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Hidden dynamics in the unfolding of individual bacteriorhodopsin proteins

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Abstract

Protein folding occurs as a set of transitions between structural states within an energy landscape. An oversimplified view of the folding process emerges when transiently populated states are undetected because of limited instrumental resolution. Using force spectroscopy optimized for 1- μ s resolution, we reexamined the unfolding of individual bacteriorhodopsin molecules in native lipid bilayers. The experimental data reveal the unfolding pathway in unprecedented detail. Numerous newly detected intermediates—many separated by as few as 2–3 amino acids—exhibited complex dynamics, including frequent refolding and state occupancies of $<10 \mu$ s. Equilibrium measurements between such states enabled the folding free-energy landscape to be deduced. These results sharpen the picture of the mechanical unfolding of membrane proteins and, more broadly, enable experimental access to previously obscured protein dynamics.

Elucidating the process by which a protein folds into its native structure is a large, interdisciplinary field (1). Proper identification of the intermediate states along the protein folding pathway is essential to describe the folding process accurately. Advances in ensemble techniques have led to the characterization of previously “invisible” folding intermediates (2). Single-molecule techniques, including fluorescence (3, 4) and force spectroscopy (5–7), have proven especially valuable for detecting sparsely populated intermediates. In particular, force spectroscopy studies have yielded kinetic insights into the unfolding pathways of diverse macromolecules, including globular proteins (8), membrane

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proteins (9), multi-protein complexes (10), and ribozymes (11). These studies have identified the energy barriers between states and distinguished among obligatory, non-obligatory, and off-pathway intermediates. However, critical kinetic information is obscured whenever closely spaced and/or transiently occupied intermediates remain undetected (Fig. 1A). For example, limited experimental precision can lead to two closely spaced states being misinterpreted as a single composite state exhibiting non-exponential lifetimes (8). Moreover, limited temporal resolution can lead to artifacts in force spectroscopy studies (12, 13), and theoretical simulations suggest that state occupancies that are brief relative to the response time of the force probe remain undetected (12). Beyond such identified limitations, it remains challenging to characterize conformational dynamics on the microsecond-to-millisecond timescale in many single-molecule assays, particularly for subtle changes in structure.

Advanced, single-molecule force spectroscopy (SMFS) assays typically have a temporal resolution of ~50–100 μ s (14), with recent optical-trapping work opening the door to probing dynamics with ~6–10 μ s resolution (15). On still faster time scales, atomic force microscopy (AFM) using ultrashort cantilevers ($L = 9 \mu$ m) has unfolded a globular protein at high speed (4 mm/s) with 0.5- μ s time resolution (16). Here, we unfolded bacteriorhodopsin (bR), a model membrane protein, with ultrashort cantilevers optimized for 1- μ s SMFS (17) (Fig. S1) and thereby uncovered previously unobserved protein dynamics and intermediates. We elucidated the unfolding pathway with improved precision and resolved a long-standing discrepancy in the size scale of the fundamental structural elements involved in bR unfolding, as deduced from experiments (18–22) or molecular dynamics (MD) (23).

SMFS studies of bR represent an important model system to test the experimental boundaries of protein unfolding. Prior work has shown that the bR-unfolding pathway (18) has a comparable number of structural intermediates to the four-fold larger molecule Hsp90 (24). While mechanical unfolding is not the simple reversal of translocon-mediated membrane protein folding, AFM-based bR studies provide a window into quantifying the energetics of individual membrane proteins embedded in a native lipid bilayer (purple membrane for bR) (9) and the size scale of detectable structural intermediates (18, 19). Such studies have been extended to diverse classes of membrane proteins (19), including biomedically important G-protein coupled receptors (GPCRs) to which bR is homologous.

A consensus unfolding pathway for bR emerged early on (18) and this pathway has been validated over the past decade, with the number and location of bR's unfolding intermediates remaining essentially unchanged across different supporting substrates (mica, glass, and purple membrane) (19). The assay is initiated by pressing an AFM tip into the membrane to promote a nonspecific attachment of the tip to the cytoplasmic end of the G-helix of bR (Fig. 1B). The cantilever is then retracted at a fixed velocity (v) while measuring force via cantilever deflection.

When using our optimized ultrashort cantilevers to extend bR at 300 nm/s, force-extension curves displayed three major intermediates (Fig. 1C), recapitulating previous studies (9, 19). The elasticity of the unfolded segment of the protein associated with each of these states was well described by a worm-like-chain (WLC) model (Fig. 1C, colored curves). Each major

intermediate occurred after fully unfolding the previous pair of helices and was previously determined to be obligate intermediates (18–22). In other words, the unfolding pathway is dominated by the topology of the protein in the lipid bilayer. Non-obligate minor intermediates (states not fitted with the WLC model in Fig. 1C) occurred after each major state though with lower probability of occupancy (Table S1). The GF helix pair unfolds at very low extension, and it is generally excluded from analysis, due to confounding signals associated with variability in adhesion between the tip and surface (19).

Closer inspection of our resulting force-extension curves uncovered a strikingly complex, dynamic folding network (Fig. 1, D to F). The optimized ultrashort AFM cantilever gave a ~100-fold improvement in temporal resolution and a ~10-fold improvement in force precision (Fig. S1). In particular, the data showed a large increase in the number of states that could be resolved while unfolding the ED, CB, and A helices (Fig. 1, D to F, respectively). For example, whereas prior studies over the last decade reported 2 non-obligate intermediates when unfolding the ED helix pair (Fig. 1D, upper left inset) (18–22), we observed 14 intermediates (Fig. 1D and Fig. S2), denoted I_{ED}^1 to I_{ED}^{14} . Newly identified intermediates were detected throughout the unfolding pathway. For the CB helix pair (Fig. 1E) and helix A (Fig. 1F), we observed a 3-fold or larger increase in the number of resolved intermediates compared to the consensus number of observed intermediates (18–22). Changes in secondary structure associated with each intermediate were assigned based on changes in contour length (L_0) derived from WLC fits to the data, with an estimated uncertainty along the polypeptide of ± 1 amino acid (aa) (Table S1).

Most of the intermediates were closely spaced and transiently populated, making them difficult to detect. Indeed, based on the extension changes, we resolved transitions corresponding to the unwinding of just 2 aa (*e.g.*, $I_{ED}^4 \leftrightarrow I_{ED}^5$; and $I_{ED}^9 \leftrightarrow I_{ED}^{10}$; and $I_{ED}^{11} \leftrightarrow I_{ED}^{12}$) (Fig. 2, Fig. S2) or half an α -helical turn. Moreover, dwell times as short as 8 μ s could be resolved (Fig. 2B). Such fleeting times are commonly associated with transition path times between states (15, 25), rather than state occupancy times resolved in a single record. In addition, as a result of increased precision, one previously identified major obligate intermediate (I_{CB}^0), which occurs after fully unfolding the ED helix pair, is actually composed of two non-obligatory states separated by 5 aa. Interestingly the proportion of trajectories that goes directly into I_{CB}^1 without a detectable occupancy in I_{CB}^0 increases as pulling velocity is increased from $v = 30$ to 3,000 nm/s (Fig. S3). Finally, we note that a small percentage (13%) of molecules exhibited at least one continuous, rather than discrete, transition (Fig. S4). Additional data and more advanced modeling will be required to unravel the mechanism(s) underlying such continuous transitions.

Refolding of individual bR molecules has been observed as the AFM tip is brought closer to the surface ($v < 0$ nm/s)(26), which lowers the force on the unfolded molecule and thereby promotes folding. In contrast, bR refolding has not been detected while retracting the cantilever ($v > 0$ nm/s), implying that the standard rapid stretching assay was far from equilibrium. With our improved spatiotemporal resolution, we now routinely detect reversible transitions between two (Fig. 1, D to F, lower inset; Fig. S5) and even three states

(Fig. 2) while stretching. Rapid, back-and-forth transitions have been called a hallmark of equilibrium between states (27), but technically, these states are only *near* equilibrium, because the force acting on each state varies slightly in time. Unexpectedly, these near-equilibrium transitions occurred even at a high stretching rate ($v = 300$ nm/s) compared with prior reports of similar dynamics in globular proteins ($v = 1\text{--}10$ nm/s) (27, 28). Indeed, back-and-forth transitions in bR persisted up to the highest velocities examined (5,000 nm/s) (Fig. S6) and were observed in all three major regions (ED, CB, and A). These states exhibited brief lifetimes (<1 ms) and could refold against comparatively high loads ($F \approx 30\text{--}160$ pN). Refolding was most frequently observed between states located near the top of helix E and helix A (70% of molecules refolded from I_{ED}^1 to I_{ED}^0 , 90% of molecules refolded from I_A^1 to I_A^0). Moreover, refolding at $v = 300$ nm/s was ubiquitous: all records exhibited at least one refolding event in the ED helix pair.

Widespread refolding, together with the large number of resolved intermediates, led to highly varied trajectories along a multistate unfolding pathway (Fig. 1, D to F). Fig. 3 displays the unfolding (Fig. 3, orange lines) and refolding (Fig. 3, purple line) transitions observed during the unfolding of the ED helix pair. The sheer complexity of the dataset obtained here prevents broad generalizations, but the density of the intermediate states, 80% separated by just 2 or 3 aa, indicates that the structural elements associated with bR unfolding are significantly smaller than previously experimentally reported: prior reports have described the unfolding process as occurring in units of pairs of helices, a single helix, or approximately half a helix (19). Thus, the improved spatiotemporal resolution used here has uncovered an underlying free-energy landscape that is much more complex than any such landscape previously described by other experimental methods to date (29). Fully characterizing this free-energy landscape of a multistate system from dynamic experiments ($v = 0$ nm/s) requires a theoretical framework that can account for non-equilibrium interconversions among multiple states (*e.g.*, Fig. 2), such as the formalism developed by Zhang & Dudko (30). However, given the multiplicity of unfolding trajectories through the unfolding pathway (Fig. 3, Fig. S7,S8), the numerous intermediate states (>25), the brief state lifetimes (<1 ms), and the presence of refolding, a significantly larger data set is needed to apply such an analysis. Hence, the current work points to a need for further technical advances that would yield a 10–100-fold increase in the number of records at 1- μ s or better resolution.

True equilibrium folding between select rapidly interconverting states, a new regime for membrane proteins, was achieved. Specifically, the force in each state was constant in time, similar to optical-trapping studies of globular proteins (8, 29). In our AFM-based assay, we retracted the tip in a series of steps in the vicinity of I_{ED}^1 and I_{ED}^2 and thereby measured ~ 100 transitions at each stationary condition ($v = 0$ nm/s) (Fig. 4A, Fig. S9). As expected, the to equilibrium shifted from I_{ED}^1 to I_{ED}^2 as the tip-sample separation (and therefore the force) in each state increased. These states were closely spaced (~ 3 aa) and transiently populated, with dwell times of 15 μ s well resolved (Fig. 4B).

A powerful aspect of SMFS is that a one-dimensional free-energy landscape can be reconstructed from equilibrium trajectories as a function of load, with the molecular

extension serving as the reaction coordinate. A variety of theoretical methods have been developed to derive such landscapes from data (29). Here, we used p_{fold} analysis (31) because it is less sensitive to instrumental compliance issues, as shown recently using well-characterized DNA hairpins (32). In this work, we applied p_{fold} to equilibrium bR records between I_{ED}^1 and I_{ED}^2 (Fig. 4A) to calculate the free-energy landscape at $F_{1/2}$, the force where both states are equally occupied (Fig. 4C and Fig. S10). Extrapolating the energy difference between states to zero applied force yielded $G_0 = 8.0 \pm 0.4$ kcal/mol. Because this transition involves unfolding 3 amino acids (T-F-G) of the transmembrane helix, the average G_0 per aa was 2.7 kcal/mol. This result is higher than previous single-molecule (0.5–1.5 kcal/mol) measurements, which were averaged over entire transmembrane helices (26, 33). An insertion energy for a full transmembrane helix (20–30 aa) of ~12 kcal/mol is deduced from traditional ensemble measurements, yielding ~0.5 kcal/mol per aa (34). Our higher than anticipated result may be attributable, in part, to the higher energy required to solvate phenylalanines out of a lipid bilayer, to a higher G_0 per aa when starting to unfold a helix pair relative to an average over the entire helix pair, and to the fact that the angstrom-level motion of each amino acid along the stretching axis does not lead to its full solvation in the aqueous phase; rather it is positioned within the phospholipid interface (35). Future work is needed to resolve the discrepancy. We note, however, that our 1D free-energy landscape for this 3-aa transition was robust. Specifically, we independently reconstructed a 1D free-energy landscape based on an inverse-Boltzmann analysis (36) (Fig. S10C) and measured the transition barrier position ($x^\ddagger$) using a Bell analysis of the average force-dependent lifetime (37) (Fig. S11). Landscapes and landscape parameters between different methods agreed to within error, notwithstanding the well-known limitations of the Bell model (38) that assumes $x^\ddagger$ does not move as a function of applied load.

The enhanced precision achieved in these records helps to resolve a long-standing discrepancy between theoretical and experimental studies of bR unfolding, based on steered MD simulations or SMFS measurements, respectively. Specifically, prior experimental studies supplied evidence for ~1–2 intermediate unfolding states per helix pair (~50 aa), whereas steered MD simulations (23) predicted a far denser series of unfolding intermediates, occurring once every ~2–8 aa. The MD predictions, as it turns out, correspond well to the closely spaced intermediates revealed by the present work. In fact, ~60% of the unfolding intermediate states predicted by MD simulations were observed in these experiments. Expressed the other way around, ~55% of the intermediate states observed in our experiments were predicted by MD simulations (Fig. S12). For helix E, where we managed the highest resolution, a supermajority (80%) of the intermediates predicted by MD could be identified in our experimental data. Given the detail and density of unfolding intermediates, it is equally notable that SMFS detected intermediates that were otherwise absent from MD simulations, over several comparatively large regions (>5 aa; Fig. S12). We attribute this difference to the seven orders of magnitude greater pulling rates used in MD simulations, as compared to actual experiments ($v = 1$ m/s, vs. 300 nm/s, respectively). Under the circumstances, the excellent correspondence between MD and SMFS provides increased confidence for using theoretical simulations to explore the unfolding and energetics of membrane proteins.

In conclusion, by using ultrashort cantilevers optimized for improved spatiotemporal resolution, we have developed a highly detailed view of bR unfolding. Force spectroscopy has revealed a multiplicity of closely spaced, transiently occupied intermediates states, representing small changes in the molecular conformation. The widely held notion that the mechanical unfolding of bR at standard stretching rates occurred far from equilibrium is likely to be incorrect: refolding is, in fact, widespread, but masked by experimental limitations when using standard cantilevers. In retrospect, elements of bR secondary structure likely unfolded and refolded during past SMFS experiments, but did so faster than the force probe could respond. Finally, we note that the technique demonstrated here is by no means restricted to bR, but could readily be adapted to other membrane proteins studied using AFM (19) and, more generally, to studies of nucleic acid structures (39), mechanosensitive enzymes (40), and canonical globular proteins (4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

Mechanical unfolding of a membrane protein with enhanced precision reveals previously undetected intermediates and equilibrium refolding.

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Fig. 1. Single-molecule force spectroscopy of bacteriorhodopsin (bR) measured with 1- μ s temporal resolution

(A) A conceptual sketch shows a low (grey) and a high-resolution (black) representation of the same free-energy landscape. Each free-energy valley represents an intermediate, with lower energy and therefore more fully folded states depicted on the left while higher energy, more unfolded states are shown on the right. Assays with improved sensitivity enable the detection of previously hidden folding intermediates (magenta) and protein dynamics between closely-spaced states separated by low barriers (green arrows). (B) A cartoon

illustrates the unfolding of individual bR molecules by a modified ultrashort cantilever. Mechanical unfolding occurs by retracting the cantilever at a constant velocity. Each transmembrane helix is identified by its standard letter label. **(C)** A typical force-extension curve (FEC) using a modified ultrashort cantilever recapitulates the three previously detected major intermediates corresponding to pulling on the top of E (cyan), C (orange) and A (green) helices. The FEC segments associated with these major states are well described by a worm-like chain model (colored dashed lines) and labeled with their associated contour lengths. The colored bars denote the extension range in panels D to F. **(D)** Representative high-resolution FECs reveal 14 intermediates when unfolding the ED helix pair. In contrast, two intermediates were reported in prior studies (18–22) (upper left inset). This inset and the corresponding insets in panels E and F reprinted from ref (20) with permission from Elsevier. **(E)** FECs show 7 intermediates during the unfolding of the CB helix pair instead of 2 observed previously (18–22). **(F)** FECs show 3 intermediates while unfolding helix A instead of 1 observed previously (18–22). Near-equilibrium fluctuations between multiple states were observed when stretching at 300 nm/s (lower inset, D to F; see Fig. S5 for force-time curves).

Fig. 2. Improved spatiotemporal resolution details complex and rapid dynamics between closely spaced states

(A) Force-vs-time trace shows rapid back-and-forth transitions between three states determined by hidden-Markov-model analysis (black dotted lines) and correspond to I_{ED}^3 , I_{ED}^4 , and I_{ED}^5 . Data smoothed to 10 kHz (blue) and 200 kHz (pink), respectively. A highlighted portion of the trace (cyan) is shown in detail in the lower panel. (B) High-resolution force-vs-time trace illustrates rapid dynamics between I_{ED}^3 , I_{ED}^4 (green), and I_{ED}^5 . Here, two state lifetimes of 15 μ s and 8 μ s are identified by a hidden-Markov-model analysis (orange). A potentially even shorter state lifetime of 3 μ s (gray) is seen, but not identified as a state by the hidden-Markov-model analysis. Traces were smoothed to 100 kHz (light colors) and 830 kHz (dark colors).

Fig. 3. Unfolding pathway for the ED helix pair

(**Top**) Cartoon of the primary and secondary structure of bR. Locations of observed folding intermediates are shown by residues with filled in circle. (**Bottom**) Each helix pair diagram depicts an observed intermediate state, with connecting lines representing transitions observed in at least 4 (of 98) different molecules containing a total of 1,399 transitions. Orange lines represent unfolding transitions, while purple lines show refolding transitions. Line-widths represent the frequency of observing a particular transition. The analogous

unfolding pathways for the CB helix pair and the A helix are shown in Fig. S7 and an alternative matrix representation shown in Fig. S8.

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Fig. 4. Equilibrium folding of a 3-amino-acid segment of a membrane protein

(A) Force-vs-time traces show reversible transitions between two previously unresolved intermediates (I_{ED}^1 , black; I_{ED}^2 , red) at $v = 0$ nm/s. The cantilever was retracted from the surface by 0.5 nm between these two traces, increasing the force applied to the bR and thereby shifting the equilibrium towards I_{ED}^2 . Data were smoothed at 25 kHz. (B) A high-resolution section of the lower record in panel A illustrates detection of states that last only 15 μ s. Data were smoothed at 25 kHz (black and red) and 125 kHz (grey and pink). (C) A reconstructed 1D free-energy landscape at $F_{1/2}$ based on the equilibrium data shown in panel A and a p_{fold} analysis (31). The barrier position determined by p_{fold} (purple line) agrees with the result of an independent analysis based on the Bell model (green line) (Fig. S11). Error bars represent the SEM and the light green shading represents the uncertainty in determination of $\Delta x_{\text{Bell}}^{\ddagger}$.