2010). Conventionally, experiments studying the unfolding of membrane proteins use thermal or chemical denaturation. In most cases membrane proteins solubilized in detergent and/or exposed to several molar urea were studied. In vivo membrane proteins fold at different conditions. Thus, the folding pathways studied may be different from those occurring in nature.

**Figure 3.10 Refolding of the β-barrel membrane protein OmpG into a lipid membrane:** The tip of an AFM cantilever attaches the N-terminal end of OmpG and applies a mechanical pulling stress. A F-D curve records single β-hairpins unfolding one after the other. Before the last β-hairpin (grey) unfolds, the AFM tip is brought close to the membrane (≈5 nm) to relax the polypeptide. After a given refolding time the tip is withdrawn to detect the structural regions refolded. F-D curves recorded of the refolded polypeptide shows force peaks at the same position as recorded for initial unfolding. Each of these force peaks assign the unfolding of a single β-hairpin.

Single-molecule force spectroscopy (SMFS) represents a unique approach to study the refolding of membrane proteins into the lipid membrane (Kedrov et al., 2004; Kedrov et al., 2006; Kessler et al., 2006). SMFS unfolds and refolds membrane proteins in
their physiological environment such as pH, electrolyte, temperature, and importantly in absence of any chemical denaturant or detergent. In such experiments a single membrane protein is first mechanically unfolded and its polypeptide fully stretched. Then this unfolded polypeptide is relaxed to allow refolding into the membrane bilayer. Repeated mechanical unfolding assigns which structural regions of the membrane protein refolded. Allowing the polypeptide different refolding times approaches the refolding kinetics of structural regions. Thus, SMFS can detect the mechanical unfolding pathways and the equilibrium refolding pathways of a membrane protein. Previous SMFS work has investigated the mechanical unfolding and refolding of many different of water-soluble proteins (Rief et al., 1997; Dietz et al., 2004). However, compared to the variety of water-soluble proteins characterized, SMFS of membrane proteins reveals much more detailed unfolding and folding pathways. So far, the refolding of β-barrel membrane proteins into a lipid membrane has been never addressed by SMFS.

![Figure 3.11 Probing the refolding kinetics of b-hairpins of OmpG](image)

**Figure 3.11 Probing the refolding kinetics of b-hairpins of OmpG:** Probing the content of refolding in dependence of different refolding times (0.1s-5s) revealed which b-hairpins have been folded earlier than others (Figure 3.10). 189 single molecule refolding experiments have been analyzed.

### 3.2.4 Discussion

In this study we applied SMFS to unfold and refold the outer membrane protein G (OmpG) from *E. coli* (Figure 3.9). OmpG comprises 14 β-strands forming a transmembrane β-barrel pore (Yildiz et al., 2006). Six turns (T1-T6) connect individual β-strands on the periplasmic side and seven long loops (L1-L7) on the
Figure 3.12 Main refolding intermediates and pathways of OmpG Refolding intermediates of previously unfolded OmpG molecules were detected by SMFS. Each refolding step is formed by a single β-hairpin. The refolding kinetic of each refolding step (Figure 3.10) allowed reconstructing the main refolding pathway of OmpG into the lipid membrane.
extracellular side. OmpG is gated by loop L6, which in a pH dependent manner controls the permeability of the bacterial outer membrane (Yildiz et al., 2006; Conlan et al., 2000). In previous SMFS studies, we have found that the β-barrel of OmpG unfolds via many intermediates (Sapra et al., 2009). The main unfolding pathway described the stepwise unfolding of single β-hairpins. This unfolding pathway was much more detailed than that detected for water-soluble β-barrel green fluorescent protein (GFP) (Dietz et al., 2004), which at sufficiently high pulling force mainly unfolds in one step.

In our refolding experiments OmpG reconstituted in native E.coli lipid membranes was first imaged by AFM. Then, the AFM tip was pushed onto the OmpG surface to facilitate the non-specific attachment of the N-terminus (Figure 3.9). Withdrawal of AFM tip stretched the terminus and induced the unfolding of OmpG. Force-distance (F-D) curves recorded the force peaks reflecting the unfolding steps of a single OmpG (Figure 3.10). Each unfolding step represents that of a single β-hairpin of the transmembrane β-barrel (Sapra et al., 2009).

To refold the partially unfolded OmpG we stopped the mechanical unfolding before the last β-hairpin. Then, we relaxed the unfolded polypeptide by approaching the AFM tip close to the membrane (≈5 nm). After, a given time allowed the polypeptide to refold the protein was unfolded again to probe which structural regions refolded into the lipid membrane (Figure 3.13).

Individual F-D curves recorded of the refolded polypeptide showed a series of force peaks that varied in occurrence (Figure 3.9). These force peaks were detected at similar positions as upon initial unfolding of OmpG. Thus, the unfolded OmpG polypeptide folded single β-hairpins into the native E.coli lipid membrane. Probing the content of refolding in dependence of different refolding times (0.1s-5s) revealed insight into which β-hairpins refolded earlier than others (Figure 3.11). For all refolding times probed the β-hairpin IV folded fastest ($k_{\text{fold}} = 20 \, \text{s}^{-1}$). After this, β-hairpin V folded ($k_{\text{fold}} = 0.90 \, \text{s}^{-1}$), then β-hairpin VI ($k_{\text{fold}} = 0.60 \, \text{s}^{-1}$), β-hairpin III ($k_{\text{fold}} = 0.45 \, \text{s}^{-1}$), and β-hairpin II ($k_{\text{fold}} = 0.01 \, \text{s}^{-1}$). This kinetic hierarchy describes the main refolding pathway of OmpG by which the transmembrane β-barrel was assembled. A reconstruction of this main refolding pathway is shown in (Figure 3.12).
Previously, we demonstrated that by pressing the AFM tip onto OmpG the N-terminus preferentially attached to the AFM tip. Upon applying a sufficiently high mechanical pulling stress the OmpG started stepwise unfolding from the N-terminal end. The unfolding steps were defined by single β-hairpins. In our refolding experiment we used this approach to unfold the first six β-hairpins of OmpG a). The seventh β-hairpin remained embedded in the membrane bilayer (a, I and II). Without unfolding the seventh β-hairpin the movement of the AFM cantilever was reversed to approach the AFM tip to the membrane surface (a, III). To allow the unfolded polypeptide to reinsert and to refold into the lipid bilayer the AFM tip was held in close proximity (~5 nm) to the membrane surface. The time given to the unfolded polypeptide to refold varied between 0.1 and 5.0 s. After a given refolding time the AFM tip was retracted again to detect the β-hairpins that have been refolded and inserted into the membrane (a, III). Figures b to f show experimental examples of unfolding and refolding of OmpG at different refolding times.

Figure 3.13 OmpG unfolding from and refolding into the membrane: Previously, we demonstrated that by pressing the AFM tip onto OmpG the N-terminus preferentially attached to the AFM tip. Upon applying a sufficiently high mechanical pulling stress the OmpG started stepwise unfolding from the N-terminal end. The unfolding steps were defined by single β-hairpins. In our refolding experiment we used this approach to unfold the first six β-hairpins of OmpG a). The seventh β-hairpin remained embedded in the membrane bilayer (a, I and II). Without unfolding the seventh β-hairpin the movement of the AFM cantilever was reversed to approach the AFM tip to the membrane surface (a, III). To allow the unfolded polypeptide to reinsert and to refold into the lipid bilayer the AFM tip was held in close proximity (~5 nm) to the membrane surface. The time given to the unfolded polypeptide to refold varied between 0.1 and 5.0 s. After a given refolding time the AFM tip was retracted again to detect the β-hairpins that have been refolded and inserted into the membrane (a, III). Figures b to f show experimental examples of unfolding and refolding of OmpG at different refolding times.
Deviations from this main refolding pathway were possible. However, they occurred at much reduced probability. Such deviations describe that β-hairpins with low refolding kinetics can fold before β-hairpins showing higher refolding kinetics. Bulk unfolding experiments suggest that OmpG unfolds and folds reversibly (Conlan et al., 2000). The folding process is thought to be a coupled two-state membrane partition-folding reaction (Tamm et al., 2004; Kleinschmidt et al., 2006). In contrast, the mechanical unfolding of OmpG shows many sequential unfolding intermediates (Sapra et al., 2009). The unfolding intermediates and, thus, the unfolding pathway of a membrane protein sensitively depends on the environmental parameters (Kedrov et al., 2007).

For OmpG these unfolding intermediates even depend on the functional state of the protein (Damaghi et al., 2010). In our SMFS experiments OmpG was embedded in the native E.coli lipids and investigated in buffer solution at room temperature. Conventional unfolding experiments using thermal or chemical denaturants investigated unfolding scenarios of, in most cases solubilized, OmpG. Thus, it is most probable that the discrepancies in OmpG unfolding determined by SMFS and conventional denaturing are because of different experimental conditions. However, our SMFS experiments show that a partially unfolded OmpG molecule refolds via many sequential steps. The predominant refolding steps are defined by individual β-hairpins that later assemble the transmembrane β-barrel of OmpG. In contrast to the initially induced mechanical unfolding, the refolding steps occurred in different order. Whereas the sequence of unfolding steps is given by the mechanically pulling AFM tip, no externally applied force constrains the refolding of the unfolded OmpG polypeptide. Thus, the unfolded polypeptide searches its own refolding pathway. Each β-hairpin shows intrinsic folding kinetics. These kinetic parameters determine the refolding pathway of the OmpG polypeptide into the membrane. It may be interesting to investigate which factors influence the folding kinetics and, thus, the folding hierarchy of a transmembrane β-barrel. This is particularly interesting to understand how more complex β-barrel proteins such as VDAC or usher proteins of bacterial pili assemble. It may be also interesting to investigate under which conditions β-hairpins may alter their propensity to form single or cooperative folding intermediates. Searching for conditions in which β-hairpins cluster and form larger
folding intermediates may provide insight into mechanisms leading to the assembly of β-sheet like aggregates such as occurring in neurodegenerative diseases.

Specificity of the unfolding spectra for protein folding

It has been previously demonstrated, that force-distance spectra such as shown in Figure 3.9 (Top and bottom force-distance curves) are specific for the unfolding of a single OmpG molecule from native \textit{E. coli} lipid membranes. Taking the examples of various different membrane proteins, others and we have shown that force-distance spectra recorded upon unfolding of a membrane protein are specific for the folding of a membrane protein.

![Graph showing unfolding force vs refolding time](image)

**Figure 3.14 Individual β-hairpins increase their stability with increasing refolding time**: With increasing refolding time the content of β-hairpins refolded increases (Figure 3.10). Data points represent average unfolding forces and error bars their standard errors. Data points at the right represent average unfolding forces measured upon initial unfolding of OmpG. 189 refolding and 100 initial unfolding experiments were analyzed.

It has been also demonstrated that the force-distance spectrum is sensitive to misfolding events of a membrane protein (Kedrov et al., 2006). Smallest misfolding events detected so far were α-helical fragments. Moreover, it has been shown that force-distance spectra can detect the functional state of a membrane protein and sensitively react upon changing their functional state. It has also been demonstrated that the force-distance spectra of OmpG changes with the functional state of the membrane protein (Damaghi et al., 2010). This is because the pH-dependent gating of the transmembrane OmpG pore established interactions that shift individual unfolding
peaks. The force-distance spectra of membrane proteins can also change when inserting a point mutation, replacing a polypeptide loop, or changing the membrane protein assembly. Thus, if the unfolded OmpG polypeptide would adsorb onto the membrane instead of inserting into the membrane one would expect significant changes of the force-distance spectra. Even if the OmpG polypeptide would fold into a β-barrel on the surface of the protein membrane all force peaks of the force-distance spectra would be shifted by at least half the thickness of the OmpG membrane (≈6 nm). In contrast, we observe that within the accuracy of our method (± 2-4 aa) the unfolding force peaks of refolded OmpG remain at the same position as observed upon initial unfolding. Thus, we can conclude that OmpG has refolded into the same conformation and inserted back into the membrane.
INTERACTION STUDIES

4.1 pH-DEPENDENT INTERACTIONS GUIDE THE FOLDING AND GATE THE TRANSMEMBRANE PORTE OF THE β-BARREL TRANSMEMBRANE PROTEIN OmpG

4.1.2 Introduction

The physical interactions that switch the functional state of membrane proteins are not very well understood. The gating mechanism of the outer membrane proteins (Omp) from gram-negative bacteria attracts continuous interest but remains to be addressed in more detail (Schulz, et al., 1993; Schulz, et al. 2002). Recently, the structure of OmpG from *E. coli* was described in both open and closed states (Yildiz, et al., 2006). OmpG comprises of 14 β-strands that form a transmembrane β-barrel pore (Subbarao, et al., 2006; Yildiz, et al., 2006). Six short polypeptide turns on the periplasmic side and seven longer loops (L1-L7) on the extracellular side connect individual β-strands. OmpG is thought to be gated by its largest extracellular loop L6, which in a pH-dependent manner controls the flux of small molecules through the pore (Conlan et al., 2000). At acidic pH (pH<6) loop L6 folds into the pore and constricts the channel entrance. This conformational change was observed in solubilized OmpG, using X-ray crystallography (Yildiz et al., 2006). The pH-dependent conformational changes in OmpG is similar to that of observed in OmpF, where large extracellular loops collapse onto the channel entrance at low pH or upon reaching a critical voltage and close the pore (Muller et al., 1999). Experiments on maltoporin (LamB), an *E. coli* β-barrel forming porin that is specific to malto-oligosaccharides, corroborated this gating mechanism. LamB lacking the major extracellular loops L4 and L6 failed to close at lower pH (Andersen et al., 2002). These results support the model proposing conformational changes in flexible extracellular loops can gate the transmembrane channel in Omps. Although gating of OmpG and OmpF have been shown structurally,
the mechanisms guiding their conformational changes had not been described. For this reason, we structurally localized and quantified the interactions within the OmpG that are associated with the open and closed states, in order to quantitatively and mechanistically explain how pH-dependent interactions gate transmembrane pores. It also can shed light onto how stability of OmpG is optimized so that changes in pH only modify those interactions that are necessary to gate the transmembrane pore.

SMFS has been used to quantify interactions and energy levels established between and within membrane proteins (Kedrov et al., 2007; Engel et al., 2008). SMFS provides a detailed insight into the nature of these interactions and most importantly locates and quantifies these interactions with an accuracy of $\approx 4$ amino acids (aa) in membrane proteins. To conduct SMFS, the OmpG containing membranes was first localized by AFM imaging (Figure 4.1a) (Muller et al., 2007). The OmpG assembly could be observed at high resolutions, (Figure 4.1b). After the membrane was imaged, the AFM tip was pressed onto the OmpG to facilitate non-specific attachment of the terminal end to the cantilever tip (Figure 4.1c). Withdrawal of the AFM stylus stretched the polypeptide and induced unfolding of OmpG. Force-distance (F-D) curves recorded interactions that occurred upon unfolding of a single OmpG molecule (Sapra et al., 2009). It was previously determined that the non-specific attachment of OmpG to the AFM stylus occurs predominantly via the N-terminal end (Sapra et al., 2009). Here, only F-D curves that correspond to the full stretching of the unfolded OmpG polypeptide from the N-terminal end were selected. Each of these F-D curves showed a series of force peaks at distinct pulling distances (Figure 4.1d). Although the magnitude and occurrence of individual force peaks varies slightly, the superimposition of all F-D curves shows a clear pattern of evenly spaced force peaks (Figure 4.2a). The height of every force peak indicates the strength of an interaction that has been established by an OmpG unfolding intermediate (structural segments) (Sapra et al., 2009). Fitting the force peaks using the worm-like-chain model (WLC) allows us to estimate the lengths of the unfolded polypeptide (Figure 4.1d), thus locating the region at which the interaction was established (Kedrov et al., 2007; Sapra et al., 2009). The sequence of force peaks corresponding to unfolding intermediates was used to assign unfolding pathways of single OmpGs (Figure 4.1d). It has been previously shown that at neutral pH, OmpG predominantly unfolds in β-
hairpins as unfolding intermediates, and that the most probable unfolding pattern is one β-hairpin after the other (Sapra et al., 2009).

Figure 4.1. AFM and SMFS of OmpG reconstituted in native *E. coli* lipids: a) OmpG-containing lipid membrane OmpG adsorbed to mica. OmpG membranes are protruded 6.0 ± 0.4 nm (n=58) from the supporting mica. b) High resolution micrograph showing the dense assembly of OmpG porins. Topographs have full color ranges that correlate to vertical scale of 25 nm (a) and 3 nm (b). c) Single OmpGs reconstituted into a lipid membrane were non-specifically attached to the tip of an AFM cantilever. Increasing the distance between the tip and the membrane, D, establishes a force, F, which induces stepwise unfolding of OmpG. d) Force-distance (F-D) curve shows force peaks that reflect the strength of interactions established by OmpG’s unfolding intermediates. Secondary structure cartoons show the predominant mechanical unfolding pathway of OmpG at pH 7.0, i.e., β-hairpins unfold one after the other (Sapra et al., 2009; Damaghi et al., 2010).

4.1.2 Experimental procedures:

*Sample preparation:*

All procedures were described in chapter 3.1.1, the materials and methods.

*SMFS experiment:*

OmpG membranes were adsorbed onto freshly cleaved mica (=30 min) in buffer solution (pH 7.0, 25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl or pH 5.0, 25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl). The adsorbed membranes were localized by AFM (PicoForce, Nanoscope IV, Veeco, Santa Barbara, USA) extended with two server-grade PCs equipped with 16 bit data acquisition electronics (E-6036 and M-6259, National Instruments, Munich, Germany) in the same buffer solution at room
temperature. For SMFS, the AFM cantilever tip (60 µm long Biolever, Olympus) was pushed onto the OmpG membrane applying forces of ≈500-750 pN for ≈500 ms. In ≈0.1% of all cases, the OmpG terminus was attached to the AFM tip. Afterwards, the AFM tip was retracted at a pulling velocity of 600 nm/s to induce unfolding of the proteins. An F-D curve recorded the forces that were required to overcome the interaction strengths that stabilized the unfolding intermediates of the membrane protein. The F-D spectra recorded from OmpG, which was either densely packed or crystallized two-dimensionally, showed no difference in force patterns. Since OmpG was unfolded using different cantilevers, to minimize errors that may occur due to variations in cantilever spring constant, before and after each experiment the spring constant of each cantilever (≈ 0.03 N/m) was estimated from its thermal noise, using the equipartition theorem (Butt et al., 1995)

Data Analysis:
Data analysis was performed using IgorPro 5.0 software. Since membrane proteins are anchored inside the lipid membrane, the force is exclusively applied to the structural segments directly connected to the tip, so the closest segment unfolds first and so on, therefore other structural elements are not loaded until preceding elements unfold. Hence, the lifetime of a structural element is the time between two consecutive rupturing events.

4.1.3 Results and Discussion:
After recording F-D curves at neutral pH [pH 7.0], at which OmpG resides in the open state, F-D curves at pH 5.0, at which the OmpG pore closed, were recorded too (Conlan et al., 2000) (Figure 4.2a). Superimpositions of both F-D curves showed a remarkably good overlap of force peaks; with the exception of the one before the last force peak. At pH 7.0, this force peak occurred at a contour length of ≈ 204 aa, whereas at pH 5.0 this peak shifted to ≈ 213 aa. The histogram of the frequency of all force peaks detected over the contour length (Figure 4.2b) quantifies this pH-dependent shift. This shift of the one before the last force peak was observed in all pulling velocities at which OmpG was probed. The histogram of the average force showed that the interaction strengths established by OmpG did not change significantly with pH (Figure 4.2c).
Fitting of each force peak on the F-D curves, using the WLC model (Figure 4.2a), revealed the average contour lengths (Figure 4.2b) of the unfolded and stretched polypeptide. These average contour lengths allowed us to structurally locate the
interaction studies of the β-barrel protein OmpG

interactions that have been established against unfolding (Figure 4.3). At neutral pH, these interactions were exclusively located at the periplasmic end of every β-hairpin. This suggests that each β-hairpin established sufficiently strong interactions to form a stable structural segment against mechanical unfolding; with the exception of β-hairpin VI, which hosts loop L6, interactions stabilizing the other β-hairpins did not change their positions upon lowering the pH from 7.0 to 5.0.

When the pH shifts from 7.0 to 5.0, length of the unfolded polypeptide locating the interaction at β-hairpin VI was increased from ≈204 to 213 aa. A nine-aa length increase of the unfolded and stretched polypeptide must have partial unfolding of β-hairpin VI. Since the unfolding of β-sheet 11 of β-hairpin VI cannot release ≈9 aa (Figure 4.3b), the only structure that could have released a sufficiently long polypeptide stretch upon unfolding is loop L6 located on the extracellular surface. Accordingly, the interaction that resisted the pulling force was located at the opposite extracellular surface. To locate the interaction that anchored the unfolded polypeptide on the extracellular surface, the thickness of the membrane (≈4 nm) had to be added to the measured contour length of the unfolded polypeptide. This so-called ‘membrane compensation’ (Muller et al., 2002; Kedrov et al., 2007) adds ≈11 aa to the polypeptide contour length of 213 aa locates the interaction at ≈224 aa. Therefore, it was concluded that while at neutral pH the interaction stabilizing β-hairpin VI happens at the periplasmic surface, at acidic pH, this interaction shifts towards the extracellular loop L6 (Figure 4.3).

The pH-dependent shift of an interaction within OmpG has several consequences. At pH 7.0, the interactions stabilize OmpG in a way that every β-hairpin established an individual unfolding structural segment (Sapra et al., 2009). The sequential unfolding of one β-hairpin after the other described the most probable unfolding pathway for OmpG at pH 7.0. At pH 5.0 on the other hand, the force peak characterizing the unfolding of β-hairpin VI shifts from 204 to 213 aa. The missing force peak at 204 aa suggests that β-hairpin V unfolded together with β-strand S11 and the N-terminal part of loop L6. Seemingly, there were no sufficiently strong interactions that allows β-strand S11 to establish an individual unfolding barrier together with β-strand S12. One may speculate that at pH 5.0, β-strand S11 established interactions that favored cooperative unfolding with β-hairpin V. The new force peak at 213 aa reflects the
Interaction studies of the β-barrel protein OmpG

unfolding of the C-terminal part of loop L6 and β-strand S12. Thus, at pH 5.0, part of loop L6 and β-strand S12 establish different interactions that favor unfolding in a separate step. It was concluded that lowering the pH from 7.0 to 5.0 established interactions that lead OmpG to take a new unfolding pathway. When OmpG is in closed state, all β-hairpins unfold in single steps whereas, β-hairpins V and VI and loop L6 unfold differently.

The current structural model for the conformational changes of OmpG describes that its longest extracellular loop, L6, assumes two well-defined conformations depending on the pH of the buffer solution (Yildiz et al., 2006). When changing the pH from 7.5 to 5.6, loop L6 folds into the β-barrel and closes the pore. When searching for a pH-sensitive toggle that may trigger this structural rearrangement, a pair of solvent-exposed residues (His231 and His261) seems feasible. Which one of these residues are located at the C-terminal end of loop L6, close near to β-strand S12, and the other one at the N-terminal end of loop L7, close near to β-strand S13 were found. It is thought that these histidine residues act as pH sensors (Yildiz et al., 2006). Upon protonation at acidic pH, they repel each other and induce the unzipping of the hydrogen network between the β-strands S11-S13 (Yildiz et al., 2006). In agreement with this hypothesis, our SMFS data shows that at pH 5.0, β-strands S11 and S12 unfold differently. Thus, the interactions of β-strands S11 and S12 with each other and with the environment changes with pH. Furthermore, the SMFS data shows that loop L6 and the connecting β-strands unfold differently. At pH 5.0, the C-terminal part of loop L6 together with β-strand S12, established a strong interaction that was absent at pH 7.0. Thus, our experiments provided the quantitative data on the pH-dependent interactions at at loop L6 and β-strands S11-S12, that change alter their unfolding and functionally gate OmpG. It would be interesting to determine whether similar gating mechanisms can be observed in other β-barrel proteins.

The fully stretched OmpG peptide (281 aa) is ≈ 84 nm long. For data analysis, only the F-D curves that were sufficiently long (>75 nm) were selected to ensure OmpG unfolding from its terminus. F-D curves were fitted using the WLC model, and superimposed as described previously (Kedrov et al., 2007; Muller et al., 2007).

When pulling the polypeptide from the periplasmic surface, the peptide’s anchor, in case of the 213 aa force peak, had to be assumed to be at the opposite, extracellular surface. In this case, the membrane thickness (≈ 4 nm) was considered, and 11 aa (11
x 3.6 Å ≈ 4 nm) were added to the contour length determined by the WLC model (Muller et al., 2002). This ‘membrane compensation’ allowed calculating the contour length of the stretched polypeptide and to locate the interaction anchoring the polypeptide at the extracellular side.

Conclusively, Crystal structure and the pH-gating conformations of the β-barrel forming OmpG from E. coli is revealed (Yildiz et al., 2006). It was proposed that when the pH changes from neutral to acidic, the flexible extracellular loop L6 folds into the pore and subsequently closes the OmpG pore. Here, we used single-molecule force spectroscopy (SMFS) to structurally localize and quantify the interactions that are associated with the pH-dependent closure.
Interaction studies of the β-barrel protein OmpG

Figure 4.3. Interactions established in the open (pH 7.0) and closed (pH 5.0) states of OmpG: a) At pH 7, the average contour lengths of the force peaks (encircled regions) identify the β-hairpins that have been stabilized by interactions and unfolded in individual steps. β-strands (S1-S14) and extracellular loops (L1-L7) are numbered. b) At pH 5, the force peaks detected the interactions to identical structural positions except for one; the interaction that stabilized β-hairpin VI at pH 7, shifted to loop L6. Consequently the force peak at a contour length of 213 aa, structurally detected an interaction at 224 aa. At acidic pH, the β-strands S9-S11 formed an unfolding segment and loop L6 unfolded together with β-strand S12. The strengths of the interactions stabilizing the structural segments are given in Figure 4.2c. (Yildiz et al., 2006).

A pH-dependent change was detected in one of the interactions stabilizing unfolding intermediates containing L6 at acidic pH. It was also observed that this interaction changes the unfolding of loop L6 and of β-strands 11 and 12 that connect the loop. All other interactions detected within OmpG remained unaffected by changes of the pH. The results provide a quantitative and mechanistic explanation of how pH-dependent interactions gate the transmembrane pore. These results further demonstrated how the stability of OmpG is optimized so that pH-changes only modify those interactions necessary to gate the transmembrane pore.
4.2 DUAL ENERGY LANDSCAPE: THE FUNCTIONAL STATE OF THE OUTER MEMBRANE β-BARREL PROTEIN OmpG MOLDS ITS UNFOLDING ENERGY LANDSCAPE

4.2.1 Introduction:
We applied dynamic single-molecule force spectroscopy to quantify the parameters (free energy of activation and distance of the transition state from the folded state) characterizing the energy barriers in the unfolding energy landscape OmpG from *E. coli*. The pH-dependent functional switching of OmpG directs the protein along different regions on the unfolding energy landscape. The two functional states of OmpG take the same unfolding pathway during the sequential unfolding of β-hairpins I to IV. After the initial unfolding events, the unfolding pathways diverge. In the open state, the unfolding of β-hairpin V in one step precedes the unfolding of β-hairpin VI. In the closed state, β-hairpin V and β-strand 11 with a part of extracellular loop L6 unfold cooperatively, and subsequently β-strand 12 unfolds with the remaining loop L6. These two unfolding pathways in the open and closed states join again in the last unfolding step of β-hairpin VII. Also, the conformational change from the open to the closed state witnesses a rigidified extracellular gating loop L6. Thus, a change in the conformational state of OmpG not only bifurcates its unfolding pathways but also tunes its mechanical properties for optimum function.

Several of the transmembrane pores formed by Omps of *E. coli* are pH gated. Low pH induces the closing of the pores of, for example, OmpC, OmpF, OmpG, LamB and PhoE (Xu et al., 1986; Heyde et al., 1987; Todt et al., 1992; Conlan et al., 2000). What conformational changes drive pore closure has long been debated (Schulz, 1993). In 1999, for the first time, high-resolution atomic force microscopy (AFM) imaging showed that at low pH the large extracellular loops of the outer membrane protein F (OmpF) collapsed onto the pore entrance (Muller and Engel, 1999). This supported the hypothesis that Omp pores are gated by the conformational changes of the flexible extracellular loops.
Figure 4.4. Unfolding pathways of OmpG detected at pH 7.0 and 5.0 using SMFS. (A) Schematic of the SMFS experiment. OmpG reconstituted into native E. coli lipids was adsorbed to mica in buffer
solution at room temperature. Single OmpGs were non-specifically attached by their N-terminal end to the tip of the AFM cantilever (Damaghi et al., 2010). Retraction of the AFM tip stretched polypeptide and induced the unfolding of OmpG. Force-distance (F-D) curves recorded showed a saw-tooth like pattern of force peaks. Each of these force peaks recorded an unfolding event of OmpG. In previous studies (Damaghi et al., 2010; Sapra et al., 2009) we have used these force peaks to assign the unfolding pathways of OmpG exposed to (B) pH 7.0 (25 mM Tris-HCl, 25 mM MgCl$_2$, 300 mM NaCl) and (C) pH 5.0 (25 mM Na-acetate, 25 mM MgCl$_2$, 300 mM NaCl). Major unfolding pathways of OmpG are scrutinized in the top panels of (B) and (C). Yellow circled amino acids (positions labelled) indicate structural regions at which interactions stabilizing the unfolding intermediates were detected in the F-D curves. The interaction located at 224 aa (pH 5.0) is detected by the force peak at a contour length of 213 aa. Because the contour length of 213 aa suggests that the interaction lies at the membrane surface opposite to the pulling AFM tip, a membrane compensation of $\approx$11 aa had to be added to locate the interaction. Whereas at pH 7.0 single $\beta$-hairpins formed the predominant unfolding steps (equally colored $\beta$-strands), at pH 5.0 $\beta$-strands 9, 10, and 11 formed one unfolding step and $\beta$-strand 12 for itself formed one.

Experiments on the maltoporin LamB from *E. coli*, which is specific for maltodextrins, corroborated the gating model. When lacking the major extracellular loops L4 and L6, LamB failed to close at lower pH (Anderson et al., 2002).

Among the pH gated Omps, the structure and function relationship of OmpG represents possibly the best-studied example. The OmpG structure has been solved by X-ray crystallography (Yildiz et al., 2006; Subbarao et al., 2006) and NMR (Liang and Tamm 2007). It comprises 14 $\beta$-strands, 7 anti-parallel $\beta$-hairpins, that form a transmembrane $\beta$-barrel inside the membrane. On the periplasmic side the $\beta$-strands are connected by six short polypeptide turns (T1-T6). On the extracellular side the $\beta$-strands are connected by seven longer loops (L1-L7) that exhibit enhanced intrinsic flexibility (Liang and Tamm 2007). A pH-dependent gating controls the flux of small molecules through the OmpG pore (Conlan et al., 2000). X-ray structures obtained from three-dimensional OmpG crystals grown at neutral (pH 7.5) and acidic (pH 5.6) pH provide insight into the conformational changes that may guide the gating mechanism (Yildiz et al., 2006). At low pH, the largest extracellular loop L6 folds into the pore, thereby, constricting its entrance. However, the three-dimensional crystals of solubilized OmpG grown at different pHs showed different packing arrangements, and some extracellular loops formed crystal contacts with adjacent OmpG molecules. Thus, it may be assumed that the conformations observed might not represent those that naturally occur in the gating mechanism of OmpG.

Besides the gating mechanisms of the Omps, the mechanisms that guide their folding and unfolding are of pertinent interest (White and Wimmely, 1999; Tomm et al., 2004; kleinschmidt et al., 2002). So far most experiments investigating the folding of
Omps, have first denatured Omps in detergent or urea and then characterized the refolding into a lipid bilayer or in a detergent (Conlan et al., 2003; Hong et al., 2007). Such bulk unfolding experiments suggest that OmpG unfolds and refolds reversibly. The folding process of Omps is described as being coupled with membrane insertion (Tamm et al., 2004). A folding and insertion process has been recently described for the β-barrel transmembrane protein PagP from *E. coli* (Huysmans et al., 2010). PagP solubilized and denatured in 10 M urea is found to adsorb to the lipid headgroups of the bilayer, where it forms a transition state that tilts and inserts into the lipid membrane to complete the folding process. Apparently, these models contrast with the results from single-molecule force spectroscopy (SMFS) in which single OmpG molecules have been mechanically stressed to induce their unfolding from the native lipid membrane in a buffer solution (Sapra et al., 2009). These single-molecule experiments clearly show that OmpG molecules unfold via many sequential unfolding intermediates describing a detailed unfolding pathway. The unfolding step of a single β-hairpin characterizes the transition from one unfolding intermediate to the next one. However, the differences between chemical denaturation and refolding and mechanical unfolding experiments may have different origins. First, one may assume that the refolding mechanism in the absence of any external force does not reflect unfolding under an applied force. Second, it may be that the experimental conditions alter the unfolding and folding pathways chosen by β-barrel membrane proteins. On the example of α-helical transmembrane proteins it has been shown, that alterations in the temperature and buffer solution within the physiological relevant range can considerably modify their unfolding pathways (Kedrov et al., 2007). Therefore, it is not surprising, that the exposure of membrane proteins to urea, detergent and mechanical stress may force them along very different unfolding and folding pathways.

SMFS has been particularly successful in characterizing the unfolding pathways of membrane proteins, and to quantify the interactions and energies of the intermediates in the unfolding pathways (Engel and Gaub, 2008). SMFS provides detailed insights into the nature of molecular interactions and most importantly allows to locate and quantify these interactions structurally with an accuracy of ≈ 2-6 amino acids (aa). In this work we have performed dynamic SMFS (DFS) to probe the strength of the interactions that stabilize the unfolding intermediates of OmpG at different loading...
rates (applied force over time). The dependence of these interaction strengths on the loading rate allows quantifying the unfolding energy barriers of the intermediates (Evans and Kinoshita 2007). These measurements provide the position of the transition state, the transition rate of the intermediate from the folded to the unfolded state, and the energy of activation to cross the transition barrier. Because the sensitivity of SMFS permits to directly determine the sequence at which the unfolding barriers are located along an unfolding pathway, we can chart the unfolding energy landscape of OmpG in the two pH-dependent conformational and functional states. The energy landscapes reveal detailed insights into how interactions can change the unfolding pathways and the gating mechanism of OmpG. We show that the molecular interactions associated with a change in pH not only drive a conformational change but also influence the mechanical properties of the region responsible for the conformational change. We propose that the functional properties of the protein are related to its mechanical properties.

4.2.2 Experimental Procedures

DFS:

OmpG was purified from inclusion bodies, refolded in detergent and reconstituted into native E.coli lipids (Yildiz et al., 2006). Membranes showed the OmpG molecules being densely packed and assembled into two-dimensional crystals. These OmpG membranes were adsorbed onto freshly cleaved mica (≈30 min) in buffer solution (pH 7.0, 25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl or pH 5.0, 25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl). The adsorbed membranes were localized by AFM in the same buffer solution at room temperature (Muller and Engel, 2007) For SMFS, the AFM cantilever tip (60 µm long Biolever, Olympus) was pushed onto the OmpG membrane applying forces ≈500-750 pN for ≈500 ms. In ≈0.1% of all cases the OmpG terminus attached to the AFM tip. Then the AFM tip was retracted at a specific pulling velocity to induce unfolding. A F-D curve recorded the forces required to overcome the interaction strengths that stabilized the unfolding intermediates of the membrane protein. F-D spectra recorded from OmpG, which was either densely packed or crystalized two-dimensionally showed no difference in the force pattern. DFS experiments were performed at eight pulling velocities (100, 300, 600, 900, 1200, 2500, and 5000 nm/s). Before and after each experiment the spring constant of each cantilever (≈0.03 N/m) was estimated from its thermal noise using the equipartition theorem (Butt and Jascke, 1995). To minimize errors that may occur
due to uncertainties in the cantilever spring constant calibration, OmpG was unfolded using at least three different cantilevers for each pulling velocity.

**Data selection and analysis**

We analyzed only F-D curves that corresponded to the length (>75 nm) of a fully stretched and unfolded OmpG polypeptide (281 aa). This selection criterion ensured that OmpG was mechanically unfolded by stretching one of its termini. In previous section we saw that OmpG predominantly attaches with its N-terminal end to the AFM tip. All F-D curves having a length of >75 nm showed a F-D pattern similar to those published previously (Damaghi et al., 2010). Thus, we could conclude that the F-D curves were recorded upon mechanically unfolding of OmpG from the N-terminal end. For analysis each force peak of each F-D curve was fitted using the worm-like-chain model (WLC) to reveal the contour lengths of the unfolded polypeptides (Janovjak et al., 2003). Determining the unfolded polypeptide stretches allowed assigning the structural regions that form stable unfolding intermediates (Damaghi et al., 2010).

**Calculating \( x_u \) and \( k_0 \) from DFS data:**

According to the Bell-Evans theory (Bell, 1978; Evans, 1998) the most probable unfolding force \( F^* \) plotted versus \( \ln(r_f^*) \) describes the most prominent unfolding energy barriers that have been crossed along the force-driven reaction coordinate. The relation between \( F^* \) and \( r_f^* \) can be described by:

\[
F^* = \frac{k_B T}{x_u} \ln\left(\frac{x_u r_f^*}{k_B T k_0}\right)
\]

Equation 4.1

where \( k_B \) is the Boltzmann constant, \( T \) the absolute temperature, \( r_f^* \) the loading rate, \( x_u \) the distance between the free energy minimum of the folded intermediate state and transition state barrier, and \( k_0 \) the unfolding rate of the intermediate at zero force. The loading rate was calculated using \( r_f = k_{\text{spacer}} \cdot v \), \( v \) being the pulling velocity. Experimental loading rate and force histograms (Figures 4.5 and 4.6) were fitted with Gaussian distributions. The resulting \( F^* \) was semi-logarithmically plotted versus \( r_f^* \). \( x_u \) and \( k_0 \) were obtained by fitting Equation 4.1 using a non-linear least-squares algorithm. Only unfolding forces and loading rates corresponding to the main force peaks were considered for analysis.
Interaction studies of the β-barrel protein OmpG

Figure 4.5. Conceptual unfolding energy barrier tilted by an externally applied force according to the Bell-Evans theory: (Evans, 1997; Bell, 1978) a) A simple two-state model that describes the mechanical unfolding experiments. The energy potential exhibits one energy barrier separating the folded low-energy state, N, from the unfolded state, U. The activation energy for unfolding is given by $DG_u$, $x_u$ describes the distance between the folded and the transition state, $z$, and $k_u$ describes the transition rate for crossing the energy barrier. In the absence of an externally applied force, $k_u$ equals the thermal transition rate, $k_0$. b) Application of an external force, $F$, changes the thermal likelihood of reaching the top of the energy barrier. It is assumed that although for a sharp barrier the distance, $x_u$, of the folded state relative to the energy barrier does not change, but the thermally averaged projection of the energy profile along the pulling direction is tilted by the mechanical energy $(-F\cos y)x$ (shortdashed line). $y$ describes the angle of the externally applied force relative to the molecular coordinate, $x$. This tilt decreases the energy barrier (energy potential in black). At low loading rates, the thermal contribution to overcome the energy barrier is higher, and therefore, the mechanical energy that is required to overcome the barrier is smaller. With increasing loading rates, the mechanical force increases due to to a reduced lifetime of the folded state.

Calculating transition barrier height and rigidity

The height of the free energy barrier, $\Delta G^\ddagger$, separating the folded and the unfolded states, was assessed using the following Arrhenius-like expression:

$$\Delta G^\ddagger = -k_B T \ln (\tau_D k_0)$$

Where $\tau_D$ denotes the diffusive relaxation time. Typical values for $\tau_D$ found for proteins are in the order of $10^{-7}$–$10^{-9}$ s (Krieger et al., 2003). Therefore, assuming $\tau_D=10^{-9}$ s seems reasonable for determining the free energy barrier heights. This value has also been used for molecular dynamics simulations of protein folding (Grater and Grubmuller, 2007). In this work $\tau_D=10^{-9}$ s was used in all calculations. Varying $\tau_D$ within the above-mentioned range, changes the free energy of activation by <15 %. Moreover, even if $\tau_D$ were wrong by orders of magnitude, the influence of the error of
\( \tau_D \) would be the same for all conditions and values, hence, it would not affect the qualitative results. Errors in \( \Delta G^\ddagger \) were estimated by propagation of the errors of \( k_0 \). Without any information on the shape of the energy potential, it was assumed to be a simple parabola. Hence, the spring constant \( \kappa \) of the folded structure was calculated using \( \Delta G^\ddagger \) and \( x_u \) (Dietz et al., 2006; Howard, 2001):

\[
\kappa = \frac{2\Delta G^\ddagger}{x_u^2}
\]

Equation 4.3

Errors in \( \Delta G^\ddagger \) and \( x_u \) were propagated for estimation of errors in \( \kappa \).

4.2.3 Results and discussion

4.2.3.1 Functional state of OmpG directs its unfolding route

In previous experiments, SMFS-based unfolding of single OmpG molecules, which were reconstituted into membranes of native \( E. \ coli \) lipids, was established (Damaghi et al., 2010). In those experiments, OmpG membranes were first localized by AFM imaging. Then the AFM tip was pressed onto OmpG to facilitate the non-specific attachment of the terminal end (Figure 4.4a). The non-specific attachment of OmpG to the AFM tip occurred predominantly via its N-terminal end (Damaghi et al., 2010). Withdrawal of the AFM cantilever stretched the polypeptide and induced the unfolding of OmpG. During withdrawal, (F-D) curves that showed a series of force peaks were recorded. The superimposition of all F-D curves showed a clear pattern with the predominant occurrence of force peaks (Figure 4.6). Each force peak of an F-D curve reflects the strength of an interaction that has been established by an OmpG unfolding intermediate (Damaghi et al., 2010). Fitting a force peak using the worm-like-chain model (WLC) allowed estimating the length of the unfolded polypeptide and locating the structural region at which the said interaction was established (Figure 4.4b, bottom). The sequence of force peaks, or unfolding intermediates, was used to assign the unfolding pathways of single OmpG molecules (Figure 4.4b, top).

At neutral pH, OmpG predominantly unfolds one \( \beta \)-hairpin at a time until the entire membrane protein has been unfolded (Sapra et al., 2009) i.e., the unfolding intermediates of OmpG were formed by unfolding subsequent \( \beta \)-hairpins in single steps (equally colored \( \beta \)-strands in Figure 4.4b). Similarly, at pH 5.0, which induces the pore to close, most \( \beta \)-strands unfolded with an adjacent \( \beta \)-strand as \( \beta \)-hairpin (Yildiz et al., 2006). A noticeable exception was observed for the intermediate
composed of β-hairpins V (β-strands 9 and 10) and VI (β-strands 11 and 12) in the closed state of OmpG; β-strands 9, 10, and 11 unfolded together in one step followed by the unfolding of β-strand 12 in another single step (Figure 4.4c). This structural region of OmpG is also known to undergo a pH-dependent conformational change. Thus, the occurrence of new intermediates in the closed state suggests that the conformational states of the protein may be the key to the bifurcation of its unfolding pathways.

4.2.3.2 Quantifying the unfolding energy barriers of OmpG in the closed and open conformations

In our previous work, SMFS was used to characterize the unfolding pathways and interactions of OmpG in the open and closed states (Damaghi et al., 2010). Here, the strength of the interactions that stabilize the unfolding intermediates of OmpG at different loading rates were probed by using dy using DFS. This data is used to quantify the parameters that define the energy barriers (Figure 4.5) of the unfolding intermediates of OmpG in the open (at pH 7.0) and closed (at pH 5.0) conformations. Single OmpGs were unfolded at pulling speeds of 100, 300, 600, 900, 1200, 2500, and 5000 nm/s, and F-D spectra recorded at each speed were superimposed (Figure 4.6). The F-D spectra of OmpG unfolded in the open state showed seven main force peaks at contour lengths of 8, 43, 83, 126, 166, 204, and 246 amino acids (aa), while the F-D spectra of OmpG unfolded in the closed state showed seven main force peaks at contour lengths of 8, 43, 83, 126, 166, 213, and 246 aa. For both the open and the closed states, the positions of these force peaks did not change with change in the pulling speed at which OmpG was unfolded. Thus, it was concluded that the main unfolding pathways of OmpG did not depend on the loading rate.

In agreement with theoretical considerations (Evans 1998) and previous force spectroscopy studies on soluble (Carrion-Vazquez et al., 1999; Williams et al., 2003) and membrane proteins (kedrov et al., 2008), the unfolding forces increased with increasing pulling velocities. This dependency of the unfolding force became obvious after plotting the most probable unfolding force of every main unfolding peak, $F^*$, versus the logarithm of the loading rate, $r_f^*$ (Figure 4.7).
Interactions studies of the β-barrel protein OmpG

Figure 4.6. DFS of OmpG in the open (pH 7.0) and closed (pH 5.0) states. Shown are superimpositions of single F-D curves. For each pulling velocity a minimum of 100 F-D curves were superimposed. DFS was performed at pH 7.0 (25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl) and pH 5.0 (25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl).
The dynamic force spectra of each structural segment of OmpG showed one linear regime (Figure 4.7). According to the Bell-Evans model, the existence of one linear regime indicates that a single energy barrier separates the folded from the unfolded state (Evans, 1998; Bell, 1978). Using Equations 4.1 – 3 (see Materials and methods) the parameters characterizing the energy barriers stabilizing the unfolding intermediates were extrapolated (Table 4.1).

4.2.3.3 Transition state distances of unfolding energy barriers

The distance, $x_u$, separating the folded structural segments of OmpG from the transition state (Figure 4.6) ranged from 0.23 to 0.85 nm (Table 4.1). These values agree well with those measured for α-helical membrane proteins such as bacteriorhodopsin (0.32-0.77 nm) (janovjak et al., 2004), bovine rhodopsin (0.21-0.47 nm) (Sapra et al., 2008), the sodium-proton driven antiporter NhaA (0.28-0.7 nm) (Kedrov et al., 2008), and the amino acid transporter SteT (0.38-1.34 nm) (bippes et al., 2009) by DFS.

At pH 7.0 and 5.0, $x_u$ increased with an increasing number of β-strands unfolded. An exception was the unfolding intermediate of β-hairpin V, which at pH 7.0 showed the largest $x_u$ of 0.85 nm. An increase in $x_u$ with unfolding may indicate that OmpG became destabilized at an advanced unfolding stage. This interpretation may be supported by the decreasing average unfolding force of the intermediates along the unfolding pathway (Figure 4.8).

A larger $x_u$ value implies that the unfolding intermediate is located in a broad energy well (Figure 4.5). Thus, the structural segment has to be stretched further to reach the transition state that if it is sitting in a narrow energy well. The accompanying decrease in the spring constant, $\kappa$, (Table 4.1) signifies the highly frustrated nature of the energy landscape.
Figure 4.7. DFS plots of OmpG unfolded in the open (pH 7.0) and closed (pH 5.0) states: Loading rate dependence of interactions that stabilize the individual unfolding intermediates of OmpG at pH 7.0 (red) and pH 5.0 (black). Each data point represents the most probable unfolding force of a structural segment at the given loading rate. Structural segments unfolded are shown in Figures 1B (pH 7.0) and 1C (pH 5.0). Fitting the loading rate-dependent force (lines) using Equation 1 provides the parameters of the energy barriers that stabilize the unfolding intermediates of OmpG (Table 1). Fits were weighted using the standard error (S.E.) of the most probable force. Error bars represent the S.E. of force and loading rate. The data was extracted from the F-D spectra shown in Figure S1. Experiments were performed at pH 7.0 (25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl) and pH 5.0 (25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl).
A greater flexibility of the structure denotes more conformations being trapped by many local minima of the rough energy landscape. In contrast, a more rigid structure is indicative of a minimally frustrated, smooth energy landscape. Recently, the unfolding energy landscapes of bacteriorhodopsin and bovine rhodopsin has been compared (Park et al., 2009; sapra et al., 2006). The comparison showed that the $x_u$ values increased as the unfolding of bacteriorhodopsin progressed whereas no specific trend was observed during the unfolding of rhodopsin. Also, rhodopsin showed the presence of rigid structural cores suggesting the presence of long-range interactions, which make the folding and unfolding of multiple structural segments a cooperative process (Park et al., 2009; sapra et al., 2006). Such rigid structural cores were absent in bacteriorhodopsin suggesting that the $\alpha$-helices of bacteriorhodopsin, which unfold independent of each other, are stabilized mainly by short-range interactions. Similar to bacteriorhodopsin, OmpG shows a clear trend of increasing $x_u$ values as the unfolding progresses and an absence of rigid structural cores. The rigidity of the structural segments decreases as observed in the case of bacteriorhodopsin. Thus, it can be suggested that the folding of OmpG is guided by short-range interactions and not by long-range interactions.

**4.2.3.4 Activation free energy of $\beta$-strands and $\beta$-hairpins**

At pH 7.0 and 5.0, the height of the free energy barriers, $\Delta G^\ddagger$, separating the folded from the unfolded state of the $\beta$-strands and $\beta$-hairpins ranged from 20 to 23 $k_B T$. $\Delta G^\ddagger$ of transmembrane $\alpha$-helices of bacteriorhodopsin range from 20 to 26 $k_B T$ (Sapra et al., 2008a), of bovine rhodopsin from 20 to 26 $k_B T$ (Sapra et al., 2008b), and of SteT from 20 to 36 $k_B T$ (Bippes et al., 2009). Thus, the height of the free energy barriers stabilizing transmembrane $\beta$-strands and $\beta$-hairpins is similar to the activation free energies of transmembrane $\alpha$-helices. Assuming that a transmembrane $\alpha$-helix has in average $\approx 20 \text{ aa}$ and a $\beta$-strand $\approx 10 \text{ aa}$, the average energy per residue stabilizing a $\beta$-strand is approximately 2 fold higher than that stabilizing an $\alpha$-helix. The height of the free energy barrier was approximately the same whether a single $\beta$-strand or a single $\beta$-hairpin (two $\beta$-strands) were unfolded. Thus, we assume that the height of
Figure 4.8 Average force of interactions detected upon unfolding OmpG in the open (pH 7.0) and closed (pH 5.0) state: To determine the average force every force peak of every F-D curve (superimposed in Figure 4.6) was fitted using the WLC model. This allowed extracting the contour length at which the force peak occurred and the force at which the interaction stabilizing the unfolding intermediate ruptured. Experiments were performed at pH 7.0 (25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl) and pH 5.0 (25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl).
the free energy barrier to be overcome to initiate the unfolding of a β-hairpin and a β-strand is independent of the number of amino acids in the structural segment.

Bulk unfolding experiments denaturating β-barrel membrane proteins in the presence of urea or detergent suggest a surprisingly low stability (<10 kcal/mol) (Tamm et al., 2007). Similar to that of the water-soluble β-barrel protein GFP (>10–25 kcal/mol) (Ishii et al., 2007). Our results show that the mechanical unfolding of single β-hairpins and β-strands requires overcoming energy differences between 20 and 23 $k_B T$, i.e., $\approx 50$ to 57 kcal/mol at room temperature. Assuming that we have to unfold seven β-hairpins (at pH 7.0) the energy required to unfold OmpG entirely sums up to $\approx 140$-160 kcal/mol.
**Interaction studies of the β-barrel protein OmpG**

Figure 4.9 Most probable forces of interactions detected upon unfolding OmpG in the closed conformation (pH 5.0) (black) and the open conformation (pH 7.0) (red):

Histograms show force distributions for the seven major peaks (compare Figure 4.4 and 4.6) over different pulling velocities. The bin size of the histograms is 10 pN. Curves represent Gaussian fits of force distributions. These Gaussian fits were used to determine the most probable forces of each force peak. Data (Figure 4.6) was performed at pH 7.0 (25 mM Tris-HCl, 25 mM MgCl₂, 300 mM NaCl) and pH 5.0 (25 mM Na-acetate, 25 mM MgCl₂, 300 mM NaCl).

However it may be quite reasonable that the experimental conditions used for chemical denaturing solubilized OmpG and those used here to mechanically unfold OmpG embedded in the lipid bilayer force the membrane protein along very different unfolding pathways (janovjak et al., 2003; Kedrov et al., 2007). Therefore, one may only ‘compare’ the unfolding free energies of both experimental conditions with caution.
4.2.3.5 Mechanical properties of OmpG

Structural rigidity defines the resistance of a material to structurally deform in response to a mechanical force. The structural rigidity of a protein depends on the curvature of the potential well of the energy profile, the height of the energy barrier, $\Delta G^\ddagger$, and the distance $x_u$ separating its folded state from the transition state (Figure 4.5). An energy landscape describes the energy of a protein structure as a function of its conformational entropy (Dill, 1997). Accordingly, the width of an energy valley defines the conformational entropy of a protein structure. With increasing (decreasing) width of an energy valley a protein structure can adopt more (less) conformational substates owing to an increase (decrease) in its flexibility (Wolynes et al., 1995; Dill, 1997). Because different functional states have different conformational states, structural rigidity is intrinsically related to the function of a protein.

We assumed a parabolic potential of the energy minimum and a sharp transition barrier for all unfolding intermediates of OmpG (To approximate the rigidity of the individual structural segments ($\beta$-hairpins and $\beta$-strands) of the unfolding intermediates we calculated their spring constants using Equation 4.3. The spring constants of the $\beta$-hairpins and $\beta$-strands of OmpG ranged from 0.3 to 2.6 N/m (Table 4.1). The spring constants of transmembrane $\alpha$-helices of bacteriorhodopsin ranged from 0.5 to 7.3 N/m (Sapra et al., 2008a), of bovine rhodopsin from 0.9 to 3.8 N/m (Sapra et al., 2008b) and of SteT from 0.2 to 2.8 N/m (Bippes et al., 2009). Thus, we conclude that $\beta$-hairpins and $\beta$-strands of OmpG that show a similar structural flexibility as transmembrane $\alpha$-helices. Changing OmpG from the open to the closed state did not significantly affect the mechanical properties of most of the $\beta$-hairpins.

<table>
<thead>
<tr>
<th>Table 4.1. Parameters characterizing the energy barriers and spring constants of the unfolding intermediates of OmpG in the open (pH 7.0) and closed (pH 5.0) states</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural segment (folded state conformation)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>$\beta$-hairpin I (86aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin II (47aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin III (81aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin V (143aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin VI (143aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin VII (143aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin VII and part of loop I (145aa)</td>
</tr>
<tr>
<td>$\beta$-hairpin VII and part of loop II (215aa)</td>
</tr>
</tbody>
</table>
and β-strands.

As discussed in the previous section, the average rigidity of the unfolding intermediates decreased with the increasing number of secondary structures unfolded.

Figure 4.10 Unfolding energy landscape of OmpG being set in the open (pH 7.0, red) and closed (pH 5.0, black) conformation: Upon applying a sufficiently high mechanical force to the N-terminal end, the structural segments of OmpG start unfolding sequentially. Individual unfolding intermediates of OmpG are trapped in energy valleys (rainbow colored). Structures forming the unfolding intermediates along the unfolding pathway are shown for each energy valley. For simplicity we have assumed the energy valleys stabilizing the folded native state of OmpG in the open (pH 7.0) and closed (pH 5.0) state to be the same (valley indicated by black number 1). Overcoming each energy barrier that separates two energy valleys from each other induces the unfolding of a β-hairpin. The first four unfolding steps (stepwise unfolding of β-hairpins I, II, III, and IV) that guide one unfolding intermediate to the other are the same for OmpG in the open and closed states. After this, the unfolding pathway differs for OmpG being set in the open (top, red pathway at pH 7.0) and the closed (bottom, black pathway at pH 5.0) state. In the open conformation, unfolding of β-hairpin IV is followed by the unfolding of β-hairpins V (transition from energy valley numbered with black 5 to energy valley numbered red 6), then VI (transition from energy valley numbered with red 6 to energy valley numbered black 7), and finally VII (transition from energy valley numbered with black 7 towards completely unfolded OmpG). In the closed conformation, unfolding of b-hairpin IV is followed by the unfolding of β-hairpin V together with β-strand S11 (transition from energy valley numbered with black 5 to energy valley numbered black 6), then of β-strand S12 (transition from energy valley numbered with black 6 to energy valley numbered black 7), and finally of β-hairpin VII (transition from energy valley numbered with black 7 towards completely unfolded OmpG). Because the parameters characterizing the unfolding of β-hairpin VII do not differ between OmpG being set in the open or in the closed state we assume that the last unfolding step is the same for both unfolding pathways. The schematic representation of the unfolding energy landscape was reconstructed from parameters revealed by SMFS and DFS (Fig. 4.4 and Table 4.1). Shown is only the predominant (main) unfolding pathway for each functional conformation of OmpG at pH 7.0 and pH 5.0.
A possible reason for this decrease in the rigidity could be the breaking of the inter-strand hydrogen bond network as unfolding proceeds.

The first β-hairpin is the most rigid, and as hydrogen bonds are broken the remaining β-strands get flexible. The contribution of the inherent rigidity of each structural segment, i.e., the rigidity of structural segments in the absence of hydrogen bonds, is a daunting experimental task. However, it may be assumed that the rigidity of the last β-hairpin VII is the approximate inherent rigidity of a β-hairpin in OmpG, and that the rigidity increases with increasing complexity of the hydrogen bond network. A greater flexibility of the structure denotes more conformations being trapped by many local minima in a rough energy landscape. The accompanying decrease in the spring constant, κ, (Table 4.1) also signifies the highly frustrated nature of the energy landscape. In contrast, a more rigid structure is indicative of a minimally frustrated, smooth energy landscape. The rigidity or the spring constant of the structural segment constituted of β-strands S9-S11 and part of loop L6 at pH 5 was calculated to be three times more than that of β-hairpin V (β-strands S9-S10) at pH 7. It may be assumed that the extra mechanical rigidity of the gating region in the closed state reduces its structural fluctuations to ensure that the gated pore remains closed. In the open conformation, however, this may not be a necessity and a flexible loop L6 can be tolerated by the organism. Thus, a change in conformation of OmpG changes its mechanical properties to ensure an efficient functional state.

4.2.3.6 Mapping the unfolding energy landscapes of OmpG in the open and closed states

It is worth noting that a change in the mechanical properties during the pH-dependent conformational change of OmpG also led to a bifurcation in the unfolding pathways, i.e., specifically the structural region gating the pore unfolded via different intermediates at pH 7.0 and 5.0 (Figure 4.4 a and b). Based on the F-D spectra it was possible to construct the sequence of unfolding events that occurred until single OmpG molecules were entirely unfolded to a fully stretched conformation (Figure 4.4). The DFS spectra enabled to quantify the parameters associated with the energy barriers of the main unfolding intermediates of OmpG (Table 4.1). Based on these data the unfolding energy landscape was mapped for OmpG in the two functional conformations corresponding to the open and the closed states (Figure 4.10). In both
the states OmpG shows similar unfolding events for the first four β-hairpins I, II, III and IV (Figure 4.4), i.e., the same intermediates populated the unfolding pathway from β-hairpin I to IV. All parameters characterizing the energy barriers of these first four unfolding events did not show significant differences (Table 4.1). Thus, the first four β-hairpins of OmpG unfolded via the same pathway. Unfolding of β-hairpins V and VI depended on whether OmpG resided in the open or closed state. β-hairpins V and VI unfolded in individual events when OmpG was in the open state (Figure 4.4). When OmpG was in the closed conformation, β-hairpin V unfolded together with β-strand 11. In this case, β-strand 12 unfolded in an individual event. Thus, the unfolding pathways of OmpG bifurcated upon reaching the unfolding of β-hairpins V and VI, and depended on whether OmpG was in the closed or in the open conformation. Accordingly, the parameters, $x_u$ and $k_u$, characterizing the unfolding energy barriers of both the pathways also differed. The last unfolding event of OmpG in the open and in the closed states was characterized by the unfolding of the β-hairpin VII. Within experimental error the parameters characterizing the energy barrier of this unfolding event did not depend on the conformational state of OmpG (Table 4.1). Because we did not observe any significant difference in $x_u$ and $k_u$ at pH 5 and pH 7, we assumed that the last unfolding event of OmpG is very similar or even the same whether OmpG is in the open state or the closed state. Both the scrutinized unfolding pathways of OmpG in the open and closed state show how the mechanical unfolding of OmpG follows the same pathway. For β-hairpins V and VI this pathway separates until both pathways join again in the final unfolding step of the last β-hairpin.

4.2.4 Conclusion:

In this work we have quantified the parameters that characterize the unfolding energy barriers of OmpG in its open and closed conformations. The unfolding intermediates of OmpG depend on the functional state of the transmembrane pore. Deciphering the unfolding pathways showed that the initial unfolding intermediates are the same for both the open and closed conformational states of OmpG. Till the unfolding of β-hairpin IV, OmpG takes the same pathway in both the states. After the unfolding of β-hairpin IV the unfolding pathways bifurcate on the energy landscape. Crucially, only the structural region involving extracellular loop L6, which shows pH-dependent gating, unfolds via different intermediates. The parameters characterizing the last
unfolding intermediate, i.e., the unfolding of β-hairpin VII, do not differ in both the functional states of OmpG. Besides the unfolding pathways, the spring constants of most structural segments of OmpG remain the same. However, a rigid extracellular loop L6 in the closed state becomes more flexible in the open state suggesting a crucial role in maintaining the functional integrity of the protein. Finally, we propose that the two conformational states of OmpG have different mechanical properties, which are intricately related to the protein’s unfolding pattern and function.
<table>
<thead>
<tr>
<th>Structural segment</th>
<th>pH 7.0</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
<th>pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_u (nm)</td>
<td>0.24 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>3.3 ± 4.0</td>
<td>3.4 ± 1.0</td>
<td>19.5 ± 0.9</td>
<td>19.5 ± 0.9</td>
<td>2.6 ± 1.0</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>k_u (s^{-1})</td>
<td>0.28 ± 0.04</td>
<td>0.26 ± 0.02</td>
<td>0.9 ± 1.0</td>
<td>1.2 ± 0.7</td>
<td>20.7 ± 1.8</td>
<td>20.5 ± 0.6</td>
<td>2.1 ± 1.7</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>ΔG^‡ (kJ/mol)</td>
<td>0.31 ± 0.05</td>
<td>0.30 ± 0.03</td>
<td>0.5 ± 0.6</td>
<td>0.7 ± 0.4</td>
<td>21.5 ± 1.0</td>
<td>21.0 ± 0.5</td>
<td>1.8 ± 0.7</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>
| Barrier heights, ΔG^‡, and spring constants, κ, were calculated as described under “Materials and methods.

Table 4.1 Parameters characterizing the energy barriers and spring constants of the (pH 7.0) unfolding intermediates of OmpG in the open and closed (pH 5.0) states. Parameters were derived from fitting the DFS data shown in Figure 3. Errors represent S.D. X_u measures the distance from the energy well of the native state to the transition state, and k_u describes the kinetic transition rate at which the structural segment unfolds at zero force. Barrier heights, ΔG^‡, and spring constants, κ, were calculated as described under “Materials and methods.
Chapter 5

OUTLOOK

5.1 INTRODUCTION

Despite of being a rather newborn technology, AFM has been the basis for the invention of a variety of new techniques to investigate both biological and non-biological events and interactions in the field of single molecule study. Since 1986, AFM has been under constant improvement and progress on both instrumentation and experimental design to address different kinds of questions. Although AFM was invented for high resolution imaging of surface topology of solid specimens in early 1990s, since then new aspects of the AFM application has been arisen as a result of the magnificent work done in the Gaub lab on measuring forces of single ligand-receptors (Florin et al., 1994) and simultaneously on direct measurements of the forces between complementary strands of DNA developed in the Colton lab (Lee et al., 1994). Later, this technique was applied on membrane proteins and revolutionized the field of mechanical unfolding studies of membrane proteins (Osterhelt et al., 2000).

Force spectroscopy experiments provided intriguing insight into the underlying interactions stabilizing membrane proteins and their (un)folding mechanisms, specifically how these interactions are related to function of the system under investigation. SMFS has been a valuable technique for mechanical unfolding studies of macromolecules such as DNA, polysaccharides, and proteins (Florin et al., 1994; Damaghi et al., 2010a). It has been proven to be a good tool for evaluating the interactions stabilizing membrane proteins as well as their unfolding energy landscapes (Damaghi et al., 2010b). In the following pages, my ideas for how to tackle more enigmas in membrane protein interactions and folding mechanism using AFM based SMFS will be briefly described.
5.2 INTERACTION STUDY AND UNFOLDING ENERGY LANDSCAPE

The gating mechanisms of the β-barrel forming outer membrane proteins (Omps) is a focus of interests these days because of their importance in Biosensor studies and also in the pathology of bacterial toxins (Chen et al., 2008). Several of the transmembrane pores formed by Omps in the outer membrane of *Escherichia coli* are pH gated. The conformational changes that drive pore gating have been described in detail (Schulz, 1993). In 1999, AFM imaging showed, for the first time, that the large extracellular loops of the outer membrane protein F (OmpF) close the pore (Muller and Engel, 1999). This supported the hypothesis that Omp pores are gated by the conformational changes of the flexible extracellular loops. Later on, it was shown that the maltoporin LamB mutants from *E. coli*, lacking the major extracellular loops L4 and L6, fail to close at lower pH, supporting the loop-associated gating model (Anderson et al., 2002).

For this study, OmpG was used for several reasons, namely that it is a monomer with no proof of oligomerization, it does not contain any disulfide bonds, and it is a pure β-barrel protein. These reasons made OmpG the best candidate as a model for those kinds of studies that focus on β-barrel proteins and particularly our first SMFS experiment on β-barrel proteins. Besides, OmpG is a new candidate for the biosensors construction as a main pore. It has a very easy nature for biological engineering and very robust structure against harsh environmental condition (Yildiz et al., 2006).

SMFS has been particularly successful in characterizing the unfolding pathways of membrane proteins, as well as in quantifying the interactions and energies of the intermediates in unfolding pathways of macromolecules (Engel and Gaub, 2008). SMFS provides a detailed insight into the nature of molecular interactions and most importantly allows us to structurally locate and quantify these interactions with an accuracy of ≈ 2-6 amino acids (aa).

We used SMFS in this thesis to structurally localize and quantify the interactions that are associated with the pH-dependent closure of OmpG. At acidic pH, a pH-dependent interaction was detected at loop L6. This interaction changed the unfolding of loop L6 and β-strands 11 and 12, which connect loop L6. All other interactions detected within OmpG were unaffected by changes in pH. These results provide a quantitative and mechanistic explanation of how pH-dependent interactions change the folding of an extracellular loop to gate a transmembrane pore. We further
demonstrate how the stability of OmpG is optimized so that pH changes only modify those interactions that are necessary to gate the transmembrane pore. It is intriguing to determine whether similar gating mechanism is exploited in other β-barrel membrane proteins.

The next step would be to understand how more complex β-barrel proteins, such as VDAC, gate and regulate their function under different conditions. It might also be interesting to investigate under which conditions β-hairpins may alter their propensity to form single or cooperative unfolding intermediates that define mechanical properties of the correlated structural segments. Searching for conditions in which β-hairpins cluster and form larger folding intermediates will provide insight into mechanisms leading to the assembly of β-sheet-like aggregates such as those occurring in neuro-degenerative diseases.

We also applied dynamic SMFS (DFS) at different loading rates (applied force over time) in order to probe the strength of the interactions that stabilize the unfolding intermediates of OmpG. Dependence of these interaction strengths on loading rates allows us to quantify the unfolding energy barriers of the intermediates (Evans and Kinoshita 2007). These measurements provide the position of the transition state along the reaction coordinate, the transition rate of the intermediates from the folded to the unfolded states, and the energy of activation to cross the transition barrier. Because the sensitivity of SMFS permits direct determination of the sequence at which the unfolding barriers are located along an unfolding pathway, it would be possible to chart the unfolding energy landscape of OmpG in two pH-dependent conformational and functional states. The energy landscapes reveal a detailed insight into these interactions and how they can change the unfolding pathways and the gating mechanism of OmpG. We discovered that the molecular interactions associated with a change in pH not only drive a conformational change but also influence the mechanical properties of the region responsible for the conformational changes. We also proposed that the functional states of the protein are related to its mechanical properties.

The next step of unfolding energy landscape study would be deciphering the energy landscape of different OmpG mutants in order to observe the effect of each region or amino acid on the shape of the energy landscape. To study this, mutant samples are being designed and produced. We are also interested to see how the energy landscape
roughness would change in different functional state. Applying energy landscape in different temperature would answer this question.

5.3 MEMBRANE PROTEIN FOLDING

Most approaches to study insertion and folding mechanism of membrane proteins have been carried on α-helical proteins. Wimley and White established a scale based on amino acid hydrophobicity by measuring the partitioning of small peptides into the membrane inter-phase (Wimley and White, 1996). Later on, von Heijne and co-workers tested the capability of different peptide segments to adopt a transmembrane topology using an in vitro translation system. Based on these findings, they proposed a model for insertion and folding of α-helical membrane proteins (chapter 1). Meanwhile, investigations carried on β-barrel proteins led to proposing the coupled insertion and folding model (Tamm et al., 2004; Huysmans et al, 2010).

SMFS studies of folding mechanism of membrane proteins are limited to BR and NhaA (Kedrov et al., 2004; Kedrov et al., 2006; Kessler et al, 2006). The refolding of these proteins initiated from partially unfolded proteins, i.e. the last structural segment kept into the membrane, which makes it proper to study the refolding of the unfolded peptide refolding inside the membrane. However, the weak point would be missing the insertion part and also interference of surrounded proteins in the folding procedure.

The mechanisms that guide the folding and unfolding of Omps are of pertinent interest (White and Wimmely, 1999; Tomm et al., 2004; Kleinschmidt et al., 2002). So far, most experiments investigating the folding of Omps have first denatured Omps in detergent or urea and then characterized the refolding process into a lipid bilayer or detergent (Conlan et al., 2003; Hong et al., 2007). Such bulk unfolding experiments suggest that OmpG unfolds and refolds reversibly. The folding process of Omps is described as being coupled with membrane insertion (Tamm et al., 2004). Folding and the insertion process of β-barrel transmembrane protein PagP from E. coli has been recently described (Huysmans et al., 2010). PagP, solubilized and denatured in 10 M urea, was found to adsorb to the lipid head groups of the bilayer, where it forms a transition state that tilts and inserts into the lipid membrane to complete the folding process. However, these models contrast with the SMFS results, in which single OmpG molecules in a buffer solution were mechanically stressed to induce unfolding from the native lipid membrane (Sapra et al., 2009). These single-molecule
experiments clearly show that OmpG molecules unfold via many sequential unfolding intermediates, and detailed unfolding pathways were described. The unfolding step of a single β-hairpin characterizes the transition from one unfolding intermediate to the next. However, the difference between chemical denaturation and refolding and mechanical unfolding experiments may be due to a different reason. First, it may be assumed that the refolding mechanism in the absence of external force does not reflect unfolding under an applied force. Second, the experimental conditions may alter the unfolding and folding pathways taken by β-barrel membrane proteins. In the case of α-helical transmembrane proteins, it has been shown that alterations in the temperature and buffer solution, within the physiologically relevant range, can considerably modify unfolding pathways (Kedrov et al., 2007). Therefore, it is not surprising that exposure of membrane proteins to urea, detergents and mechanical stress may force them along very different unfolding and folding pathways.

SMFS experiments showed that a partially unfolded OmpG molecule refolds via many sequential steps. The predominant refolding steps are defined by individual β-hairpins that later assemble to the transmembrane β-barrel of OmpG. Each β-hairpin shows intrinsic folding kinetics, and these kinetic parameters determine the refolding pathway of that the OmpG polypeptide will take. It is interesting to investigate which factors (such as different environment or biological chaperones) influence the folding kinetics and, thus, the folding hierarchy of a transmembrane β-barrel. It would also be interesting to study the effects of various lipid compositions on refolding kinetics. Another approach would be to study the role of adjacent proteins on the refolding process and kinetics of target protein. This can open an era in single protein-protein interaction studies.

Nevertheless, the main goal would be to understand the folding mechanism of β-barrel proteins inside bacteria or mitochondria. To mimic in vivo conditions, a new SMFS experiment was designed in order to study the refolding of single β-barrel protein in the presence of β-barrel assembly machinery. To do so, β-barrel assembly machinery (BAM) complex reconstituted in a membrane would be adsorbed on the Mica and the β-barrel protein will be picked from different patches on the same support. This required a complicated movement of AFM piezo, which is now possible by new software that was designed specifically for this task by JPK.
This software adds an extra layer of control on the piezo movement in the microscope set up that enables us to pick a protein from a spot on the mica and move it to a different position\textsuperscript{10}, i.e. in this case, where the BAM complex is located. Using this new SMFS design makes it also possible to study the mechanism of membrane protein insertion and folding into the membrane bilayer much more representative to \textit{in vivo} conditions (Figure 5.1).

\textbf{Figure 5.1 Schematic demonstration of the new SMFS experiment on membrane protein folding design:} This new technique enables us to study the folding mechanism of a single membrane protein in different membrane compositions, with the presence of chaperons, and with the presence of other membrane proteins or large complexes.

Another option is to modify the AFM tip and covalently attach the protein onto the tip via a linker such as polyethylene glycol (PEG) molecule. The linker will reduce the interference of the AFM tip and the protein while inserting or folding inside the membrane. One of the difficulties of this experiment is the aggregation of membrane protein in aqueous solutions. Furthermore, it should be ensured that only interactions of single proteins are measured. The experimental procedure would be exactly the same as what we did in normal refolding experiments (described in chapter 3). In

\textsuperscript{10} It used to be impossible to pull from a position on the sample and move it horizontally to somewhere else all in one step.
brief, we would approach the modified tip to the membrane and held it for a certain amount of time close to the membrane surface to allow the unfolded polypeptide to bind, insert, and fold into the bilayer. By withdrawing the cantilever in different time scales, we can watch any of the three mentioned steps.
Appendix

References:


Appendix


Appendix


Appendix


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Appendix


ABBREVIATIONS

°C  Degree Celsius
µm  Micrometer (10⁻⁶ m)
2D  Two-dimensional
3D  Three-dimensional
Å  Ångström (10⁻¹⁰ m)
aa  Amino acid
AFM  Atomic force microscope
ATP  Adenosine triphosphate
bR  Bacteriorhodopsin
cm  Centimeter (10⁻² m)
Da  Dalton (1.66054·10⁻²⁷ kg)
DFS  Dynamic force spectroscopy
DMPC  1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DNA  Deoxyribonucleic acid
DOPC  1,2-Dioleoyl-sn-glycero-3-phosphocholine
DSPE  1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
E. coli  Escherichia coli
EDTA  Ethylenediaminetetraacetic acid
EGTA  Ethylene glycol-bis(2-aminoethylether)-N,N′,N′,N-tetraacetic acid
EM  Electron microscopy
eWLC  Extended wormlike chain
F-D  Force-distance
FRET  Förster resonance energy transfer
FTIR  Fourier transforme infrared
GPCR  Guanine nucleotide-binding protein coupled receptor
h  Hour
I27  27th immunoglobulin domain of the giant muscle protein titin
IgG  Immunoglobulin G
J  Joule
kcal  Kilocalories (10³ cal; 1cal = 4.1868 J)
kDa  Kilodalton
kHz  Kilo Hertz (10³ Hz)
min  Minute
mm  Millimeter (10⁻³ m)
mM  Millimolar (10⁻³ mol/l)
MT  Magnetic tweezers
NhaA  Sodium:proton (Na⁺:H⁺) antiporter from Escherichia coli
nm  Nanometer (10⁻⁹ m) NMR Nuclear magnetic resonance
PBS  Phosphate buffered saline
PC  Phosphatidylcholine
PDB  Protein data bank
PE  Phosphatidylethanolamine
pN  Piconewton (10⁻¹² N)
POPC  1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PS  Phosphatidylserine
PSD  Power spectral density
RNA  Ribonucleic acid
s  Second
S/N  Signal-to-noise ratio
SD  Standard deviation
SDS  Sodium dodecyl sulphate
SEM  Standard error of the mean
SFA  Surface force apparatus
Appendix

SM  Sphingomyeline
SMFS  Single-molecule force spectroscopy
SNOM  Scanning nearfield optical microscope
SOPC  1-Stearoyl-2-oleoyl-sn-glycero-3-phosphocholine
SPM  Scanning probe microscope
STM  Scanning tunneling microscope
TAPS  N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid
Tris  2-Amino-2-(hydroxymethyl)-1,3-propanediol
WLC  Wormlike chain
wt  Wild-type

SYMBOLS

B  Measurement bandwidth [s^{-1}]
D  Differential error [V]
d  Distance between two surfaces [m]
dg  Differential gain
e  Elementary charge [C]
F  Force [N]
F*  Most probable force [N]
I  Integral error [V]
Ig  Integral gain
k_{(F)}  Force-dependent unfolding rate
k_0  Unfolding rate in absence of force [s^{-1}]
k_B  Boltzmann constant (1.38·10^{-23} J/K)
Lc  Contour length [m]
L_{cant}  Length of a cantilever [m]
l_K  Kuhn length [m]
l_p  Persistence length [m]
m  Mass [kg]
p  Pressure [Pa]
p  Proportional error [V]
Pg  Proportional gain
R  Radius of a cantilever tip [m]
Re  Reynolds number
r_f  Loading rate [N/s]
r_f*  Most probable loading rate [N/s]
T  Temperature [K]
t  Time [s]
tss  Tip-sample separation [m]
v_p  Pulling velocity [m/s]
w_{cant}  Width of a cantilever [m]
x_{mol}  Molecule extension [m]
x_u  Distance between native and transition state corrected for deviations from the direction of force (x_u = x_β \cosθ) [m]
x_β  Distance between native and transition state along the reaction coordinate [m]
z_{corr}  Correction value for z-piezo position [V]
zp  Piezo movement [m]
γ  Damping coefficient [Ns/m]
ΔG  Free energy change [J]
ΔG‡  Free energy of activation [J]
η_f  Fluid viscosity [Ns/m^2]
κ_{bond}  Elasticity of a bond or structural segment [N/m]
### Appendix

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\kappa_{\text{cant}}$</td>
<td>Cantilever spring constant [N/m]</td>
</tr>
<tr>
<td>$\kappa_{\text{segment}}$</td>
<td>Segment elasticity [N/m]</td>
</tr>
<tr>
<td>$\kappa_{\text{spacer}}$</td>
<td>Spring constant of the polymeric handle connecting cantilever tip and molecule [N/m]</td>
</tr>
<tr>
<td>$\lambda_D$</td>
<td>Debye length [m]</td>
</tr>
<tr>
<td>$\rho_f$</td>
<td>Fluid density [kg/m$^3$]</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Standard deviation (SD)</td>
</tr>
<tr>
<td>$\sigma_i$</td>
<td>Surface charge density [C/m$^2$]</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Molecular relaxation time [s]</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>Diffuse relaxation time [s]</td>
</tr>
<tr>
<td>$\nu_0$</td>
<td>Resonance frequency [s$^{-1}$]</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Specific polymer stiffness [N]</td>
</tr>
<tr>
<td>$\chi$</td>
<td>Optical lever deflection sensitivity [m/V]</td>
</tr>
<tr>
<td>$\psi$</td>
<td>Surface potential [V]</td>
</tr>
<tr>
<td>$\omega_0$</td>
<td>Angular resonance frequency [s]</td>
</tr>
</tbody>
</table>
Publications:
*Equal contribution.


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Declaration:
I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 01/09/2008 to 18/01/2012 under the supervision of Prof. Dr. Daniel J. Müller at the Biotechnology Center, University of Technology, Dresden, Germany and Department of BioSystems Science and Engineering (BSSE) ETH Zurich, Basel, Switzerland.

Mehdi Damaghi

Dresden, January 2012

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