Folding mechanisms of β-barrel outer membrane proteins and their catalysis by natural holdases and foldases

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Summary. Although membrane proteins are essential for every basic process of life, the folding mechanism of a freshly synthesized polypeptide into the functional membrane protein is hardly understood. This PhD project aims at determining molecular aspects of the folding mechanisms of integral outer membrane proteins by a combination of solution NMR spectroscopy and single-molecule atomic force microscopy (AFM). The project aims at a characterization of the folding mechanisms of polypeptides into membrane mimicking environments. Furthermore, we will characterize the effect of chaperones (holdases) and folding catalysts (foldases) on the folding kinetics and thermodynamics. This combination of structural biology and nanoscience will provide new insight into the outer membrane biogenesis and establish nanomechanical methods for the characterization and manipulation of integral membrane proteins on the single-molecule level.

Introduction. β-barrel membrane proteins are essential components of the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts. Membrane proteins have highly interesting folding properties, since they fold in an external environment that comprises hydrophobic and hydrophilic phases. The biogenesis of these outer membrane proteins (Omps) poses a complex biophysical challenge to the pro- and eukaryotic cell, because the Omps are synthesized at locations distant from their target membrane (1). In the absence of a membrane, the Omp polypeptide chains are prone to aggregation in aqueous cellular compartments. They can thus not reach their cellular destination by self-diffusion, but require to be conveyed by molecular chaperones. This overall essential biological function is accomplished by molecular chaperones that pass the unfolded substrates from the ribosome to the destination membrane (2). In the gram-negative bacterium E. coli, the periplasmic chaperones SurA and Skp transport the substrate to the Bam complex, which folds and inserts the substrates into the outer membrane (3). The in vitro and the in vivo folding mechanisms of β-barrel Omps from mitochondria or Gram-negative bacteria are so far not understood at atomic resolution. In contrast to the in vivo situation, the same polypeptide chains can refold in vitro in the absence of chaperones and other proteins, but resulting in the same three-dimensional β-barrel structures. Typical folding times for these proteins are on a timescale of minutes to hours. Since the three-dimensional protein structure as well as an autonomous folding pathway is encoded entirely in the amino acid sequence of β-barrel Omps, a detailed understanding of the in vitro folding process will be very helpful, if not essential, to fully understand chaperone mediated folding in vivo.

Goal. The goal of this thesis is to describe molecular folding mechanisms of integral membrane proteins and the effect of varied external conditions, including the effect of native holdases and foldases on these mechanisms. We will use a combination of AFM and NMR spectroscopy to unravel the folding pathways of model substrates.

Single-molecule AFM. Using AFM-based single-molecule force spectroscopy (SMFS) we will investigate the insertion and folding of completely unfolded Omp polypeptides (OmpG, OmpA, and FhuA) into lipid membranes. In the past we have established SMFS to unfold and refold partially unfolded Omps (Figure 1, refs. 4–6). Bottlenecks of this previous approach will be overcome by covalently tethering the unfolded Omp polypeptide to the AFM tip and by approaching the tethered polypeptide the membrane. Deflections of the AFM cantilever

will detect insertion and folding attempts of the unfolded polypeptide into the membrane. Time-resolved measurements will measure the insertion kinetics, the free energy of insertion, and of the secondary structures folded after certain time spans. To approach the complexity of the cell the folding and insertion of Omps into lipid membranes will be characterized in the presence of chaperons and foldases. In combination with NMR experiments this approach will help us to understand the factors that control and support the insertion and folding of membrane proteins into membranes.

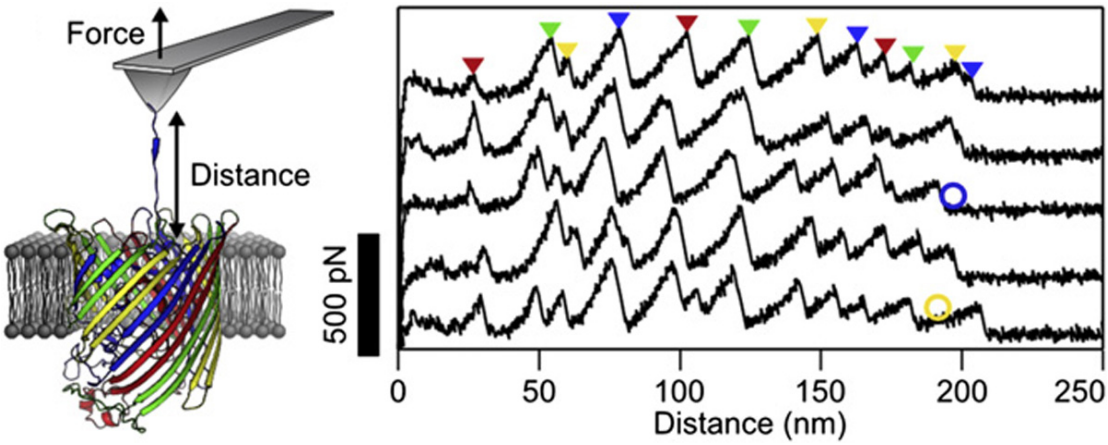

Figure 1. Schematic view of the AFM experimental steup to unfold integral membrane proteins. The barrel is pulled from them membrane by the cantilever (left panel). Thereby, the force is measured as a function of the distance. Right panel: Typical data set for an outer membrane protein. The colored arrows indicate separation events of individual strands from the barrel.

NMR spectroscopy. We will adapt the proton/ deuterium exchange labeling technique (7) to membrane proteins. Protein refolding is initiated by rapid dilution of a denatured protein sample with micelle solution. After a variable folding time τ, the buffer is rapidly mixed with a larger volume of D_2O. In the folded state, all amide protons involved in the β-barrel are strongly protected with exchange times of the order of weeks or longer and we can thus quantify the proton occupancy for each residue in the β-barrel using 2D [^{15}N,^{1}H]-TROSY NMR experiments (8). In subsequent experiments, τ is scanned through the entire folding time scale. Initial experiments have established that refolding at decreasing folding times leads to increased incorporation of deuterium into the barrel, which can be quantified by solution NMR spectroscopy. The chaperone-stabilized state occurring in vivo is thus fully refolding-competent in vitro (Figure 2, Ref. 9). We will study the impact of the membrane mimic, as well as external and solution parameters on the refolding kinetics. The final phase of the project will relate the in vitro folding mechanism to the situation in vivo. We thus will be able to compare the kinetic data of the in vivo and the "in vitro"/Skp-mediated folding mechanisms.

Sample preparations. As starting points for the experiments, we have the following systems biochemically available: The chaperones Skp and SurA, the foldase BamA–E, as well as several Omp substrates, including OmpA, OmpX and OmpF. For all of these, biochemical expression and purification protocols are established and samples can be produced in mg amounts.

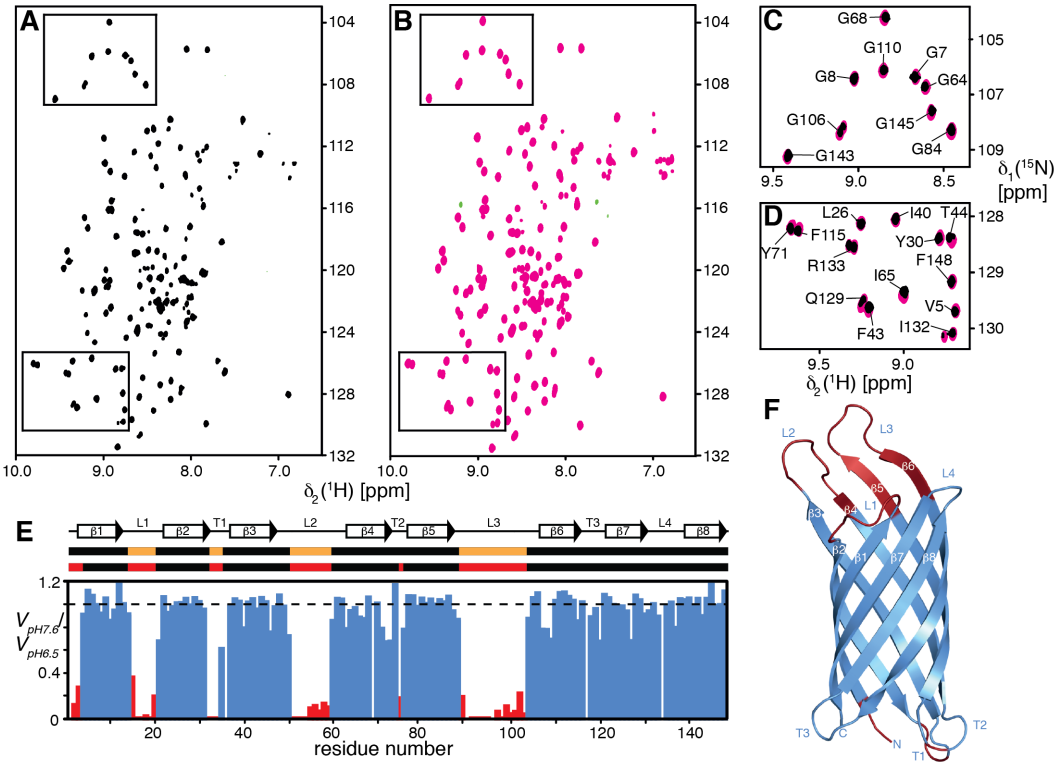

Figure 2. OmpX refolding from different denatured states. A) 2D NMR spectrum of OmpX in LDAO micelles, refolded from the chaperone Skp and (B) from 6 M guanidine hydrochloride solution. C), D) The perfect overlay of the two spectra indicates that the protein folds into the same 3D structure in both experiments. E) Amide exchange as measured by pH variation. (F) Solution NMR structure of OmpX refolded in DHPC micelles in ribbon representation (PDB 1Q9F; ref. 10).

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