Membrane Proteins

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One β Hairpin after the Other: Exploring Mechanical Unfolding Pathways of the Transmembrane β-Barrel Protein OmpG**

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Single-molecule force spectroscopy (SMFS) is a unique approach to study the mechanical unfolding of proteins.[1,2] 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),[3] 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 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.[2] 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 SMFS.

The structure of OmpG comprises 14 β strands that form a transmembrane β-barrel pore.[4] Six short loops (T1–T6) on the periplasmic side and seven longer loops (L1–L7) on the extracellular side connect the individual β strands. OmpG is gated by loop L6, which controls the flux of small molecules through the pore and the permeability of the bacterial outer membrane in a pH-dependent manner.[4,5] Being able to withstand rather harsh environmental conditions, OmpG forms a robust pore, which makes it suitable for application as a biosensor.[6]

In our SMFS experiments, OmpG reconstituted in *E. coli* lipid membranes were first imaged by AFM.[7] The AFM tip was then pushed onto the OmpG surface to facilitate the nonspecific attachment of the N or C terminus (Figure 1a).

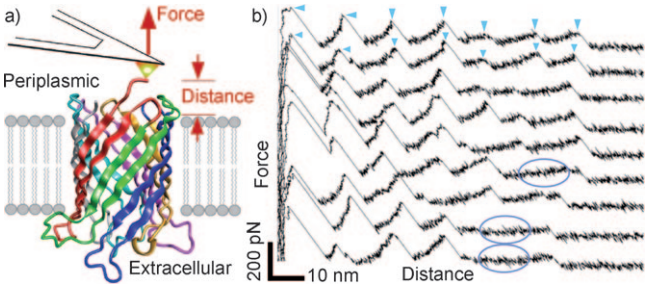

Figure 1. Mechanical unfolding of the β-barrel membrane protein OmpG. a) Structural representation of a single OmpG molecule non-specifically attached to the tip of an AFM cantilever. An increase in the distance between the tip and the membrane establishes a force that induces unfolding of OmpG. b) Force–distance (F–D) curves recorded during the unfolding show certain force peaks (marked by arrows) that correspond to the unfolding intermediates of OmpG (Figure 2). Occasionally, F–D curves lacked force peaks (marked by ellipses); this indicates alternating unfolding pathways.

Withdrawal of the AFM tip stretched the terminus and induced unfolding of OmpG. Force–distance (F–D) curves recorded the interactions that occured upon unfolding of a single OmpG molecule (Figure 1b). We analyzed only F–D curves that correspond to the fully stretched length (> 75 nm) of an unfolded OmpG polypeptide (281 amino acids). This selection criterion ensured that OmpG was mechanically unfolded by stretching one of its termini.[2]

Individual F–D curves showed a series of force peaks that varied in occurrence (Figure 1b). 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 2a). The superimposition of all of the F–D curves showed a clear pattern of predominant force peaks (Figure 2b). After determination that OmpG unfolded from pulling on the N terminus (Supporting Information, Figure S1), each force peak was fitted by using the wormlike-chain model (WLC) to reveal the lengths of the unfolded

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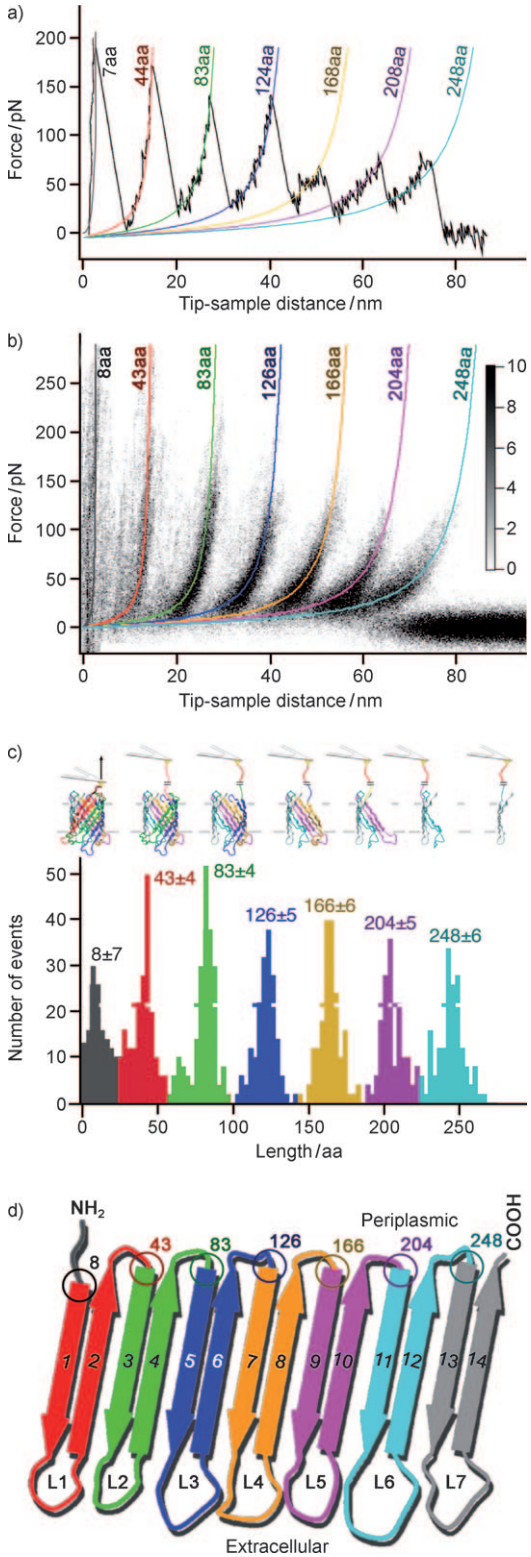

Figure 2. Unfolding intermediates and pathways of OmpG. a) The F–D curve obtained from the unfolding of a single OmpG molecule. The colored lines are wormlike-chain model (WLC) fits to individual force peaks. The numbers denote the contour lengths (amino acids, aa) of the unfolded polypeptide chain obtained 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, β hairpins unfold individually. d) The average contour lengths of the force peaks (encircled regions) identify the β hairpins (equally colored) that unfold individually. The β strands and extracellular loops (L1–L7) are numbered.

polypeptide stretches (Figure 2b,c). Determination of the unfolded polypeptide stretches allowed assignment of the structural segment that unfolded and to describe the unfolding intermediate (Figure 2c,d). Each unfolded structural segment was created by two β strands forming one β hairpin. Although the heterogeneous population of the F–D curves indicated multiple unfolding pathways that coexist, the common pattern from superimposition of the curves showed that the unfolding intermediates taken by the protein were similar. However, whereas some individual unfolding pathways described by some F–D curves suggested that every β hairpin unfolded individually, other F–D curves suggested that two adjacent β hairpins could also unfold collectively. With 14 β strands forming 7 β hairpins, OmpG showed structural segments that could unfold individually. This results in 6 unfolding intermediates. The probability that every β hairpin unfolded individually was 31%, that one pair of β hairpins unfolded cooperatively was also 31%, that two pairs of β hairpins unfolded cooperatively was 23%, and that three pairs of β hairpins unfolded cooperatively was 8% (Supporting Information, Table S1). 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.

Bulk unfolding experiments suggest that OmpG unfolds and folds reversibly.[8] The folding process is thought to be a coupled two-state membrane partition–folding reaction.[9] 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}),[9] similar to that of GFP (> 10–25 kcal mol^{-1}).[10] Our results shows 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[2] and than those (ca. 100 pN) required to unfold the entire β-barrel protein GFP[3] at similar conditions. Thus, our experiments suggest that OmpG is an 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.[15]

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.[3] 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.[1] 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. In the case of transmembrane α-helical proteins, it was shown that the mechanically unfolded polypeptide can fold back into the membrane bilayer.[11,12] Whether the mechanical unfolding of transmembrane β-barrel proteins is reversible too is a question currently under investigation.

Each β hairpin of the OmpG β barrel can unfold individually, or cooperatively with an adjacent β hairpin. This variety yields a diversity of unfolding structural segments, intermediates, and pathways that is not observed in the mechanical unfolding of water-soluble β-barrel proteins.[3] 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.[2,13] 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.[2] It remains to be investigated under which conditions β hairpins may alter their propensity to unfold individually. 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.

Experimental Section

SMFS: OmpG was purified from inclusion bodies, refolded in detergent, and reconstituted into native *E. coli* lipids.[4] Study of the membranes showed the OmpG molecules being densely packed and assembled into two-dimensional crystals.[14] These OmpG membranes were adsorbed onto freshly cleaved mica (30 min) in buffer solution (pH 7; 25 mM tris(hydroxymethyl)aminomethane–HCl, 25 mM $MgCl_2$, 300 mM NaCl). After this, the membranes were localized by AFM in the same buffer solution at room temperature.[7] For SMFS, the AFM cantilever tip (60 μm long Biolever, Olympus) was pushed onto the OmpG membrane with the application of 500–750 pN of force for 500 ms. In approximately 0.1 % of all cases the OmpG terminus attached to the AFM tip. The AFM tip was then retracted at 600 $nm\,s^{-1}$ to induce unfolding. 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 being either densely packed or crystallized two-dimensionally showed no difference in pattern. Before and after each experiment, the spring constant of each cantilever (0.03 $N\,m^{-1}$) was estimated from its thermal noise by using the equipartition theorem.[16]

Data analysis: 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 >75 nm long. The F–D curves were fitted by using the WLC model and superimposed as previously described.[2,7]

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[1] A. Borgia, P. M. Williams, J. Clarke, *Annu. Rev. Biochem.* **2008**, *77*, 101.
[2] A. Kedrov, H. Janovjak, K. T. Sapra, D. J. Muller, *Annu. Rev. Biophys. Biomol. Struct.* **2007**, *36*, 233.
[3] H. Dietz, M. Rief, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16192.
[4] O. Yildiz, K. R. Vinothkumar, P. Goswami, W. Kühlbrandt, *EMBO J.* **2006**, *25*, 3702.
[5] S. Conlan, Y. Zhang, S. Cheley, H. Bayley, *Biochemistry* **2000**, *39*, 11845.
[6] M. Chen, S. Khalid, M. S. Sansom, H. Bayley, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6272.
[7] D. J. Müller, A. Engel, *Nat. Protoc.* **2007**, *2*, 2191.
[8] S. Conlan, H. Bayley, *Biochemistry* **2003**, *42*, 9453.
[9] L. K. Tamm, H. Hong, B. Liang, *Biochim. Biophys. Acta Biomembr.* **2004**, *1666*, 250.
[10] M. Ishii, J. S. Kunimura, H. T. Jeng, T. C. Penna, O. Cholewa, *Appl. Biochem. Biotechnol.* **2007**, *137–140*, 555.
[11] A. Kedrov, H. Janovjak, C. Ziegler, W. Kühlbrandt, D. J. Muller, *J. Mol. Biol.* **2006**, *355*, 2.
[12] M. Kessler, K. E. Gottschalk, H. Janovjak, D. J. Muller, H. E. Gaub, *J. Mol. Biol.* **2006**, *357*, 644.
[13] H. Janovjak, M. Kessler, H. E. Gaub, D. Oesterhelt, D. J. Muller, *EMBO J.* **2003**, *22*, 5220.
[14] M. Behlau, D. J. Mills, H. Quader, W. Kühlbrandt, J. Vonck, *J. Mol. Biol.* **2001**, *305*, 71.
[15] X. Kang, L.-Q. Gu, S. Cheley, H. Bayley, *Angew. Chem.* **2005**, *117*, 1519; *Angew. Chem. Int. Ed.* **2005**, *44*, 1495.
[16] H. J. Butt, M. Jaschke, *Nanotechnology* **1995**, *6*, 1.

