**Free Energy Landscape of Bacteriorhodopsin Reveals Local Variations in Unfolding Energy**

P. Heenan, H. Yu, M. Siewny , T. Perkins

# ABSTRACT

Precisely quantifying the energetics that drive the folding of membrane proteins into a lipid bilayer remains challenging. More than 15 years ago, atomic force microscopy (AFM) emerged as a powerful tool to mechanically extract individual proteins from a lipid bilayer. Concurrently, fluctuation theorems, such as the Jarzynski equality, were applied to deduce equilibrium free-energies () from non-equilibrium single-molecule force spectroscopy (SMFS) records. These two advances in single-molecule studies determined the free-energy of the model membrane protein bacteriorhodopsin in its native lipid bilayer. To elucidate this free-energy landscape at a higher resolution, we applied two recent developments. First, as an input to the reconstruction, we acquired force-extension curves with a 100-fold higher time resolution and 10-fold higher force precision than traditional AFM studies of membrane proteins. Next, by using the inverse Weierstrass transform of the Jarzynski free-energy integral, we avoided convolving the free energy associated with the force probe with the landscape of the molecule under study, bacteriorhodopsin. The resulting landscape with a ~2 Å resolution yielded an average unfolding free-energy per amino acid (aa) of 0.7 0.1 kcal/mol, in agreement with past bulk and single-molecule studies. This high-resolution landscape derived from a non-equilibrium measurement also agreed with the one prior equilibrium measurement of for a membrane protein, which investigated a particular three aa transition and yielded 2.7 kcal/mol/aa. Hence, while average unfolding per aa is a useful metric, our high-resolution study highlighted significant local variation from the mean.

# Introduction

Membrane proteins perform critical biological functions, such as light harvesting, signaling, and transport. To carry out these and other diverse functions, they fold into equilibrium structures within a lipid bilayer. Yet, predicting the structure and stability of membrane proteins significantly lags success in globular proteins.[1](#ref-stanley_process_2008) For instance, models still struggle to predict the tilt and overall packing of transmembrane (TM) -helices, Predicting locations of TM kinks remains even less successful.[2](#ref-kessler_unfolding_2006)–[4](#ref-janovjak_molecular_2005) Hence, there is an ongoing need to better quantify the molecular forces that drive and stabilize the folding of membrane proteins. As in the folding of globular proteins,[5](#ref-onuchic_theory_1997) the energy landscape provides the fundamental theoretical framework for understanding protein folding and stabilization.[6](#ref-janovjak_valleys_2008)

Single-molecule force spectroscopy (SMFS) has emerged as important tool in determining the energetics underlying the folding and unfolding individual proteins.[7](#ref-rief_reversible_1997)–[10](#ref-neuman_single-molecule_2008) For membrane proteins, atomic force microscopy (AFM) is the modality of choice,[11](#ref-bippes_high-resolution_2011) since individual proteins can be mechanically extracted from their native bilayer.[12](#ref-oesterhelt_unfolding_2000) In the canonical assay, the tip of a cantilever is pressed into bacteriorhodopsin (BR) embedded in its native lipid bilayer to promote nonspecific attachment.[12](#ref-oesterhelt_unfolding_2000) The tip is then retracted at constant velocity while force is deduced by bending of the cantilever, revealing a series of unfolding peaks. The largest of these peaks correspond to pulling on the top of TM helix pairs or a terminal helix, notable the ED and CB helix pair and the A helix (Figure 1a-b). and to a rote with a nanometer-scale tip attaches to a surface-bound molecule and moves away from the surface at a known velocity (see Figure 1). During this process, the force is recorded as a function of molecular end-to-end distance, or extension. Importantly, these AFM-based studies avoid non-native structures arising from solubilizing membrane proteins in detergent and provide a means to fully unfold the protein to a well defined state, fully extended. As a result, such studies bypass the confounding effects in standard bulk biochemical assays where the “unfolded” state still contains -helical content.

In order to determine properties of the free energy landscape, single molecule force spectroscopy (SMFS) techniques repeatedly unfold surface-bound molecules. The energy barriers and distances to transition states in the absence of force can be obtained from the velocity-dependence of force distributions at transition points.[13](#ref-dudko_theory_2008),[14](#ref-zhang_transformation_2013) In addition, the entire energy landscape as a function of molecular extension can be obtained through fluctuation theorems such as Crook's fluctuation theorem[15](#ref-crooks_entropy_1999) or Jarzynski's equality[16](#ref-jarzynski_nonequilibrium_1997). As discussed below, removing the effect of the force probe on the landscape is required for accurate insights into membrane protein folding.

SMFS-AFM experiments have determined dominant folding pathways of the membrane protein bacteriorhodopsin in its lipid bilayer, purple membrane. Measured data include barriers between the major states of the protein, distances between these states, and even the protein's entire molecular energy landscape.[6](#ref-janovjak_valleys_2008) Although many paths are possible along the energy landscape, not all pathways are equally likely; bacteriorhodopsin may spend only microseconds in an unstable state before transitioning.[17](#ref-yu_hidden_2017) Quantifying the location and lifetime of these short-lived states is required for a detailed understanding of the energy landscape's shape and roughness.

Using AFM to measure rarely occupied states during an unfolding pathway has traditionally been problematic due to poor time resolution[6](#ref-janovjak_valleys_2008). Although energy barriers between major states of bacteriorhodopsin have been measured as a function of point mutation;[6](#ref-janovjak_valleys_2008) C- or N-terminal pulling geometry;[2](#ref-kessler_unfolding_2006) and environmental conditions[18](#ref-park_stabilizing_2007),[19](#ref-preiner_free_2007), these studies either failed to reconstruct the entire energy landscape; failed to remove the perturbing effect of the force probe on the energy landscape; or were limited in detecting transient states due to poor temporal resolution.

Our order-of-magnitude advances in AFM cantilever force precision and time resolution have accelerated identification of intermediate protein unfolding states.[17](#ref-yu_hidden_2017) Ten-fold higher force precision and 100-fold improvements in time resolution were facilitated by removing the gold coating of commercially available cantilevers and using a focused ion-beam to modify the cantilever geometry[20](#ref-sullan_atomic_2013),[21](#ref-edwards_optimizing_2017). By taking advantage of these improvements, our experiments reconstructed the unfolding energy landscape of bacteriorhodopsin in its native lipid bilayer as a function of molecular extension by repeated mechanical dissociation (see Figure 1a). The reconstruction applied the inverse Weierstrass transform of the Jarzynski free-energy integral[16](#ref-jarzynski_nonequilibrium_1997), which relates the free energy landscape to an ensemble of non-equilibrium SMFS experiments, correcting for the effect of the force probe. This work reports the first energy landscape of bacteriorhodopsin which is reconstructed with single-amino acid resolution and which removes the effect of the force probe on the energy landscape.

# Methods

## Experimental

### Sample preparation

The details of sample preparation are discussed elsewhere.[17](#ref-yu_hidden_2017) Briefly, bacteriorhodopsin in its native lipid bilayer, purple membrane, was purified from *Halobacterium salinarium* and stored at C at 2.4 mg/mL. For use, this bR stock was diluted to 1.2 g/mL in 10mM Tris-HCl (pH 7.8), 300mM KCL and sonicated for 3 minutes. A 50 L volume was deposited on freshly-cleaved, V1 mica for 1 hour, then rinsed 5 times with a total of 1 mL of imaging buffer (10mM Tris-HCl (pH 7.8), 150 mM KCL).

### Data acquisition

Modified BioLever Fast cantilevers (AC10DS, Olympus) were used for data acquisition. The cantilever modification procedure is discussed elsewhere[21](#ref-edwards_optimizing_2017) and results in cantilevers with improved force precision, lower force drift, and microsecond temporal resolution. The modified cantilevers were pressed into the purple membrane at 900 pN for 1 s and retracted from the surface at a constant velocity of 300 nm/s. Data were recorded at 5MHz, anti-aliased to 2.5MHz, then smoothed with a second-order, 500-point Savitsky-Golay filter for all subsequent analysis. All 168 force-extension curved used are shown in Figure 1. The force-extension curves were filtered as previously described to remove uninterpretable data and an interference artifact.[17](#ref-yu_hidden_2017) The unfolding of the GF helical pair was masked due to variable tip-membrane non-specific adhesion; only the ED, CB, and A helical pairs were used for energy landscape reconstruction.

## Landscape reconstruction

Energy landscape reconstruction of the ED, CB, and A helical pairs of bacteriorhodopsin applied the inverse Weierstrass transform of the Jarzynski free energy integral. The Jarzynski free energy integral reconstructs the free energy of the system (*i.e.* cantilever and molecule) as a function of molecular extension, and the inverse Weierstrass transform approximately removes the contribution of the cantilever to the landscape. Jarzynski's equality[16](#ref-jarzynski_nonequilibrium_1997) is a thermodynamic relationship between the Helmholtz free energy of a system, , along a reaction coordinate to the measured work done along that coordinate, :

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where the average is taken over many independent experiments, each starting and ending at the same choice of z. Jarzynski's equality is exact only in the limit as , but is approximately true for finite . The equality is remarkable because it relates the work done during many repetitions of a non-equilibrium process to the equilibrium free energy difference as a function of the reaction coordinate. In practice, SMFS experiments apply Jarzynski's equality by repeatedly folding or unfolding a single molecule of interest using a force probe. In this case, the work is the integral of the force as a function of the reaction coordinate , where is the position of the cantilever. For example, a linear ramp moving from with constant velocity would have .

The energetic contribution of the force probe must be removed for an accurate energy landscape reconstruction. The inverse Weierstrass transform of Jarzynski's free-energy integral, hereafter referred to as the inverse Weierstrass transform, determines molecular free energy as a function of molecular extension by removing the perturbation of the force probe. The correction modifies Jarzynski's equality assuming a stiff, harmonic pulling apparatus.[22](#ref-hummer_free_2010) The weighted histogram analysis method[23](#ref-minh_optimized_2008) can be used when the probe is not a harmonic spring (*e.g.* with DNA molecules linking the probe to the system of interest) or when the probe stiffness, including possible linkers, is not much greater than the system stiffness.

Figure 2 applies the inverse Weierstrass to bacteriorhodopsin. Figure 2a is a heatmap of 168 force-extension curves. This heatmap represents the ensemble of measurements needed to apply Jarzynski's inequality and the inverse Weierstrass transform. Figure 2b shows the Helmholtz free energy, A(z), and the inverse Weierstrass corrections which are functions of A(z), the stiffness , and the temperature. The corrections were obtained as described previously.[22](#ref-hummer_free_2010),[23](#ref-minh_optimized_2008) Figure 2c represents the free energy of the ED, CB, and A helical pairs of bacteriorhodopsin. Shaded regions represent the standard deviation around the mean using 300 rounds of bootstrapping.

# Discussion and Results

The unfolding energy of bacteriorhodopsin depends on the structural element being unfolded. Figure 3 reports the energy landscape and unfolding energy per amino acid as a function of molecular extension for the ED, CB, and A helices. Of these, the ED helix has the highest associated average unfolding energy per amino acid, followed by the CB and A helices. Although this energy landscape provides a good estimation for the CB and A helices, the reconstruction of the ED helical pair is limited by its extremely high stiffness.

The large stiffness of the ED helical pair of bacteriorhodopsin, compared to the force probe, introduced error in its energy landscape reconstruction. The inverse Weierstrass transform requires that stiffness of the probe be much greater than the stiffness of the reconstructed landscape. As shown in Figure 3, the greatest stiffness of the bacteriorhodopsin, at the top of the ED helix, is about 8 kcal/(mol ) 50 pN/nm, larger than the cantilever stiffness of 20 pN/nm. Therefore, the top of the ED Helix was poorly reconstructed by the inverse Weierstrass and represents a lower bound on the true landscape. For the CB and A helices, where the protein's stiffness was at least an order of magnitude lower, the stiff-spring approximation of the inverse Weierstrass transform was much better and yielded a more accurate landscape reconstruction. In addition, the almost-negligible correction to the landscape from the term in the inverse Weierstrass transform outside of the ED Helix (Figure Figure 2) confirmed that higher-order corrections were unlikely to effect the landscape of the CB and A helices.

The energy landscape of Figure 3 shows significant variation in unfolding energy per amino acid as a function of extension. The average unfolding energy per amino acid is 0.58 0.03 kcal/mol, in close agreement with past single-molecule results of 0.7 kcal/mol per amino acid[6](#ref-janovjak_valleys_2008) and bulk denaturation studies which report between 0.5 and 0.8 kcal/mol per amino acid[24](#ref-white_membrane_1999). However, in our work the unfolding energies per amino acid varied by more than an order of magnitude, from 4 kcal/mol at the C-terminal end of the ED helix to 0.2 kcal/mol at the C-terminal end of the A helix. The average unfolding energy per amino acid near the top of the ED helix, 3 1 kcal/mol, supported a previous equilibrium result for a specific three amino acid portion of that region of 2.7 0.1 kcal/mol.[17](#ref-yu_hidden_2017) This agreement between the unfolding energies obtained by equilibrium and dynamic force spectropy in this region underscores the local variability in unfolding cost in bacteriorhodopsin.

# Conclusions

The energy landscape reconstruction presented here highlighted that not all amino acids within bacteriorhodopsin have the same unfolding energy. The reconstruction could be improved by increasing the stiffness of the cantilever, providing a more accurate reconstruction of the stiff ED helix; adopting site-specific chemistry to reconstruct the GF helical pair of bacteriorhopsin, a region currently uninterpretable due to tip-surface adhesion; or analyzing refolding experiments, in which the molecule is unfolded and then refolded repeatedly. Refolding experiments would focus on specific portions of bacteriorhodopsin that can reversibly fold and unfold, such as the top of the ED helix, and would provide an independent method for verifying equilibrium energy landscape reconstructions. In addition, the energy cost of disassociating higher-order structure within the protein could be obtained by subtracting the less-interesting energetic contribution of stretching already-unfolded polypeptide chain. Finally, reconstructing the energy landscape with high resolution as a function of buffer conditions, pulling geometry, or point mutations would dramatically improve the current understanding of the energy landscape of bacteriorhodopsin.

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| **Figure 1.** **Bacteriorhodopsin's energy landscape determined by single-molecule force spectroscopy.** (**A**) A cartoon of the pulling geometry used to unfold bR. (**B**) A representative force versus molecular extension curve, or force-extension curve. The first 20nm of all curves are not analyzed due to non-specific surface adhesion. The colored bars correspond to the regions where the helical pairs from (A) rupture. Many such curves are used for reconstruction an energy landscape via an inverse Weierstrass transform. (**C**) A detailed plot of the A helix, which exhibits transitions between states. (**D**) A plot detailing many force extension curves of bR with worm-like chain polymer fits overlayed onto major states. (**E**) An equilibrium assay demonstrates rapid transitions between intermediate states in the E helix. (**F**) The energy landscape obtained via applying to equilibrium data as in (E) (XXX). Figures (D-F) are reproduced with permission from XXX. |
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| **Figure 2.** **Energy landscape reconstruction of bacteriorhodopsin reveals significant intra-molecule variation in unfolding energy.** (**A**) A heat map of all force-extension curves used in this work. Data within 20nm of the surface are excluded due to surface adhesion. (**B**) The Helmholtz free energy, the red, dashed and dotted line , was corrected[22](#ref-hummer_free_2010) to obtain the free energy as a function of molecular extension, the black dotted line . The derivative and second derivative of the Helmholtz free energy, denoted by a dot, are with respect to extension. (**C**) The from (B), where the shaded region gives the standard deviation from three non-overlapping subsets of the data in (A). |
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| **Figure 3.** **Unfolding energies vary within a single bacteriorhodopsin molecule.** This figure shows the energy landscape applied to each helical region of Bacteriorhodopsin separately. The left axis is the change in free energy, plotted as a dotted line which is color-coded by the helix. The standard deviation is shown as a shaded region around the mean. Errors were determined by applying the inverse Weierstrass transform to each of three equal-sized subsets. The right axis denotes free energy change, denoted by a thick purple line. |

1. Stanley, A. M. & Fleming, K. G. Process of folding proteins into membranes: Challenges and progress. *Archives of Biochemistry and Biophysics* **469,** 46–66 (2008).

2. Kessler, M. & Gaub, H. E. Unfolding Barriers in Bacteriorhodopsin Probed from the Cytoplasmic and the Extracellular Side by AFM. *Structure* **14,** 521–527 (2006).

3. Leonhard, K. *et al.* Membrane Protein Degradation by AAA Proteases in Mitochondria. *Molecular Cell* **5,** 629–638 (2000).

4. Janovjak, H., Müller, D. J. & Humphris, A. D. L. Molecular Force Modulation Spectroscopy Revealing the Dynamic Response of Single Bacteriorhodopsins. *Biophysical Journal* **88,** 1423–1431 (2005).

5. Onuchic, J. N., LutheySchulten, Z. & Wolynes, P. G. Theory of protein folding: The energy landscape perspective. *Annual Review of Physical Chemistry* **48,** 545–600 (1997).

6. Janovjak, H., Sapra, K. T., Kedrov, A. & Mueller, D. J. From valleys to ridges: Exploring the dynamic energy landscape of single membrane proteins. *Chemphyschem* **9,** 954–966 (2008).

7. Rief, M., Gautel, M., Oesterhelt, F., Fernadez, J. M. & Gaub, H. E. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science; Washington* **276,** 1109–12 (1997).

8. Kellermayer, M. S. Z., Smith, S. B., Granzier, H. L. & Bustamante, C. Folding-unfolding transitions in single titin molecules characterized with laser tweezers. *Science; Washington* **276,** 1112–6 (1997).

9. Borgia, A., Williams, P. M. & Clarke, J. Single-molecule studies of protein folding. in *Annual Review of Biochemistry* **77,** 101–125 (Annual Reviews, 2008).

10. Neuman, K. C. & Nagy, A. Single-molecule force spectroscopy: Optical tweezers, magnetic tweezers and atomic force microscopy. *Nature Methods* **5,** 491–505 (2008).

11. Bippes, C. A. & Muller, D. J. High-resolution atomic force microscopy and spectroscopy of native membrane proteins. *Reports on Progress in Physics* **74,** 086601 (2011).

12. Oesterhelt, F. *et al.* Unfolding pathways of individual bacteriorhodopsins. *Science (New York, N.Y.)* **288,** 143–146 (2000).

13. Dudko, O. K., Hummer, G. & Szabo, A. Theory, analysis, and interpretation of single-molecule force spectroscopy experiments. *Proceedings of the National Academy of Sciences* **105,** 15755–15760 (2008).

14. Zhang, Y. & Dudko, O. K. A transformation for the mechanical fingerprints of complex biomolecular interactions. *Proceedings of the National Academy of Sciences of the United States of America* **110,** 16432–16437 (2013).

15. Crooks, G. E. Entropy production fluctuation theorem and the nonequilibrium work relation for free energy differences. *Physical Review E* **60,** 2721–2726 (1999).

16. Jarzynski, C. Nonequilibrium Equality for Free Energy Differences. *Physical Review Letters* **78,** 2690–2693 (1997).

17. Yu, H., Siewny, M. G. W., Edwards, D. T., Sanders, A. W. & Perkins, T. T. Hidden dynamics in the unfolding of individual bacteriorhodopsin proteins. *Science* **355,** 945–950 (2017).

18. Park, P. S.-H. *et al.* Stabilizing Effect of Zn2+ in Native Bovine Rhodopsin. *The Journal of biological chemistry* **282,** 11377–11385 (2007).

19. Preiner, J., Janovjak, H., Rankl, C., Knaus, H. & al, et. Free Energy of Membrane Protein Unfolding Derived from Single-Molecule Force Measurements. *Biophysical Journal; New York* **93,** 930–7 (2007).

20. Sullan, R. M. A., Churnside, A. B., Nguyen, D. M., Bull, M. S. & Perkins, T. T. Atomic force microscopy with sub-picoNewton force stability for biological applications. *Methods (San Diego, Calif.)* **60,** 131–141 (2013).

21. Edwards, D. T. & Perkins, T. T. Optimizing force spectroscopy by modifying commercial cantilevers: Improved stability, precision, and temporal resolution. *Journal of Structural Biology* **197,** 13–25 (2017).

22. Hummer, G. & Szabo, A. Free energy profiles from single-molecule pulling experiments. *Proceedings of the National Academy of Sciences* **107,** 21441–21446 (2010).

23. Minh, D. D. L. & Adib, A. B. Optimized Free Energies from Bidirectional Single-Molecule Force Spectroscopy. *Physical Review Letters* **100,** 180602 (2008).

24. White, S. H. & Wimley, W. C. Membrane protein folding and stability: Physical principles. *Annual Review of Biophysics and Biomolecular Structure; Palo Alto* **28,** 319 (1999).