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Characterizing folding, structure, molecular interactions and ligand gated activation of single sodium/proton antiporters

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Abstract Using the example of sodium/proton antiporter from *Escherichia coli* NhaA, we review the capabilities of single-molecule atomic force microscopy and force spectroscopy to observe structural and functional insights of a membrane protein, which are not attainable by other traditional methods. While atomic force microscopy provides high-resolution topographs of single membrane proteins, their oligomeric state and assembly, single-molecule force spectroscopy experiments detect molecular interactions of the protein. The sensitivity of this method makes it possible to detect and locate interactions that stabilize secondary structures such as transmembrane α -helices, polypeptide loops and segments within them. Controlled refolding experiments using single-molecule force spectroscopy observed individual secondary structure segments folding into the functional protein. Various folding pathways of NhaA were detected, each one exhibiting a certain probability to be taken. Time-lapse refolding experiments enabled determining the folding kinetics and hierarchy of individual secondary structural elements. Recent examples detected and located the ligand binding of an antiporter. Similarly, inhibitor binding and location can be detected which in future may guide towards comparative studies of agonist and antagonist altering the functional state of a membrane protein. We review current and future potentials of these approaches to characterize the action of pharmacological molecules on the antiporter function.

Keywords Ion exchange · Protein folding · Single-molecule approach · Stable segments · Two-stage folding model

Abbreviations AFM: atomic force microscopy · SMFS: single-molecule force spectroscopy · aa: amino acid · F-D: force-distance · pN: pico-Newton · nm: nanometer

Introduction

Secondary transporters mediate the reactions of uniport, symport or antiport of ions or small molecules. As they facilitate solute accumulation and toxin removal against concentration gradients by converting energy supplied by ion gradients across the cell membrane, this class of membrane proteins plays a central role in human health and disease. Regulation of proton and sodium gradients is involved in virtually every physiological process. Na^+/H^+ antiporters regulate intracellular pH, cellular Na^+ concentration and cell volume of eukaryotic and prokaryotic organisms. In *Escherichia coli* two antiporters, NhaA and NhaB specifically exchange Na^+ and Li^+ ions for H^+ allowing the cell to adapt to high environmental salinity and to grow at an alkaline pH (Padan et al. 2001). The activity of NhaA is highly dependent on intracellular pH and increases 2000-fold upon enhancing the pH from 7.0 to 8.0 (Taglicht et al. 1991). The structural analysis of NhaA reconstituted into two-dimensional (Williams 2000) or grown in three-dimensional crystals (Hunte et al. 2005) revealed that the NhaA polypeptide folds into 12 transmembrane α -helices.

Despite the intense focus secondary transporters have received, an accurate mechanistic description of their action remains unsolved. Understanding the transporter function at a molecular level poses an important challenge to modern biology, medicine, and pharmacology. It is of equal importance to understand the molecular origins of transporter folding, stabilization, ligand binding and activation. Understanding the interactions that fold antiporters, of the forces that stabilize antiporters, and of the spatial and structural formation of dynamic complexes with signaling molecules, are fundamental to our understanding of their function. Here, we review the capabilities of two new techniques: single-molecule atomic force microscopy (AFM) (Binnig et al.

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Fig. 1 Native membrane protein surfaces imaged by AFM. **(a)** AFM topograph of sodium driven rotors of the F-ATP synthase from *I. tartaricus*. The sample was imaged in buffer solution (300 mM KCl, 10 mM Tris, pH 7.8) at room temperature (22°C) (Stahlberg et al. 2001). **(b and c)** Extracellular connexon (Cx26) surface from rat liver epithelial cells. **(b)** Connexons imaged in absence of calcium. The central channel entrances of the hexameric pores were clearly resolved. **(c)** Upon addition of 0.5 mM calcium the pores closed their channel. The central channel closure was reversible as proved by their opening upon removal of calcium in the buffer solution (Müller et al. 2002a). The unprocessed topographs were observed in buffer solution (1 mM phenyl methylsulfonylfluoride, 0.5 mM

CaCl₂ (for c), 5 mM Tris) at room temperature. *Upper right insets* of **(b and c)** represent correlation averaged topographs of the raw data. Averages are displayed in a perspective view (5° tilt). The *lower right insets* represent the corresponding standard deviation (SD) maps. Maxima of the SD maps represent regions of the protein exhibiting an enhanced structural flexibility. **(d)** AFM topograph of NhaA molecules, which were reconstituted into a lipid bilayer and assembled into a two-dimensional crystal. The topograph was recorded in buffer solution. The correlation average of the topograph (*insets*) shows the structural assembly of the NhaA molecules in more detail (Kedrov et al. 2004)

1986) and single-molecule force spectroscopy (SMFS) to reveal unique insights into the structure-function relationship, the folding kinetics and pathways, and the ligand binding and activation of individual antiporters. As this approach is applicable to many other membrane proteins, we draw a scenario of the future impact of this technology to study secondary transporters under fully variable physiological conditions, which are of medical or pharmacological importance.

While AFM allows the observation of single proteins at sub-molecular resolution (Engel and Müller 2000; Müller et al. 2002b; Frederix et al. 2003), SMFS enables detecting their inter- and intramolecular interactions (Fisher et al. 2000; Rief and Grubmüller 2002). In the first part of this review, we give an overview of high-resolution AFM imaging, which enables visualizing the oligomeric states and the structural assemblies of functional proteins in the membrane bilayer. Functionally related conformational changes could be directly observed on single native membrane proteins at molecular resolution (Fig. 1). Then, we discuss recent advances in SMFS performed on membrane proteins. Such experiments enabled detecting the stability of various membrane proteins (Oesterhelt et al. 2000; Moller et al. 2003; Kedrov et al. 2004; Cisneros et al. 2005), probing their energy landscape (Janovjak et al. 2004) and refolding kinetics (Kedrov et al. 2005b). Protein structures were observed to contain stable segments such as those represented by transmembrane α -helices, polypeptide loops or fragments thereof. It was observed that collective

interactions of amino acids (aa) within these structural segments ensure their stability. Once the force externally applied by the SMFS reaches a certain critical value, all aa within these segments unfold cooperatively. Initial experiments allowed investigation into how environmental variations such as temperature changes (Janovjak et al. 2003), pH variations (Müller et al. 2002c; Kedrov et al. 2005a), or oligomeric assembly (Sapra et al. 2005; Janovjak et al. 2005a) influence the strength of molecular interactions establishing stable structural segments within the protein. It has become possible to directly observe the various pathways and to determine the folding steps and kinetics of a polypeptide folding into the final antiporter. Comparing the molecular interactions established within two different membrane proteins having almost identical structures allowed insights into their origin (Cisneros et al. 2005). Comparative experiments detecting molecular interactions of a membrane protein being in different well-defined functional states reveals insights into molecular mechanisms associated with its activation (Kedrov et al. 2005a). Recent measurements on the G-protein coupled receptor (GPCR) rhodopsin allowed the assigning of the structural and functional role of such localized molecular interactions stabilizing and holding highly conserved residues at their location (Sapra et al., submitted). That these molecular interactions have not just a mechanistic rule stabilizing the protein and its substructures, were shown by recent experiments in which the binding of a ligand activating the antiporter NhaA could be detected and located as well (Kedrov et al. 2005a).

High-resolution AFM imaging of membrane proteins

Within the last decade AFM provided high-resolution topographs of many transmembrane and of membrane associated proteins in their native environment at subnanometer resolution (Müller et al. 1995b, 2002a; Scheuring et al. 2001; Engel and Müller 2000; Fotiadis et al. 2003). AFM imaging is based on a simple mechanical principle similar to a record player: a sharp stylus mounted at the end of a flexible cantilever raster scans over the sample surface. While contouring the sample surface, the cantilever deflection measures the force interacting locally between stylus and sample. Most high-resolution AFM topographs of native proteins were recorded using the constant-force imaging mode. In this mode, the forces interacting between stylus and biological sample are held constant during scanning. In the ideal case, the imaging force lies in the pN range, which is required to prevent irreversible or reversible deformation of the native protein sample (Müller et al. 1999a). After carefully adjusting the imaging parameters, biomolecular surfaces can be observed at a spatial resolution of <1 nm and a signal-to-noise ratio better than achieved by any optical microscopy technique. AFM does not require the sample to be labeled or fixed and allows observing the biological system in a wide range of buffer solutions and temperatures (Marti et al. 1988; Drake et al. 1989). In summary, the exceptionally high spatial resolution and the ability to observe membrane proteins under physiological relevant conditions allows the direct observation of dynamic biological processes (Müller et al. 1996, 2002a, 2003; Müller and Engel 1999).

To establish AFM for high-resolution imaging of single proteins, it had to be demonstrated that the topographs correlate to the native protein structure. In the first step, preparation methods had to be developed which would prevent any functional or structural distortion of the sample (Karrasch et al. 1993; Müller et al. 1997; Shao et al. 1995; Wagner et al. 1994). High-resolution AFM topographs of the preparations could be recorded to demonstrate that the delicate structure-function relationship of the biological sample remained unchanged. It was shown, that depending on the contact force between AFM stylus and membrane proteins could be reversibly or irreversibly deformed (Müller et al. 1995a, 1999b). Thus, successful approaches to establish reproducible conditions to observe protein substructures focused on minimizing the interacting forces between the AFM stylus and the biological sample (Moller et al. 1999; Müller et al. 1999a). By following these protocols, membrane proteins could be observed at subnanometer resolution and topographs of single proteins correlated well with structural information elucidated using X-ray crystallography and electron microscopy (Engel and Müller 2000; Heymann et al. 1997; Karrasch et al. 1994; Schabert et al. 1995). Examples showed that even individual polypeptide loops on single membrane protein surfaces could be observed by AFM and correlated with their atomic models. However, it was also observed that changing the membrane protein assembly could change individual loop conformations as well (Müller et al. 1999b).

Single-molecule force spectroscopy

Forced denaturation represents a general approach for studying the stability of proteins. Denaturants of chemical (urea, Gdn-HCl, pH) (Anderson et al. 1990; Alonso and Dill 1991; Bennion and Dagget 2003) or physical (high temperature, pressure) (Makhatadze and Privalov 1992; Silva and Weber 1993; Sturtevant 1987) natures change interactions within a protein finally leading to the loss of its functional structure. Alterations of molecular conformations change macroscopic properties of the molecular assembly which can be observed for example as changes in their fluorescence, heat capacity, and their viscosity. Such observations allow for following the denaturation reaction and quantifying kinetic or thermodynamic processes. Statistical analysis of the experimental data provides insights into populations of different molecular conformations (Ptitsyn 1995; Privalov 1996). Since the pioneering works of Anfinsen (1973), bulk techniques such as fluorescent spectroscopy, circular dichroism spectroscopy, and calorimetry have been intensively applied and developed to study the conformation and stability of water-soluble and membrane proteins. However, some general limitations of these approaches for measuring the properties of the bulk may be considered. Measurable macroscopic parameters originate from averaging over a significant amount of molecules. Individual conformations within these assemblies are somehow distributed and cannot be directly resolved. The co-existence of different molecular populations, such as intermediate states, complicates the analysis of experimental data, and even a small experimental error can cause low-populated fractions to be totally missed. A possible way to overcome these challenges is to focus the research on single proteins. Until recently, the existing technology did not allow detecting subtle changes at the single molecule level. However, the rapid progress of bionanotechnology within the past two decades has provided an intensive burst of new approaches such as Forster resonance energy transfer spectroscopy, fluorescent correlation spectroscopy, and AFM allowing the assessing properties of individual macromolecules and providing fascinating insights on their dynamics (Rief et al. 1997; Medina and Schwille 2002; Lipman et al. 2003).

During the last decade, a novel AFM-based approach to assessing the mechanical stability of single macromolecules was applied (Lee et al. 1994; Rief et al. 1997; Marszalek et al. 2001), which exploited an externally applied force as a denaturant. The multidomain protein titin was covalently attached to the AFM stylus with one of its peptide end, while its other end was attached to a supporting surface. Then, stylus and surface were separated stretching the polypeptide chain. At a mechanical tension of a few hundred pN, non-covalent bonds within the titin molecule ruptured, inducing the unfolding of individual proteins. Measuring the length of the polypeptide chain and the applied force allowed for describing the unfolding process. The final state of the unfolded protein is represented by a fully stretched polypeptide chain and therefore lacks any tertiary or secondary interactions. The power of the approach was instantly appreciated, as it assesses the stability of single molecules and

provides information on intramolecular interactions of and between structural domains (Rief and Grubmüller 2002). Experimentally recorded force-distance (F-D) traces can be specific for each type of molecule since they provide a characteristic force spectrum. In certain applications a single force spectrum provides not only a characteristic pattern, but also reveals the energy landscape of the protein. Further developments may allow for time resolved analysis of the forces measured during unfolding and to discriminate between the various interactions contributing to single force peaks. In the meantime this AFM-based approach is commonly referred to as single-molecule force spectroscopy (SMFS), which has been applied to studying dozens of different water-soluble and membrane proteins.

Probing molecular interactions of single membrane proteins

Initial SMFS studies had been focused on proteins, whose biological functions required mechanical resistance. However, further experiments showed that mechanical unfolding could also provide plenty of valuable information on proteins, which are not normally suggested to withstand the mechanical stress (Best et al. 2001; Ohta et al. 2004). Membrane proteins are a perfect example of this case. Mechanical pulling of single bacteriorhodopsins from their native membrane induces the step-wise unfolding of the protein structure (Oesterhelt et al. 2000). In contrast to titin, where the unfolding event of individual domains is probability-driven, the secondary structure elements of a membrane protein unfold sequentially with each unfolding event resulting in characteristic unfolding peak. Due to thermal fluctuations at the molecular level, individual molecules demonstrate certain deviations in their stability. Variations in stability lead to a dispersion in unfolding force for identical domains. Thus, the stability of a domain in SMFS experiments is commonly described by its average unfolding force. Force peak heights and positions in the F-D trace allow for directly determining the strength and locating the position of molecular interactions within the protein and scrutinizing individual unfolding pathways of the protein (Müller et al. 2002c). In some unfolding pathways, transmembrane α -helices unfold in pairs together with their connecting loop. The co-existence of other pathways, however, suggests that transmembrane helices or loops have certain probabilities to unfold in a single event or stepwise via intermediates. This suggests the presence of distinct molecular interactions establishing stable structural segments within the membrane protein. These molecular interactions are collectively formed by all aa in a segment and can be related to local structural features, such as single helical domains (Müller et al. 2002c), helical kinks (Janovjak et al. 2005b), Pi-interactions (Cisneros et al. 2005). It was, however, also possible to detect molecular interactions originating from protein-protein interactions (Sapra et al. 2005) and ligand binding and protein activation (Kedrov et al. 2005a).

Probing mechanical stability of NhaA

Many of above insights on molecular interactions being established within membrane proteins have been originally revealed on bacteriorhodopsin (Müller et al. 2002c; Oesterhelt et al. 2000). At the same time, most of these insights could also be obtained on other membrane proteins, such as halorhodopsin from *H. salinarum* (Cisneros et al. 2005), human aquaporin-1 from red blood cells (Moller et al. 2003), bovine rhodopsin from native disc membranes (Sapra et al., submitted), or NhaA from *E. coli* (Kedrov et al. 2004). Entire unfolding of single NhaA molecule by SMFS resulted in a characteristic force spectrum of ~ 100 nm length (Fig. 2a, b). NhaA could be unfolded by pulling either its N- or C-terminal end, which were both facing the same side of the membrane (Hunte et al. 2005). Accordingly, the resulting F-D curves showed two major classes, each representing pulling from one terminal end. Experiments on cleaved NhaA allowed assign one class to pulling from the C-terminal end and the other one to pulling from the N-terminal end. However, the analysis also showed that the majority of unfolding events reflected pulling the C-terminal end of NhaA; an effect, which may be explained by the different accessibility of both terminal ends to the AFM stylus (Venturi et al. 2000).

To analyze F-D traces, the worm-like-chain (WLC) model (Bustamante et al. 1994) is applied. It describes the entropic stretching x of a polymer, i.e. the polypeptide chain, under an applied force F :

$$F(x) = \frac{k_b T}{l_p} \left[\frac{1}{4} \left(1 - \frac{x}{L_0} \right)^{-2} - \frac{1}{4} + \frac{x}{L_0} \right],$$

where k_b represents the Boltzmann's constant, T the temperature, and l_p the persistence length (for polypeptide chain $l_p = 0.4$ nm). The above equation can be applied to fit every peak in the F-D trace with a WLC model curve (Fig. 2b, red traces). This provides the contour length L_0 , which stands for the physical length of the polypeptide chain stretched. After determining L_0 , trivial counting of the corresponding number of amino acid residues from the stretched polypeptide end localizes the molecular interaction within the protein. The recently resolved structure of NhaA (Hunte et al. 2005) allows mapping molecular interactions onto the protein structure (Fig. 3). These structurally stable segments overlap reasonably well with secondary structure elements. In such cases all aa residues within a secondary structure element collectively establish the stabilizing molecular interactions. As soon as the externally applied force overcomes their stability, the whole segment unfolds cooperatively. At the same time, deviations from the trend were observed. Thus, short unwound region within the α -helix XI (Fig. 3) leads to the loss of cooperativity in unfolding and separates the helix into two stable segments (peaks at 47 and 60 aa). Other detected stable segments (peaks at 125 and 225 aa) cannot be directly correlated with the features of NhaA structure and have been in focus of further studies.

Fig. 2 Detecting and locating molecular interactions within NhaA by forced unfolding experiments. (a) Schematic drawing of the single-molecule force microscopy set-up (not to scale). NhaA molecules embedded into a lipid membrane are immobilized on to a supporting surface and mounted on top of a piezoelectric actuator. Pushing the AFM stylus onto the native protein forces the adsorption of the terminal end (here the C-terminal end) to the stylus. With this molecular bridge the protein could be mechanically pulled leading to its unfolding and extraction from the membrane. (b) The experimental force curve representing an unfolding spectrum of a single NhaA. Fitting individual force peaks in the spectrum with worm-like-chain (WLC) model curves (red lines) (Bustamante et al. 1994) provides length of the polypeptide

stretched at individual unfolding steps (given for each curve) and allows to reconstruct the unfolding pathway of a single molecule. The first force peaks detected at stylus-sample separations below 20 nm indicate the unfolding of transmembrane helices XII and XI. After unfolding these two helices, a 91 aa long polypeptide chain links the AFM stylus and membrane. Separating the stylus further from the membrane stretches the polypeptide thereby exerting a force on helix X. At a certain critical load, the mechanical stability of helices X and IX is overcome and they unfold either together with their connecting loop or via intermediate states (peaks at 107 and 125 aa). Consequent separation of the molecule from the AFM stylus leads to sequential unfolding of the whole NhaA structure. The unfolding pathway is shown on top of the figure

Fig. 3 Single-molecule force spectroscopy locates stable segments within NhaA. Secondary structure of NhaA from *E. coli* (Hunte et al. 2005) contains 12 α -helical domains marked above and their connecting loops. The structure is mapped with stable structural segments (shown in shades of grey) that established barriers against

mechanical unfolding of the protein. The structural regions establishing molecular interactions against unfolding were detected by pulling the C-terminal end of NhaA. Numbers given were determined from WLC fitting the force peaks (Fig. 2b) and locate starting points of barriers

Activation and ligand binding of NhaA

The most remarkable feature of NhaA is its functional regulation by pH. While NhaA is inactive at acidic conditions, it rapidly activates if the cytosolic pH increases to values between 7 and 8 (Taglicht et al. 1991). This makes the protein an emergency and adaptation tool essential for cellular survival in alkaline and saline environment (Padan et al. 2001). Upon adjusting the intracellular pH, NhaA removes sodium ions from the cytoplasm in exchange for protons and reduces the sodium toxicity. The functioning of the protein and its homologues has been a subject of numerous studies during the last decade, as the sodium/proton exchange is crucial in the development of ischemia heart disease and reperfusion (Blatter and McGuigan 1991; Fliegel 1999). While certain functional insights were achieved via biochemical and mutational studies (Gerchman et al. 1999; Tzuber et al. 2004; Zhang et al. 2002), detailed molecular mechanisms of ion transport and regulation of sodium/proton exchange still challenges structural biology. X-ray diffraction on NhaA crystals has recently provided detailed insights into the packing of transmembrane domains (Hunte

et al. 2005) and, particularly, the architecture of Na^+ -binding pocket formed by α -helices IV, V and XI (Fig. 4). In agreement with previous mutational studies (Inoue et al. 1995), a few conserved aa residues are located within the pocket: Asp-133 (α -helix IV), Asp-163 (α -helix V), and Asp-164 (α -helix V). The reported structure of the pH-locked NhaA conformation gives hints on the activation mechanism. It is assumed that upon pH increase NhaA forms an open conformation, and the Na^+ -binding pocket undergoes a structural rearrangement. This conformational change is regulated by a pH-sensor within the cytoplasmic loop VIII-IX (Gerchman et al. 1999) and, most probably, by the neighboring α -helix IX.

To reveal insights into these mechanisms, SMFS was applied to characterize molecular interactions of inactive and active NhaA forms (Kedrov et al. 2005a). Firstly, the force spectra showed that the stability of structure segments established within single antiporters was unaffected upon the activation. This suggested that the antiporter remained properly folded within the membrane. However, one significant change was detected in the case of α -helix V (peak 225 aa in Fig. 2), which is involved in forming the Na^+ -binding pocket

Fig. 4 Na^+ -binding sites of sodium transporters. The functional domain of NhaA (on the *left*) involves α -helix V, and two partly unwound α -helices IV and XI. Core of leucine/sodium transporter LeuT_{Aa} (on the *right*) contains two Na^+ -binding sites embedded by

partly unwound α -helices I and VI, and neighboring α -helix VIII. Positions of a bound Na^+ (yellow) and a leucine molecule are shown. Functionally important residues of both proteins were highlighted

Fig. 5 Activation of NhaA detected in SMFS experiments. (a) Changing the pH increases the stability of the Na^+ -binding domain located at α -helix V. It is accompanied by the increase of the corresponding peak appearance at 225 aa (b). Both tendencies are fitted by a sigmoid curve, and show their mid-point at pH ~ 5.6 . Grey shaded area represents pH values at which the full activity of NhaA was detected using biochemical methods

and hosts the negatively charged residues Asp-163 and Asp-164. The stability of this structural region was increased from 74 ± 29 to 107 ± 26 pN by increasing the pH from 3.8 to 7.7 (Fig. 5a). The process of establishing these molecular interactions at the ligand-binding site was reversible and the protein stability decreased by lowering the pH to acidic values. The increase in strength of molecular interactions was accompanied by their enhanced probability to be detected in F-D traces (Fig. 5b). Being plotted versus pH, the force and probability demonstrated a clear sigmoidal transition with a mid-point at pH 5.6 reaching their maximum values at pH 6.5–7.

To explain the observed phenomena, we ought to assume that alternative molecular interactions were formed at the functional site of the antiporter. These interactions were most probably directly related to the NhaA activation. The pH value at which these molecular interactions reached their full strength lay about 1 pH unit below the reported pH range of NhaA activation (Taglicht et al. 1991). Further enhancing the pH to that of full NhaA activity resulted in the majority of all NhaA molecules establishing the full strength of molecular interactions. The NhaA structure does not suggest that α -helix V plays an active part opening the Na^+ -binding pocket. However, the two adjacent helices IV and XI have short unwound regions which may serve as flexible joints (Fig. 4). Thus, it is assumed that the helices may change their orientation within the membrane plane upon NhaA activation. Such correlations between molecular interactions established within a ligand binding protein may in future provide a novel approach to characterize the protein activation.

SMFS experiments showed that exchanging Na^+ ions for K^+ , but not for Li^+ , caused significant destabilization of helix V at pH 7.7 (Kedrov et al. 2005a). The protein stabilization in the presence of Na^+ or Li^+ was initially assigned to the binding of the ligand to NhaA, and currently available structural information would allow simulating and modeling such a process. Structural constraints of the NhaA ligand-binding site suggests that only non-hydrated sodium and lithium ions are sufficiently small to pass the narrow funnel within the protein and to reach the core of the binding pocket (Hunte et

al. 2005). This structural constriction ensures the selectivity of transport, but does not explain the role of Na^+/Li^+ in establishing the measured molecular interactions. To explain the underlying mechanism, recent structural information on LeuT_{Aa}, a bacterial homologue of human neurotransmitter sodium symporters (Yamashita et al. 2005), may be taken into account. The LeuT_{Aa} structure, which was resolved in complex with sodium ions and the natural substrate leucine, provides fascinating insights into the substrate docking mechanism and at the same time reveals a network of interactions established between the protein domains, sodium ions and a leucine molecule formed within the binding site (Fig. 4). Na^+ ions may play a key role stabilizing the core of the protein, as their electrostatic interactions with negatively charged residues may restrict the structural flexibility and damp its thermal motions. Similar to NhaA, the Na^+ -binding sites of LeuT_{Aa} involve two partly unwound α -helices I and VI in anti-parallel orientation. Both helices form charge dipoles within the hydrophobic core of the membrane. The common specificity for monovalent cations and a clear resemblance in architecture of ligand binding sites of NhaA and LeuT_{Aa}, suggests that similar molecular interactions may be formed within NhaA thereby increasing the domain stability in a similar manner.

SMFS provides a novel avenue to characterize molecular mechanisms being involved in activating a protein. In the first example, ligand-binding to a antiporter could be detected and located. Further single-molecule studies may be focused on the interactions of membrane proteins with specific compounds affecting their activity. Dynamic SMFS may be applied to explore the energy landscape of ligand binding and membrane protein activation. Similarly as SMFS experiments revealed the binding stoichiometry and equilibrium binding constant of DNA-protein interactions (Koch et al. 2002), we here assume that future developments may allow to spatially and temporally observe ligand-binding to native membrane proteins. SMFS may be also applied to directly probe and quantify interactions between a potent drug and a targeted protein (Edwardson and Henderson 2004). The first SMFS experiments characterizing the inhibitor binding to NhaA provides insights into the competition between inhibitor and activator of an antiporter (Kedrov et al., in preparation). For pharmacological purposes one may focus on membrane proteins playing major roles in human health and disease, which are already focused on drug development and targeting.

Observing single NhaA molecules folding

Protein folding, i.e. formation of its unique functional structure, has been a focus of elaborate biochemical and biophysical studies for the last 30 years. Significant progress was achieved in understanding folding mechanisms of globular proteins (Dill 1990; Daggett and Fersht 2003) and transmembrane porins showing a β -barrel fold (Eppens et al. 1997; Kleinschmidt 2003). Meanwhile, the sequence of events leading to a proper fold of a helical membrane protein within the highly anisotropic lipid bilayer is generally un-

clear. Evolutionarily cells have developed enhanced mechanisms of insertion of newly synthesized polypeptide chain into the membrane of the endoplasmatic reticulum (Gier 2005; White and von Heijne 2004). The insertion typically exploits translocon machineries, i.e. ATP-dependent complexes of proteins, which form a pore through the membrane where the nascent polypeptide is inserted. Such controlled folding pathways ensure high folding efficiency and a quality control. At the same time, biophysical studies demonstrated that a number of model polypeptides (Wimley and White 2000; Yano and Matsuzaki 2002) and even the entire polypeptide of a protein (Hunt et al. 1997; Booth 2000) are able to insert into the lipid bilayer spontaneously and to acquire a proper α -helical fold without the help of assisting machineries. Such spontaneous folding of integral membrane proteins has been shown to occur *in vivo* (Andersson and von Heijne 1993; Thompson et al. 1998). Theoretical considerations support these observations as they predict the process to be energetically favorable. A free energy release of $\sim 20 k_b T$ was suggested for folding and membrane partitioning of a single transmembrane α -helix (Popot and Engelman 2000). Both enthalpic and entropic factors play a role in these processes. Folding into an α -helix requires a polypeptide to establish hydrogen bonds between adjacent helical turns, while insertion of the helix into the hydrophobic core of the membrane has entropic origins, as it avoids the unfavorable polar environment of water molecules. The model of Popot and Engelman (2000) divides the folding process of membrane proteins in two distinct steps. Firstly, the polypeptide chain forms helical domains in the membrane-water interface and inserts into the membrane. At this stage, α -helices play a role of basic structural domains, which are independently stable. In the second step, α -helices acquire proper orientation within the membrane and establish mutual tertiary interactions leading to the functional conformation of the protein. However, other observations imply that folding of integral membrane proteins might be more complex. For example, polypeptide chains can adopt intermediate conformations within the membrane or membrane-water interface region (Riley et al. 1997). However, the nature of these intermediates is far from clear. On the other hand, non-native contacts established within the polypeptide can guide the protein to a misfolded conformation (Sanders and Nagy 2000), which lacks functional activity and may even be pathogenic for the organism.

Single-molecule approaches have been intensively applied to protein folding studies (Lipman et al. 2003; Leite et al. 2004). They allow for assessment of conformational dynamics of an individual polypeptide chain along a folding pathway, while measuring its molecular properties, such as effective radius, flexibility, or intramolecular interactions. Here, SMFS measurements have become a useful approach in folding studies, as they provide well-defined information on protein stability and the underlying molecular interactions (Carrion-Vazquez et al. 1999; Fernandez and Li 2004). Once a protein is unfolded using SMFS, the polypeptide chain attached to the AFM stylus is relaxed, as soon as the stylus is moved back to the membrane surface. This leads to a rapid coiling of the polypeptide

chain and allows it to form secondary and tertiary contacts to reduce the free energy. If the polypeptide chain is relaxed for a sufficient time, formation of specific interactions brings it to the energy minimum at the bottom of the energy funnel (Dill and Chan 1997), when all the structural domains are folded and the protein acquires its native state. In other cases, the polypeptide chain may adopt partly folded conformations. Repeatedly pulling of the molecule ruptures re-established molecular interactions and produces a F-D trace of the refolded protein. Comparison of this trace to the one recorded on the native protein provides information regarding the refolding extent of individual structural regions. Initial force spectroscopy experiments on subsequent unfolding and refolding of immunoglobulin domains (Rief et al. 1997; Carrion-Vazquez et al. 1999) resolved their folding kinetics on a time scale of hundreds of milliseconds, which were in reasonable agreement with biochemical data. Further development of the SMFS technique led to observation of folding pathways followed by single molecules, as well as detection of intermediate states and misfolded conformations of globular proteins (Marszalek et al. 1999; Schwaiger et al. 2004; Oberhauser et al. 1999).

SMFS can be also applied to characterize the folding pathways of single integral membrane proteins. In an attempt to show these capabilities, NhaA was chosen since its (un-) folding pathways have been characterized previously by SMFS (Kedrov et al. 2004). In these experiments, the peptide of a single antiporter was unfolded and extracted from the membrane except for the last helices I and II (Fig. 6a). In the following step, the polypeptide chain was relaxed in close proximity (≈ 10 nm) to the native membrane. After a certain relaxation time allowed the peptide to fold and re-insert into the membrane, the peptide end was repeatedly separated from the membrane surface. The F-D spectrum of the refolded peptide typically contained several force peaks at positions being to those observed upon initial unfolding of functional NhaA (Fig. 6b). Since the peaks exhibited the same position it was concluded that certain regions of the polypeptide chain folded and inserted into similar structural conformations as observed for initial unfolding of native NhaA. In most cases, the refolded structures were represented by helical pairs. It is concluded that the pairwise folding and insertion of transmembrane α -helices is a dominant folding pathway of the membrane protein. This finding of pairwise folding and insertion of transmembrane helices strongly supports its hypothesis which was proposed 25 years ago (Engelman and Steitz 1981), and experimental studies pointing towards this hypothesis (Kuhn 1987; Johnson et al. 2004).

The probability of observing certain force peaks in unfolding curves showed a clear dependence on the refolding time (Fig. 6c). This correlation allowed first insights into folding kinetics of individual structures. Measuring refolding probabilities of structural segments within an extended time range (0.01–15 s) enabled us to estimate the folding rate for every segment (Kedrov et al. 2005b). Folding rates varied between 0.3 s^{-1} and 2 s^{-1} , which were in good agreement with the values coming from bulk experiments. The ob-

Fig. 6 Refolding a single membrane protein. (a) Schematic drawing of the experiment. (b) Experimental data. After tethering the C-terminal end of a single NhaA to the AFM stylus, the protein was unfolded (top red curve) except for the last two helices, which were left in the membrane. The AFM stylus was then lowered towards the membrane surface (blue curve) thereby allowing the protein to refold. After a certain refolding time, the molecule was unfolded again (red curves) to detect the refolding of the helices. The force spectrum

contained a set of peaks, which were assigned to refolded stable segments. WLC fitting values are shown above peaks. Refolding extents strongly depended on refolding time. (c) Refolding kinetics of NhaA helical pairs. Fitting by single exponential dependence gives folding rate values (see text). *Inset*: ultra-fast folding kinetics of the α -helix V, Na^+ -binding domain. (d) NhaA stability is dependent on folding time suggesting that tertiary interactions are formed between refolded domains

served dispersion of folding rates of structural segments such as α -helices may originate from local features of primary structure. This assumption may be reflected by the helical pair V and VI, which demonstrate the highest folding rate and yield among other helical pairs of NhaA (Fig. 6c) and at the same time represent the most hydrophobic helices being connected by a very short periplasmic loop of 6 residues (Fig. 3). In this case, it is straightforward to assume that the insertion of these hydrophobic residues into the hydrophobic core of the membrane is highly favorable. Additionally, helices V and VI represent the only helical pair of NhaA lacking proline residues. The isomerization of proline residues slows down the proper folding of α -helices (Lu et al. 2001). Since the observed folding of helical pairs can be described as a single exponential dependence over time, it may be suggested that they fold independently of each other. Interestingly, the measured stability of the refolded domains

showed a gradual increase over time (Fig. 6d). The origin of this trend is not yet clear, but it is tempting to speculate that the refolded domains mutually stabilize each other and thus the stability increases with the refolded structures.

In contrast to the rest of the protein, the helical pair V and VI folded via an intermediate state (Kedrov et al. 2005b). Within the first 50 ms of folding the domain formed a stable structural segment within the membrane resulting in a force peak at aa 225. As described above, this force peak is assigned to the Na^+ -binding pocket of NhaA located at α -helix V being located within the hydrophobic core of the membrane. This domain, which is characterized by an ultra-fast folding rate of 47 s^{-1} (Fig. 6c, inset), was shown to acquire its folded state even against an externally applied force of ~ 30 pN. The formation of the ligand binding domain is then followed by the much slower folding of the helical pair V and VI. Both helices mostly contain hydrophobic

alanine and isoleucine residues, which promote the formation of a transmembrane domain. Presence of functionally important aspartates Asp-163 and Asp-164 (Inoue et al. 1995) suggests that the high hydrophobicity of both helices is required to compensate the unfavorable insertion of both negatively charged residues. The observations of this folding hierarchy and priority may further imply that the formation of the Na⁺-binding pocket of NhaA was finely “tuned” during evolution, which ensured its proper fold and enhanced stability.

The recent development of SMFS allows for observation of the behavior of a polypeptide chain under constant force, a technique called force-clamp spectroscopy (Fernandez and Li 2004). If the clamped force is sufficiently low, small globular proteins are able to follow their folding pathway and to acquire their functional conformation. Measurements on ubiquitin showed a presence of several distinct phases of the folding process, which were characterized by different molecular dynamics and flexibility (Fernandez and Li 2004). In the case of membrane proteins, the approach is potent to provide deeper insights on different folding stages, which have been described by bulk techniques (Booth 2000). Thus, the approach may assess the dynamics of pre-folded coiled state and to distinguish folding phases of individual structural domains into the functional membrane protein.

Membrane protein misfolding

The energy landscape is a convenient model that describes all possible conformations of a protein in terms of their free energies (Dill and Chan 1997). It suggests the presence of multiple separated energy minima corresponding to stable compact states which a polypeptide can acquire. Only one of these conformations represents a functional form of the protein, while others may represent stable intermediate states on the folding pathway, or kinetically trapped misfolded forms (Aguzzi and Haass 2003). The pathogenic role of misfolded forms of protein, their origins and structure, belong to the most important topics in current biology. Studies performed on membrane protein misfolding suggest possible

origins of the phenomenon, such as the formation of non-native polar contacts, alternative conformations of transmembrane domains, influence of lipid environment or point mutations (see, e.g., Wigley et al. 2002; Forloni et al. 2002). As we are still far from general understanding of the folding and stabilization processes, protein misfolding challenges modern approaches which would reveal its molecular mechanisms and regulation.

SMFS experiments on NhaA folding have provided certain insights on membrane protein misfolding (Kedrov et al. 2005b). Here again, the capability to assess the stability and location of every structural domain was exploited. The most prominent observation was the appearance of new unexpected force peaks in unfolding spectra of refolded NhaA molecules. The probability to observe such force peaks did not depend on the refolding time, so evidently the proteins were trapped in alternative conformations. A non-characteristic peak at 180 aa was detected for ~15% of refolded NhaA molecules (Fig. 6b) suggesting a relatively high propensity of the helical pair VII and VIII to misfold. This helical pair demonstrates the slowest folding rate of 0.3 s⁻¹ and a very low folding yield, reaching 20% after 15 s. The helical pair is formed by two unusually short α -helices of 14 residues each and a connecting loop of 6 residues (Fig. 3). Thus, these helices could not protrude the whole lipid bilayer, as this would require at least 20 residues per helix. It may be therefore concluded that the segment may insert into the membrane in some alternative compact conformation, when polar residues of the loop VII–VIII are buried in a core, or it forms an elongated 3₁₀-helix bringing the hydrophilic loop to the periplasmic membrane surface. Another possible pathway for membrane proteins to misfold may be their aggregation in membrane-water interface. Multiple polar and non-polar interactions could be formed within the polypeptide chain, which could possibly result in a non-native conformation. We assume that further SMFS studies, particularly those involving force-clamp approach, may provide further insights on processes occurring in the interface region and reveal their role in formation of a functional protein structure.

Single-molecule force measurements on membrane proteins

STRUCTURE-FUNCTION RELATION	ORIGINS OF MOLECULAR INTERACTIONS	FOLDING	LIFE-TIME OF MOLECULAR INTERACTIONS	ENERGY LANDSCAPE OF A PROTEIN
Oligomeric state	Structural features (helical kinks, salt bridges etc.)	Protein folding and membrane insertion	Studying interactions on microsecond time scales	Location and geometry of transition states
Conformational changes	Protein-lipid & protein-protein interaction	Folding pathways and intermediates	Molecular interactions under periodic and constant loads	Unfolding rates of individual domains
Ligand binding	Type of molecular interactions	Folding kinetics		Free energy of unfolding
Protein inhibition and drug screening	Influence of environmental factors on molecular interactions	Origins of protein misfolding		Roughness of the energy landscape
Binding constants				

Fig. 7 Major research frontiers achieved characterizing membrane proteins by single-molecule atomic force microscopy and force spectroscopy. The information provided by both techniques allows detailed insights into the structure–function relationship of mem-

brane proteins, their molecular interactions, their folding pathways and kinetics, the origins of molecular interactions and into their energy landscapes

Summary and Outlook

Single-molecule AFM imaging and force spectroscopy of native membrane proteins provide powerful approaches to characterizing the structure-function relationship and their underlying molecular interactions as summarized in Fig. 7. While AFM imaging allows characterizing surface structures of native membrane proteins at subnanometer resolution, single-molecule analysis reveals their individual conformations. The structural variability and flexibility of structural regions such as polypeptide loops, channel entrances, or domains can be assigned and functionally related conformational changes observed. The oligomeric state and the complex formation of membrane proteins can be directly followed depending on physiologically relevant conditions. These approaches have been demonstrated on various different membrane proteins. In the future AFM imaging may be applied to observe conformational changes of transporters, as well as their complexes, their structural variability and flexibility. It is only a question of time until the newest ultrafast AFM imaging methods will be capable of directly visualizing the various processes of transporters in realtime.

SMFS detects individual unfolding pathways of single membrane proteins under physiological relevant conditions. Molecular interactions stabilizing structural segments within the functional antiporter NhaA were localized and quantified. Taking examples of other membrane proteins, it could be shown that the strength of these molecular interactions sensitively depend on the environment of the protein, such as structural modifications, temperature, or alterations of the macromolecular assembly. It may be expected that the molecular interactions established within NhaA react similarly on environmental changes. Small changes of these molecular interactions can cause the protein to populate certain unfolding pathways differently. The ability of SMFS to operate within a wide range of environmental factors (pH, salinity, temperature) makes the technique powerful in studying the mechanisms (de-)stabilizing native transporters and determining their (un-)folding pathways (Fig. 7).

On the example of NhaA it could be shown for the first time that SMFS is capable of detecting molecular interactions associated with ligand binding and functional activation. Moreover, it was possible to locate the molecular interaction on the three-dimensional structure of the transporter. The single-molecule study allows the determination of the percentage of NhaA molecules having established the activating molecular interaction at their ligand-binding site. It was observed that some NhaA molecules could already establish the full strength of this molecular interaction at pH values below that determined to fully activate the antiporter activity. However, at pH 7–8 fully activating the sodium/proton transport, all antiporters have established the full strength of this characteristic molecular interaction at the ligand-binding site. In future, such experiments may be applied to observe and to locate the binding of certain molecules or drugs to transporters. Ongoing experiments already indicate that SMFS also allows for detecting and locating the binding of an inhibitor to NhaA. Competing measurements having

both the activating ligand and the inhibitor exposed to the transporter may in future allow revealing insights into the underlying activating and inhibiting mechanisms. Such insights may be in particular interesting for pharmaceutical research on certain members of the secondary transporter family.

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