# Transmembrane Helices Have Rough Energy Surfaces

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Protein folding is one of the most challenging areas in biochemical and biophysical research.[1] Multidimensional energy landscapes and folding funnels provided new theoretical frameworks for protein folding in vitro and in vivo[2,3] as well as for molecular binding mechanisms and dynamic switching between functional protein conformations.[4] The topography of their free-energy surfaces, whether they are smooth or rough, determines the dynamics of virtually all of these fundamental processes. Reasonably smooth energy landscapes are, for example, linked to the fast and robust folding of small water-soluble proteins,[3] while rough surfaces designate transient trapping in local energy minima.[2,5] The folding of transmembrane (TM) proteins is a particularly long-standing problem.[6] Historically, experimental studies were hindered by difficulties associated with anisotropic lipid bilayer environments, while computational studies had to deal with increased complexity in protein-membrane systems.[7] Thus it may not be surprising that relatively little biophysical information on the energy landscapes of membrane proteins are available.

Hyeon and Thirumalai[8] recently established the basis for experimentally probing the roughness of the energy landscapes of two-state (un-)folders. They proposed to determine the strengths of single proteins or RNA molecules at different temperatures in forced unfolding experiments. Here, we apply a modified form of their model[9] to determine the energy landscape roughness of single transmembrane α-helices of bacteriorhodopsin (BR). Membrane proteins like BR reside and function in the highly anisotropic environment of lipid bilayers. BR is composed of seven TM helices that form small ($\approx 20-30$ amino acids (aa) length) and highly confined structures with different sequences and different conformational restriction (also see later).

Using an atomic force microscope (AFM), individual BR molecules can be mechanically unfolded by applying a pulling force to their C-termini (Figure 1A).[10] Similar to this experimental setup, mechanical pulling forces are also believed to play a central role in membrane protein unfolding in vivo (see later). Since single proteins unfold sequentially and in different unfolding pathways, mechanical energy landscapes can be obtained for the unfolding of individual helices and hairpins.[11] The unfolding forces of the helices show a logarithmic dependence on the force loading rate (Figure 1C), indicating that a sharp energy barrier is crossed during two-state unfolding (Figure 1B).[11] Different approaches exist for extracting the widths, $x_\beta$, and natural transition rates, $k_u$, of the energy barriers. We applied the established model of Evans[12] and Monte Carlo simulations[13] (also see Supporting Information). Both approaches yield similar results (Table 1S) with barrier widths between $\approx 3$ and 8 Å and unfolding rates between $10^{-4}$ and $10^{-1}$ s$^{-1}$ at a temperature of 27 °C.

As intuitively expected, the unfolding rates increase with enhanced temperature. For example, helices B and C unfold with a rate of $2.7 \times 10^{-5}$ s$^{-1}$ at 18 °C, $7.0 \times 10^{-4}$ s$^{-1}$ at 27 °C, and $8.1 \times 10^{-2}$ s$^{-1}$ at 42 °C (Figure 1C; see Tables 1S and 2S for a complete list). The temperature-dependence of the transition rates
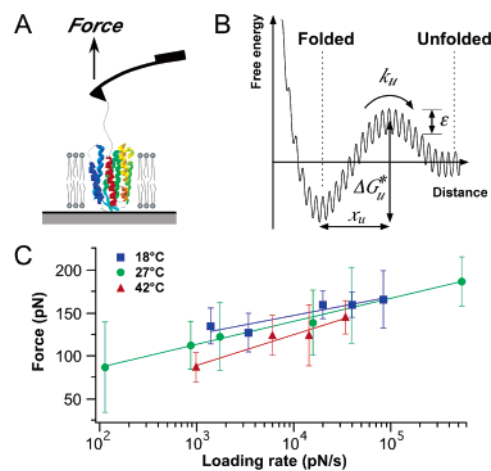


***Figure 1.*** (A) Pulling on their C-terminus with an AFM unfolds single BRs. (B) A simple two-state potential describes the unfolding of single α-helices and α-helical pairs. (C) Monitoring the most probable unfolding forces vs force loading rate determines the width of the barrier, $x_u$, and transition rate, $k_u$. The pairwise unfolding of helices B and C is shown.

can be explained by the thermally activated nature of the unfolding process and/or by a destabilization of the protein (i.e., a change in barrier height). To address this point, we applied the Arrhenius equation to determine the free energy of activation, $\Delta G_u^*$ (methods in SI). Despite the large uncertainties when estimating transition rates in forced unfolding experiments[14] no clear temperature dependence of the activation energies could be identified (Tables 1S and 2S), in line with earlier studies that reported no significant changes in the structure or heat capacity of BR in this physiological temperature range.[15] We also observed a temperature-dependent reduction in the widths of the energy barriers. For example, the barrier to pairwise unfolding of helices B and C is located at 7.67 ± 0.03 Å at 18 °C, 6.52 ± 1.65 Å at 27 °C, and 4.11 ± 0.40 Å at 42 °C from the folded states (Figure 1C; Tables 1S and 2S).

From these values, we determined the energy surface roughness of BR helices. Zwanzig[16] suggested that a size-dependent roughness superimposed on a smooth energy landscape reduces diffusion coefficients, especially at low temperatures. Hyeon and Thirumalai accounted for the applied force and Nevo and co-workers for variations in $x_\beta$ with temperature.[8,9] A root-mean-square energy roughness scale, $\epsilon$, is proposed, which is considered to be independent of the position along the reaction coordinate and Gaussian distributed. For small $\epsilon/(k_B T)$ and $\epsilon/\Delta G_u^* < 1$, $\epsilon$ is given by

$$\epsilon^2 \approx \frac{x_\beta(T_2)k_B T_2 x_\beta(T_1)k_B T_1}{x_\beta(T_2)k_B T_2 - x_\beta(T_1)k_B T_1}\left[\Delta G_u^*\left(\frac{1}{x_\beta(T_1)} - \frac{1}{x_\beta(T_2)}\right) + \frac{k_B T_1}{x_\beta(T_1)}\ln\left(\frac{r_f(T_1)x_\beta(T_1)}{k_u(T_1)k_B T_1}\right) - \frac{k_B T_2}{x_\beta(T_2)}\ln\left(\frac{r_f(T_2)x_\beta(T_2)}{k_u(T_2)k_B T_2}\right)\right] \quad (1)$$

**Table 1.** Energy Surface Roughness of BR Helices (Data Recorded at 18 and 42 °C; 50 pN (75 pN) Threshold Force)

| secondary structure element(s)[a] | $\epsilon$ (pN nm) |
|---|---|
| pairwise unfolding of helices[b] | |
| helices E and D | 22.33 (22.33) |
| helices C and B | 22.68 (22.68) |
| unfolding of single secondary structure elements | |
| helix E[c] | 23.92 (23.92) |
| helix D[d] | 23.82 (23.81)/10.86 (10.88) |
| helix C[d] | 22.82 (22.81)/38.09 (38.05) |
| helix B[d] | 28.24 (28.25)/no value[f] |
| helix A[e] | 16.71 (16.71) |
| loop BC | 27.68 (27.68) |

[a] Five of seven BR helices are resolved in mechanical unfolding experiments (see methods in SI). [b] Including their connecting loops. [c] Including the 3 aa long loop DE. [d] Elements unfold in two different unfolding pathways and two values were obtained for $\epsilon$. [e] Including the 7 aa long N-terminus. [f] No value is obtained for helix B in one pathway because of a negative term in eq 1.

where $r_f$ denotes force loading rates yielding identical unfolding forces at temperatures $T_1$ and $T_2$.[9] The roughness values in Table 1 were obtained using data recorded at 18 and 42 °C and threshold forces of 50 and 75 pN. For single and pairs of helices, $\epsilon$ was between 15.45 and 26.68 pN nm ($\approx 4-6$ $k_B T$). These values corresponds to $\epsilon/\Delta G_u^* \approx 0.2$ and $\epsilon/(k_B T) \approx 5$.

Since minor membrane phase transitions were proposed in this temperature range,[17] we also applied eq 1 to datasets obtained at 18 and 27 °C (Table 3S). With only two exceptions, the determined roughness changed by $<1.5$ $k_B T$, indicating a small influence of lipid phase transitions. Second, we tested to what extent uncertainties in $\Delta G_u^*$ influence our calculations and found that an under- or overestimation of $\Delta G_u^*$ by 20% changes $\epsilon$ by less than 11% (data not shown). Finally, we also excluded an influence of temperature on the optical sensitivity or spring constant of the cantilever force sensor (Figure 1S).

The determined energy surface ruggedness for the BR helices ($\approx 5$ $k_B T$) is of the same magnitude as for short peptides ($\approx 2$ $k_B T$), small globular proteins ($<5$ $k_B T$), or the force-induced dissociation of two interacting proteins ($\approx 5$ $k_B T$) or DNA strands ($\approx 10$ $k_B T$),[9,18] suggesting similar dynamic properties and local energy features in TM helices and globular proteins. The mechanically probed BR helices differ in their lengths, sequences, and neighborhoods. Through the design of the forced unfolding experiments, helices E and D unfold in an environment surrounded by more helices and a more rigid lipid bilayer than helices B and C or the last helix A. However, in all cases similar amounts of roughness were detected indicating that our results can be related to fundamental properties of TM helices.

Rough energy surfaces are prerequisites for functionally related conformational changes and have pronounced effects on protein stability and folding.[3,4,9] We directly measured the energy surface roughness of TM helices, the structural and functional building blocks in most membrane proteins, and found considerable local energy corrugations. Interestingly, pulling forces (like those applied here) are believed to be central in membrane protein degradation in vivo.[19] The proposed mechanical extraction of misfolded proteins is thus likely to occur on similarly rugged energy surfaces as reported here. Our results also provide an energetic framework for small vertical motions of functionally relevant TM helices, such as marginally stable voltage-sensing helices.[20] Membrane proteins

perform complex tasks within highly confining lipid bilayers as expressed in the narrow distribution of lengths and packing angles of their helices.[21] For a different biological system, the chaperonin-assisted folding of water-soluble proteins, the confinement in chaperonin-cavity (the Anfinsen cage) was shown to increase folding rates by reducing the energy roughness.[22] Similar effects were observed in simulations.[23] Our results extend these views by showing that TM structures exhibit rough energy surfaces despite topological confinement. Since lipid bilayers severely restrict conformational dynamics, topological and curvature frustration[24] may be origins of rough-free energy landscapes in membranes.

**Supporting Information Available:** Additional energy parameters and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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