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Structure and Thermal Stability of Monomeric Bacteriorhodopsin in Mixed Phospholipid/ Detergent Micelles

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ABSTRACT **Thermal unfolding experiments on bacteriorhodopsin in mixed phospholipid/detergent micelles were performed. Bacteriorhodopsin was extracted from the purple membrane in a denatured state and then renatured in the micellar system. The purpose of this study was to compare the changes, if any, in the structure and stability of a membrane protein that has folded in a nonnative environment with results obtained on the native system, i.e., the purple membrane. The purple membrane crystalline lattice is an added factor that may influence the structural stability of bacteriorhodopsin. Micelles containing bacteriorhodopsin are uniformly sized disks 105 ± 13 Å in diameter (by electron microscopy) and have an estimated molecular mass of 210 kDa (by gel filtration HPLC). The near-UV CD spectra (which is indicative of tertiary structure) for micellar bacteriorhodopsin and the purple membrane are very similar. In the visible CD region of retinal absorption, the double band seen in the spectrum of the purple membrane is replaced with a broad positive band for micellar bacteriorhodopsin, indicating that in micelles, bacteriorhodopsin is monomeric. The plot of denaturational temperature vs. pH for micellar bacteriorhodopsin is displaced downward on the temperature axis, illustrating the lower thermal stability of micellar bacteriorhodopsin when compared to the purple membrane at the same pH. Even though micellar bacteriorhodopsin is less stable, similar changes in response to pH and temperature are seen in the visible absorption spectra of micellar bacteriorhodopsin and the purple membrane. This demonstrates that changes in the protonation state or temperature have a similar affect on the local environment of the chromophore and the protein conformation. We conclude that the tertiary structure of the bacteriorhodopsin monomer is essentially the same in micelles and the purple membrane. On the other hand, in the synthetic mixed micelle system, the packing between the nonnative amphiphiles and bacteriorhodopsin is probably not optimal, protein–protein interactions have been lost, and the helical packing may be looser because the crystalline lattice is absent. It is likely that a combination of these effects leads to the decreased stability of micellar bacteriorhodopsin.**

Key words: reconstitution, membrane protein folding/unfolding, pH effects, differential scanning calorimetry, circular dichroism, electron microscopy, HPLC

INTRODUCTION

As an integral membrane protein, light-transducing bacteriorhodopsin is unique in its ability to refold to a functional structure from a denatured and delipidated state.[1] Preparations of the reconstituted protein in a variety of detergents and/or phospholipids have visible absorption and far-UV CD spectra that are very similar to the native protein in the purple membrane.[2–4] When bacteriorhodopsin is renatured in the presence of phospholipids and dialyzed to remove the detergent, sealed unilamellar vesicles are formed in which bacteriorhodopsin is fully active as a pump for light-driven proton translocation across the membrane.[5]

These studies demonstrate that amphiphiles other than natural membrane lipids can support a folded structure for bacteriorhodopsin that is sufficiently like the natural structure, in nonnative lipid vesicles, to be functional; but is the structural stability of the protein altered when it is removed from a native environment? Also, how do the differences in macromolecular organization between the reconstituted systems and the purple membrane affect the stability of the protein? We address these questions

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Abbreviations: CD, circular dichroism; CHAPS, [3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate]; DMPC, dimyristoyl phosphatidylcholine; DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; ΔH_{cal}, calorimetric enthalpy; ΔH_{vH}, van't Hoff enthalpy; λ_{max} absorbance maximum; SDS, sodium dodecyl sulfate; T_m, midpoint temperature; UV, ultraviolet.

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here by comparing previous studies on the stability of the purple membrane[6] with our present studies on the monomeric protein. A convenient system for studying the monomeric protein is detergent micelles. The micelle system is also useful for exploring the importance of amphiphile–protein interactions for the structural stability of the protein. Furthermore, because detergents are used for crystallizing membrane proteins,[7] understanding the forces involved in stabilizing detergent-solubilized membrane proteins is important to our interpretation of the derived crystal structures and their relationship to the structure in the native system.

We have used electron microscopy, HPLC, and UV/VIS circular dichroism to analyze the native structure of monomeric bacteriorhodopsin reconstituted into mixed phospholipid/detergent micelles. Thermal unfolding of micellar bacteriorhodopsin as a function of pH was followed by differential scanning calorimetry and UV/VIS absorption spectroscopy. Our results support a model for bacteriorhodopsin structure in which the protein monomer is folded similarly in the micelle and the native purple membrane. Micellar bacteriorhodopsin is less stable than bacteriorhodopsin in the purple membrane and possible reasons for this will be discussed. The information gained from this work is a prerequisite to our future studies on the structure and stability of site-specific mutants of the protein. Our goal is to establish the relative importance of membrane-embedded residues to the stability of the folded structure.

MATERIALS AND METHODS

Micellar Bacteriorhodopsin Preparation

To effectively remove the native purple membrane lipids, organic extraction of the membrane was carried out using a modification of Braiman,[8] which is based on standard methodology for extraction of membrane lipids.[9] A 10-mg pellet of purified purple membrane was dissolved in 10 ml of chloroform: methanol:triethylamine (100:100:4) and 1 ml 10 M hydroxlamine hydrochloride was added to remove the bound retinal. Two phases were formed by adding 10 ml distilled water, and the bacterioopsin (now referred to as "-opsin" rather than "-rhodopsin" because retinal has been removed) was collected at the interface between the lower organic phase and the upper aqueous phase following centrifugation at 10,000 rpm for 15 minutes. The precipitated bacterioopsin was subjected to a second two-phase extraction by first dissolving it in chloroform:methanol:triethylamine and then again adding water to form two phases. The delipidated protein isolated from this extraction was then twice dispersed in water and pelleted by centrifugation. The precipitated bacterioopsin is difficult to solubilize and 10% SDS (Fluka) was found to be most convenient for making a concentrated stock solution of the protein. The stock is used to prepare micellar bacteriorhodopsin containing 8.8%, 130 mM DMPC (Avanti Polar Lipids), 7.2%, 117 mM (cmc < 10 mM) CHAPS (Boehringer Mannheim), pH 6 (adjusted with 0.1 M NaOH before adding the retinal). These preparations contain 0.5% (17 mM) SDS, which did not interfere with quantitative chromophore regeneration. The molar ratios of protein to amphiphile (protein:SDS:DMPC:CHAPS) used for calorimetry were 0.5–1:300:2200:2000. An ethanolic solution of all-*trans*-retinal, approximately 1.2 eq (Kodak), added to micellar bO nearly quantitatively regenerates the native chromophore, which has a calculated extinction coefficient of 56,600 M^{-1} cm^{-1} at 558 nm (determined by the method of Rehorek and Heyn[10]).

Calorimetry

DSC scans were obtained with a Microcal MC-2 high sensitivity calorimeter (North Hampton, MA) at a scanning rate of 60°/hour. The bacteriorhodopsin concentration was typically between 30 and 60 μM. The pH of the sample was adjusted with a stock solution of 1 M buffer to the desired pH and to give a final buffer concentration of 50 mM. The buffers used were found from previous experiments on the purple membrane[6,11] to have no noticable temperature-dependent changes in pK_a: glycine, pH 2.4–3.7; acetate, pH 4–5; citrate, pH 5–7.4; phosphate, pH 8; carbonate-bicarbonate, pH 9–9.6. A solution of 0.5% SDS, 8.8% DMPC, 7.2% CHAPS, 50 mM buffer at the appropriate pH was used as a reference for DSC. The baseline subtracted was obtained from a repeat scan of the denatured sample since the transitions were irreversible. The transition T_m was taken as the point of maximum excess heat capacity, and the calorimetric enthalpy was estimated from the total area under the transition peak.

Absorption Spectroscopy

Spectra were recorded with a Gilford Response spectrophotometer in a 1-cm cell. The instrument is equipped with a thermoelectric cell holder for heating and monitoring the temperature of the cuvette.

Circular Dichroism

The CD spectra were recorded with a Jasco J-500A spectropolarimeter interfaced to a DP-500N data processor. Spectra were recorded on 55 μM bacteriorhodopsin, 0.4% SDS, 8.8% DMPC, 7.2% CHAPS, 50 mM citrate, pH 6.4; 59 μM bacterioopsin, 0.4% SDS, 8.8% DMPC, 7.2% CHAPS, 50 mM citrate, pH 6.4; and 49 μM purple membrane, 50 mM citrate, pH 6.4.

HPLC

Micellar bacteriorhodopsin was chromatographed using a Beckman instrument with 110B solvent delivery modules on a 300 × 7.5 mm BioGel TSK 20 gel filtration column (no guard column). Micellar bacte-

riorhodopsin was eluted with 1% DMPC, 0.8% CHAPS, 60 mM phosphate, 150 mM sodium sulfate, pH 6.5 at 0.4 ml/minute. Bio-Rad HPLC gel filtration standards were used to calibrate the column: thyroglobulin, MW 670,000; γ-globulin, 158,000; ovalbumin, 44,000; myoglobin, MW 17,000; vitamin B_{12}, 1,350. We found no difference in the retention times of the standards whether or not the elution buffer contained DMPC/CHAPS. A standard line was constructed from a plot of the log (molecular weight of standard) versus retention time and the linear least squares fit of the data gave the equation $y = 8.082 - 0.144x$; $r^2 = 0.98$.

Electron Microscopy

Micellar bacteriorhodopsin was stained with 2% (w/v) potasium phosphotungstate, pH 5.9, and examined with a Philips EM400 microscope on Formvar grids.

Estimating the Number of Amphiphiles per Micelle

The number of amphiphiles per micelle can be estimated from either the molecular mass determined by HPLC or the size of the micelle determined from electron microscopy. (We have assumed the molar ratio of amphiphiles is the same in the micelle and the bulk solution and the number of proteins per micelle is one for these calculations.) We can determine if a molecular mass of 210,000 Da corresponds to a 105-Å-diameter bilayer disk by comparing the estimated values obtained by the two techniques.

HPLC

The molecular mass of the micelles is about 210,000 Da from HPLC. The molecular weight of bacteriorhodopsin is 26,000, and with one protein per micelle, the remaining mass of 184,000 is due to the amphiphiles. The molar ratio of the amphiphiles is 1:7.5:6.8 for SDS:DMPC:CHAPS. From their respective molecular weights (228:678:615) and a total mass of 184,000, the number of molecules of each per micelle is calculated to be 19:145:131.

Electron Microscopy

We have used the bilayer disk model for the bacteriorhodopsin micelle[12,13] (see Results), and this yields a calculated cross-sectional surface area (parallel to the bilayer plane) of 8,659 $Å^2$ for a 105-Å-diameter disk. The cross-sectional surface area of bacteriorhodopsin parallel to the bilayer plane is about 875 $Å^2$, based on the reported dimensions of the protein, 35 Å × 25 Å × 45 Å[14]; and the remaining cross-sectional surface area equals 8,659 − 875 = 7,784 $Å^2$. Since the amphiphiles do not extend the entire thickness of the micelle bilayer (half reside in each of the two monolayers of the micelle) the total surface area available for the amphiphiles is 2 × 7,784 = 15,568 $Å^2$. To calculate the number of amphiphiles per micelle in this case, a knowledge of the molecular cross-sectional area is needed for each. In the case of DMPC, we have used 72 $Å^2$, which was obtained from X-ray diffraction data of maximally hydrated phosphatidylcholine bilayers.[15] For SDS and CHAPS, an approximate value of the cross-sectional areas can be obtained from diffraction data on (1) the hydrated lamellar phase of a sodium C-8 fatty acid (36.65 $Å^2$ [16]), and (2) the cross-sectional area of cholesterol (37 $Å^2$ [16]). These values are used with the molar ratio of the amphiphiles and the surface area available to them to calculate the number of amphiphiles per micelle: 19:141:127 for SDS:DMPC:CHAPS, respectively. These values agree with the values calculated from HPLC to within 4%.

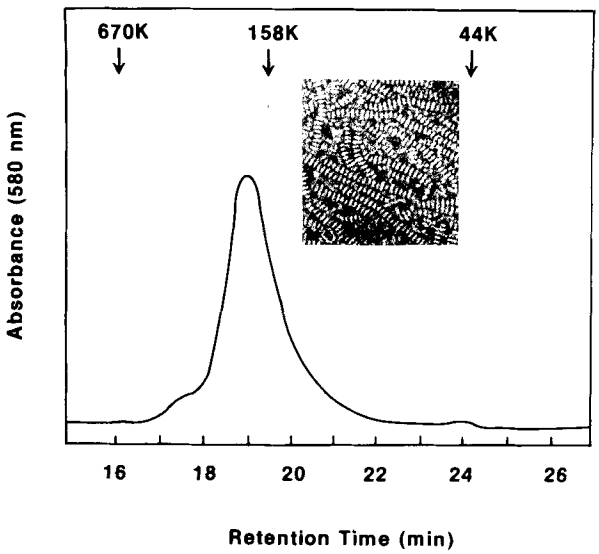

Fig. 1. HPLC gel filtration of micellar bacteriorhodopsin monitored at 580 nm. A 300 × 7.5 mm BioGel TSK 20 column was used at 0.4 ml/minute of 1% DMPC, 0.8% CHAPS, 60 mM phosphate, 150 mM sodium sulfate, pH 6.5. Molecular masses of standards are indicated. **Inset:** Electron micrograph of micellar bacteriorhodopsin, pH 6; bar equals 500 Å. The discoidal micelles can be seen stacked as rouleaux.

RESULTS

Structure of Micelles Containing Bacteriorhodopsin

The apoprotein was renatured after delipidation with the zwitterionic bile acid analog CHAPS, and the phospholipid DMPC.[8] The shape of the micelle observed from electron microscopy (Fig. 1, inset) is consistent with the bilayer disk model of Small[12] and Mazer et al.[13] for bile salt/phospholipid micelles. In these models, a bilayer disk of phospholipid is surrounded on its rim with the bile salt, hydrophilic side pointing toward the water. A bilayer disk would mimic the geometric constraints of a natural membrane (in respect to the radius of curvature perpendicular to the major axis) and may, in part, explain the successful renaturation of bacterio-

rhodopsin in other bile salt/phospholipid systems.[1] The dimensions of the micelles containing bacteriorhodopsin are 105 ± 13 Å in diameter by 43–45 Å^2 thick, which agrees with the average thickness of a DMPC bilayer.[17,18] The micelle molecular mass was estimated to be 210 kDa, by interpolation from a standard line (see Fig. 1 and Materials and Methods for details). The size determined by electron microscopy correlates well with the molecular mass estimated by HPLC, based on one protein per micelle (see Materials and Methods for details). It has been shown that detergent solubilization of the purple membrane forms mixed micelles with Stokes radii consistent with a single protein per micelle.[19,20] Although the size of the DMPC/CHAPS micelles does not preclude the possibility that more than one protein is present, the only reasonable conclusion from the data, when combined with the visible CD and DSC results discussed below, is that there is a single protein per micelle.

Structural Changes Studied by Differential Scanning Calorimetry

DSC scans typical for micellar bacteriorhodopsin between pH 4.4 and 9.0 are shown in Figure 2. In the DSC scans we usually saw two peaks, although sometimes the first was undetected. We inferred from spectroscopic measurements that these two peaks involve changes in the protein conformation (Fig. 6, see discussion below), and that the transition at higher temperature reflects bacteriorhodopsin denaturation. In all DSC and spectral experiments, the reference was the micellar preparation without protein and we saw no evidence for pH or temperature effects originating from protein-free micelles within the temperature (25–95°C) and pH ranges studied.

Pretransition

A predenaturational calorimetric transition has been seen for bacteriorhodopsin in the purple membrane.[6,21] Through the temperature region of the pretransition, changes occur in the visible CD[22] and X-ray diffraction pattern[22,23] that are caused by a loss of the long range ordering in the crystalline lattice, i.e., a loss of inter-trimer interactions.

A calorimetric pretransition is also observed for micellar bacteriorhodopsin. A comparison between the spectral changes associated with this transition in the two systems suggests that the same protein conformational change is responsible for the calorimetric pretransition of bacteriorhodopsin in micelles and the purple membrane. The spectral changes will be discussed below.

Denaturation

The curves in the lower panel of Figure 2 are replots of the high temperature denaturational transition of scans 1–4 shown in the upper panel. Although denaturation is irreversible in the DSC experiment, theoretical curve fitting of the data indicates that these transitions fit fairly well to a two-state model for the unfolding of a monomer. In other words, an equilibrium two-state transition between a native bacteriorhodopsin monomer and a denatured monomer was assumed, and a theoretical curve was calculated. The theoretical curves are superimposed on the data curves in Figure 2, and in each case, the curve just below was obtained by subtracting the theoretical curve from the data curve. The agreement between the theoretical and data curves supports the application of equilibrium thermodynamics to an irreversible process.[24] For the pH

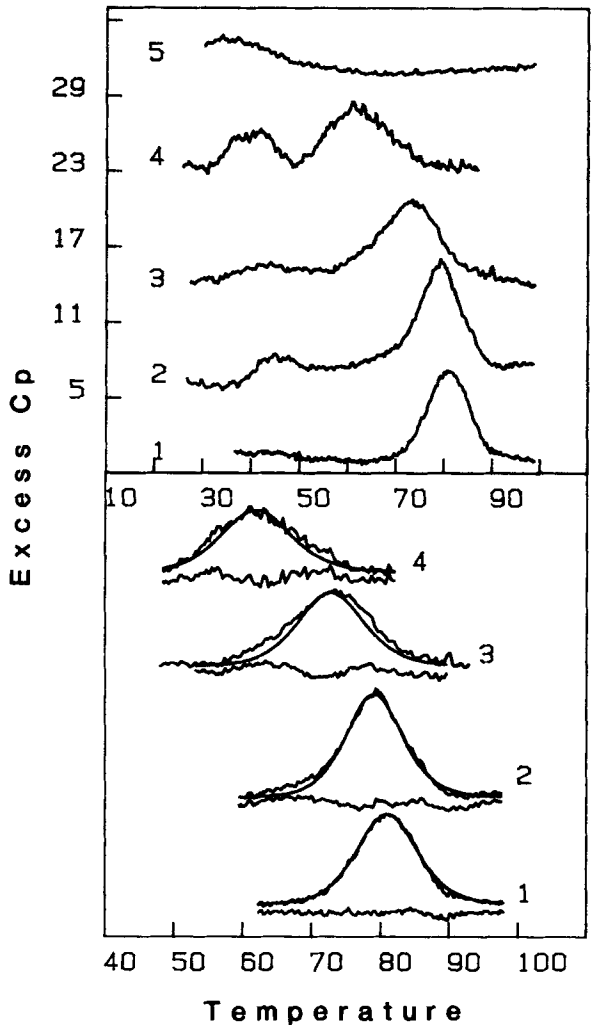

Fig. 2. Differential scanning calorimetry of micellar bacteriorhodopsin (30–60 μM) at the indicated pH. Heat capacity units are kcal mol^{-1} deg^{-1}. Scans have had baselines subtracted from them. **Upper panel:** scan 1, pH 4.4; scan 2, pH 4.9; scan 3, pH 6.9; scan 4, pH 9.0; scan 5, pH 6.9, a typical baseline. **Lower panel:** Scans 1–4 are replots of the high temperature transition shown in the respective scans of the upper panel. Superimposed on each scan is a theoretical curve that simulates a two-state transition for monomeric bacteriorhodopsin unfolding (see text for details). Subtracting the calculated curve from the data curve yields the difference curve just below.

TABLE I. Analysis of the DSC Denaturational Transition of Monomeric Bacteriorhodopsin*

n	pH	Tm	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)
4	4.7 ± .22	79.9 ± 1.0	94 ± 15	87 ± 10
3	5.7 ± .06	81.6 ± 1.6	90 ± 8	98 ± 8
4	6.3 ± .16	74.9 ± 1.4	104 ± 10	78 ± 17
5	7.1 ± .15	72.4 ± 1.8	95 ± 10	71 ± 14
2	7.4 ± 0	67.5 ± 1.8	85 ± 2	76 ± 7

*Values listed in columns 2–5 are the mean ± standard deviation of the number of DSC experiments listed in column 1. For the purple membrane, ΔH_{cal} = 92 ± 2, Tm = 98.4 ± 0.7C° at pH 6.1 ± 0.7 (n = 3).

range from 4.4–7.4, the T_ms and enthalpies of denaturation obtained by DSC are summarized in Table I. The van't Hoff enthalpies can also be obtained from the calorimetric data[25,26] and these are listed in Table I, as well. If the unfolding of micellar bacteriorhodopsin is a two-state process, then the calculated ratio $\Delta H_{vH}/\Delta H_{cal}$, will equal 1. This ratio is an indication of the cooperativity of the process and is often called the cooperative unit. This ratio is equal to 1 for many small soluble proteins.[27] The average value of $\Delta H_{vH}/\Delta H_{cal}$, determined from 18 DSC scans between pH 4.4 and pH 7.4, is calculated to be 0.9 ± 0.2. Although this value is close to 1, a value of less than 1 indicates that the process is not a perfect two-state transition, and may indicate the presence of intermediate states.

The temperature of micellar bacteriorhodopsin denaturation depends on the pH, as was observed for the purple membrane (Fig. 3). The thermal stability of soluble proteins is also pH dependent, due mostly to the titration of surface groups. By analogy, the surface charge of the membrane amphiphiles and/or protein is likely to play a role in the structural stability of bacteriorhodopsin. Micellar bacteriorhodopsin and the purple membrane are thermally most stable between pH 5 and 6, and the shape of the plots of denaturational T_m versus pH are similar. At any given pH, however, micellar bacteriorhodopsin denatures at a lower temperature. This indicates that incorporating bacteriorhodopsin into micelles destabilizes the protein.

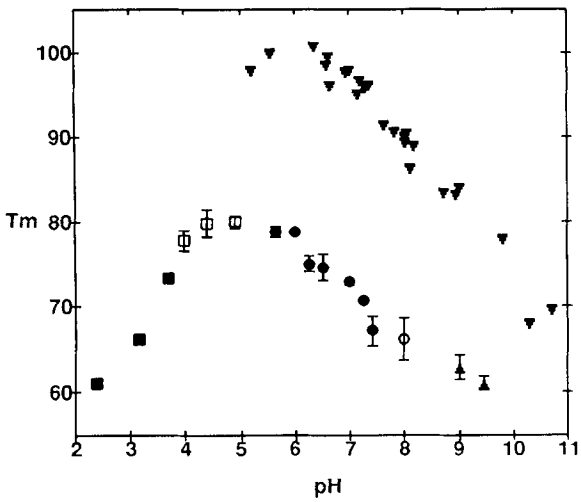

Fig. 3. pH dependence of the denaturational midpoint temperature (T_m), determined by calorimetry. purple membrane ▼ (data depicted for the purple membrane here and in Fig. 7 has been reported in ref. 6); micellar bacteriorhodopsin—lower curve. With the exception of pH 2.4, each point represents the average of from two to four determinations. Error bars represent the standard deviation and are present on all points but do not extend beyond the symbol in some cases. Different buffers were used to adjust the sample pH (see Materials and Methods for details): glycine ■; acetate □; citrate •; phosphate ○; carbonate-bicarbonate ▲.

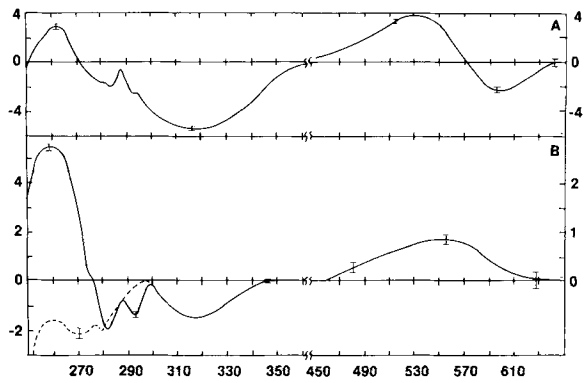

Fig. 4. Circular dichroism spectra. **A:** 49 μM purple membrane, 50 mM citrate, pH 6.4. **B:** 55 μM micellar bacteriorhodopsin, 50 mM citrate, pH 6.4 ——; 59 μM micellar bacterioopsin, 50 mM citrate pH 6.4 – – –. The noise in the spectra is indicated by perpendicular bars.

Absorption Spectroscopy: Similarities between Purple Membrane and Micellar Bacteriorhodopsin

Information concerning the tertiary structure of the protein can be obtained from circular dichororism in the near UV and visible region. The aromatic amino acids absorb in the 250–300 nm region and bands in the 300–600 nm region can be attributed to the retinal moiety.[28–30] These regions of the CD spectrum are shown in Figure 4 for the purple membrane and micellar bacteriorhodopsin (because of the CHAPS amide group, the far-UV CD cannot be obtained). There is a close similarity between the two in the fine structure of the bands originating from the aromatic amino acids. In the region from 250 to 350 nm, the peaks and valleys of the CD spectra occur at approximately the same wavelengths. The biggest differences between the CD spectra are seen in the regions of retinal absorption. The magnitude, but not position, of the 317 nm peak in the purple membrane is much larger and the double band in the 500–650 nm region is replaced by a broad positive ellipticity in micellar bacteriorhodopsin. The absence of exciton coupling in the visi-

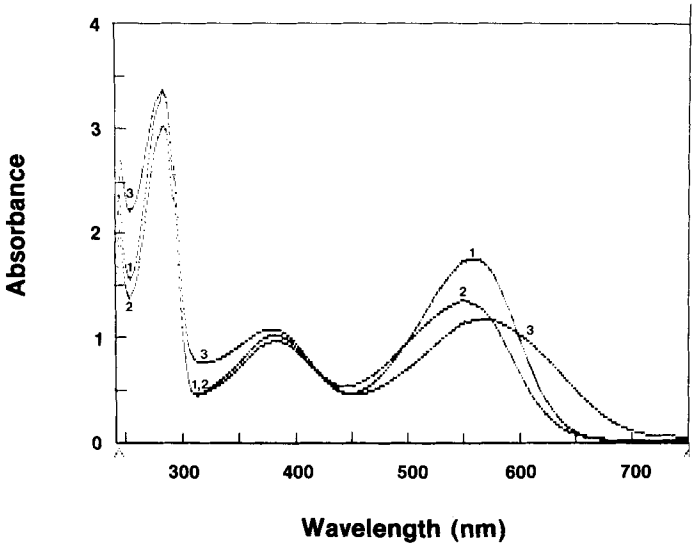

Fig. 5. Absorbance spectroscopy of micellar bacteriorhodopsin. The pH was adjusted with either 0.1 M NaOH or 0.1 M HCl. Spectrum 1, pH 6.4; spectrum 2, pH 8.0; spectrum 3, pH 2.7.

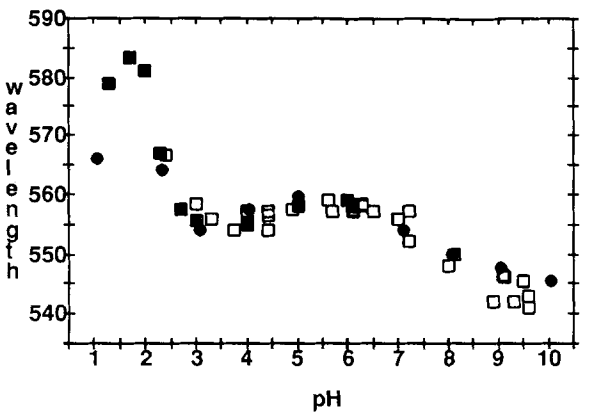

Fig. 6. pH dependence of the wavelength of maximum absorbance for micellar bacteriorhodopsin. Each open square represents a different sample prepared for DSC. Closed symbols represent a sequential titration of two individual samples.

ble CD spectrum distinguishes the monomeric protein from an aggregate.[20,28,29] The bands at 317 nm and 500–650 nm result from the induced optical activity of retinal when bound to the protein.[31] Overall, the differences in the CD spectra can be attributed to some change in the nature or extent of the interaction between retinal and the protein.

pH effects

Absorbance spectra for micellar bacteriorhodopsin at three different pH values are shown in Figure 5 and a plot of the visible λ_{max} as a function of pH is shown in Figure 6. All spectra in Figures 5 and 7 show a free retinal peak because micellar bacteriorhodopsin is regenerated with an excess of retinal. There is an intermediate pH range, from 4.4 to 7.4, where small changes are observed. As the pH drops below 4, micellar bacteriorhodopsin is converted to a species blue in color, which appears analogous to the acid-blue or cation-depleted pigment of the purple membrane.[30,32,33] This shift to longer wavelengths has also been observed for detergent-solubilized purple membrane.[34,35] Above pH 7, micellar bacteriorhodopsin becomes more pink in color as the visible λ_{max} shifts to shorter wavelengths. The visible spectrum changes with pH are much the same for the purple membrane, however the purple membrane spectrum remains unchanged over a slightly more basic pH range—5 through 8.5.[30] The pH-induced changes in the visible absorption spectrum of the purple membrane, along with changes in the near- and far-UV and CD spectra, have been taken as evidence that small changes in the tertiary structure occur below pH 5 in purple membrane and relatively larger changes occur above pH 8.5. The secondary structure of purple membrane is essentially preserved throughout the pH range of 2.4 to 11.8.[30] It is likely that the similarities between the spectral changes for micellar bacteriorhodopsin and purple membrane are due to similar changes in the tertiary structure.

Pretransition

The changes which occur in the absorption spectrum through the temperature range of the DSC experiment are shown in Figure 7. For clarity and the purpose of discussion, the spectra have been divided into two temperature ranges to illustrate pretransitional changes (panel A) and denaturational changes (panel B). For comparison, the pretransitional changes of the purple membrane are shown in the inset of panel A.

As previously mentioned, a predenaturational transition is observed in the purple membrane by DSC and a change in the visible absorption occurs in the temperature region of this pretransition. The spectral changes have been interpreted as a change in the environment around the retinal moiety as the visible λ_{max} shifts from a species absorbing at about 560 nm to one absorbing at 460 nm.[6] This absorption shift probably reflects a transition between two conformational states of the protein, since it appears to proceed through an isobestic point at 520 nm. For micellar bacteriorhodopsin, a similar shift to shorter wavelengths is observed through the temperature range of the DSC pretransition. This transition appears to be associated with an isobestic point at 545 nm, suggesting that in this case also, the transition is between two conformational states of the protein. The similarity between the spectral and calorimetric pretransitions in both systems suggests that in the purple membrane, a conformational change within a monomer indirectly affects protein–protein interactions and results in a disordering of the crys-

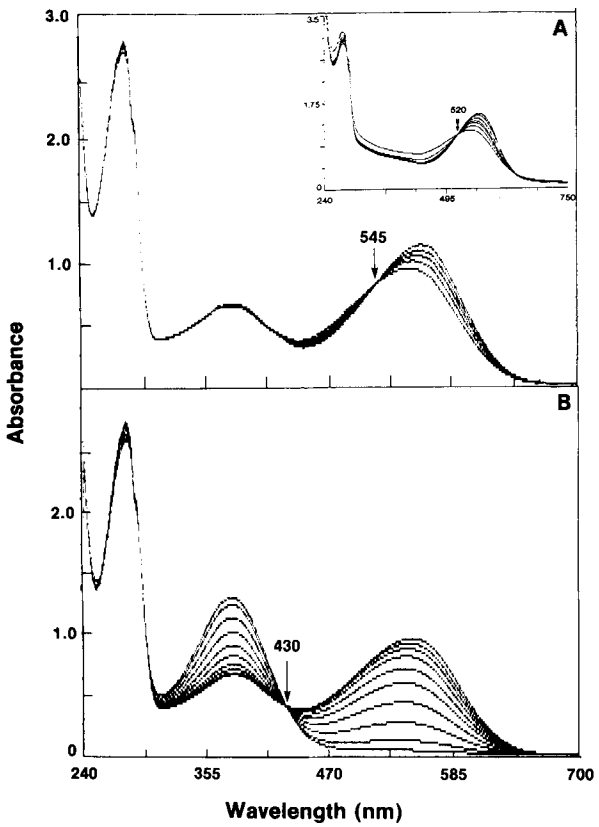

Fig. 7. Absorbance spectroscopy as a function of temperature for micellar bacteriorhodopsin at pH 7.4. A single sample was used. The time between recording each spectrum was 4 minutes. **A:** Pretransitional changes. Spectra from top to bottom were taken from 5–45°C, respectively, in 10° increments. **Inset:** Pretransitional changes for the purple membrane, pH 8.3. Spectra from top to bottom were taken at 10, 30, 50, 65, 70, 75, and 80°C, respectively. **B:** Denaturational changes. Spectra from top to bottom were taken at 45, 50, 53, 56, 59, 61, 64, 67, 70, and 73°C, respectively.

talline lattice (an observation made using temperature-dependent X-ray diffraction and visible CD.[22,23])

Denaturation

The visible absorption spectrum of bacteriorhodopsin reports on the environment of the covalently linked chromophore, retinal, in the binding pocket of the protein. When micellar bacteriorhodopsin unfolds, the visible absorption is completely lost, as it is in the purple membrane, because retinal-protein contacts are broken. Spectroscopically (Fig. 7B), this transition is evident as an apparent two-state transition between the native 558 nm species and the denatured 380 nm species, the wavelength at which free retinal absorbs. An isobestic point is seen at 430 nm. This transition is reminescent of the two-state transition between the native and hydroxylamine-bleached purple membrane.[31] In the purple membrane, the absorption band at 567 nm is lost and replaced with one at 366 nm, and an isobestic point at 414 nm is observed. The product of bleaching is the folded retinal-free protein, bacterioopsin, and retinaloxime. The micellar species most closely analogous to bleached purple membrane is micellar bacterioopsin—the DMPC/CHAPS reconstituted protein before retinal is added. The near-UV CD spectrum of micellar bacterioopsin, shown in Figure 4, has little in common with the native micellar bacteriorhodopsin, as expected, but does resemble the relatively featureless 250–300 nm region of the bleached purple membrane.

CONCLUSIONS

In this paper, we have used various indirect means to show the tertiary structure of bacteriorhodopsin in micelles and the purple membrane is close to the same. The visible CD spectrum and analysis of the DSC data indicate that micellar bacteriorhodopsin is monomeric. The near-UV CD spectrum is an indicator of tertiary structure, and for these two systems, this region is quite similar. The 317 nm band originates from a magnetically allowed transition of retinal (and therefore it is absent from the absorption spectrum) and has been interpreted to be due to a constraint imposed on retinal by the asymmetric environment of the protein binding pocket.[30,31] Because the magnitude of this band is diminished, but the position and shape are the same in micellar bacteriorhodopsin, it seems that removing bacteriorhodopsin from the crystalline lattice of the purple membrane allows the structure to "breath" and consequently loosen its hold on retinal. Disrupting the bacteriorhodopsin trimer may also contribute to this effect. The observation that tryptophan fluorescence increases more than 2-fold during octylglucoside solubilization of the purple membrane,[20] (presumably due to a reduction in energy transfer to the retinal), is consistent with our results. In addition to the similarities between the CD spectra of the two systems, the responses of the purple membrane to pH and temperature are mimicked by micellar bacteriorhodopsin. Both systems show similar pH-dependent changes in their absorption spectra and thermal stabilities, demonstrating that changes in the protonation state have a similar affect on the local environment of retinal and protein conformation in the two systems. Both systems also exhibit predenaturational transitions and their pretransitions are associated with similar spectral changes. It is difficult to imagine how these data could result from structures that were not the same or nearly so. Taken together, these results also suggest that the surface charge of the membrane (contributed by either or both the amphiphiles and protein) is important to the structural stability of the membrane protein, as it is for soluble proteins.

Substantial differences arise when comparing the

relative thermal stabilities of the two systems. Micellar bacteriorhodopsin denaturation is closely approximated by a two-state transition between the monomeric native and denatured protein from a thermodynamic analysis of the DSC data. A $\Delta H_{vH}/\Delta H_{cal}$ ratio of 0.9, however, may indicate that intermediates are present in low concentrations. Temperature-dependent spectroscopy is also consistent with a two-state denaturation of the monomer. On the other hand, in the purple membrane, it is the trimer that appears to denature as a cooperative unit.[6] It is tempting to compare the unfolding ΔH_{cal} of the purple membrane and micellar bacteriorhodopsin. A direct comparison is not possible now, however, because we do not know the structure of the denatured state in micelles (in the purple membrane, bacteriorhodopsin does not completely unfold).

The stability gained through the crystalline lattice of the purple membrane, and protein–protein and protein–lipid interactions derived from it, could account for the differences in stability between these two systems. A recently described[36] 2.8 Å resolution density map of the purple membrane is of sufficient detail to show lipid molecules within the center of the bacteriorhodopsin trimer and surrounding it. In reconstituting bacteriorhodopsin, *Halobacterium* lipids were found to be necessary for reforming the P_3 lattice.[37] The favorable packing interactions between the proteins and native lipids in the purple membrane are not likely to be duplicated in the synthetic environment of the mixed micelle. In addition to lost intermolecular interactions, intramolecular interactions may not be optimal in the micellar bacteriorhodopsin either. Our CD data suggest that micellar bacteriorhodopsin has a looser or more flexible structure than that in the purple membrane. With no alteration in the equilibrium position of the residues relative to the purple membrane, the looser structure of micellar bacteriorhodopsin would translate into a structure with a higher free energy, a model which has been proposed for destabilizing mutations of T4 lysozyme.[38,*] We favor these explanations for the decreased stability of micellar bacteriorhodopsin over a possible specific destabilizing effect of our reconstitution system, because several different preparations of monomeric bacteriorhodopsin have been shown to be less stable than the purple membrane. Monomeric bacteriorhodopsin has been shown to denature 31° lower than the purple membrane in octylglucoside micelles at pH 7[19], 20° lower in lauryl maltoside micelles at pH 6[†], and 15° lower in DMPC vesicles.[6]

Bacteriorhodopsin is a single polypeptide chain which threads back and forth through the membrane. In the low resolution electron diffraction image of the purple membrane,[14] the protein appears something like a sandwich of three helices packed against four, all roughly perpendicular to the membrane plane. As we learn more about the topography of other membrane proteins, a seven helix bundle begins to emerge as a possible folding motif for many of them.[39,40] Structure–function studies on the prototype, bacteriorhodopsin, are likely to further our understanding of this entire class of membrane proteins.

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*In their 1984 paper, Hawkes et al. propose several mechanisms by which a mutation would destabilize the native structure: "This model merely states that the intrusion of the mutation into the wild-type structure raises the energy and loosens the structure by disrupting the packing pattern of the molecule."

†C. Brouillette, unpublished observation.

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