Probing the Folding and Unfolding of Wild-Type and Mutant Forms of Bacteriorhodopsin in Micellar Solutions: Evaluation of Reversible Unfolding Conditions†

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ABSTRACT: Wild-type and mutant forms of bacteriorhodopsin (sbR) from *Halobacterium salinarium*, produced by *Escherichia coli* overexpression of a synthetic gene, were reversibly unfolded in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 3-[(3-cholamidopropyl)dimethylamino]-2-hydroxyl-1-propane (CHAPSO), and sodium dodecyl sulfate (SDS) mixed micelles. To study the effect on protein stability by substitutions on the hydrophobic surface with polar residues, the unfolding behavior of a G113Q, G116Q mutant [sbR(Q2)] was compared to the wild-type sbR [sbR(WT)]. sbR(Q2) was more sensitive to SDS-induced unfolding than sbR(WT) under equilibrium conditions, and kinetic experiments showed that sbR(Q2) was more sensitive to acid-induced denaturation and thermal unfolding than sbR(WT). Since the mutations in sbR(Q2) were on the detergent-embedded hydrophobic surface of sbR, protein destabilization by these mutations supports the concept that the membrane-embedded segments are important for the stability of sbR. Our experiments provide the basis for studying the thermodynamic stability of sbR by evaluating reversible folding and unfolding conditions in DMPC/CHAPSO/SDS mixed micelles.

There is relatively little experimental information concerning the folding and thermodynamic stability of integral membrane proteins in comparison to the wealth of data available on water-soluble proteins (*1*). The major problem for integral membrane proteins is the difficulty in finding conditions in which the folded and unfolded states are in equilibrium. Thermal unfolding of membrane proteins often results in irreversible denaturation due to protein aggregation, as in the cases of cytochrome *c* oxidase (*2*), photosystem II (*3*), and erythrocyte band 3 (*4*). Since some membrane proteins can be refolded after denaturation by urea, guanidine hydrochloride, SDS,[1] or sarkosyl (*5*−*10*), exploration of the kinetic and thermodynamic properties of membrane proteins has begun (*9*, *11*−*15*). Bacteriorhodopsin (bR), an integral membrane protein that functions as a proton pump in *Halobacterium salinarium* (*16*), is an excellent candidate for integral membrane protein folding and thermodynamic stability studies for the following reasons: (i) the full-length protein or various fragments can be folded from the SDS-denatured state (*5*, *6*, *17*−*19*); (ii) the retinal chromophore is a sensitive probe of the tertiary structure (*20*) and thus can be used to monitor the folding and unfolding reactions; (iii) high-resolution tertiary structural information is available (*21*−*23*); and (iv) there are extensive mutagenesis data (*24*).

Unfolding of bR by urea (*25*), guanidine hydrochloride (*26*), organic solvent (*27*), organic acids (*5*), or heat (*15*, *28*, *29*) was either incomplete or irreversible. Although it has been known for many years that bR can be denatured completely by SDS and the SDS-denatured bO can be refolded in DMPC/CHAPS micelles (*5*, *6*, *17*−*19*), and the kinetics of the folding and unfolding reactions have been studied (*13*, *30*−*32*), the folding and unfolding equilibrium measured under a range of denaturant concentrations has not been explored. Based on the fact that the SDS-denatured sbR can be completely folded in DMPC/CHAPSO mixed micelles, we have focused on obtaining unfolding equilibrium data in order to study the thermodynamic stability of sbR. To determine if mutagenesis in the membrane-embedded regions affects the thermodynamic stability, a mutant in which glycine residues at positions 113 and 116 on the hydrophobic surface of helix D were substituted with glutamine residues [sbR(Q2); *15*] was subjected to unfolding reactions. Similar experiments were carried out for the wild-type protein [sbR(WT)], and the results were compared. Folding and unfolding equilibrium measurements of sbR(WT) and sbR(Q2) were carried out in DMPC/CHAPSO/

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[1] Abbreviations: bO, bacterioopsin; bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propane; CHAPSO, 3-[(3-cholamidopropyl)dimethylamino]-2-hydroxyl-1-propane; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MBP, maltose binding protein; MBP−sbO, sbO fused to the C-terminus of MBP; NaOAc, sodium acetate; NG buffer, 10 mM sodium phosphate, pH 5.6, 18 mM β-nonyl glucoside; sbO, synthetic bO from *E. coli* expression; sbR, synthetic bR from in vitro folding of sbO; sbR(Q2), synthetic bR with mutations of glycine 113 to glutamine (G113Q) and glycine 116 to glutamine (G116Q); sbR(WT), wild-type bR derived from our synthetic gene; SDS, sodium dodecyl sulfate; UV/VIS, ultraviolet and visible absorption.

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SDS mixed micelles. On the basis of the highly similar UV/VIS spectra of the folded proteins, we suggest that sbR(Q2) and sbR(WT) have a similar tertiary structure. Because sbR was resistant to denaturation by high concentrations of urea (8 M) or guanidine hydrochloride (6 M) and by low pH (pH 1.2), and sbR(Q2) was more sensitive to SDS-induced and acid-induced denaturation, we conclude that accessibility of the denaturant to the detergent-embedded regions is important for unfolding integral membrane proteins such as sbR.

MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate (SDS) was purchased from J. T. Baker Inc. (Phillipsberg, NJ). 3-[(3-Cholamidopropyl)dimethylamino]-2-hydroxyl-1-propane (CHAPSO), β-nonyl glucoside, and β-octyl glucoside were from Anatrace (Maumee, OH). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was from AVANTI Polar Lipids, Inc. (Alabaster, AL). Retinal and reduced Triton X-100 were from Sigma (St. Louis, MO).

Protein Expression, Purification, Reconstitution, Characterization, and Quantitation. Details of experimental procedures of protein expression, purification, and folding were described previously (*15*, *33*). Briefly, the wild-type and mutant sbOs were expressed as maltose binding protein (MBP) fusions which formed inclusion bodies in DH5α cells. After removal of MBP by limited trypsin cleavage, sbO was purified by size exclusion chromatography and folded in DMPC/CHAPSO mixed micelles in the presence of retinal. The folded sbR was purified by Q-Sepharose followed by Red A column chromatography. The UV/VIS absorption spectra were measured on a Lambda 6 UV/VIS spectrometer after the protein was incubated in NG buffer (10 mM sodium phosphate, pH 5.6, 18 mM β-nonyl glucoside) in the dark overnight. The protein concentrations were determined spectrophotometrically using the absorption of bR at 555 nm ($\epsilon = 52\,000\ M^{-1}\ cm^{-1}$; *20*).

Unfolding and Folding Reactions of sbR in the Presence of Urea and Guanidine Hydrochloride. The unfolding reaction mixtures were prepared by addition of the purified sbR(WT) to a series of solutions that resulted in different final concentrations of urea (0−8 M) and guanidine hydrochloride (0−6 M). The unfolding reaction mixtures were incubated at room temperature for 2 h before their UV/VIS spectra were taken. For the folding reaction in the presence of urea, the SDS-denatured sbR(WT) was incubated with a solution of 10 mM NaOAc, pH 5.8, 15 mM DMPC, 16 mM CHAPSO, 5 mM SDS, 7 M urea at room temperature for 2 h. The final protein concentration was 20 μg/mL.

Folding and Unfolding Equilibrium of sbR in DMPC/CHAPSO/SDS Mixed Micelles. Unfolded sbO was prepared by trypsin cleavage of the MBP fusion (MBP−sbO) in the presence of SDS, and the monomeric unfolded sbO was purified on a preparative Superose 12 column (*33*). For sbR refolding reactions monitored by UV/VIS spectra, the unfolded protein was prepared by addition of SDS (35 mM final concentration) to the folded protein in NG buffer and incubation at room temperature for 30 min. The absorbance at 555 nm of the unfolded material was zero, and the absorbance at 380 nm reached a maximum value, indicating that the tertiary structure of sbR was disrupted. The final concentrations of DMPC (15 mM) and CHAPSO (16 mM) were kept constant in all folding and unfolding reactions. Different final SDS concentrations ranging from 0.7 to 87 mM in the reaction mixture were reached by inclusion of the calculated SDS concentrations in the reaction buffers before addition of the protein. In the refolding reactions, the SDS-denatured protein was diluted into a DMPC/CHAPSO/SDS solution. In the unfolding reaction, the folded sbR was added into a DMPC/CHAPSO/SDS solution. The same amount of folded sbR was diluted into the DMPC/CHAPSO solution without SDS as a control of 100% folded sbR. The final protein concentration was 3 μg/mL for the fluorescence and 15 μg/mL for the UV/VIS measurements. The same buffers without protein were used as references in the UV/VIS and fluorescence measurements. The refolding and unfolding reaction mixtures were incubated at room temperature in the dark for 2 h before the UV/VIS or fluorescence spectral measurements were taken. The excitation wavelength for fluorescence was 285 nm, emission spectra were recorded between 300 and 400 nm, and the emission maximum was at 326 nm.

Incubation of sbR(WT) in DMPC/CHAPSO/SDS Mixed Micelles at a Low SDS Concentration at Raised Temperatures. The solution containing 15 mM DMPC, 16 mM CHAPSO, 7 mM SDS, 25 μg/mL sbR(WT), 10 mM NaOAc, pH 6.0, was incubated at different temperatures between 35 and 75 °C for approximately 10 min. The temperature was controlled by a constant-temperature circulator (VWR) which was connected to the cuvette holder. The A_{555} changes of the heated protein solution were measured by a time-drive operation of the UVDM program (Perkin-Elmer). To analyze the reversibility of the spectral changes, after the reaction solution was preincubated at 45 °C, the temperature was raised to 65 °C for 10 min and then cooled back to 45 °C. UV/Vis spectra at different incubation stages were measured.

Kinetic Measurements of sbR Thermal Unfolding in Different Detergent Micelles. To search for reaction conditions that minimized protein aggregation during heating, thermal unfolding experiments of sbR(WT) in a reaction buffer containing 5 mM NaOAc, pH 5.0, 250 mM NaCl with different additives were carried out. The additives were 30% glycerol, 4 M urea, and 2 mM SDS. The protein concentration was 25 μg/mL, and the reaction temperature was 72 °C. To measure the spectra of the reaction mixture, the thermal unfolding reaction was carried out in 5 mM NaOAc, pH 5.0, 250 mM NaCl, 18 mM β-nonyl glucoside, 2 mM SDS at 50 °C. For kinetic measurements, 1 volume of the purified sbR(WT) in NG buffer was added to 9 volumes of the premix which was preheated at 60 °C for 5 min. The reaction solutions contained 10 mM NaOAc, pH 6.0, 20 μg/mL sbR(WT), 2 mM SDS, and different detergents. The detergent concentrations were 1 mM dodecyl maltoside, 34 mM β-octyl glucoside, 33 mM β-nonyl glucoside, and 8 mM reduced Triton X-100. The spectrophotometer, loaded with the premix solutions, was zeroed at 555 nm prior to the addition of the protein. Measurements began after the entire unfolding reaction mixture was incubated for 30 s. According to the first-order reaction kinetics, $\ln(A_{t2}/A_{t1}) = -k(t_2 - t_1)$, where k is the unfolding rate constant and A_{t2} and A_{t1} are the OD_{555} at time points t_2 and t_1, respectively.

Acid-Induced Unfolding of sbR. One volume of the purified sbR in NG buffer was mixed with 9 volumes of a series of

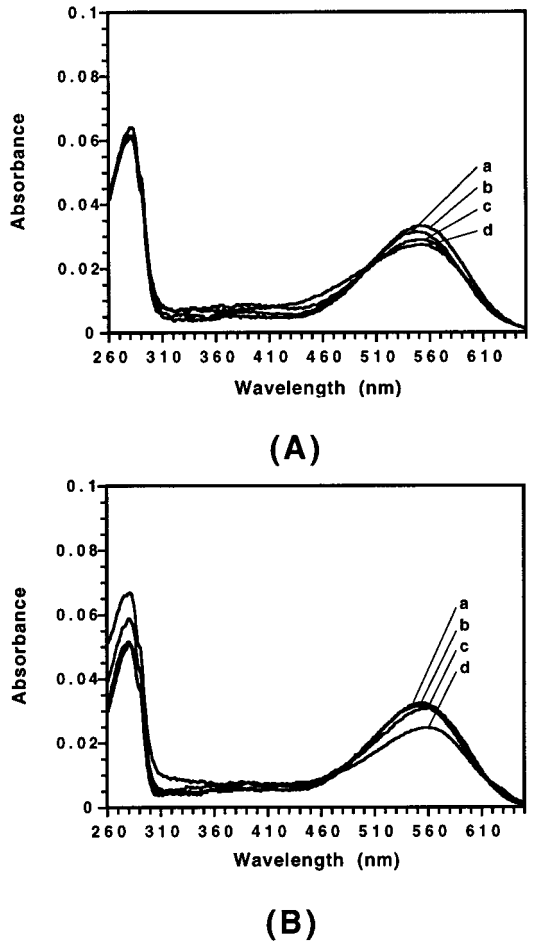

FIGURE 1: UV/VIS spectra of sbR(WT) in the presence of different concentrations of urea (A: a, 1 M; b, 3 M; c, 6 M; d, 8 M) and guanidine hydrochloride (B: a, 1 M; b, 2 M; c, 4 M; d, 6 M) in 10 mM sodium phosphate, 18 mM β-nonyl glucoside, pH 5.6.

solutions at different pH values (5.8 and 1.2) with different SDS concentrations (0, 2, 4, 7, 10, 14, 17, and 21 mM). After the reaction mixture was incubated at room temperature for 15 min, the UV/VIS spectrum was measured. For kinetic experiments, the acidified protein solution was incubated at room temperature for 10 min, then SDS was added to a final concentration of 6 mM, and the changes of OD_{580} were measured.

RESULTS

Urea and Guanidine Hydrochloride Are Not Efficient Denaturants of sbR. The UV/VIS spectra of sbR(WT) in the presence of different concentrations of urea and guanidine hydrochloride are illustrated in Figure 1. The absorbance of the bound retinal at 555 nm, which is a sensitive probe of the tertiary structure of bR, was not significantly disrupted by high concentrations of urea (8 M) or guanidine hydrochloride (6 M). The λ_{max} of the chromophore had only a ~5 nm blue shift and a ~5 nm red shift in the presence of 8 M urea and 6 M guanidine hydrochloride, respectively. In addition, the presence of 7 M urea did not significantly inhibit folding of SDS-solubilized sbR(WT) in DMPC/CHAPSO mixed micelles. These results demonstrated that the conventional denaturants urea and guanidine hydrochloride did not dramatically change the three-dimensional conformation of sbR.

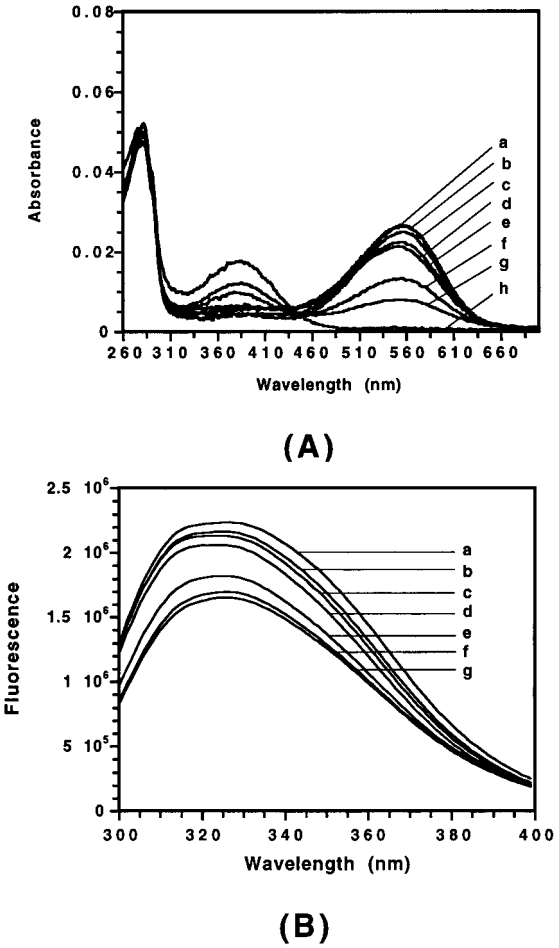

FIGURE 2: Unfolding equilibrium of sbR(WT) and sbO(WT) in DMPC/CHAPSO/SDS mixed micelles. The buffer contained 10 mM sodium phosphate, pH 6.0, 15 mM DMPC, 16 mM CHAPSO, and different SDS concentrations. (A) UV/VIS spectra of sbR(WT) with SDS concentrations of (a) 7 mM, (b) 14 mM, (c) 21 mM, (d) 28 mM, (e) 35 mM, (f) 42 mM, (g) 48 mM, (h) 69 mM. (B) Fluorescence spectra of sbO(WT) with SDS concentrations of (a) 4 mM, (b) 14 mM, (c) 21 mM, (d) 28 mM, (e) 35 mM, (f) 42, (g) 69 mM.

Folding and Unfolding Equilibrium of sbR Induced by SDS in DMPC/CHAPSO Mixed Micelles. In contrast to urea and guanidine hydrochloride, SDS denatured sbR(WT) efficiently. The unfolding equilibrium of sbR(WT) and sbO-(WT) at different SDS concentrations was followed by UV/VIS and fluorescence spectroscopy, respectively (Figure 2). Fluorescence was used to monitor the conformational changes of bO in previous studies (*30*). The λ_{max} of the sbR chromophore had a 3 nm blue shift as the SDS concentration increased. Reversibility of the unfolding reaction was demonstrated by comparing unfolding and the folding reactions under identical final conditions. The percentage of folded sbR(WT) reached the same value in the unfolding and folding reactions as shown in Figure 3. For example, the percentage of folded protein for sbR(WT) was about 75% in both refolding and unfolding reactions when the final SDS concentration was 35 mM. Unfolding of sbO(WT) measured by fluorescence showed that the apoprotein was more easily denatured by SDS than the retinal-bound protein. For example, the percentage of the folded protein was below 10% for sbO(WT) and about 60% for sbR(WT) when the SDS

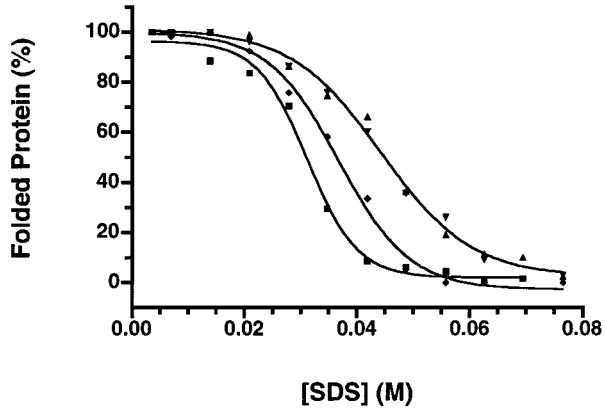

FIGURE 3: Comparison of the UV/VIS data for sbR(WT) unfolding (▲), sbR(WT) folding (▼), and sbR(Q2) unfolding (◆) and fluorescence data of sbO(WT) folding (■). The unfolding reactions were carried out by addition of SDS to the folded sbR in DMPC/CHAPSO micelles. The folding reactions were performed by dilution of the SDS-denatured sbR or sbO into the DMPC/CHAPSO buffer.

concentration was 42 mM based on the fluorescence and UV/VIS spectral data.

Spectral Changes of sbR in DMPC/CHAPSO/SDS Micelles at Raised Temperatures. As shown in Figure 4A, the A_{555} decreased when the temperature of the solution of sbR(WT) in DMPC/CHAPSO/SDS mixed micelles was raised. The A_{555} remained steady when the temperature was kept constant, and cooling the heated reaction mixture recovered the A_{555} (Figure 4B), suggesting a certain equilibrium state was obtained at a specific temperature. The temperature shift from 45 to 65 °C resulted in an 18% decrease in A_{555} and a 12 nm λ_{max} red shift (Figure 4C). However, the significant changes of A_{555} and λ_{max} did not accompany the changes of A_{380}, indicating the population of different conformational states of sbR at 65 °C did not include completly denatured sbO plus free retinal.

Increased Hydrophilicity on the Molecular Surface Resulted in Higher Sensitivity to SDS-Induced Unfolding of sbR. Substitution of Gly-113 and Gly-116 with glutamine residues in helix D of sbR increased the hydrophilicity of the detergent-bound molecular surface (*15*). The purified double mutant sbR(Q2) had UV/VIS spectra very similar to the wild-type protein [sbR(WT)], as shown in Figure 5. For example, the λ_{max} values of the chromophore for sbR(WT) and sbR(Q2) were 555 and 556 nm, respectively. The $A_{280}/A_{\lambda max}$ values were 1.62 for both sbR(WT) and sbR(Q2). However, the polar mutant was more sensitive to SDS-induced denaturation; the percentage of folded protein was about 30% for sbR(Q2) and about 60% for sbR(WT) when the SDS concentration was 42 mM according to the UV/VIS spectral data (Figure 3). The polar mutant sbR(Q2) also showed lower stability than sbR(WT) in acid-induced unfolding reactions, as described below.

Thermal Unfolding of sbR in Different Detergent Micelles. In a previous study (*15*), we reported that thermal unfolding of sbR(WT) and a number of polar mutants resulted in irreversible unfolding reactions that followed first-order kinetics. Inclusion of 2 mM SDS in the thermal unfolding reaction mixture minimized protein precipitation during heating as shown by the UV/VIS spectra (Figure 6A,B). The assumption that the membrane-embedded segments make

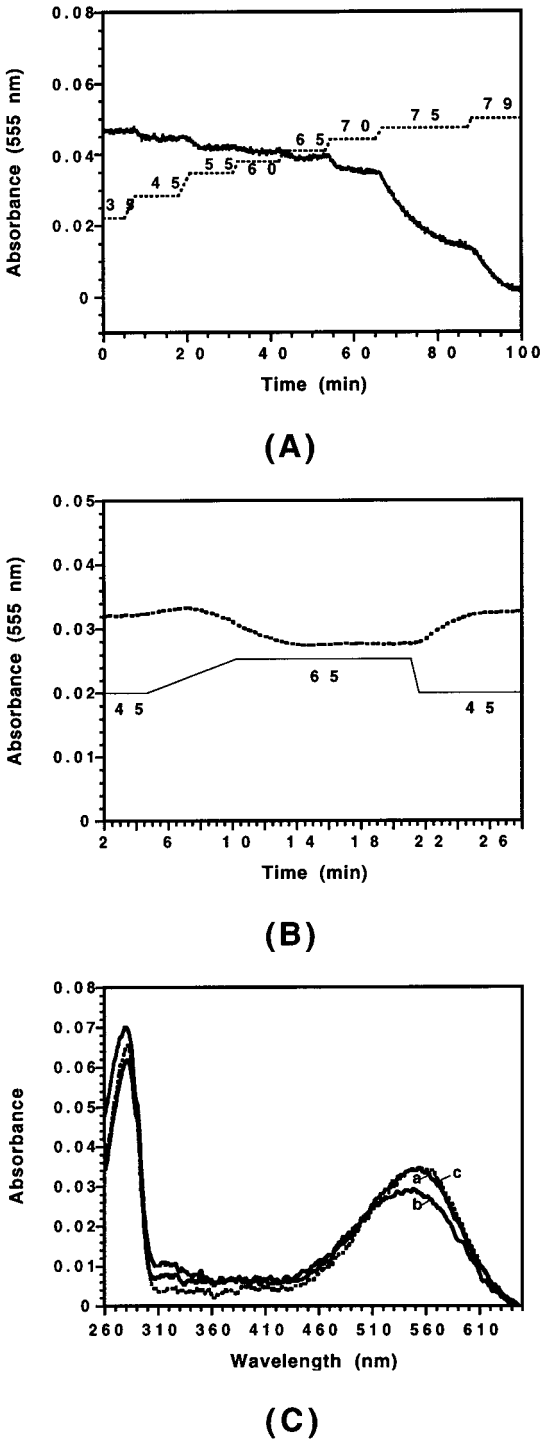

FIGURE 4: (A) Spectral changes of sbR(WT) in SDS/DMPC/CHAPSO mixed micelles at different temperatures. The dotted lines represent the temperature changes, and the numbers above them are the temperatures in a time course. (B) Reversibility of sbR(WT) spectral changes between 45 and 65 °C. The dotted line represents the changes of light absorbance at 555 nm. The temperature changes are illustrated by the solid line, below which are the temperature values. (C) UV/VIS spectra of the thermal unfolding reaction mixture: a, 45 °C; b, 65 °C; c, 45 °C after 65 °C.

important contributions to the stability of sbR was tested by performing chromophore melting experiments in different detergents. As shown in Figure 6C, the thermal unfolding reaction rate constant was strongly dependent on the detergent. The unfolding rate constants of sbR(WT) based on the decrease in OD_{555} at 60 °C were 0.095 min^{-1} in

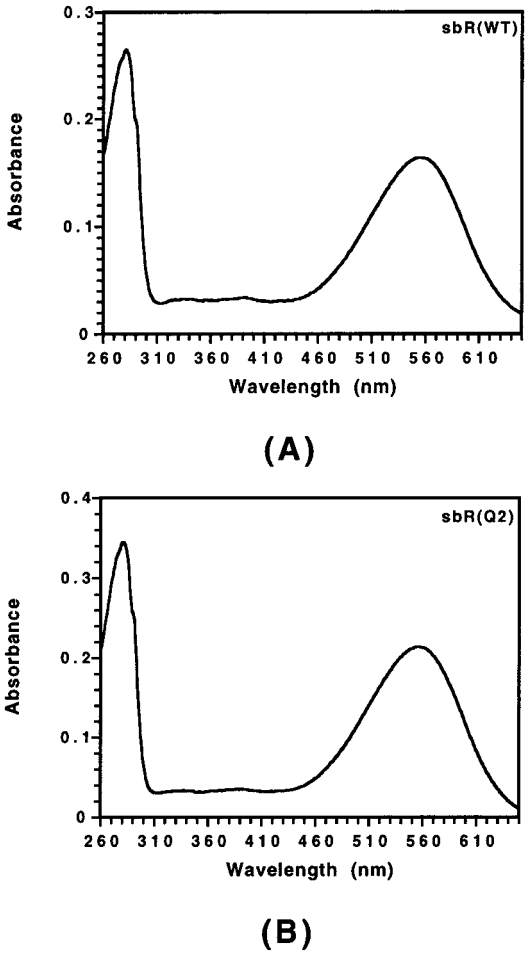

FIGURE 5: UV/VIS spectra of purified sbR(WT) and sbR(Q2). The λ_{max} values of the chromophore for sbR(WT) and sbR(Q2) are 555 and 556 nm, respectively. The $A_{280}/A_{\lambda max}$ values for both sbR(WT) and sbR(Q2) are 1.62.

dodecyl maltoside, 0.26 min^{-1} in β-nonyl glucoside, 0.77 min^{-1} in β-octyl glucoside, and 0.95 min^{-1} in reduced Triton X-100 micelles.

Denaturation of sbR in the Presence of SDS at a Low pH. Incubation of sbR(WT) in DMPC/CHAPSO micelles at pH 1.2 resulted in a blue shift of the chromophore λ_{max} (24 nm) without loss of the retinal (*34*). As illustrated in Figure 7A, inclusion of 10 mM SDS in the pH 1.2 solution led to complete acid-induced denaturation. As previously determined, the acid-induced denaturation of bR yielded a species with a λ_{max} of ca. 446 nm which was an unfolded bR with a protonated Schiff base between the apo-protein and retinal (*35*). Acidification of the compeletly SDS-denatured sbR-(WT) did not produce the species derived from the acid-denatured protein but yielded unfolded protein with decomposition of the Schiff base and gave free retinal with a λ_{max} at 380 nm. The unfolding rate at pH 1.2 in the presence of 6 mM SDS (Figure 7B,C) was 3-fold faster for sbR(Q2) than that for sbR(WT), indicating that the polar mutant was more sensitive to acid-induced denaturation than the wild-type protein.

DISCUSSION

Folding and unfolding studies of α-helical membrane proteins present particular challenges in part because some of the most useful denaturation agents, such as SDS,

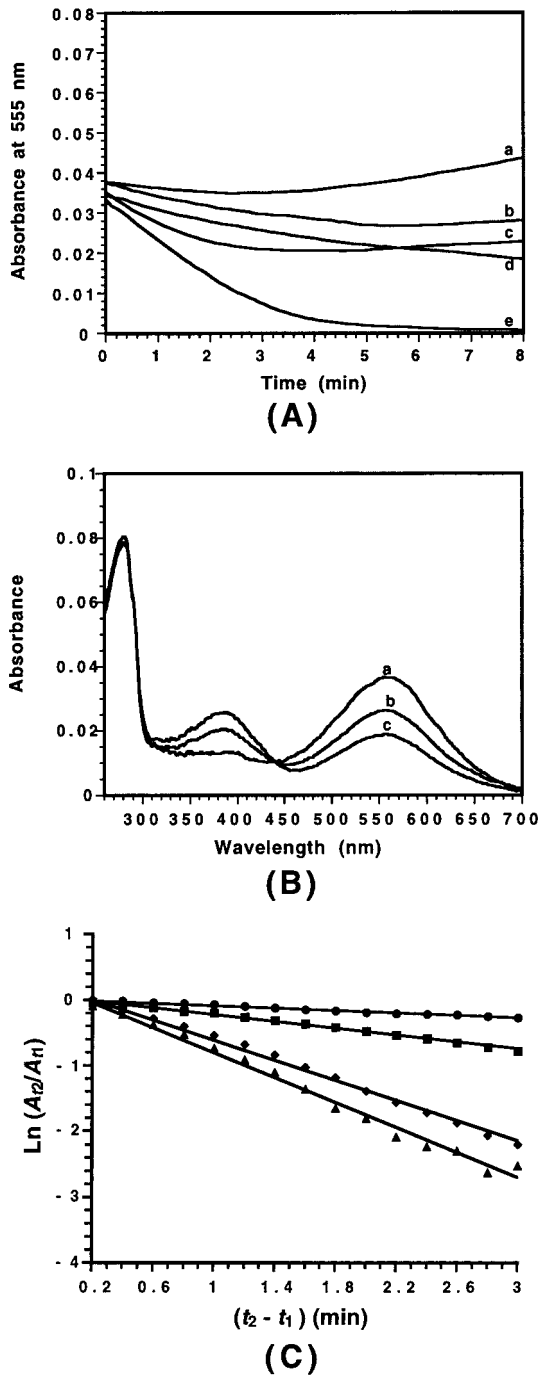

FIGURE 6: Irreversible thermal unfolding of sbR(WT) in different detergent micelles followed by loss of absorbance at A_{555}. (A) Protein aggregation during heating was minimized by addition of 2 mM SDS to the reaction mixture. The buffer was 5 mM NaOAc, pH 5.0, 250 mM NaCl, and one of the following detergent mixtures: (a) 18 mM β-nonyl glucoside; (b) 18 mM β-nonyl glucoside and 30% glycerol; (c) 18 mM β-nonyl glucoside and 4 M urea; (d) 15 mM DMPC and 16 mM CHAPSO; (e) 18 mM β-nonyl glucoside and 2 mM SDS. (B) UV/VIS spectra of the thermal unfolding reaction mixture in 18 mM β-nonyl glucoside and 2 mM SDS at 50 °C at different time points: (a) 15 min; (b) 75 min; (c) 120 min. (C) Thermal unfolding reactions of sbR(WT) in different detergents: 1 mM dodecyl maltoside (●); 33 mM β-nonyl glucoside (■); 34 mM β-octyl glucoside (◆); 8 mM reduced Triton X-100 (▲). A_{t2} and A_{t1} are the OD_{555} at time points t_2 and t_1, respectively. According to the first-order reaction kinetics, $\ln (A_{t2}/A_{t1}) = -k(t_2 - t_1)$; the unfolding rate constant k was obtained by the slope of the linear relationship between $\ln (A_{t2}/A_{t1})$ and $(t_2 - t_1)$.

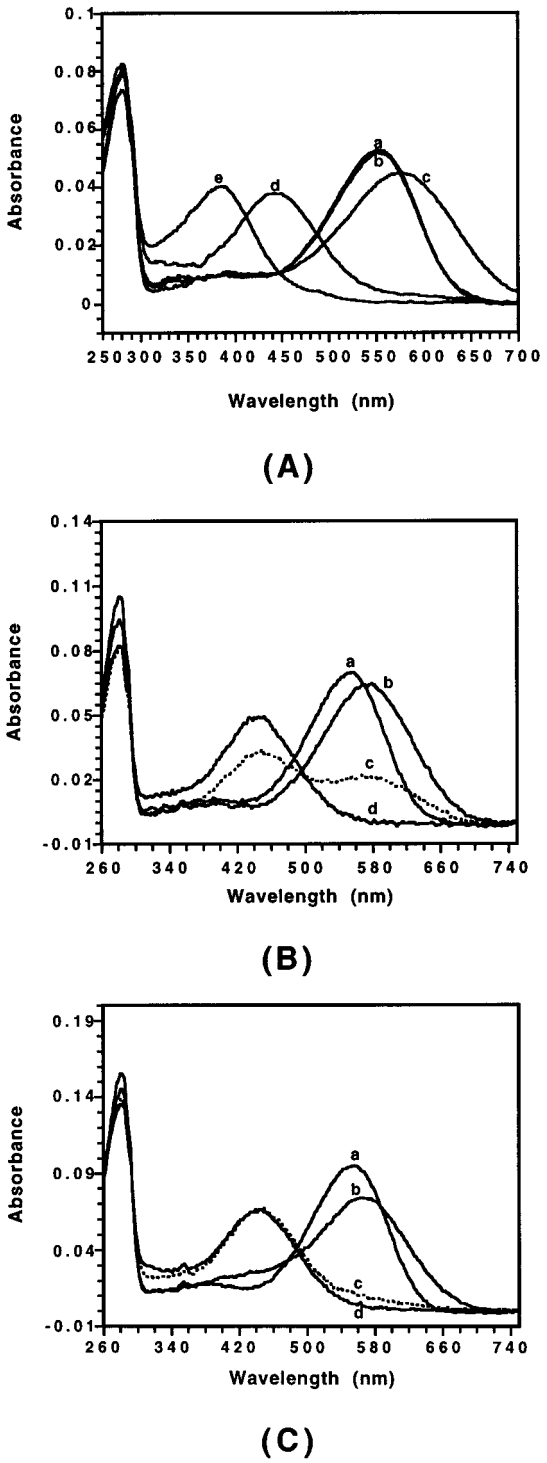

FIGURE 7: Acid-induced unfolding of sbR(WT) and sbR(Q2) catalyzed by SDS. The unfolding reaction was carried out at room temperature. (A) UV/VIS spectra of sbR(WT) in different solutions: (a) pH 5.8, no SDS; (b) pH 5.8, 10 mM SDS; (c) pH 1.2, no SDS; (d) pH 1.2, 10 mM SDS; (e) pH 5.8, 69 mM SDS. Comparison between sbR(WT) (B) and sbR(Q2) (C) under the same acid-induced unfolding conditions: (a) pH 5.8, no SDS; (b) pH 1.2, no SDS; (c) pH 1.2, 6 mM SDS; (d) pH 1.2, 21 mM SDS. The unfolding rate constants for sbR(WT) and sbR(Q2) were 0.070 and 0.21 min^{-1} under condition (c).

destabilize tertiary interactions while at the same time stabilizing or even promoting the formation of α-helical secondary structure. Here, the unfolding of sbR by SDS specifically refers to the disruption of the tertiary structure of bR that is required for the formation of the retinal binding site. Since the native tertiary structure of sbR is essential to form the chromophore which has a λ_{max} of 555 nm (*20*, *36*), the loss of absorbance at 555 nm reflects the denaturation of the tertiary structure of sbR. Although SDS effectively unravels the tertiary structure of sbR and is therefore of value for probing interactions such as helix−helix contacts, SDS-denatured sbR might retain a certain degree of nativelike secondary structure because (i) native bR contains a majority of α-helix (*21*−*23*) and SDS promotes the formation of α-helix, and (ii) the CD spectra of the folded and SDS-denatured bO are similar (*37*). Nevertheless, the α-helix structure of SDS-denatured sbR is not necessarily the same as that of the native sbR since SDS also promotes α-helix formation in all β-strand proteins such as porin (*8*). Indeed, SDS may act as a denaturant not only by perturbing tertiary interactions but perhaps also by promoting the formation of non-native α-helical structure.

The unfolding state of the SDS-denatured sbR in DMPC/CHAPSO/SDS mixed micelles was examined by acidification of the denatured product. Acidification of the completely SDS-denatured sbR did not yield a species with a λ_{max} at 446 nm, which is the signature of a protonated Schiff base and a denatured protein. Rather, the acidification of completely SDS-denatured sbR yielded a species with an absorbance at 380 nm, which corresponds to the λ_{max} of free retinal. Therefore, the denatured state of sbR was denatured sbO plus free retinal in a completely SDS-denatured sbR solution.

Determination of the extent to which a two-state model applies to the folding and unfolding of membrane proteins in general and to bR in particular is another challenging area of investigation. In the case of bR, the results from differential scanning calorimetric studies in DMPC/CHAPS/SDS micelles support a simple two-state model (*28*). On the other hand, kinetic studies have shown that the folding reaction of bR from SDS-denatured bO and retinal is a multiple-step process (*13*, *30*−*32*). The sbR folding and unfolding reactions at different SDS concentrations reported here showed a slight λ_{max} shift, suggesting that some intermediates may be populated in the folding and unfolding reactions. Furthermore, the UV/Vis spectra (Figure 2A) showed that the A_{555} decreases did not correspond to significant A_{380} increases at low SDS concentrations (7−28 mM).

To further investigate the unfolding intermediates formed at low SDS concentrations, unfolding reactions of sbR(WT) at a SDS concentration of 7 mM were carried out at different temperatures. A change of temperature from 45 to 65 °C, for example, a significant A_{555} decrease, and a wide λ_{max} red shift were observed. Although the detailed structural basis for the A_{555} and λ_{max} changes at different temperatures is not clear, the significant A_{555} and λ_{max} changes correponded to some degree of diversity in the confomational states of sbR. Since the A_{555} decreases did not result in A_{380} increases when the temperature was raised to 65 °C, the population of different confomational states unlikely included the completely denatured products. Detailed characterization of the intermediate states and mechanistic studies of sbR unfolding in DMPC/CHAPS/SDS micelles are required for further thermodynamic analysis.

In addition, the structure and dynamic properties of the detergent micelle and its interaction with sbR, as a function

of SDS concentration, may change. Thus, the effective molar concentrations of substances interacting with the protein may be difficult to determine, and calculation of free energies may be complicated. Nevertheless, previous experiments suggest that the structure and aggregation state of DMPC/CHAPS or DMPC/CHAPSO mixed micelles can be altered so as to favor micelles or bicelles by varying the ratio between the two components (*38*). For instance, when the phospholipid concentration was above 50 mM and the CHAPSO:DMPC ratio was higher than 1:3, a bicelle structure was formed. When the CHAPSO:DMPC ratio was about 1:1, spherical micelles were formed (*38*). The DMPC and CHAPSO concentrations in our folding and unfolding experiments were 15 and 16 mM, yielding a ca. 1:1 ratio, respectively. The 16 mM concentration of CHAPSO employed in the studies reported here was sufficient to produce micelles, since the critical micelle concentration (CMC) of CHAPSO is 4 mM at an ionic strength of 0.15 (*39*). Although further biophysical experiments are required to thoroughly characterize the reagents, we suggest that the DMPC/CHAPSO/SDS mixture used here formed micelle aggregates. In fact, a system with a DMPC:CHAPS:SDS molar ratio of 7.5:6.8:1 formed micelles with a molecular mass of 210 kDa (*28*).

An understanding of the structure and aggregation state of the detergent micelle surrounding sbR is important because the hydrophobic, transmembrane regions make important contributions to the stability of sbR, as indicated by a number of pieces of evidence. First, classical hydrophilic denaturants such as 8 M urea, 6 M guanidine hydrochloride, or protons (pH 1.2) did not efficiently unfold sbR. In contrast, the protein was efficiently denatured by SDS. The resistance of sbR unfolding to high concentrations of urea, guanidine hydrochloride, or acid is related to the inability of these hydrophilic reagents to penetrate the hydrophobic regions of sbR. Since SDS has both hydrophilic and hydrophobic properties, it can more effectively partition into and disrupt the structure of the hydrophobic, transmembrane segments. SDS combined with dodecyl maltoside has recently been used for unfolding studies of other membrane proteins such as diacylglycerol kinase (*14*). Second, the thermal stability of sbR was strongly detergent-dependent. For example, 35 mM SDS completely denatured sbR(WT) in NG buffer which contained 18 mM β-nonyl glucoside, while the same SDS concentration gave only about 20% unfolding yield in a buffer containing 15 mM DMPC and 16 mM CHAPSO. In the thermal unfolding experiments, the order of sbR stability in different alkyl saccharide detergents was dodecyl maltoside > β-nonyl glucoside > β-octyl glucoside, suggesting that a longer alkyl chain stabilized sbR more than the shorter ones did. This similar trend in membrane protein stability has been documented in rhodopsin, which also has seven α-helical transmembrane segments (*40*). Third, introduction of polar residues on the lipid- or detergent-exposed surface of the transmembrane domain affected the thermal stability of sbR. The results presented here show that sbR(Q2) was more easily denatured by SDS, acid, and heat (*15*) than sbR(WT). Since the mutations in sbR(Q2) increase the hydrophilicity of the exposed surface of helix D (*15*), the diminished stability of the polar mutant may result from an increase in the accessibility of the transmembrane region to denaturants. Alternatively, the introduction of polar residues on the surface of helix D may destabilize sbR by perturbing protein/micelle interactions or by destabilizing the helix itself via side chain−side chain interactions.

In conclusion, we have studied the reversible folding and unfolding reactions of sbR in DMPC/CHAPSO/SDS mixed micelles and have applied these reagents to probe the stability of this integral membrane protein. The resistance of sbR unfolding to high concentrations of urea, guanidine hydrochloride, and acid, the detergent-dependent stability of sbR, and the relative instability of sbR(Q2) compared to sbR(WT) suggest that denaturant accessibility to the membrane-embedded segments is critical for unfolding of sbR and, by extension, other α-helical integral membrane proteins. The detergent and denaturant properties of SDS and its application to the folding and unfolding of sbR support the conclusion that SDS and related surfactants are promising reagents for in vitro folding and thermodynamic studies of integral membrane proteins.

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