Membrane Proteins

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One β Hairpin Follows the Other: Exploring Refolding Pathways and Kinetics of the Transmembrane β-Barrel Protein OmpG**

*Mehdi Damaghi, Stefan Köster, Christian A. Bippes, Özkan Yildiz, and Daniel J. Müller**

Despite their enormous relevance to cellular vitality, the folding mechanisms of only a few transmembrane proteins have been studied. From these studies, only a handful of β-stranded membrane proteins were characterized.[1] Current models describe that transmembrane β barrels fold into the lipid membrane in two major steps. Firstly, the unfolded polypeptide interacts with the lipid surface where it folds, tilts, and then inserts into the membrane.[1b,2] Consequently, it is thought that single β strands and β hairpins form unstable units, and that β-barrel proteins (pre-)fold prior to their insertion into the cellular membrane.[3] Experiments studying the (un-)folding of membrane proteins are conventionally carried out by using thermal or chemical denaturation. In most cases, membrane proteins that were solubilized in detergent and/or exposed to approximately 4–10 M urea were studied. In vivo membrane proteins fold under different conditions. Thus, the folding pathways studied may be different from those that occur in nature.

Single-molecule force spectroscopy (SMFS) represents a unique approach to studying the refolding of membrane proteins into the lipid membrane.[4] SMFS is used to unfold and refold membrane proteins under conditions typical for their physiological environment such as pH, electrolytes, temperature, and, importantly in the absence of any chemical denaturant or detergent.[5] In such experiments, a single membrane protein is first mechanically unfolded and its polypeptide is fully stretched. Then this unfolded polypeptide is relaxed to allow refolding into the membrane bilayer. Repeated mechanical unfolding is used to determine which structural regions of the membrane protein are refolded. Allowing the polypeptide different refolding times addresses the refolding kinetics of structural regions. Thus, SMFS can be used to detect the mechanical unfolding pathways and the equilibrium refolding pathways of a membrane protein. In previous SMFS work, the mechanical unfolding and refolding of many different water-soluble proteins have been investigated.[6] However, compared to the variety of water-soluble proteins that were characterized, SMFS of membrane proteins reveals much more detailed unfolding and folding pathways.[4] To date, the refolding of β-barrel membrane proteins into a lipid membrane has never been addressed by SMFS.

Herein we report the application of SMFS to unfold and refold the outer membrane protein G (OmpG) from *Escherichia coli* (Figure 1). The structure of OmpG comprises 14 β strands that form a transmembrane β-barrel pore.[7] Six short turns connect individual β strands on the periplasmic side and seven longer loops (L1–L7) on the extracellular side. In vitro experiments show that OmpG is gated by loop L6, which controls the permeability of the pore in a pH-dependent manner.[7,8] In previous SMFS studies, we found that the β barrel of OmpG unfolds via many intermediates.[9] The main unfolding pathway described the stepwise unfolding of single β hairpins. This unfolding pathway was much more detailed than that detected for the water-soluble β-barrel green fluorescent protein (GFP),[6b] which mainly unfolds in one step when a sufficiently high pulling force was applied.

In our refolding experiments, OmpG that had been reconstituted in native *E. coli* lipid membranes was first imaged by AFM.[8b] Then, the AFM tip was pushed onto the OmpG surface to facilitate the nonspecific attachment of the N terminus (Figure 1).[9] Withdrawal of the AFM tip stretched the terminus and induced the unfolding of OmpG. Force–distance (F–D) curves recorded the force peaks that reflect the unfolding steps of a single OmpG (Figure 1). Each unfolding step represents that of a β hairpin of the transmembrane β barrel.[9] To refold the partially unfolded OmpG, we stopped withdrawal before unfolding the last β hairpin VII. Then, we relaxed the unfolded polypeptide by approaching the AFM tip close to the membrane (ca. 5 nm). After a given time to allow the polypeptide to refold, the protein was unfolded again to probe which structural regions refolded into the lipid membrane (see Figure S1 in the Supporting Information).

Individual F–D curves of the refolding polypeptide showed a series of force peaks that varied in occurrence (Figure 1). These force peaks were detected at similar positions as upon initial unfolding of OmpG. If β hairpins had folded without inserting or had attached to the membrane surface, the force peaks would have been detected at shifted positions (see the Supporting Information, Part 2). Similarly, force peaks which are characteristic for the folding of membrane proteins, would have changed their position if misfolding events had occurred.[4b,5] Thus, the unfolded OmpG polypeptide folded and inserted single β hairpins into the native *E. coli* lipid membrane. Probing the content of refolding in dependence of different refolding times (0.1–5 s)

[*] M. Damaghi, Dr. C. A. Bippes, Prof. Dr. D. J. Müller
ETH Zürich, Dept. of Biosystems Science and Engineering
4058 Basel (Switzerland)
Fax: (+41) 61-387-3994
E-mail: daniel.mueller@bsse.ethz.ch
Dr. S. Köster, Dr. Ö. Yildiz
Max-Planck-Institute of Biophysics
60438 Frankfurt am Main (Germany)

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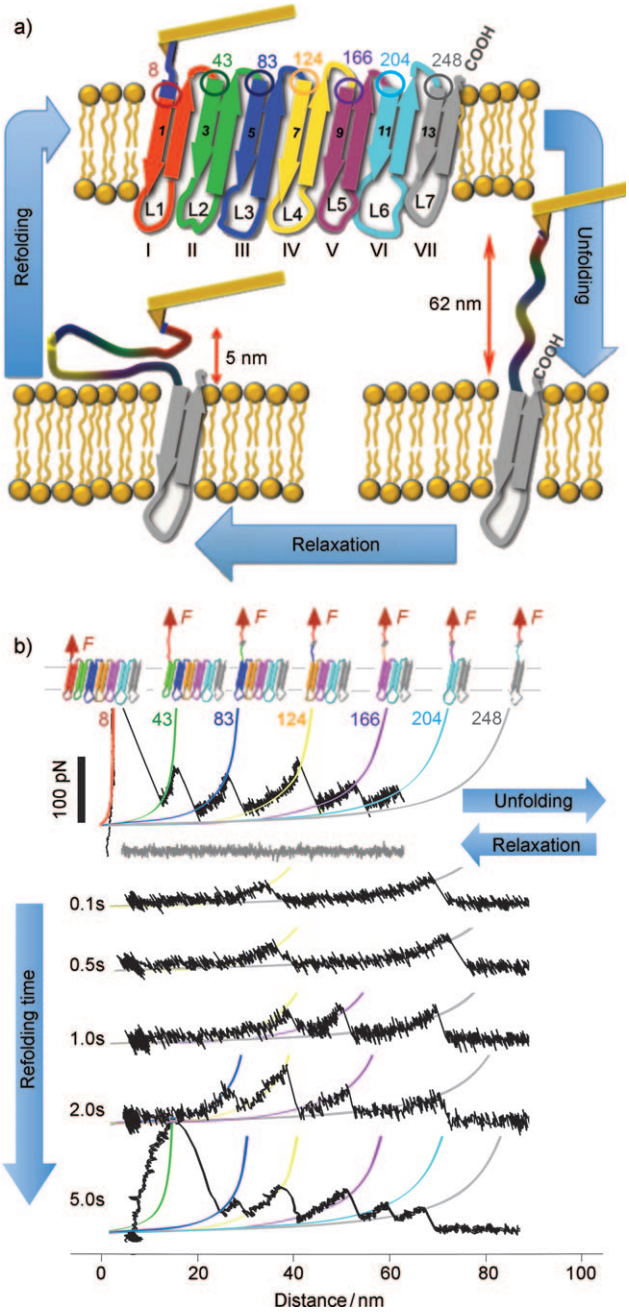

Figure 1. a) Experimental procedure and b) results for refolding of the β-barrel membrane protein OmpG into a lipid membrane. The tip of an AFM cantilever attaches to the N-terminal end of OmpG and applies a mechanical pulling stress. An F–D curve (black) shows the consecutive unfolding of single β hairpins (I–VII). Each of the force peaks marks the unfolding of a single β hairpin.[9] Fitting every force peak using the wormlike chain model (colored curves) reveals the contour length of the unfolded peptide (number of amino acids (aa) given at every fit) and marks the unfolded β hairpin (secondary-structure model in (a)). Before the last β hairpin (gray) unfolds, the AFM tip is brought close to the membrane (ca. 5 nm) to relax the polypeptide. After a given refolding time, the tip is withdrawn to detect which structural regions have refolded. F–D curves recorded of the refolded polypeptide show force peaks at the same positions as recorded for initial unfolding.

revealed insights into which β hairpins refolded before others (Figure 2). Of all refolding times probed, β hairpin IV folded

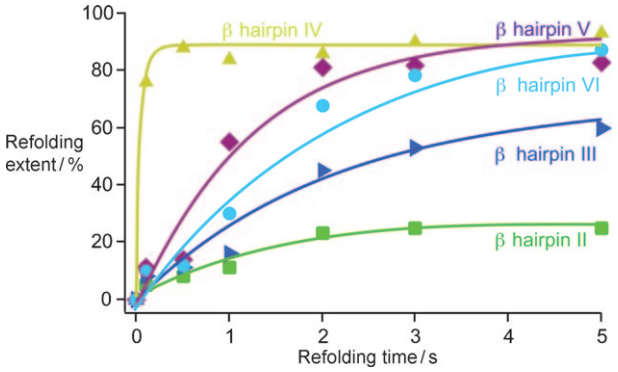

Figure 2. Refolding kinetics of β hairpins of OmpG. 189 refolding experiments were analyzed.

fastest ($k_{fold} = 20\ s^{-1}$). After this, β hairpin V folded ($k_{fold} = 0.90\ s^{-1}$), then β hairpin VI ($k_{fold} = 0.60\ s^{-1}$), β hairpin III ($k_{fold} = 0.45\ s^{-1}$), and β hairpin II ($k_{fold} = 0.01\ s^{-1}$). This kinetic hierarchy describes the main refolding pathway of OmpG by which the transmembrane β barrel assembles. A reconstruction of this main refolding pathway is shown in Figure 3. The interactions that stabilize each β hairpin increased with the refolding time and, thus, with the number of hairpins folded (see Figure S2 in the Supporting Information). As the refolding time and content increased the refolded β hairpins approached similar stability, as observed upon initial unfolding of the β barrel.

Bulk unfolding experiments suggest that OmpG unfolds and folds reversibly.[8a] The folding process is thought to be a coupled two-state membrane partition-folding reaction.[1] In contrast, the mechanical unfolding of OmpG occurs via many sequential unfolding intermediates.[9] The unfolding intermediates and, thus, the unfolding pathways of membrane proteins depend on the environmental parameters.[5] For OmpG, these unfolding intermediates even depend on the functional state of the protein.[10] In our SMFS experiments, OmpG was embedded in native *E. coli* lipids and investigated in a physiological buffer solution at room temperature. Conventional unfolding experiments that use thermal or chemical denaturants investigated unfolding scenarios of (in most cases solubilized) OmpG. Thus, it is most probable that

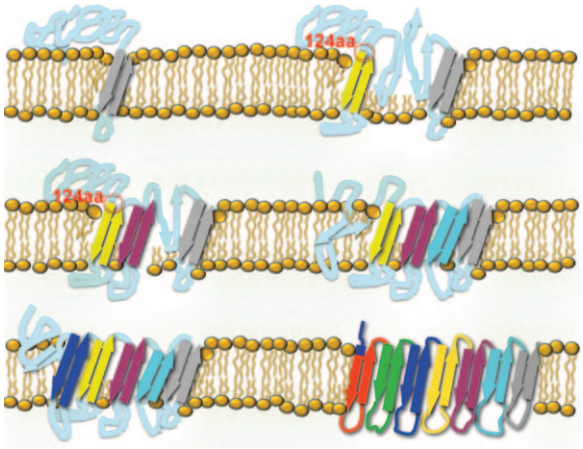

Figure 3. Main refolding intermediates and pathway of OmpG.

the discrepancies in OmpG unfolding determined by SMFS and conventional denaturing are a result of different experimental conditions.

Our SMFS experiments show that a partially unfolded OmpG molecule refolds over many sequential steps. Predominant refolding steps are defined by individual β hairpins that later assemble to the transmembrane β barrel. In contrast to the initially induced mechanical unfolding, the refolding steps occur in a different order. Whereas the sequence of unfolding steps is strongly influenced by the mechanically pulling AFM tip, no externally applied force constrains the refolding of the unfolded OmpG polypeptide. Thus, the unfolded polypeptide could follow its refolding pathway. Each β hairpin showed intrinsic folding kinetics that determined the refolding pathway of the OmpG polypeptide into the membrane.

The finding that β hairpins independently form stable folding units in a lipid membrane is unexpected.[1,3] Our experiments suggest that once inserted into the bilayer, the β hairpins assemble with each other to form the β barrel. It may be suggested that the first stage of β-hairpin insertion and folding is driven by hydrophobic interactions. Consequently, backbone hydrogen bonds that stabilize the β barrel must be formed after the β hairpin is inserted. This hypothesis is supported by the increasing stability of individual β hairpins with completion of folding (see Figure S3 in the Supporting Information).

Our results suggest a fundamentally different folding mechanism of transmembrane β-barrel proteins. In contrast to the two-stage folding and insertion model of a transmembrane β barrel, we observe distinct folding steps for β hairpins. Our findings extend the three-stage folding model of α-helical membrane proteins introduced by Popot and Engelmann.[11] In their model, single α helices fold and insert independently into the membrane, where they assemble into the functional protein. Our experiments suggest that similar folding steps occur with β hairpins. A further approach must test whether other β-barrel membrane proteins show a similar behavior, and whether a completely unfolded β barrel inserts and folds into the membrane through similar mechanisms. Only then we can be sure that the folding mechanism presented here resembles the mechanism that occurs in vivo.

It may be interesting to investigate which factors influence the folding kinetics and, thus, the folding hierarchy of β hairpins into a transmembrane β barrel. This is particularly important to understand how more complex β-barrel proteins assemble. It may also be interesting to investigate under which conditions β hairpins alter their propensity to form different folding intermediates. Searching for conditions under which β hairpins cluster and form larger folding intermediates may provide insights into mechanisms leading to the assembly of β-sheet-like aggregates such as those occurring in neurodegenerative diseases.

Experimental Section

SMFS: OmpG (281 aa) was purified from inclusion bodies, refolded in detergent, and reconstituted into native *E. coli* lipids.[7,9] Membranes showing densely packed OmpG molecules were adsorbed to freshly cleaved mica (ca. 30 min) in buffer solution (pH 7, 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 25 mM $MgCl_2$, 300 mM NaCl). Membranes were localized by AFM in the same buffer solution at room temperature.[8b] For SMFS, the AFM tip (60 μm 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.[9] Then the AFM tip was retracted at 600 $nm\,s^{-1}$ 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 and relaxed the polypeptide 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 $N\,m^{-1}$) was estimated from its thermal noise using the equipartition theorem.[12]

Data analysis: F–D curves were fitted by using the WLC model as described.[5,9] For analysis we selected only experiments of which the final F–D curves were sufficiently long (> 70 nm) to ensure that OmpG was completely unfolded from its terminus. The folding rate (k_{fold}) for every refolded β hairpin was acquired from single-exponential fit (continuous lines) of folding extent: $y = y_0 + A\exp(-t\,k_{fold})$.

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