Revisiting the folding kinetics of bacteriorhodopsin

Jonathan P. Schleich,^{1,2} Zheng Cao,³ James U. Bowie,³
and Chiwook Park^{1,2,4*}

¹Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907

²Interdisciplinary Life Science Graduate Program, Purdue University, West Lafayette, Indiana 47907

³Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095-1569

⁴Bindley Bioscience Center, Purdue University, West Lafayette, Indiana 47907

Received 16 September 2011; Revised 5 November 2011; Accepted 7 November 2011

DOI: 10.1002/pro.766

Published online 16 November 2011 proteinscience.org

Abstract: The elucidation of the physical principles that govern the folding and stability of membrane proteins is one of the greatest challenges in protein science. Several insights into the folding of α -helical membrane proteins have come from the investigation of the conformational equilibrium of *H. halobium* bacteriorhodopsin (bR) in mixed micelles using SDS as a denaturant. In an effort to confirm that folded bR and SDS-denatured bR reach the same conformational equilibrium, we found that bR folding is significantly slower than has been previously known. Interrogation of the effect of the experimental variables on folding kinetics reveals that the rate of folding is dependent not only on the mole fraction of SDS but also on the molar concentrations of mixed micelle components, a variable that was not controlled in the previous study of bR folding kinetics. Moreover, when the molar concentrations of mixed micelle components are fixed at the concentrations commonly employed for bR equilibrium studies, conformational relaxation in the transition zone is slower than hydrolysis of the retinal Schiff base. As a result, the conformational equilibrium between folded bR and SDS-denatured bR cannot be achieved under the conventional condition. Our finding suggests that the molar concentrations of mixed micelle components are important experimental variables in the investigation of the kinetics and thermodynamics of bR folding and should be accounted for to ensure the accurate assessment of the conformational equilibrium of bR without the interference of retinal hydrolysis.

Keywords: bacteriorhodopsin; membrane protein; protein folding; folding kinetics; conformational relaxation; mixed micelles; retinal hydrolysis

Abbreviations: bR, *H. halobium* bacteriorhodopsin; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Institutes of Health Grant; Grant number: R01 GM063919.

*Correspondence to: Chiwook Park, 575 Stadium Mall Drive, Purdue University, West Lafayette, IN 47907.
E-mail: chiwook@purdue.edu

Introduction

α -Helical membrane proteins account for about a quarter of the human proteome and represent the majority of cellular drug targets.^{1,2} However, relatively little is known about the folding and stability of this important class of proteins.^{3–7} Many of the pioneering efforts in the exploration of the physical properties of α -helical membrane proteins have been focused on *H. halobium* bacteriorhodopsin (bR). A principal reason why bR has served as a popular model for the folding and stability of an α -helical membrane protein is that bR is one of few α -helical membrane proteins for which reversible unfolding conditions are known^{8,9}; bR fully recovers its native

structure from SDS-denatured state under folding conditions with a lower SDS concentration.^{10,11}

Denaturation of bR by SDS has been commonly studied in mixed micelles containing a phospholipid (DMPC) and a non-denaturing detergent (CHAPSO or CHAPS). As the SDS concentration is increased in this system, native bR (bR_F) is converted to SDS-denatured bR (bR_U) in a cooperative fashion.¹² The absorbance of the retinal cofactor of bR at 560 nm (A₅₆₀) disappears upon the conversion to bR_U and serves a convenient spectroscopic probe to monitor unfolding of the protein. The free energy of unfolding (ΔG_{unf}^0) determined with A₅₆₀ in the transition zone was shown to be linearly proportional to the mole fraction of SDS (X_{SDS}) as ΔG_{unf}^0 of soluble proteins is linearly proportional to the molar concentration of a chemical denaturant.¹³ The effect of various mutations on the thermodynamic stability of bR has been studied based on this empirical relationship between ΔG_{unf}^0 and X_{SDS} .^{13,14} Kinetics of folding and unfolding of bR in mixed micelles has been also studied by using SDS as a denaturant. The effect of SDS on the folding and unfolding kinetics of bR is quite similar to the effect of urea or guanidinium chloride on the folding and unfolding kinetics of soluble proteins; the logarithm of folding and unfolding kinetic constants is linearly dependent on X_{SDS} .^{15–17} This relationship allows the application of simple two-state kinetics model to determine ΔG_{unf}^0 from the kinetic constants. Moreover, ΔG_{unf}^0 determined from the kinetic constants is consistent with that determined by equilibrating bR in varying X_{SDS} .¹⁵ This consistency between the equilibrium data and kinetic data has strongly suggested that the folding and unfolding of bR is reversible, and that the conformational equilibrium between bR_F and bR_U is experimentally accessible.

Though the thermodynamics and kinetics of bR unfolding in SDS have been investigated thoroughly, the reversibility of bR unfolding has not been directly demonstrated. A rigorous test of the reversibility of protein unfolding and the achievement of equilibrium is to superimpose the equilibrium data obtained by unfolding of folded protein and refolding of unfolded protein in varying concentrations of a denaturant.¹⁸ To provide direct evidence for establishment of equilibrium between bR_F and bR_U, we monitored the refolding of bR_U in varying X_{SDS} under the identical condition where we have characterized unfolding of bR_F previously.¹⁹ On the basis of the previous kinetics study of bR folding, we predicted that equilibrium should be achieved rapidly under this experimental condition. To our surprise, we find that refolding is much slower than previously reported, and as a result, hydrolysis of the retinal Schiff base occurs to the significant degree during refolding. Further investigation of the discrepancy between our result and the published

result on bR folding kinetics reveals a previously unknown dependence of bR folding kinetics on the molar concentrations of mixed micelle components. We discuss the implication of this finding in the studies of the conformational equilibrium of bR in SDS.

Results

Test of the reversibility of bR unfolding in SDS

To test the reversibility of bR unfolding in SDS, we attempted to monitor the equilibrium achieved by refolding of SDS-denatured bR (bR_U). For this assay, we chose pulse proteolysis as a conformational probe. We previously demonstrated the successful application of pulse proteolysis in the determination of the fraction of native bR (f_N) after unfolding of bR_F in mixed micelles containing DMPC, CHAPSO, and varying concentrations of SDS.¹⁹ Pulse proteolysis is an experimental method by which one can determine the f_N of a protein under a given condition simply by digesting unfolded protein selectively with a short pulse of proteolysis and quantifying the remaining intact protein.^{20,21} For its simplicity, this method is a convenient alternative to conventional spectroscopic methods to monitor protein unfolding. In our previous study, we incubated bR_F in mixed micelles with varying X_{SDS} for 3 min. The 3-min incubation was known to be sufficient for bR to achieve steady state, and the sufficiency of this short incubation was also supported by a study on the folding and unfolding kinetics of the protein.¹⁵ Therefore, in the refolding experiment we also incubated bR_U for 3 min in mixed micelles with varying X_{SDS} .

Surprisingly, the f_N values determined after refolding of bR_U are significantly less than those determined after incubation of natively folded bR under the same condition (Fig. 1). The transition was observed at $\sim 0.4 X_{\text{SDS}}$, which is significantly lower than the C_m value (the midpoint of the conformational transition) determined from the unfolding experiment ($0.75 X_{\text{SDS}}$). Refolding is close to completion only when X_{SDS} is as low as 0.3. Also, the transition is significantly broader than that of the unfolding experiment. This result suggests that refolding of bR may not be complete under this experimental condition.

Incomplete refolding could result from slow refolding or from low refolding yields. To determine whether refolding takes longer than 3 min, we monitored bR refolding by A₅₆₀ at $0.50 X_{\text{SDS}}$ [Fig. 2(A)]. The refolding was indeed slow under this condition. The folding rate constant was determined to be $1.4 \times 10^{-3} \text{ s}^{-1}$. Therefore, we did not achieve complete refolding after the 3-min incubation simply because refolding takes significantly longer than 3 min. The folding rate constant is ~ 40 -fold smaller than that previously reported by Curnow and Booth ($\sim 5.2 \times$

Figure 1. Folding of bR monitored by pulse proteolysis. The amount of refolded bR was determined by pulse proteolysis after equilibration of unfolded bR at varying X_{SDS} for 3 min ($\circ$). The f_N values were determined by dividing the band intensities of remaining intact bR after pulse proteolysis by the intensity of the folded bR treated with pulse proteolysis. For comparison, the f_N values determined by pulse proteolysis after equilibration of folded bR at varying X_{SDS} ($\bullet$)¹⁹ are shown. The f_N values determined after equilibration of folded bR were fit to a two-state equilibrium model (solid line).

10^{-2} s^{-1}) at the same X_{SDS} .¹⁵ The folding of bR is somehow significantly slower in our experiments than that observed by Curnow and Booth under an apparently similar condition.

Effect of micelle component concentrations on the folding kinetics of bR

To identify the origin of the discrepancy in the folding kinetics, we carefully examined the differences in experimental conditions. The difference we first noticed was the non-denaturing detergent used in the assay. We have used CHAPSO, whereas Curnow and Booth used CHAPS.¹⁵ However, when we monitored bR refolding in CHAPS instead of CHAPSO, the folding rate constant was $1.4 \times 10^{-3} \text{ s}^{-1}$ (data not shown), which is identical to that determined in CHAPSO. The use of CHAPSO rather than CHAPS in our experiments does not account for the significant difference in the folding kinetics of bR.

Another difference in the experimental conditions we noticed was the concentrations of DMPC and CHAPSO. We have monitored refolding of bR in the presence of 15 mM DMPC and 16 mM CHAPSO (we will refer to this condition as 15/16 buffer). However, the molar concentrations of DMPC and CHAPS were not fixed in Curnow and Booth's experiment because they decreased X_{SDS} by mixing SDS-denatured bR with a concentrated DMPC/CHAPS solution.¹⁶ Only the final X_{SDS} after dilution was considered without regard for the concentrations of DMPC and CHAPSO. Their experimental design is based on the widely accepted notion that the energetics of bR unfolding is only dependent on X_{SDS} , not on the

Figure 2. The effect of DMPC and CHAPSO concentrations on folding and unfolding kinetics of bR. bR unfolding and refolding were monitored by the absorbance at 560 nm (A_{560}) in buffers containing the same X_{SDS} but different molar concentrations of DMPC and CHAPSO. A: bR was refolded at 0.50 X_{SDS} in buffers containing 15 mM DMPC and 16 mM CHAPSO (15/16 buffer, dashed line) or 29 mM DMPC and 31 mM CHAPSO (29/31 buffer, solid line). B: bR was unfolded at 0.81 X_{SDS} in 15/16 buffer (dashed line) and in 29/31 buffer (solid line). C: The rate constants for bR refolding at 0.50 X_{SDS} ($\bullet$) and for bR unfolding at 0.81 X_{SDS} ($\circ$) were determined in varying molar concentrations of DMPC and CHAPSO. The ratio of the molar concentration of DMPC to that of CHAPSO was fixed to 15:16. The plots of the logarithms of the rate constants versus X_{SDS} are fitted to a linear function.

molar concentrations of lipids and non-denaturing detergents.¹³ Nevertheless, we tested the effect of the variation in the concentrations of DMPC and CHAPSO on bR folding kinetics. We monitored bR refolding at 0.50 X_{SDS} in the same buffer but with about two-fold higher molar concentrations of DMPC and CHAPSO (29 mM DMPC, 31 mM CHAPSO, which will be referred to as 29/31 buffer). Surprisingly, refolding of bR is about 10-fold faster under this condition than in 15/16 buffer [Fig. 2(A)]. Therefore, folding kinetics of bR is dependent not only X_{SDS} but also the concentrations of DMPC and CHAPSO. It is likely that folding of bR was faster in Curnow and Booth's experiments because their dilution with concentrated DMPC/CHAPS solution resulted in significantly higher concentrations of DMPC and CHAPS in their assay.

To determine whether the unfolding of bR is also dependent on the molar concentrations of DMPC and CHAPSO, we monitored bR unfolding by A_{560} in 15/16 buffer and in 29/31 buffer at 0.81 X_{SDS} [Fig. 2(B)], a condition at which bR is known to be unfolded.¹⁵ The rate constants for bR unfolding in 15/16 buffer and in 29/31 buffer were $2.1 \times 10^{-2} \text{ s}^{-1}$ and $2.4 \times 10^{-2} \text{ s}^{-1}$, respectively. This result shows that, unlike folding kinetics, unfolding kinetics is not dependent on the molar concentrations of DMPC and CHAPSO.

To elucidate a quantitative description of the effect of molar concentrations of DMPC and CHAPSO, we determined folding kinetic constants of bR in buffers containing varying molar concentrations but fixed mole fractions of DMPC and CHAPSO ($X_{\text{DMPC}} = 0.24$, $X_{\text{CHAPSO}} = 0.26$, and $X_{\text{SDS}} = 0.50$) in which the ratio of the molar concentration of DMPC to that of CHAPSO is maintained at 15:16. The logarithm of the observed folding rate constants shows a clear linear dependence on the molar concentrations of DMPC and CHAPSO [Fig. 2(C)]. To test the possibility that the difference in the folding kinetics results from the dilution of bR in the micellar phase by increasing molar concentrations of DMPC and CHAPSO, we determined the folding rates with 2-fold lower concentration of bR (0.050 mg/mL instead of 0.10 mg/mL) at 0.40 X_{SDS} in 15/16 buffer. The folding kinetic constant determined with 0.050 mg/mL bR ($5.2 \times 10^{-3} \text{ s}^{-1}$) was virtually identical from that determined with 0.10 mg/mL bR ($5.0 \times 10^{-3} \text{ s}^{-1}$), which suggests that dilution of the protein in the micellar phase does not likely affect the folding kinetics. We also monitored unfolding in buffer containing varying molar concentrations but fixed mole fractions of DMPC and CHAPSO ($X_{\text{DMPC}} = 0.092$, $X_{\text{CHAPSO}} = 0.098$, and $X_{\text{SDS}} = 0.81$) in which the ratio of the molar concentration of DMPC to that of CHAPSO is again maintained at 15:16. The rate constants for unfolding under this condition are independent of variations in the molar concentrations within the experimental range [Fig. 2(C)].

Figure 3. The kinetics of conformational relaxation of bR at varying X_{SDS} with fixed concentrations of DMPC and CHAPSO. The natural logarithm of the observed rate constant (k_{obs}) for unfolding ($\diamond$) and refolding ($\circ$) in 15/16 buffer as well as the natural logarithm of k_{obs} for unfolding ($\diamond$) and refolding ($\bullet$) in 29/31 buffer are plotted against X_{SDS} . The rate constant for the hydrolysis of the retinal Schiff base (k_h) is indicated for comparison (dashed line). The fitting of $\ln k_{\text{obs}}$ versus X_{SDS} reported by Curnow and Booth is also shown for comparison (solid line).¹⁵

The dependence of the folding kinetics on the concentrations of DMPC and CHAPSO reveals the origin of the faster folding observed by Curnow and Booth. According to the kinetic parameters reported by Curnow and Booth, the rate constant for bR folding is predicted to be $5.2 \times 10^{-2} \text{ s}^{-1}$ at 0.50 X_{SDS} . The empirical relationship shown in Figure 2(C) predicts that bR folding would occur with this rate constant in 38 mM DMPC and 40 mM CHAPSO. This condition is realistic, considering that Curnow and Booth diluted bR in SDS with a buffer containing 60 mM DMPC and 64 mM CHAPS.

Folding and unfolding kinetics of bR at fixed concentrations of DMPC and CHAPSO

Because the previous study on the folding and unfolding kinetics of bR was performed without controlling the molar concentrations of lipids and detergents, we reinvestigated the effect of X_{SDS} on the folding and unfolding of bR in buffers with fixed molar concentrations of DMPC and CHAPSO (15/16 buffer and in 29/31 buffer) (Fig. 3). The rate constants determined with Curnow and Booth's fitting parameters¹⁵ are shown for comparison. Unfolding rate constants determined in 29/31 buffer are again similar to those determined in 15/16 buffer as discussed above. The unfolding rate constants are somewhat smaller than Curnow and Booth's data,¹⁵ but the difference is not dramatic. The plot of $\ln k_{\text{obs}}$ versus X_{SDS} shows a downward curvature in the unfolding region, which has not been observed in Curnow and Booth's result.¹⁵ The folding rate constants determined in 29/31 buffer are all greater

than those determined in 15/16 buffer as shown at 0.50 X_{SDS} [Fig. 2(C)]. In both buffers, the rate constants are significantly smaller than those determined by Curnow and Booth. Interestingly, the natural logarithm of the folding rate constants determined in 15/16 buffer shows a kink at 0.50 X_{SDS} . The folding kinetics in this buffer appears to be independent to X_{SDS} for $X_{\text{SDS}} = 0.50$ – 0.65 . Moreover, we observed in this region that the folding yield decreases significantly as X_{SDS} increases (Supporting Information Fig. S1). At 0.70 X_{SDS} , we did not observe any detectable refolding. This independence of folding kinetics to X_{SDS} is not observed in the corresponding range of X_{SDS} in 29/31 buffer. However, folding of bR at 0.70 X_{SDS} in 29/31 buffer was also incomplete (data not shown), and the rate constant cannot be determined reliably.

Hydrolysis of the retinal Schiff base during bR refolding

Interestingly, the folding rate constants in 15/16 buffer for $X_{\text{SDS}} = 0.50$ – 0.65 , which are independent of X_{SDS} , are similar to the rate constant for hydrolysis of the retinal Schiff base (k_h). We determined k_h to be $1.0 \times 10^{-3} \text{ s}^{-1}$ at 0.83 X_{SDS} by monitoring the change in the absorbance at 390 nm (A_{390}), which results from the formation of free retinal after hydrolysis. This k_h value is consistent with the previously reported value that is determined under a similar condition ($9 \times 10^{-4} \text{ s}^{-1}$).¹⁵ The determined k_h value is indeed close to the folding rate constants (dashed line in Fig. 3). As X_{SDS} increases, the folding rate constant seems to converge to k_h .

The convergence of the apparent rate constants for folding to k_h in 15/16 buffer is explained by a simple kinetic model in which SDS-denatured bR undergoes partitioning between bR_F and bacterioopsin (bO), apoprotein without retinal, during folding.

In this model, the folding of bR is treated as an irreversible reaction, which is a reasonable simplification under strong folding conditions ($X_{\text{SDS}} \ll C_m$). From the kinetic model, the rate equations for bR_F, bO, and bR_U are derived as following:

$$[\text{bR}_F]_t = \frac{k_f}{(k_f + k_h)} [\text{bR}_U]_0 (1 - e^{-(k_f + k_h)t}), \quad (1)$$

$$[\text{bO}]_t = \frac{k_h}{(k_f + k_h)} [\text{bR}_U]_0 (1 - e^{-(k_f + k_h)t}), \quad (2)$$

$$[\text{bR}_U]_t = [\text{bR}_U]_0 e^{-(k_f + k_h)t}, \quad (3)$$

where $[\text{bR}_U]_0$ is the concentration of bR_U at $t = 0$. As shown in Eq. (1), the apparent rate constant for the formation of bR_F is $k_f + k_h$. Therefore, when folding is

much slower than hydrolysis ($k_f \ll k_h$), the apparent rate constant would converge at k_h . This prediction is consistent with our observation that k_{obs} converges to k_h in $X_{\text{SDS}} = 0.50$ – 0.65 in 15/16 buffer. In 29/31 buffer, folding is faster than hydrolysis of retinal, and the contribution of k_h to the apparent rate constant is minimal within the experimental range of X_{SDS} . We also observed that the amplitudes of the refolding reactions in 15/16 buffer decrease significantly as X_{SDS} increases (Supporting Information Fig. 1). This observation is also consistent with the kinetic model, which predicts that the amplitude of refolding is proportional to $k_f/(k_f + k_h)$ [Eq. (1)]. When folding is slower than hydrolysis, a significant population of bR loses its retinal by hydrolysis, which results in the lower yield of bR_F.

The kinetic model suggests that a significant population of bO would form when folding is comparable to or slower than hydrolysis of the retinal Schiff base. To confirm the formation of bO, we took absorbance spectra at several time points during folding at 0.65 X_{SDS} in 15/16 buffer. The spectrum taken after the initiation of refolding consists of a single absorbance peak with a maximum absorbance near 440 nm, which is the characteristic spectrum of bR_U [Fig. 4(A)]. As the reaction progresses, two absorbance peaks with maxima near 390 nm and 560 nm grow simultaneously, which indicates that both bO and bR_F form. We also took absorbance spectra at several time points during folding at 0.40 X_{SDS} in 15/16 buffer, where folding is significantly faster than hydrolysis. Under this condition, the single peak with a maximum near 440 nm decays as a peak with a maximum near 560 nm grows [Fig. 4(B)]. Though the slight shift of the maximum of the absorbance peak at 440 nm towards 390 nm suggests that a small fraction of bO may form, this result clearly shows that bR_F is the primary product. The formation of less bO at 0.40 X_{SDS} than at 0.65 X_{SDS} is consistent with higher refolding yield at lower X_{SDS} . (Supporting Information Fig. S1).

Our proposed kinetic model stipulates that the concentration of each species changes with the same rate constant in any given refolding condition [Eqs. (1–3)]. The presence of isosbestic points in the absorbance spectra taken during refolding at 0.65 X_{SDS} and at 0.40 X_{SDS} demonstrates that the concentration of all species change with the same rate in each reaction (Fig. 4). Indeed, the rate constants for the change in the absorbance at 560 nm, 440 nm, and 390 nm are quite similar to one another under each condition (data not shown). These data provide further evidence for the kinetic model.

In the kinetic model, we treat bR folding and the hydrolysis of the retinal Schiff base as irreversible processes for simplicity. However, despite the fact that the reverse reactions of both processes are quite slow under our experimental condition, neither

Figure 4. The formation of bacteriorhodopsin during bR folding. Absorbance spectra were taken at several time points following the initiation of refolding in 15/16 buffer under conditions in which bR folding is similar to the rate of retinal hydrolysis ($0.65 X_{\text{SDS}}$) (A) and under conditions in which bR folding is significantly faster than the rate of retinal hydrolysis ($0.40 X_{\text{SDS}}$) (B). The arrow indicates the order of the spectra taken over time during refolding. Vertical lines indicate the absorbance maxima of native bR (bR_F , 560 nm), SDS-denatured bR (bR_U , 440 nm), and bacteriorhodopsin + free retinal (bO , 390 nm).

folding nor hydrolysis of retinal is irreversible. When bR is incubated in SDS for several days, equilibrium between bR_F and bO + retinal is established.²² Therefore, it is likely that the initial partitioning of bR_U into bR_F and bO + retinal (kinetically controlled process) results in a steady state, which is followed by slow relaxation between bR_F and bO + retinal (thermodynamically controlled process). We have indeed observed that the initial incomplete folding of bR in 15/16 buffer is followed by a slow increase in A_{560} at $0.55 X_{\text{SDS}}$ and a slow decrease in A_{560} at $0.65 X_{\text{SDS}}$ (Supporting Information Fig. S1). This slow change in A_{560} may represent relaxation to equilibrium between bR_F and bO + retinal.

Discussion

Our results show clearly that the folding kinetics of bR is dependent on the molar concentrations of DMPC and CHAPSO and that the folding of bR is much slower than previously reported. Importantly,

our kinetics data show that the conformational relaxation between bR_F and bR_U is slower than hydrolysis of retinal Schiff base under the condition commonly used to study the thermodynamic stability of bR (15/16 buffer). This finding suggests that the previous studies on the thermodynamic stability of bR may have been performed without achieving conformational equilibrium. Because of the hydrolysis of the retinal Schiff base of bR_U , the equilibrium between bR_F and bR_U can be considered as a pre-equilibrium before the hydrolysis, which is practically irreversible under unfolding conditions:

A quantitative assessment of the pre-equilibrium between bR_F and bR_U is only possible when the equilibrium is achieved significantly faster than the following irreversible hydrolysis reaction ($t_{1/2} \sim 12$ min). However, our kinetics data show that hydrolysis is faster than conformational relaxation in 15/16 buffer (Figs. 3 and 4). Therefore, 15/16 buffer is not a valid condition to assess the pre-equilibrium between bR_F and bR_U .

Previously, the conformational relaxation of bR has been believed to be fast in 15/16 buffer, and only a short incubation time (~ 3 min) has been employed for equilibrium studies. This short incubation was also chosen to ensure that the measurement is completed before significant hydrolysis occurs. The sufficiency of this short incubation was supported by the observation that the variation in the incubation time does not affect the C_m value significantly, unless the incubation is long enough to allow hydrolysis of retinal Schiff base.²² However, our data shows that conformational relaxation of bR is not achieved in this short time (Fig. 3). When folded bR is incubated at varying X_{SDS} for an insufficient time, the C_m value that we determine is not the true thermodynamic C_m . This apparent C_m is just the X_{SDS} at which half of bR unfolds during the incubation, which is determined mostly by the unfolding kinetics. The steep dependence of $\ln k_u$ on X_{SDS} (Fig. 3) explains why the apparent C_m value is insensitive to the moderate variation in the incubation time. The X_{SDS} at which half of bR unfolds during a range of incubation times does not change much because the rate of unfolding increases dramatically within a small range of X_{SDS} .

The difference in bR folding kinetics between 15/16 buffer and 29/31 buffer implies that the thermodynamic stability of bR is likely to be dependent on the molar concentrations of DMPC and CHAPSO. This implication contradicts the previous belief that thermodynamic stability of bR is dependent only on the mole fraction of SDS, not on the molar concentrations of DMPC and CHAPSO. This belief was originally formed by the observation that C_m of bR is independent to the molar concentrations of DMPC

and CHAPSO.¹³ Again, the apparent C_m values are independent to the concentrations of DMPC and CHAPSO because the apparent C_m value is determined by the unfolding kinetics alone, not by the equilibrium. The unfolding kinetics of bR is independent to the concentrations of DMPC and CHAPSO, and the apparent C_m values determined by unfolding kinetics would, therefore, also be identical in 15/16 buffer and 29/31 buffer.

Recently, we have also shown that bR cannot refold at the apparent C_m in a buffer containing 15 mM DMPC and 6 mM CHAPSO because refolding is slower than retinal hydrolysis. However, we have also shown that, though the apparent C_m values are determined by the unfolding kinetics, the effect of mutations on ΔG_{unf}^0 determined by the apparent C_m is consistent with that on ΔG_{unf}^0 for equilibrium between bR_F and bO + retinal.²² Prolonged incubation of bR in SDS leads to an equilibrium between bR_F and bO + retinal,¹² and the ΔG_{unf}^0 value of bR can be determined using bO instead of bR_U as a reference state.²² This consistency demonstrates that, even in case that equilibrium is not achieved during titration, the apparent C_m may provide valuable information on the effect of mutations on the stability of bR.

Despite the complication from retinal hydrolysis, it seems to be possible to identify a condition under which we can investigate the equilibrium between bR_F and bR_U reliably. Our result shows that the folding rate of bR increases exponentially as concentrations of DMPC and CHAPSO increases [Fig. 2(C)]. With sufficiently high concentrations of DMPC and CHAPSO, it may be possible to increase the folding rate enough to enable complete relaxation between bR_F and bR_U before retinal hydrolysis occurs. Based on our data, we can predict the concentrations of DMPC and CHAPSO that would satisfy this requirement. When only the linear portions of the folding ($X_{\text{SDS}} = 0.40\text{--}0.60$) and unfolding ($X_{\text{SDS}} = 0.74\text{--}0.81$) kinetics data in 29/31 buffer (Fig. 3) are used, the kinetic C_m , where folding and unfolding rates are identical, is estimated to be $0.73 X_{\text{SDS}}$ (Fig. 5). The relaxation rate constant at the C_m is estimated to be $5.2 \times 10^{-3} \text{ s}^{-1}$. According to our results shown in Figure 2(C), the folding rate would increase by 10-fold, if 45 mM DMPC and 48 mM CHAPSO is used instead of 29/31 buffer (Fig. 5). By assuming that the dependence of $\ln k_f$ on X_{SDS} remains same, we predict that the kinetic C_m would be $0.79 X_{\text{SDS}}$ and the relaxation kinetic constant at the C_m would be 0.030 s^{-1} in 45 mM DMPC and 48 mM CHAPSO. Therefore, the conformational relaxation under this condition would be ~ 30 -fold faster than retinal hydrolysis ($k_h = 0.0010 \text{ s}^{-1}$). Because relaxation is faster in this condition, bR is likely to reach its conformational equilibrium before retinal hydrolysis becomes significant.

The physical origin of the effect of the concentrations of DMPC and CHAPSO on the folding

Figure 5. Relaxation under higher concentrations of DMPC and CHAPSO. The folding kinetics of bR in 45 mM DMPC and 48 mM CHAPSO (45/48) is predicted based on the folding kinetics in 29/31 buffer shown in Figure 3 and the effects of the concentrations of DMPC and CHAPSO on the folding kinetics of bR shown in Figure 2(C). Only the linear portions of the folding ($X_{\text{SDS}} = 0.40\text{--}0.60$, ●) and unfolding ($X_{\text{SDS}} = 0.74\text{--}0.81$, ○) kinetics data in 29/31 buffer are used for the analysis. The kinetic C_m in 45 mM DMPC and 48 mM CHAPSO is estimated to be $0.79 X_{\text{SDS}}$. Also, the relaxation rate constant at the C_m value in 45 mM DMPC and 48 mM CHAPSO is estimated to be 0.030 s^{-1} , which 30-fold greater than retinal hydrolysis constant ($k_h = 0.0010 \text{ s}^{-1}$, dashed line).

kinetics of bR is puzzling. The concentrations of DMPC and CHAPSO affects only the rate of folding, not the rate of unfolding [Fig. 2(C)]. The acceleration of folding without a change in the rate of unfolding occurs because increased concentrations of DMPC and CHAPSO either stabilize the native state and the transition state to the same degree or destabilizes the SDS-denatured state alone. It is not clear at this point how the change in the concentrations of DMPC and CHAPSO can affect the free energy of any of these states.

The primary effect of the increase in the concentrations of micellar components is the dilution of bR in the micellar phase. Because membrane proteins are confined in the hydrophobic environment, dilution of membrane proteins by increasing micellar components may affect the chemical potential of the proteins.²³ If folding of SDS-denatured bR is hindered by aggregate formation, dilution of bR may increase the folding rates. However, the folding rate independent to the bR concentration (*vide supra*) suggests that dilution of bR in the micellar phase is not likely a cause of the faster folding in higher concentrations of micellar components. Another tempting explanation is that bR folding is limited by the encounter rate of denatured bR with empty micelles. In this case, the refolding rate would be linearly proportional to the number of micelles. However, the refolding rate increases exponentially, not linearly,

as the concentrations of micellar components are increased [Fig. 2(C)], suggesting that the encounter with micelles is not likely to limit the folding kinetics. Another possibility is that micelle mixing is rate-limiting. However, the establishment of equilibrium in micelle composition in similar phospholipid/detergent mixed micelles has been shown to be quite fast (typically, $t_{1/2} < 1$ s).^{24,25} Therefore, micelle mixing is not likely to be limiting the rate of bR folding either.

Does the difference in the concentrations of DMPC and CHAPSO modify the properties of the mixed micelles? It is known that the properties of the mixed micelles are described well by mole fractions of micelle components. Once micelles form above the critical micelle concentration, the molar concentrations of micelle components are somewhat irrelevant to the properties of mixed micelles. For instance, it is known that combining multiple detergents in solution leads to non-ideal mixing of the components,²⁶ which suggests that mixtures of SDS, DMPC, and CHAPSO may yield heterogeneous populations of micelles with varying compositions. However, the degree of non-ideal mixing is dependent only on the mole fractions, not the molar concentrations, of the components. Because the molar ratios of the components remain constant when we vary their molar concentrations, it is unlikely that the dependency of the folding rate constant on the molar concentrations of DMPC and CHAPSO [Fig. 2(C)] results from any major changes in the size or the compositions of mixed micelles.

The more obvious change in the solution of mixed micelles from the increased molar concentrations of micelle components is the increase in the number of the micelles. As the size of the mixed micelles is relatively independent to the molar concentrations of the components, the number of micelles would be linearly dependent on the molar concentrations of the components. We do not have any reasonable explanation on how this increase in the number of micelles may affect the folding rates of bR. However, we can consider the possibility that the negatively charged mixed micelles destabilize negatively charged SDS-denatured bR by Coulombic repulsion. SDS-denatured bR is likely to have more extended conformations than folded bR and to have greater negative charge density on the surface due to the association of SDS molecules.^{14,27} Refolding of this SDS-denatured bR should require compaction of the structure as well as release of SDS molecules. The presence of negatively charged mixed micelles may promote this refolding process by destabilizing SDS-denatured bR. In addition to this Coulombic destabilization of extended SDS-denatured states, micelles may destabilize extended denatured states by macromolecular crowding. A crowding agent at high concentration affects the rates and equilibria of

many biochemical reactions by excluding volume to other molecules.²⁸ DMPC (29 mM), CHAPSO (31 mM), and SDS (60 mM; 0.50 X_{SDS} in 29/31 buffer) would occupy more than 6% of the volume of the solution. With a greater number of mixed micelles, SDS-denatured states are destabilized further by this volume exclusion as well as Coulombic repulsion. Further investigation of the properties of the mixed micelles and their interactions with SDS-denatured bR may provide valuable insight into the effect of the micellar environment on the folding of membrane proteins.

bR has long served as a key model protein for the investigation of the folding and stability of α -helical membrane proteins. A number of investigations have led to a cohesive outlook on the factors governing the kinetics and thermodynamics of bR folding. Unexpectedly, our experiments reveal that the kinetics of bR folding depends not only on the mole fraction of SDS but also on the molar concentrations of mixed micelle components. Moreover, conformational relaxation of bR is much slower than previously known when concentrations of mixed micelle components are controlled. As a result of slow relaxation, hydrolysis of the retinal Schiff base occurs during bR folding, and conformational equilibrium between bR_F and bR_U cannot be achieved under the condition conventionally employed for the studies of the equilibrium unfolding of bR. Insights contained in this work pave the way for a more comprehensive analysis of the kinetics and thermodynamics of bR folding. Also, the study presented herein suggests that the molar concentrations of lipid components should be considered as an experimental variable in investigations of membrane protein folding.

Materials and Methods

Materials

bR was prepared as described previously.²⁹ 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), sodium phosphate, phenylmethylsulfonyl fluoride (PMSF), and subtilisin A from *Bacillus licheniformis* were purchased from Sigma Chemical (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from J.T. Baker (Phillipsburg, NJ).

Measurement of bR refolding by pulse proteolysis

Pulse proteolysis was employed to measure f_N of bR after the incubation of SDS-denatured bR at varying X_{SDS} as we monitored bR unfolding previously.¹⁹ Briefly, 2.0 mg/mL bR was equilibrated in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM

DMPC and 16 mM CHAPSO in the dark at 25°C for at least an hour before refolding experiments. bR was then unfolded by a two-fold dilution into the same buffer but with a high concentration of SDS (final $X_{\text{SDS}} = 0.82$). After a 3-min incubation, refolding was initiated by diluting SDS-denatured bR 10-fold with 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO but with varying concentrations of SDS. The final protein concentration in the refolding reaction was 0.10 mg/mL. Following 3-min incubation, pulse proteolysis was initiated by the addition of subtilisin to 50 µg/mL. After 1 min, the reactions were quenched by the addition of PMSF to 10 mM. Reactions were then run on an SDS-PAGE gel, and the amount of the remaining intact protein in each condition was determined by integration of band intensity using ImageJ (<http://rsbweb.nih.gov/ij/>). The f_N value in each condition was determined as the ratio of the band intensity of the remaining intact protein to that of folded bR after pulse proteolysis.

Refolding kinetics

bR was equilibrated at 2.0 mg/mL in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO in the dark at 25°C for at least an hour before each experiment. bR was unfolded by a two-fold dilution into 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO and a high concentration of SDS (final $X_{\text{SDS}} = 0.82$) before refolding. After a 3-min incubation, refolding was initiated by a 10-fold dilution into 10 mM sodium phosphate buffer (pH 6.0) containing the specified concentrations of DMPC, CHAPSO, and SDS. The final protein concentration was 0.10 mg/mL. The molar concentrations of DMPC and CHAPSO were adjusted by mixing with appropriate volume of a solution containing 60 mM DMPC and 64 mM CHAPSO. Absorbance measurements and spectral scans were made in a 1-cm glass cuvette using a Cary 3 Bio UV-Visible spectrophotometer (Varian, Melbourne, Australia). The observed rate constant for refolding was determined by fitting a plot of the absorbance versus time with the appropriate number of exponential phases. We observed a slow minor phase after the major refolding phase in some reactions. Only when the rate constant of the slow phase could be determined with reasonable accuracy on the experimentally observed reaction time, we fit the plot of absorbance versus time to a biphasic rate equation. Because the origin of this slow minor phase is not clear, we use the rate constant of only the major phase for our analysis.

Unfolding kinetics

bR was equilibrated at 1.0 mg/mL in 10 mM sodium phosphate buffer (pH 6.0) containing 15 mM DMPC and 16 mM CHAPSO in the dark at 25°C for at least

an hour before each experiment. Unfolding was then initiated by a 10-fold dilution of the preincubated bR into 10 mM sodium phosphate buffer (pH 6.0) containing the specified concentrations of DMPC, CHAPSO, and SDS. The final protein concentration was 0.10 mg/mL. The molar concentrations of DMPC and CHAPSO were adjusted by mixing with appropriate volume of a solution containing 60 mM DMPC and 64 mM CHAPSO. After the initiation of the unfolding reactions, the absorbance was monitored as described above. The observed rate constant for unfolding was determined by fitting a plot of the absorbance versus time to a first-order rate equation.

Acknowledgments

The authors thank Pei-Fen Liu, Joseph R. Kasper, and Mark W. Hinzman for helpful comments on this manuscript. They also thank Karen G. Fleming for encouraging them to investigate bR refolding by pulse proteolysis.

References

1. Fagerberg L, Jonasson K, von Heijne G, Uhlen M, Berglund L (2010) Prediction of the human membrane proteome. *Proteomics* 10:1141–1149.
2. Yildirim MA, Goh KI, Cusick ME, Barabasi AL, Vidal M (2007) Drug-target network. *Nat Biotechnol* 25: 1119–1126.
3. Bowie JU (2005) Solving the membrane protein folding problem. *Nature* 438:581–589.
4. Oberai A, Ihm Y, Kim S, Bowie JU (2006) A limited universe of membrane protein families and folds. *Protein Sci* 15:1723–1734.
5. Stanley AM, Fleming KG (2008) The process of folding proteins into membranes: challenges and progress. *Arch Biochem Biophys* 469:46–66.
6. White SH (2009) Biophysical dissection of membrane proteins. *Nature* 459:344–346.
7. Booth PJ, Curnow P (2006) Membrane proteins shape up: understanding *in vitro* folding. *Curr Opin Struct Biol* 16:480–488.
8. Barrera FN, Renart ML, Molina ML, Poveda JA, Encinar JA, Fernandez AM, Neira JL, Gonzalez-Ros JM (2005) Unfolding and refolding *in vitro* of a tetrameric, alpha-helical membrane protein: the prokaryotic potassium channel KcsA. *Biochemistry* 44:14344–14352.
9. Hong H, Joh NH, Bowie JU, Tamm LK (2009) Methods for measuring the thermodynamic stability of membrane proteins. *Methods Enzymol* 455:213–236.
10. Huang KS, Bayley H, Liao MJ, London E, Khorana HG (1981) Refolding of an integral membrane protein: denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. *J Biol Chem* 256:3802–3809.
11. London E, Khorana HG (1982) Denaturation and renaturation of bacteriorhodopsin in detergents and lipid-detergent mixtures. *J Biol Chem* 257:7003–7011.
12. Chen GQ, Gouaux E (1999) Probing the folding and unfolding of wild-type and mutant forms of bacteriorhodopsin in micellar solutions: evaluation of reversible unfolding conditions. *Biochemistry* 38:15380–15387.
13. Faham S, Yang D, Bare E, Yohannan S, Whitelegge JP, Bowie JU (2004) Side-chain contributions to membrane protein structure and stability. *J Mol Biol* 335: 297–305.

14. Joh NH, Min A, Faham S, Whitelegge JP, Yang D, Woods VL, Bowie JU (2008) Modest stabilization by most hydrogen-bonded side-chain interactions in membrane proteins. *Nature* 453:1266–1270.
15. Curnow P, Booth PJ (2007) Combined kinetic and thermodynamic analysis of α -helical membrane protein unfolding. *Proc Natl Acad Sci USA* 104:18970–18975.
16. Curnow P, Booth PJ (2009) The transition state for integral membrane protein folding. *Proc Natl Acad Sci USA* 106:773–778.
17. Curnow P, Booth PJ (2010) The contribution of a covalently bound cofactor to the folding and thermodynamic stability of an integral membrane protein. *J Mol Biol* 403:630–642.
18. Moon CP, Kwon S, Fleming KG (2011) Overcoming hysteresis to attain reversible equilibrium folding for outer membrane phospholipase a in phospholipid bilayers. *J Mol Biol* 413:484–494.
19. Schleich JP, Kim MS, Joh NH, Bowie JU, Park C (2011) Probing membrane protein unfolding with pulse proteolysis. *J Mol Biol* 406:545–551.
20. Park C, Marqusee S (2005) Pulse proteolysis: a simple method for quantitative determination of protein stability and ligand binding. *Nat Methods* 2:207–212.
21. Park C, Marqusee S (2006) Quantitative determination of protein stability and ligand binding by pulse proteolysis. *Curr Protoc Protein Sci* 46:20.11.21–20.11.14.
22. Cao Z, Schleich JP, Park C, Bowie JU (in press) Thermodynamic stability of bacteriorhodopsin mutants measured relative to the bacterioopsin unfolded state. *Biochim Biophys Acta*.
23. Fleming KG (2002) Standardizing the free energy change of transmembrane helix-helix interactions. *J Mol Biol* 323:563–571.
24. Soltys CE, Roberts MF (1994) Fluorescence studies of phosphatidylcholine micelle mixing: relevance to phospholipase kinetics. *Biochemistry* 33:11608–11617.
25. Thomas MJ, Pang K, Chen Q, Lyles D, Hantgan R, Waite M (1999) Lipid exchange between mixed micelles of phospholipid and triton X-100. *Biochim Biophys Acta* 1417:144–156.
26. Sehgal P, Mogensen JE, Otzen DE (2005) Using micellar mole fractions to assess membrane protein stability in mixed micelles. *Biochim Biophys Acta* 1716:59–68.
27. Pan Y, Brown L, Konermann L (2009) Mapping the structure of an integral membrane protein under semi-denaturing conditions by laser-induced oxidative labeling and mass spectrometry. *J Mol Biol* 394:968–981.
28. Minton AP (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* 276:10577–10580.
29. Oesterhelt D, Stoekenius W (1974) Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Method Enzymol* 31:667–678.