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Fast slow folding of an outer membrane porin

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In comparison to globular proteins, the spontaneous folding and insertion of β-barrel membrane proteins are surprisingly slow, typically occurring on the order of minutes. Using single-molecule Förster resonance energy transfer to report on the folding of fluorescently labeled outer membrane protein G we measured the real-time insertion of a β-barrel membrane protein from an unfolded state. Folding events were rare and fast (<20 ms), occurring immediately upon arrival at the membrane. This combination of infrequent, but rapid, folding resolves this apparent dichotomy between slow ensemble kinetics and the typical timescales of biomolecular folding.

outer membrane porins | protein folding | single-molecule FRET

It is well established that water-soluble proteins rapidly fold through a funnel-like energy landscape to the lowest energy state via a collection of pathways (1–4); but in comparison, the folding mechanisms of membrane proteins (5–7), and in particular β-barrel proteins (8, 9), are relatively poorly understood. β-barrel proteins are specific to the outer membranes of chloroplasts, mitochondria, and gram-negative bacteria, playing a crucial role in processes as varied as energy production (10), photosynthesis, nutrient transport (11), enzymatic activity (e.g., protease (12) and lipase (13)), cellular adhesion (14), membrane anchoring (15), complement binding (16) and drug efflux (17). Arguably the most important β-barrel proteins are the bacterial outer membrane proteins (OMPs) that, as their name suggests, reside in the outer membrane of gram-negative bacteria. Most OMPs form pores of some kind (8) and are characterized by a cylindrical topology, high thermodynamic stability, and (typically) an even number of β-strands. Typically conserved across gram-negative bacteria, OMPs act as gatekeepers between the bacterium and its environment and are thus promising candidate targets in the development of new antibiotics (18).

The biogenesis of OMPs in vivo is distinct from that of the α-helical transmembrane proteins of the inner membrane: Unfolded OMPs enter the periplasm via the Sec translocon, where they are protected from aggregation by a variety of molecular chaperones (19). Folding and insertion into the outer membrane are assisted by the β-barrel assembly machinery (BAM) complex (20–22), which is thought to help OMPs overcome the energetic barrier to folding by local destabilization of the outer membrane (23). Although BAM acts to accelerate OMP folding kinetics in a manner analogous to classical chaperones, β-barrel folding and insertion also occur spontaneously (24). Much of our understanding of the folding of β-barrel proteins is built on in vitro studies in the absence of BAM, in which the folding of OMPs from denaturants into lipid membranes or detergent micelles can be measured precisely. The overall OMP folding pathway has been reviewed thoroughly elsewhere (8, 9); in summary, the spontaneous folding and insertion of β-barrel proteins follow a sequential pathway involving the rapid formation of a collapsed state before membrane absorption and then insertion of β-hairpins (25, 26). The available biochemical and biophysical evidence supports a concerted mechanism, whereby the final folding step and membrane insertion occur simultaneously (27–30). There is, however, evidence that for some OMPs insertion occurs via intermediate states (31). The membrane itself plays a key role in the kinetics of folding and insertion of OMPs: Folding rates are dictated by membrane fluidity, thickness, curvature, and head-group composition (8, 24, 28, 32–35). Overall, the picture for OMPs spontaneously folding and inserting into lipid membranes is one where kinetics are remarkably slow, typically on the order of minutes (32).

Although OMP folding kinetics are slow, it seemed implausible to us that the folding event itself could take so long at a molecular scale. To gain further insight we turned to single-molecule methods, which allow for the interrogation of heterogeneous molecular processes and provide the means to resolve individual kinetic steps without the need to synchronize events.

Single-molecule Förster resonance energy transfer (smFRET) has proved to be an invaluable tool to measure protein folding kinetics over a wide range of timescales.

Significance

Outer membrane porins play a crucial role in processes as varied as energy production, photosynthesis, and nutrient transport. They act as the gatekeepers between a gram-negative bacterium and its environment. Understanding how these proteins fold and function is important in improving our understanding and control of these processes. Here we use single-molecule methods to help resolve the apparent differences between the fast folding expected on a molecular scale and the slow kinetics observed in ensemble measurements in the laboratory.

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and protein systems (23, 36). Confocal smFRET measurements have been particularly insightful, enabling the examination of folding dynamics of cytoplasmic (37–41) and membrane (42–45) proteins. These methods have recently been applied to the folding and aggregation states of OMPs mediated by detergent (46, 47) and chaperonins (48). However, due to the fast diffusion in solution, confocal single-molecule measurements generally lack the ability to directly observe transitions between such states. In contrast, continuous measurements of immobilized, confined, or encapsulated molecules permit transitions between conformational states to be measured (49, 50) (typically in exchange for time resolution). Continuous smFRET measurements have been used with great effect to probe functional dynamics (51–54) and association dynamics (55–58) of membrane proteins in lipid bilayers. However, their application to membrane protein folding in a lipid bilayer environment is, to our knowledge, yet to be demonstrated; such a study was recently proposed by Krainer et al. (59) and here we seek to address that call.

To study single-molecule β-barrel folding we identified the Escherichia coli outer membrane protein G (OmpG) (60) as a promising candidate. OmpG is a 14-stranded β-barrel notable for its monomeric status and flexible loops at the entrance to the pore (61, 62). In comparison with trimeric OMPs, these characteristics simplify its folding landscape and have led to applications in nanopore sensing (61–63). Detergent-mediated refolding of OmpG from a urea-unfolded state has been well characterized (64, 65), and kinetics of refolding in lipid vesicles, also in the presence of the BAM complex, occur on the order of minutes (66).

Using FRET-labeled OmpG we report on β-barrel folding into model membranes from urea using single-molecule total internal reflection fluorescence (smTIRF) microscopy.

**Results**

**FRET-Labeled OmpG Reports on β-Barrel Folding.** Maleimide-functionalized Cy3 (donor) and Cy5 (acceptor) fluorophores were stochastically conjugated to two engineered cysteine residues on the otherwise cysteine-free native OmpG. Labeling sites near the N and C termini were selected to optimize the change in FRET efficiency between unfolded and folded states (Fig. 1A).

Since OMPs are known to spontaneously fold at a higher rate and to a greater extent in bilayers with short hydrocarbon chains (32, 66), we refolded from 8 M urea into small unilamellar vesicles (SUVs) of the short-chain (10 carbon) phospholipid 1,2-dicetyl-sn-glycero-3-phosphocholine (DCPC). The extent of folding was assessed by both spectrometry (Fig. 2B) and cold sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) band shift (Fig. 1B). Folded OMPs retain their structure in the presence of SDS at room temperature and migrate faster than their unfolded counterparts because the folded state has a more compact structure (67). Both (labeled) OmpG-Cy3Cy5 and (unlabeled) OmpG displayed a lipid-dependent band shift from the unfolded to the folded state, characteristic of OMPs. Variations in total band intensity, on account of slight loading volume variability, are normalized in the densitometry analysis. OmpG-Cy3Cy5 FRET was also visualized directly in the SDS-PAGE gel where the efficiency of the (lower) folded protein bands is, as expected, consistently higher than that of the (upper) unfolded protein bands.

Electrophoresis was also performed to assess the functional state of OmpG following labeling. OmpG-Cy3Cy5 was refolded first in detergent and then reconstituted into a droplet interface bilayer (DIB) following our previously published protocol (68). We observed OmpG-Cy3Cy5 switching between open and closed states at neutral pH, with a conductance of 0.7 nS (Fig. 1C), consistent with previous reports by both ourselves (69, 70) and others (65, 71, 72), indicative that the construct is capable of folding and forming a functional, native state.

Further ensemble measurements sought to establish whether FRET efficiency could be used as a direct readout for the folded state of OmpG-Cy3Cy5. An increase in FRET was observed when OmpG-Cy3Cy5 was diluted in the presence of SUVs compared to an SUV-free sample, denoted by a shift in donor and acceptor peaks in the emission spectra (Fig. 2A). Titrating the lipid:protein (L:P) as before, and correcting the spectra for FRET-independent emission from both fluorophores (SI Appendix, Fig. S3), the FRET efficiency increased from 0.25 to 0.4 with dependence on L:P consistent with a two-state binding model (K_A = 101 ± 17) (Fig. 2B). Quantification of the folded fraction of the same samples by densitometry analysis of the band shift showed a similar dependence on L:P ratio (K_A = 125 ± 10). By comparison, (unlabeled) OmpG-2xCys appeared to fold more readily at lower L:P than OmpG-Cy3Cy5 (K_A = 64 ± 2) and with a higher overall efficiency (maximum folded fraction 0.8 vs. 0.6).

![Figure 1](https://www.pnas.org)
Single-Molecule Folding of OmpG from Urea. Next we examined folding of OmpG-Cy3Cy5 from urea. During image acquisition, OmpG-Cy3Cy5 (8 M urea, 250 mM NaCl, 25 mM Tris HCl, pH 7.0) was added to the solution above a preformed DCPC SLB, yielding a dilution to 0.1 M urea and a final L:P of 1.5 × 10^6 (Fig. 4D). Using the same trajectory selection criteria as with prefolded OmpG, we identified trajectories of high-FRET spots from the point of their arrival at the bilayer, a distance of 100 to 200 nm given the laser power used.

The majority of trajectories (91%) showed high (E > 0.5) FRET efficiencies for their entire duration (Fig. 4B). The dominant high-FRET population lies well within the SD of FRET values seen for prefolded OmpG-Cy3Cy5 (Fig. 4D). In addition, a small, low-FRET population was observed, as well as a subset of trajectories (9%) containing fluctuations at low (0 to 0.5) FRET efficiency values (Fig. 5D) not observed with prefolded OmpG. Over the 20 min following injection, there was no significant change in the dominant high-FRET population (SI Appendix, Fig. S4). There was also no significant change in FRET 20 min postinjection in the sample that had been imaged periodically, compared to one that had been allowed to fold in the dark (SI Appendix, Fig. S4D). Fluctuations between high- and low-FRET states were no longer observed once arrival of new OmpG molecules to the bilayer had ceased.

As with prefolded OmpG-Cy3Cy5, following renaturation from urea OmpG-Cy3Cy5 were essentially immobile at the bilayer. The rate at which OmpG-Cy3Cy5 arrived at the bilayer in our measurements was constant for ~50 s after injection and a majority of spots were found to have arrived during the 84-s measurement (SI Appendix, Fig. S5), reflecting the time taken for a majority of OmpG molecules to diffuse from the pipette tip to the SLB, a distance of 2 to 3 mm. Although the frequency of low-FRET OmpG-Cy3Cy5 arriving at the bilayer was 20-fold greater than that of the high-FRET trajectories, the cumulative frequency distributions of both are identical, indicating that arrival rate is the same regardless of observed folded state (Fig. 5A and B).

Assuming each OmpG molecule associates with the SLB long enough to be detected once and is distributed evenly throughout the SLB, the expected spot density (0.33 μm^−2) closely matches...
the observed total spot density (0.27 μm$^{-2}$). Comparing total spot density to the spot density in the acceptor channel over the same period (0.012 μm$^{-2}$), we can estimate the fraction of molecules that fold on arrival at the bilayer (method detailed in SI Appendix). Taking into account the population of FRET-irrespective donor-only labeled species and assuming that acceptor-only labeled species are not visible, we estimate the probability of insertion of an individual molecule upon encountering the bilayer to be low, \( \approx 0.07 \).

OmpG was immobile in our SLB experiments (Fig. 5C). We were concerned that bilayer defects might be the cause of this immobility and thus affect our kinetics: The mobility of the lipids in the SLB was assessed by incorporating a small fraction of fluorophore-labeled lipid and single-particle tracking (SPT) was used to track the two-dimensional diffusion before and after injection of OmpG Cy5 (Fig. 5C). The lipids remained mobile, with no evidence of anomalous subdiffusion, where previously we have exploited PEG-induced bilayer defects to control anomalous subdiffusion in SLBs (76). Here we saw no dependence of lipid diffusion on observation time (SI Appendix, Fig. S6), indicating that the PEG-cushioned SLBs in this work were, as expected, free from such defects. A second possible cause for the lack of mobility of OmpG-Cy3Cy5 could be interactions between the protein and underlying glass substrate. Previous reports suggest that spontaneous insertion occurs such that N and C termini do not traverse the bilayer (24). We therefore expect that injected OmpG would be oriented with the large periplasmic loops situated between the lower leaflet and glass substrate and the N and C termini situated in the bulk solution.

**Discussion**

Overall, our experimental evidence supports a simple two-state folding model for OmpG, absent from long-lived (>20 ms) folding intermediates. Our band-shift assays and ensemble FRET yield folding rates identical within experimental error. smFRET reveals just a single folded state, consistent with what Rath et al. (77) similarly observed for OmpX and Huysmans et al. (30) for PagP.

Consistent with previous reports, our OmpG ensemble measurements indicated folding kinetics on the order of several tens of seconds. These kinetics were reflected in single-molecule measurements where the arrival rate of OmpG at the bilayer occurred on a similar timescale, with a majority of the protein being found to arrive during the course of the 84-s recording (SI Appendix, Fig. S5). However, individual folding events observed at the single-molecule level, regardless of arrival time, are orders of magnitude faster (<20 ms).

Single-molecule measurements are limited by photobleaching time; in our case fluorophores bleached 1 to 10 s after arrival at the bilayer. We can therefore only comment on the folding kinetics of proteins that fold within this observation window. Ninety percent of the OmpG that we observed in a folded state were detected immediately in their final folded state. Thus these events must have occurred faster than the time resolution of our measurements, within 20 ms of arrival of an OmpG molecule at the bilayer. Clearly future insights stand to be gained by single-molecule techniques capable of improved temporal resolution.

Our results indicate the probability of OmpG insertion upon membrane binding is low, only 7%. This implies that a majority of binding events are not productive and that productive binding events, however rare, proceed to fold rapidly.

In the absence of a direct functional readout, we cannot explicitly rule out the possibility of misfolded or intermediate species, which cannot be distinguished from fully folded OmpG-Cy3Cy5 by FRET efficiency. Further mutants designed to detect specific folding intermediates, as has been done previously using tryptophan quenching (27), have the potential to resolve further kinetic steps in the folding pathway.
Fig. 4. Spontaneous folding and insertion of OmpG in DCPC SLBs: smFRET and SPT. (A) Urea-unfolded OmpG-Cy3Cy5 was injected into the bulk solution surrounding a DCPC SLB. The injection coincided with a rapid dilution of urea, allowing for spontaneous folding and insertion. (B) Representative trajectories of tracked spots. (Upper) Intensity of the spot in donor (green) and acceptor (red) channels, including baseline intensities directly before and after the trajectory. (Lower) Corresponding FRET efficiency for duration of each trajectory. Vertical dashed line indicates acceptor photobleaching event. (C) Image sections of acceptor (Upper) and donor (Lower) channels at key points in a trajectory shown in B, vii (symbols represent location indicators), each showing three consecutive frames: first two frames of the trajectory, acceptor photobleaching event, and last two frames of the trajectory. Frame time: 20 ms. Image shown is 6 × 6 μm. (D) All spots FRET efficiency (excluding datapoints after acceptor photobleaching event), calculated from 53 trajectories. Mean and SD of Gaussian fit: 0.73 ± 0.15.

Using timecourse band-shift assays, Burgess et al. (32) reported folding kinetics for a range of OMPs into DCPC membranes that were on the order of several tens to hundreds of seconds, but they noted that some OMPs displayed a significant folded fraction that existed by their first measured timepoint (5 s). They termed these rapid folding events a “burst phase,” which was

Fig. 5. Arrival rates and rare events. (A) Spot arrival times of injected OmpG-Cy3Cy5 landing on a DCPC SLB from a urea-unfolded state, detailed in Fig. 3. Green: spots detected in the donor channel (3,596 in total). Red: spots detected in the acceptor channel (169 in total). (B) Mean FRET vs. start time for high-FRET trajectories of injected OmpG-Cy3Cy5 in DCPC SLBs. (C) Mean-squared displacement vs. observation time interval with linear fit for SPT of fluorophore-labeled lipids in DCPC SLBs before (solid circles) and 10 min after (open circles) addition of urea-unfolded OmpG Cy5 (diamonds). (D) Trajectories of injected OmpG-Cy3Cy5 that have unusual (not consistently greater than 0.5) FRET efficiency on encountering the DCPC bilayer.
found to account for up to around 80% of folding for some OMPs (e.g., OmpX) but was not detectable in others. We did not require the inclusion of a burst phase to fit OmpG band-shift timecourse data, so we conclude that the proportion of OmpG burst folding in our ensemble assay must be low. Overall, our single-molecule measurements might also be considered as a burst phase, as they too generally occur faster than our temporal resolution. However, some caution should be exercised when comparing these experiments—as different methods often place very different restrictions on protein to lipid ratio. In both ensemble and single-molecule setups, diffusion to the membrane is rate limiting.

Our single-molecule assay is sensitive to rare events, such as the small number of folding events that displayed a fluctuating FRET efficiency (Fig. 5 D). However, given the infrequent nature of these events, it is difficult to speculate further. Perhaps these represent instances where the protein transitioned to a misfolded state in which the N and C termini were in close proximity? Whether or not this transition is reversible is not known. We would not expect to see these rare, short-lived events in the band-shift or bulk FRET measurements, as they would be obscured by averaging of the signal. It is not possible to draw firm conclusions about the nature of a misfolded state from so few examples, other than to acknowledge the possibility of its existence.

It is known that the rate of folding of OMPs into membranes is influenced by the accessibility of the hydrophobic bilayer interior; more rapid folding is observed for thinner bilayers, those with increased curvature, or those at their transition temperature (32, 78). We therefore interpret the folding events that we observed are those in which OmpG landed in an orientation and region of the membrane, leading to a rapid (millisecond) folding event.

These experiments highlight the utility of single-molecule tools for dissecting difficult biological processes such as membrane protein folding. Fast, infrequent, folding provides a consistent simple explanation bridging both ensemble and single-molecule observations of kinetics. Here we have deliberately chosen OmpG as a simple testbed with “uncomplicated” kinetics and structure; the real challenge is to bring these tools to bear to a wider array of complex challenges in membrane protein folding.

Materials and Methods

Detailed materials and methods are provided in SI Appendix.

Expression, Purification, and Labeling of OmpG-2xCys. A double-cysteine mutant of full-length OmpG (E2C and C281) was produced by site-directed mutagenesis and confirmed by DNA sequencing. Constructs were expressed and purified using a previously described method (69).

Samples were stored in denaturing buffer (8 M urea, 250 mM NaCl, 1 mM TritonX-100, glycerophosphate [TECP], 25 mM Tris·HCl, pH 8.0) at −80°C until required.

Fluorescent Labeling of OmpG-Cy3/Cy5. OmpG-2xCys was purified by gel filtration to remove TCEP. Labeling was performed immediately afterward by incubation with a 10-M excess each of Cy3- and Cy5-smaleimide for 60 min at room temperature. Excess label was removed by gel filtration. OmpG-Cy3/Cy5 was stored at −80°C until required.

Electrophysiology. For single-channel recordings, OmpG-Cy3/Cy5 was refolded in detergent micelles and then reconstituted in a droplet interface bilayer (DIB). Droplet interface bilayers were produced as described in ref. 77. Voltage-clamped recordings of ionic current were made at room temperature and digitized at a rate of 1 kHz.

Supported Lipid Bilayers. SLBs were prepared on glass coverslips by vesicle fusion (80) from SUVs consisting of 1.77 mM DCPC with 1.0 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] ammonium salt (and 3 × 10−6 mol% Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt for lipid tracking experiments). For injection measurements, OmpG-Cy3/Cy5 was diluted to 7 pM in denaturing buffer (8 M urea, 250 mM NaCl, 10 mM Tris, pH 7) and then 2 μL was added to the bulk solution during image acquisition. For SLBs containing prefolded OmpG-Cy3/Cy5, the protein stock was diluted to 700 pM in denaturing buffer and then 5 μL was mixed with 45 μL SUV stock and incubated for 1 h at 37°C prior to SLB formation. For lipid tracking measurements, SLBs were imaged before and 10 min after the addition of OmpG-Cy5, as described for OmpG-Cy3/Cy5 injection.

Data Availability. All study data are included in this article and/or SI Appendix.

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