Combined kinetic and thermodynamic analysis of α -helical membrane protein unfolding

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The analytical toolkit developed for investigations into water-soluble protein folding has yet to be applied in earnest to membrane proteins. A major problem is the difficulty in collecting kinetic data, which are crucial to understanding any reaction. Here, we combine kinetic and thermodynamic studies of the reversible unfolding of an α -helical membrane protein to provide a definitive value for the reaction free energy and a means to probe the transition state. Our analyses show that the major unfolding step in the SDS-induced denaturation of bacteriorhodopsin involves a reduction in α -helical structure and proceeds with a large free-energy change; both our equilibrium and kinetic measurements predict that the free energy of unfolding in the absence of denaturant is +20 kcal·mol⁻¹, with an associated m -value of 25 kcal·mol⁻¹. The rate of unfolding in the absence of denaturant, $k_u^{\text{H}_2\text{O}}$, is surprisingly very slow ($\approx 10^{-15}$ s⁻¹). The kinetics also give information on the transition state for this major unfolding step, with a value for β ($m_f/[m_f + m_u]$) of ≈ 0.1 , indicating that the transition state is close to the unfolded state. We thus present a basis for mapping the structural and energetic properties of membrane protein folding by mutagenesis and classical kinetics.

bacteriorhodopsin | folding | free energy | transition state

The folding and assembly of integral membrane proteins is a fundamental process within biological systems, yet surprisingly few quantitative studies of membrane protein folding have been performed. Many transmembrane α -helical proteins are unstable once they are displaced from the biological membrane, and our limited understanding of the factors affecting folding and stability presents a major barrier to studies of structure and function.

Bacteriorhodopsin (bR), a light-driven proton pump from the purple membrane (PM) of *Halobacterium salinarum*, is the current paradigm for studies of α -helical membrane protein folding (1, 2). The native purple chromophore is formed by the covalent attachment of the cofactor retinal at K216 and offers a direct, quantitative measure of the folded state of the protein because it is strongly influenced by retinal isomerization and noncovalent binding pocket interactions (i.e., refs. 3 and 4).

The unfolding and refolding of bR in lipid/detergent mixtures was first reported in the seminal work of London and Khorana (5). bR was found to unfold in SDS from the native purple state by way of an intermediate to give denatured apoprotein, bacteriorhodopsin (bO), and bR was efficiently reconstituted from bO by replacing SDS with renaturing micelles. The kinetics of refolding have been investigated in considerable detail (1, 6, 7). A recent equilibrium study of this denaturation of bR (8) has also demonstrated that the free-energy change of unfolding could be measured. A full kinetic and thermodynamic study of unfolding and folding is, however, vital for a complete understanding of the reaction mechanism, but it has yet to be achieved. Here, we present such a study and probe the reversible unfolding of bR in greater detail and over wider time scales than before. We identify a series of intermediate states as well as the point when helical structure is lost. We further highlight the intermediate states involved in the major unfolding step of bR and determine the free-energy change for this step from both kinetic rates and

the equilibrium constant. The reaction can be approximated to a two-state process; both kinetic- and equilibrium-derived free-energy values are linear with SDS concentration and with comparable gradients (m values), and extrapolate to remarkably similar free-energy changes at zero denaturant. Analysis of the kinetic m -values gives information on the transition state. Thus, we obtain a robust value for the free-energy change and information on the transition state for the formation of the final fold of an α -helical membrane protein. This advances understanding of this final folding step that is notoriously difficult to achieve *in vitro*.

Results

Equilibrium Measurements. Equilibrium measurements of unfolding were performed as in refs. 8 and 9 by titrating bR in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) micelles with increasing concentrations of SDS (expressed throughout as bulk mole fraction SDS, χ_{SDS}) and monitoring the loss of the native purple chromophore (bR₅₆₀). The overall free energy of unfolding was then determined by fitting the entire dataset to a two-state equation (Fig. 1*a*) as well as by linearly extrapolating individual free-energy values around the transition region (Fig. 1*b*). These two analyses were consistent (Table 1), with the latter, linear method giving a free energy of unfolding in the absence of denaturant, $\Delta G_u^{\text{H}_2\text{O}}$ of $+20.57 \pm 0.20$ kcal·mol⁻¹ with $m_u^{\text{H}_2\text{O}}$ of -28.30 ± 0.27 kcal·mol⁻¹. The transition midpoint ($K_U = 1$) was found to be at $0.725 \chi_{\text{SDS}}$, higher than the value of $\approx 0.60 \chi_{\text{SDS}}$ found in work using SDS/DMPC/CHAPSO micelles (8). The DMPC/CHAPS system used here may impart greater stability to the protein.

CD spectroscopy was used to determine changes in secondary structure on unfolding (Fig. 1*a* *Inset*). At low SDS, the percentage of secondary structure present as α -helix, β -sheet, β -turn, and random coil, respectively, was 78%, 7%, 9%, and 6%. These values are characteristic of native bR. A sharp transition was observed at $>0.73 \chi_{\text{SDS}}$ and the secondary structure composition changed to 53%, 21%, 9%, and 17% of the motifs above. These values correlate with those found during formation of the partially unfolded apoprotein bO at high SDS concentrations (5, 10). The sloping baseline seen in the absorbance signal (0–0.6 χ_{SDS}) was not associated with a change in the CD spectra. The reduction in helicity on SDS unfolding, monitored at 226 nm, agrees well with the loss of bR₅₆₀ (Fig. 1*a*). (Note that the unfolding time used means that the SDS-unfolded state in the CD measurements corresponds to bO₄₄₀ below.)

Kinetic Measurements. Spectral changes during bR unfolding over short time scales (milliseconds to seconds) were analyzed by

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Fig. 1. SDS-induced equilibrium unfolding of bR monitored by absorbance and CD spectroscopy. (a) The reduction in the native chromophore band at 560 nm (open circles) and helical secondary structure, as reflected by the intensity at 226 nm (filled triangles). A_{560} data were fit to a two-state equation (solid line). Error bars where shown represent deviation from the mean average of duplicate experiments. (Inset) CD spectra of bR (solid line) and unfolded bO (at 0.82 χ_{SDS} , dashed line). (b) Linear dependence of the free-energy change (ΔG_u) with respect to SDS.

using stopped-flow mixing and diode array detection. On mixing with $\chi_{\text{SDS}} > 0.72$, the native bR band at 560 nm broadens and shifts to 600 nm within the 16-ms experimental dead time (Fig. 2a). This 600-nm band (bR_{600}) then decays coincident with the appearance of a band at 490 nm (bR_{490}) with a single isosbestic point at 530 nm. Subsequently, bR_{490} decays to a band at 440 nm with a single isosbestic point at 480 nm. This 440-nm band arises from denatured protein that has lost secondary structure as well as retinal binding pocket structure and interactions with retinal (see below) and is termed bO_{440} . No wavelength shifts or photodegradation of the native bR band were observed in control samples recorded at $\chi_{\text{SDS}} < 0.70$ (data not shown).

Analysis at individual wavelengths (Fig. 2b) shows that bR_{560}

Fig. 2. Fast unfolding kinetics. (a) Changes in bR spectra over time on addition of 0.882 χ_{SDS} . After an immediate red shift to 600 nm (solid black line; recorded at 16 ms), the signal decays to a 490-nm band (dashed line; recorded at 208 ms). This subsequently decays to a band at 440 nm (dotted line; recorded at 11.58 s). The gray lines show intermediate traces, demonstrating the single isosbestic point between each species. Arrows indicate loss or gain of band during reaction. (b) Changes at individual band maxima over time: 600 nm (open circles), 490 nm (filled inverted triangles), and 440 nm (open triangles).

decays sequentially through the 600-nm and 490-nm states to give bO_{440} . This conclusion is reinforced by a global data analysis of the spectra in Fig. 2a in which the data were best fit to a sequential reaction model with three species ($A \rightarrow B \rightarrow C$) (data not shown). These three species corresponded to bR_{600} , bR_{490} , and bO_{440} respectively.

Single-wavelength measurements, with increased data density, were used for more accurate determination of unfolding rates. Decay of bR_{560} was found to fit well to the sum of two exponential functions (Fig. 3a): $k_{u1} = 14.9 \text{ s}^{-1}$ and $k_u = 0.54 \text{ s}^{-1}$ at 0.882 χ_{SDS} , in good agreement with the rates of formation of bR_{490} (11.4 s^{-1}) and bO_{440} (0.40 s^{-1}), respectively, determined from the diode array spectra. The magnitude of both k_{u1} and k_u

Table 1. Summary of kinetic and equilibrium data

Data Type	Method	Folding*		Unfolding*		$\Delta G_u^{\text{H}_2\text{O}, \dagger}$ kcal·mol ⁻¹	$m_u^{\text{H}_2\text{O}, \dagger}$ kcal·mol ⁻¹	$\beta^\ddagger$
		$k_f^{\text{H}_2\text{O}}, \text{s}^{-1}$	$m_f, \text{kcal·mol}^{-1}$	$k_u^{\text{H}_2\text{O}}, \text{s}^{-1}$	$m_u, \text{kcal·mol}^{-1}$			
Kinetic	Linear fit	0.200 ± 0.014	-2.69 ± 0.21	$2.65 \times 10^{-13} \pm 1.2 \times 10^{-14}$	32.08 ± 1.58	+16.14	20.51	0.13
	Chevron plot	0.307 ± 0.07	-3.55 ± 0.42	$9.27 \times 10^{-16} \pm 4.3 \times 10^{-16}$	38.53 ± 0.50	+19.72	24.83	0.14
Equilibrium	Global fit	—	—	—	—	+15.94 $\pm$ 0.95	-22.28 $\pm$ 1.30	—
	Linear transformation	—	—	—	—	+20.57 $\pm$ 0.20	-28.30 $\pm$ 0.27	—

*Folding and unfolding rates from bO_{440} .

[†]For equilibrium data, $\Delta G_u^{\text{H}_2\text{O}}$ and m value are derived from the global fit of a denaturation curve (as in Fig. 1a) or from the same data but by a linear transformation of individual ΔG_u (Fig. 1b). For kinetic data, these parameters are calculated from Eqs. 5 and 6.

[‡] $\beta = m_f / (m_f + m_u)$

Fig. 3. Detailed bR unfolding kinetics. (a) Decay of A_{560} at $0.882 \chi_{\text{SDS}}$. (Inset) Shown are residuals from single (Upper) and double (Lower) exponential fits to the data, the latter resolving k_{u1} and k_u . (b) Dependence of $\ln k_u$ and $\ln k_f$ on χ_{SDS} . (Inset)

depended on SDS concentration (Fig. 3b), but the ratio of the amplitudes of the two phases (40:60, respectively) was independent of SDS.

k_u represents the unfolding of bR to bO_{440} and $\ln k_u$ shows a clear linear dependence on χ_{SDS} (Fig. 3b). Accordingly, extrapolation to the y axis gives a rate of unfolding in the absence of denaturant, $k_u^{\text{H}_2\text{O}}$, of $2.65 \times 10^{-13} \text{ s}^{-1}$ with an associated m value, mk_u , of $32.08 \text{ kcal}\cdot\text{mol}^{-1}$ (Eq. 2 and Table 1).

Slow Unfolding Events. Slow unfolding events (10–2,500 s) were monitored at multiple wavelengths after hand mixing. Over these longer time scales, bO_{440} slowly decays coincident with the emergence of a band at 390 nm (bO_{390}). This transition occurs with a single isosbestic point at 413 nm (Fig. 4 Inset). The rate

Fig. 4. Slow unfolding kinetics and retinal dissociation. Free retinal (A_{390}) is formed from bO_{440} by a single exponential phase (filled diamonds; Inset, spectra of same) at $\approx 9 \times 10^{-4} \text{ s}^{-1}$. Error bars represent deviation from a mean average of two experiments. Addition of hydroxylamine (open diamonds) leads to formation of a retinaloxime (A_{365}) and increases the rate of retinal dissociation ≈ 15 -fold.

Fig. 5. Kinetics of refolding from bO_{440} analyzed by sequential stopped-flow mixing. (open squares, upper curve), recovery of A_{560} when refolding is induced by concentrated DMPC/CHAPS (after 2 min unfolding to give bO_{440}); a monoexponential fit is shown. (Inset) Full-spectrum scans of the same reaction: first trace (dashed line); intermediate traces (gray lines), and final trace (solid black line). Arrows indicate decay or growth of absorbance bands. Controls: denatured protein mixed with a dilute DMPC/CHAPS solution and thus no refolding (X; lowest trace) and bR_{560} unfolded only for 10 ms (thus no bO_{440} formed) (+).

of growth of the bO_{390} band fit well to a single exponential function (Fig. 4) with a rate constant of $\approx 9 \times 10^{-4} \text{ s}^{-1}$. The addition of hydroxylamine increased the rate of growth of bO_{390} to 0.015 s^{-1} (Fig. 4) and shifted the band to 365 nm, corresponding to the formation of a retinaloxime (11). These data suggest that the retinal binding pocket is significantly disrupted in bO_{440} but that retinal remains covalently bound. This disruption leaves the Schiff base accessible to the bulk solvent, leading to slow hydrolysis of the covalent bond in water and more rapid hydrolysis by hydroxylamine (5).

Refolding from bO_{440} . Refolding was initiated from bO_{440} by introducing concentrated DMPC/CHAPS such that SDS was reduced to subdenaturing concentrations ($\leq 0.63 \chi_{\text{SDS}}$). The refolding reaction proceeded through a single isosbestic point at 490 nm (Fig. 5 Inset) and the purple chromophore was recovered at 560 nm with a refolding yield of $>95\%$. The unfolding intermediates bR_{490} and bR_{600} are thus not populated on refolding, as noted for refolding from apoprotein (bO) in SDS (12).

A sequential mix stopped-flow experiment was then used to analyze the folding kinetics from bO_{440} (i.e., rapid unfolding to the 440-nm state, followed by rapid refolding to avoid decay of bO_{440} to bO_{390}). Recovery of bR_{560} was fit well to a single exponential (Fig. 5). Fig. 5 also shows that an insignificant signal change was observed in controls where χ_{SDS} remained high (i.e., denaturing conditions) or when the delay time between unfolding and refolding was shorter than the time required to form bO_{440} (10 ms).

Chevron Plot. A chevron plot was constructed from the observed unfolding (k_u) and refolding (k_f) rates for the bR_{560} to bO_{440} transition (Fig. 6). The plot fit well to a simple two-state equation (Eq. 4) and the rates of unfolding and folding in the absence of denaturant, termed $k_u^{\text{H}_2\text{O}}$ and $k_f^{\text{H}_2\text{O}}$, were found to be $9.27 \times 10^{-16} \text{ s}^{-1}$ and 0.307 s^{-1} , respectively. We note the extensive extrapolation to the y axis required because of the limited range of χ_{SDS} accessible to experiment. The unusually low value of $k_u^{\text{H}_2\text{O}}$ indicates that the unfolding curve may follow a trend over a wider range of χ_{SDS} that is more complex than can be appreciated from our data. The total free-energy change in the absence of denaturant ($\Delta G_{u\text{kin}}^{\text{H}_2\text{O}}$ is calculated from the kinetic data to be

Fig. 6. Chevron plot showing change in the $\ln$ of the observed rates of unfolding (k_u , open circles) and folding (k_f , open squares) from bO_{440} at different concentrations of SDS. Data were fit to a two-state equation (Eq. 4). (Inset) Shown is the nonlinearity observed when refolding from bO_{390} ($\ln k_{f390}$, filled squares).

+19.72 kcal·mol⁻¹ (Eq. 5) with an associated constant, $m_{uH_2O}^{H_2O}$, of 24.83 kcal·mol⁻¹ (Eq. 6). This is in close agreement with the linear extrapolation of equilibrium data (Fig. 1, Table 1).

Refolding from bO_{390} . Refolding was also instigated from bO_{390} by incubating the unfolded protein for 1 h before refolding (to allow bO_{440} to decay to bO_{390}). Retinal was not removed after unfolding and no additional retinal was introduced. Refolding from bO_{390} (with retinal present in solution throughout) was found to be multiphasic proceeding through several intermediates as reported (6, 12, 13). The refolding yield was >95%, with an overall folding rate (k_{f390}) 20- to 100-fold slower than refolding from bO_{440} (e.g., 0.0017 s⁻¹ vs. 0.043 s⁻¹, respectively, at 0.472 χ_{SDS}). This slow rate from bO_{390} corresponds to Schiff base formation and regeneration of bR (6, 12, 13). In contrast to $\ln k_f$ (from bO_{440}), $\ln k_{f390}$ is not linear with SDS concentration; rather, there is a distinct plateau at lower concentrations (Fig. 6 Inset). This behavior arises from the rate-limiting formation of an obligatory apoprotein intermediate (6, 12, 13). We note that k_{f390} could only be resolved between 0.208 and 0.525 χ_{SDS} and k_f between 0.410 and 0.626 χ_{SDS} .

Discussion

The folding of integral membrane proteins has received relatively scant attention. This is at first surprising considering the ubiquity and importance of these proteins in biological processes and the implications for misfolding in several disease states. Membrane proteins have thus far evaded thorough analysis largely because (i) the hydrophobicity of the transmembrane regions makes these proteins difficult to express and solubilize, (ii) there are few truly reversible methods of unfolding, and (iii) the necessary complexity of the reconstitution systems used precludes the ready application of many analytical techniques. bR is currently the only α -helical membrane protein for which it is possible to monitor the kinetics and energetics of reversible folding in light of a known protein structure. We have extensively studied the folding of this protein and here turn our attention to the unfolding pathway.

Mechanistic Scheme of Unfolding. We find that unfolding of bR is a complex process with a number of spectroscopically distinct intermediate states. bR_{560} decays sequentially through bR_{600} and bR_{490} to bO_{440} , in which the protein has lost helical structure but retinal remains covalently bound (Figs. 1–3). bO_{440} then slowly decays to bO_{390} (Fig. 4). This leads to a mechanism for unfolding:

Scheme 1

bR states with absorption bands at 600 nm or 490 nm have previously been observed and seem to primarily involve changes in retinal isomerization. Treating bR with SDS has been observed to cause the formation of a “blue membrane” with an absorption band at 600 nm (5, 14, 15), presumed to be similar to the species formed at low pH (14, 16) or after the removal of cations (17, 18). The purple-to-blue transition involves the rearrangement of tertiary structure (19, 20) and protonation of the Schiff base counterion D85 (21, 22) and the blue membrane contains 70:30 *all-trans*:13-*cis*-retinal in both dark- and light-adapted states (14, 23, 24); by comparison, the native purple membrane contains *all-trans*-retinal after light adaptation and at $\approx$ 1:1 13-*cis*/*all-trans* in the dark (25). Extensive irradiation of the blue membrane by red light (26, 27) initiates decay to the “pink membrane” at 490 nm. This stable photoproduct arises from isomerization of the bound chromophore so that the 9-*cis*-form becomes the dominant isomer; the blue-to-pink transition proceeds through a single isosbestic point at 530 nm. This is consistent with our results, although light-induced formation of the pink membrane normally occurs over a time course of several hours rather than the few seconds shown here. This faster time is most likely an effect of the SDS destabilization. We therefore suggest that the intermediates bR_{600} and bR_{490} represent primarily retinal isomerizations with minor perturbations of the protein structure.

The 440-nm unfolding band was assigned to an unfolded form of the protein in which the retinal remains covalently bound but most other interactions with the protein are lost (5, 9, 15, 28, 29). Our results are consistent with this finding and confirm that in bO_{440} the retinal Schiff base is accessible to the solution environment and slowly decays to a 390 species that represents free retinal (Fig. 4; see also ref. 5). Moreover, the reaction with hydroxylamine (Fig. 4) indicates that the Schiff base is still present in bO_{440} , whereas the CD data (Fig. 1) show that the protein has unfolded with a reduction in helical structure. No change in α -helicity was observed during CD measurements over long time scales, showing that bO_{440} and bO_{390} states have the same amount of α -helix ($\approx$ 53% compared with $\approx$ 78% in bR). Thus, the formation of bO_{440} is the most significant step in unfolding because it arises from a gross structural change. In fact, there is little detailed information available on the nature of the unfolded state, but it is most likely that bO_{440} is characterized by some local unwinding of helices and the disruption of tertiary packing contacts.

A Two-State Folding Scheme for bR. Refolding from bO_{440} is highly efficient and the reaction is well fit by a single exponential phase (Fig. 5). A second, slower phase is sometimes observed that is seemingly invariant with regard to SDS concentration (remaining at $\approx$ 0.01 s⁻¹) and has low amplitude. The time scale of this phase indicates that it may represent retinal isomerization, rather than folding (12, 13).

Considering the evidence above that the major folding and unfolding events can each be represented by a single exponential rate, our kinetic data verify the earlier assumption (8) that the unfolding of bR can be approximated to a reversible two-state process:

Scheme 2

An important requisite of any two-state folding scheme is that $\Delta G_{uH_2O}^{H_2O}$ and $m_{uH_2O}^{H_2O}$ derived from equilibrium and kinetic measurements are equivalent. This condition is satisfied here (Table

1). Additionally, the log of the rate constants for folding and unfolding should be linear with regard to SDS. We find that unfolding to, and refolding from, bO₄₄₀ can be satisfactorily described by a chevron plot (Fig. 6). Both arms of this plot are linear over the experimentally accessible range of SDS concentrations, and the data are readily fit by a two-state equation.

Calculation of β -Value. The m values obtained from kinetic data (m_f , m_u ; Table 1) are most simply interpreted as being related to the degree of change in the average solvent-accessible surface area during folding and unfolding. The ratio of individual m values to the overall m value for unfolding thus provides a relative index (β , Eq. 7) of the solvent accessibility of the transition state. The value of β is a useful measure of the compactness of the transition state relative to the native and denatured states and the position of the transition state along the reaction coordinate. We find β to be ≈ 0.1 for bR (Table 1), suggesting that the transition state is relatively diffuse and similar to the unfolded state; we consider that, in this experimental context, the values for m and β reflect the accessibility of SDS to certain protein surfaces within a mixed micelle. For many water-soluble proteins exhibiting two-state kinetics, β -values are normally between 0.6 and 0.9 (30), but the low β -value found here is congruent with the high concentrations of denaturant required for unfolding. The transition state may need to be diffuse to allow the ingress and binding of SDS before unfolding.

Comparison with Other Studies. One interesting aspect of this work is the remarkably low rate of unfolding in the absence of denaturant ($k_u^{\text{H}_2\text{O}}$), which was found to be $\approx 10^{-15} \text{ s}^{-1}$ (Table 1). This is atypically low compared with $k_u^{\text{H}_2\text{O}}$ for most water-soluble proteins studied to date. This finding suggests that bR has high kinetic stability in DMPC/CHAPS micelles and that the denaturant-induced unfolded state is unpopulated under non-denaturing conditions. It is intriguing to consider whether this very slow unfolding rate is physiologically relevant and whether bR is equally stable within the cell membrane. Because misfolding of membrane proteins often dramatically affects cell function, might it be biologically advantageous that membrane proteins are relatively conformationally static?

The $\Delta G_u^{\text{H}_2\text{O}}$ obtained for bR is consistent with values obtained for small water-soluble proteins on a per-residue basis (typically $\Delta G_u^{\text{H}_2\text{O}} \approx +0.06 \text{ kcal}\cdot\text{mol}^{-1} \text{ residue}^{-1}$ (data from ref. 31); $\Delta G_u^{\text{H}_2\text{O}}$ for bR is $+0.08 \text{ kcal}\cdot\text{mol}^{-1} \text{ residue}^{-1}$). Additionally, both $\Delta G_u^{\text{H}_2\text{O}}$ and $m_u^{\text{H}_2\text{O}}$ ($+20 \text{ kcal}\cdot\text{mol}^{-1}$ and $25 \text{ kcal}\cdot\text{mol}^{-1}$, respectively, considering chevron plot and equilibrium data in Table 1) are consistent with values derived for the transmembrane domain of the 121-aa *Escherichia coli* diacylglycerol kinase, which was found to unfold in mixed SDS/detergent micelles by a single cooperative transition with $\Delta G_u^{\text{H}_2\text{O}}$ of $+16 \text{ kcal}\cdot\text{mol}^{-1}$ and $m_u^{\text{H}_2\text{O}}$ of $22 \text{ kcal}\cdot\text{mol}^{-1}$ (32). Mixed micelles might therefore offer a generic approach to the study of α -helical membrane protein folding.

Conclusions

We identify the major unfolding step of bR and show it to be a reversible, two-state process. This unfolding step is characterized by a large free-energy change and m value and a significant reduction in secondary structure. This is a demonstration that the folding of an integral membrane protein can be analyzed by applying linear free-energy relationships to kinetic data. We are able to derive important folding parameters, including the first glimpse of the transition state, and establish a platform for further investigations.

Materials and Methods

Protein Purification. bR was purified as PM (33). Concentrated PM in water was diluted into a working buffer of 15 mM DMPC, 16 mM CHAPS, and 10 mM sodium phosphate buffer (pH 6.0). bR is a monomer under these conditions (8, 34).

Equilibrium Unfolding. The unfolding of bR under equilibrium conditions was monitored by loss of the chromophore band at 560 nm according to the method of Faham *et al.* (8), except that CHAPS was used instead of 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). Small aliquots of 20% (wt/vol) SDS in DMPC/CHAPS were added to 4 μM bR in DMPC/CHAPS maintained at 25°C. After 3 min spectra were recorded on a Cary 1G UV-vis absorbance spectrophotometer (Varian). A reading of <0.02 absorbance units was considered equivalent to zero for the purposes of the work outlined here. SDS concentration is given throughout in bulk mole fraction (χ_{SDS}). Since denaturant concentration is thus dimensionless, the equilibrium and kinetic m values derived here are given in units of $\text{kcal}\cdot\text{mol}^{-1}$ ($1 \text{ kcal}\cdot\text{mol}^{-1} = 4.19 \text{ kJ}\cdot\text{mol}^{-1}$).

CD Spectroscopy. CD spectra were recorded at the Daresbury Synchrotron Radiation Source. Protein was at 12 μM in a 0.02-cm pathlength cuvette and data were collected between 185 and 270 nm at 0.5-nm intervals with a dwell time of 1 s. The temperature was maintained at 25°C. Data were analyzed with the tools available at the DICHROWEB server (35). The best fits (normalized root mean square difference [NRMSE] ≤ 0.012) for both native and unfolded protein were given by CDSSTR (36, 37) by using reference sets 3 and 6. A series of individual experiments, recorded under the same conditions at 12 μM protein, were used to monitor changes in ellipticity in the helix region at 226 nm with SDS. The absorption of buffer components was negligible at this wavelength. Changes in the 226-nm signal were in good agreement with changes in helix structure determined by analyses of the complete spectra (185–270 nm).

Kinetics of Unfolding. Kinetics of unfolding were measured at 25°C on a stopped-flow apparatus (Applied Photophysics) with 1:5 asymmetric mixing of bR and SDS solutions to a final protein concentration of 1.2 μM . A diode array detector was used to record full spectra. To compensate for the lower sensitivity of this device, protein concentration was increased to 3.2 μM postmix with an integration time of 32 ms at each data point and cell pathlength increased to 10 mm.

To resolve slow unfolding events, absorbance scans between 350 and 500 nm were recorded at two minute intervals on the CARY instrument.

Kinetics of Refolding. Refolding from the A440 state was monitored by sequential stopped-flow mixing. bR was first denatured by mixing 1:1 with an SDS solution for 2 min. Refolding was then accomplished by mixing 1:1 with a concentrated (four times) DMPC/CHAPS solution. The final protein concentration was the same as in unfolding experiments (1.2 μM). At lower concentrations of SDS ($<0.53 \chi_{\text{SDS}}$), an additional slow phase was sometimes observed. To follow refolding at multiple wavelengths, reagents were hand-mixed under identical conditions and absorbance scans were taken between 350 and 600 nm. The refolding yield is calculated from the ratio of the signals at 280 and 560 nm by using extinction coefficients of 66,000 and 55,300 $\text{M}^{-1} \text{ cm}^{-1}$, respectively (12, 38).

Data Analysis

Equilibrium Unfolding Curves. Equilibrium unfolding curves, allowing for the sloping baseline, were fit to a two-state model

according to the method of Faham *et al.* (8) and Lau and Bowie (2). This provided the free energy of unfolding in the absence of denaturant, $\Delta G_u^{\text{H}_2\text{O}}$, and an associated m value.

Free-energy values were also calculated for each data point around the midpoint of the unfolding transition and the graph of free energy of unfolding (ΔG_u) against χ_{SDS} fit to:

$$\Delta G_u = \Delta G_u^{\text{H}_2\text{O}} - m \cdot \chi_{\text{SDS}} \quad [1]$$

where $\Delta G_u^{\text{H}_2\text{O}}$ is the free energy of unfolding in the absence of denaturant and the magnitude of constant m is related to changes in solvent exposure during denaturation.

Kinetic Data. Kinetic data were fit to the sum of exponential components with a floating end point by using GraFit 5.0 (Erithacus Software). Data were fit to the fewest number of exponentials and the quality of the fits judged by χ^2 value and random residuals between the fit and the data. Global analyses were performed by using singular value decomposition (SVD) (Olis Global Works software, Olis).

The logarithms of rate constants for folding and unfolding were linear with respect to SDS. Unfolding data were thus treated according to Eq. 2:

$$\ln(k_u) = \ln(k_u^{\text{H}_2\text{O}}) + mk_u \cdot \chi_{\text{SDS}} \quad [2]$$

where k_u is the rate constant, $k_u^{\text{H}_2\text{O}}$ is the rate of unfolding in the absence of denaturant, and mk_u is the associated constant. Accordingly, for folding,

$$\ln(k_f) = \ln(k_f^{\text{H}_2\text{O}}) - mk_f \cdot \chi_{\text{SDS}} \quad [3]$$

Where k_f is the rate constant, $k_f^{\text{H}_2\text{O}}$ is the rate constant for folding in the absence of denaturant, and mk_f is the constant of proportionality. The observed rate constant, k_{obs} , is the sum of these terms and a combined graph of folding and unfolding rates, commonly termed a chevron plot, can thus be fit to Eq. 4 (31):

$$\ln k_{\text{obs}} = \ln[(k_f^{\text{H}_2\text{O}} \cdot \exp(-mk_f \cdot \chi_{\text{SDS}}) + (k_u^{\text{H}_2\text{O}} \cdot \exp(mk_u \cdot \chi_{\text{SDS}}))] \quad [4]$$

The total free energy of unfolding can then be calculated from kinetic data for comparison with equilibrium experiments, according to Eq. 5:

$$\Delta G_{u \text{ kin}}^{\text{H}_2\text{O}} = -RT \ln(k_u^{\text{H}_2\text{O}}/k_f^{\text{H}_2\text{O}}) \quad [5]$$

An overall m value for the reaction can also be derived from kinetic data by using the relationship:

$$m_{u \text{ kin}}^{\text{H}_2\text{O}} = RT(mk_u + mk_f) \quad [6]$$

where the subscript kin in Eqs. 5 and 6 is used to indicate the derivation of these values from kinetic data. A β -value is calculated from (39, 40):

$$\beta = m/(m_f + m_u) \quad [7]$$

where all values are algebraically positive.

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