Refolding of Thermally Denatured Bacteriorhodopsin in Purple Membrane†

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The change in protein conformational structure and retinal chromophore binding state have been examined by using in situ UV−vis, FTIR, and CD spectroscopies during the thermal denaturation and refolding processes in bacteriorhodopsin (bR) of purple membrane (PM), in its native trimeric and in Triton X-100 solubilized monomeric form. For the trimeric bR, it is found that heating bR through its premelting transition (T > 78 °C, T_m') does not cause any permanent damage in the protein secondary structure, and a reversible refolding occurs when it cools back to room temperature. For the monomeric bR, it is found that it is less thermally stable than the trimer. There is a significant change in its protein secondary structure and a complete dissociation of retinal occurs irreversibly at a temperature as low as 66 °C. In addition, it is found that heating the trimeric bR through its main molten state (T > 96 °C, T_m) changes the protein secondary structure so that bR does not refold fully into its original secondary structure. Upon cooling back to room temperature, about 90% of the bound retinal in native bR recovers after being heated through its premelting transition, whereas only about 12% of bound retinal recovers if bR is heated above its main melting temperature. It is also found that refolded bR molecules with their retinal chromophore rebound have a photocycle and are capable of pumping protons. Our results also suggest that from its molten state, protein secondary structure refolding precedes retinal rebinding to the Schiff base.

1. Introduction

As the simplest and well-characterized light-driven proton pump present in nature, bacteriorhodopsin (bR) provides us with an excellent model of studying bioenergetics.[1−3] Found in the purple membrane (PM) of *Halobacterium salinarum*, bR displays a seven α-helical conformation within a single polypeptide chain of molecular weight ∼27 kDa. The atomic structure of bR has become clear with atomic resolutions.[4−9] As a result of this, recent attention has been given to the function and variation of its internal bound water molecules.[10,11] Its unique purple color (with $\lambda_{max} \sim 568$ nm) in the light-adapted state is due to the covalently bound all-trans retinal (otherwise with $\lambda_{max} \sim 380$ nm in free state) with the Lys216 via a protonated Schiff base (PSB).[12,13] Beginning with the photoisomerization of the all-trans retinal to 13-cis upon light excitation, the photocycle of bR initiates and is followed by a series of intermediates distinguishable by their visible absorption spectra, such as J, K, L, M, N, and O.[14,15] During the transition from L to M, a proton is released to the extracellular surface of bR. The electrochemical energy generated from the proton gradient across the membrane is eventually used by the organism in the synthesis of ATP from ADP under low oxygen conditions.

While a considerable amount of research effort has been focused on the structure and function of membrane proteins, relatively little is known about the forces that determine protein folding and stability, as is the case of many other proteins. It is known that bR molecules form a two-dimensional hexagonal lattice (with the help of surrounding lipids), which is composed of bR in a trimeric structure.[16] By weight, approximately 25% of PM are lipid molecules and the majority of them are polar due to their phosphate headgroups. In the trimeric structure of bR, intermonomer contacts are stronger between some helices.[17,18] Considering these facts, the structural stability of bR is dependent on many factors,[19] including intramolecular and intermolecular interactions, protein−lipid interactions, and even protein−retinal interactions.

Monomeric bR can be formed by breaking the trimeric structure using neutral detergents such as Triton X-100,[20] featured by the maximum absorption at ∼550 nm in visible absorption spectra for the light-adapted bR monomer, and the monophasic CD band in the visible region. The photocycle of bR monomer is qualitatively the same as bR trimer, while the rise time of M formation is about three times faster.[21]

By using differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), and Fourier transform infrared (FTIR) spectroscopy, the thermal stability of bR has been investigated.[22−29] It has been shown that at neutral pH bR has two thermal transitions, a reversible premelting transition with a melting temperature T_m' of ∼78 °C, followed by an irreversible main transition with a melting temperature T_m of ∼96 °C;[22] During the reversible transition the protein conformation changes from its original unusual α_{II}-helical conformation to a more commonly seen α_I-helical conformation.[28,30]

Questions arise such as, does bR refold from its thermal molten state, and what is the high temperature limit heated that allows bR to refold its secondary structure to its native form? After heating to its melting or premelting temperature, does bR refold its structure first or does retinal rebind first, and what are the refolding and rebinding pathways? What are the changes

† Abbreviations: bR, bacteriorhodopsin ground state; PM, purple membrane; FTIR, Fourier transform infrared; UV−vis, ultraviolet−visible; CD, circular dichroism.

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in the protein conformation and in lipids? Does refolded bR still have a photocycle that pumps protons as that in its native form? A practical approach to answer these questions is to use various static and kinetic spectroscopic methods that are highly structure sensitive and selective to study the effect of heating on the bR structure.

In the present study, static and time-resolved UV−vis absorption spectroscopy, CD spectroscopy, and FTIR absorption spectroscopy have been used to examine the kinetic processes of thermal denaturation and renaturation of bR, as well as retinal rebinding as the protein refolds. The factors controlling protein refolding and the retinal binding have been discussed.

2. Materials and Methods

Native bR in purple membrane (PM) was prepared from the *H salinarum* strain ET1001 as described previously[31] and then dispersed in deionized H_2O, with pH ∼ 6.8. D_2O (99.9%, Aldrich) was used without further purification. The concentration of bR was controlled to give an optical density of ∼0.8 at 568 nm, without pH buffers added.

Monomeric bR was prepared by solubilizing native bR suspension in Triton X-100, according to previously described procedures.[21,32] Briefly, PM suspension in 25 mM $KHPO_4$ buffer, pH ∼ 7.0 was mixed with Triton X-100 (Aldrich) at a ratio of 1:7 (w/w). The mixture was kept overnight in the dark. Successful solubilization of bR monomer in Triton X-100 is indicated by the fact that no sediment occurs after 30 min centrifugation at 19 000 rpm. Instead, extra detergent and water were removed by using vacuumed rotor evaporation at 30 °C, to concentrate the bR monomer for optical characterization, with no further pH adjustment.

UV−vis absorption spectra were taken by using a 3101PC UV−vis spectrometer (Shimadzu); Circular dichroism (CD) spectra were taken by using a JASCO J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd.); FTIR spectra were taken by using a Nicolet Magna-IR 860 spectrometer. Flash photolysis measurements were carried out by using a system consisting of a MOPO (Spectral Physics) for laser excitation at 560 nm (10 ns, 10 Hz); a Xenon lamp was used for continuous probing, in combination with a monochromator and PMT, and a 500 MHz transient digitizer (LeCroy 9350A). The M kinetics were measured by probing absorption at 412 nm, with 600−1000 laser shots averaged.

Before spectroscopic measurements, the sample was kept in the dark for more than 30 min to allow it to reach equilibrium of the retinal isomeric ratio at the initial temperature. The sample was either in a sealed quartz cuvette (1 cm path length for UV−vis, 1−2 mm path length for far-UV CD), or a sealed liquid cell (100 μm path length for IR). Sample temperature was controlled by using a water/glycerol bathed endocal TRE-100 thermastat (Neslab Instruments Ltd.) and was probed specifically within the sample volume by a thermal diode probe (Fisher Scientific). The temperature was set at each desired value and equilibrated for ∼10 min before a spectrum was taken. After reaching the final temperature for denaturing, the kinetics of the thermal relaxation from the denatured state to the original state was followed spectroscopically as the sample was allowed to cool to room temperature. This process usually takes about 2 h.

3. Results and Discussion

3.1. Retinal Binding State Change during Thermal Denaturing and Refolding of bR. Figure 1 shows visible absorption spectra of retinal in the trimeric bR/H_2O at various temperatures, going through either the premelting transition temperature T_m' (A) or the main melting transition temperature T_m (B). The absorption of bR is at ∼562 nm, indicating a dark-adapted state of the bR bound retinal (all-trans/13-cis = 50/50). At 83 °C, it shows a decrease in absorption intensity, a slight blue shift in the absorption maximum, and a spectral broadening, indicating an increase of the 13-cis retinal, which has a smaller absorption cross section. As the system cools to 29 °C, the regenerated state is measured (curve c). It is noticed that at least 90% of the absorption intensity and spectral shape recovers (curve c) to its original preheated state (curve a), indicating a good reversibility of the thermal denaturing−refolding process during the premelting transition. Figure 1B, on the other hand, shows a complete disappearance of the retinal absorption band at 562 nm at 96 °C (curve c), as well as a significant increase in the band intensity at ∼380 nm, indicating the formation of the free retinal. Retinal dissociation out of its binding cavity inside the protein is temporarily complete at $T > T_m$, at which temperature free retinal dominates and the equilibrium of [bound retinal] ↔ [free retinal] shifts to lower wavelength. However, as the system cools, the retinal binding takes place, and the equilibrium shifts to the bound state and the protein partially recovers its purple color. The observed temperature-dependent retinal absorption is in agreement with a previous study.[23] A recovery of only ∼12% of the bound retinal is shown after the system cools to 25 °C, while the rest of the retinal stays in the free state (curve c). Our previous study[30] has shown that during the premelting transition, retinal

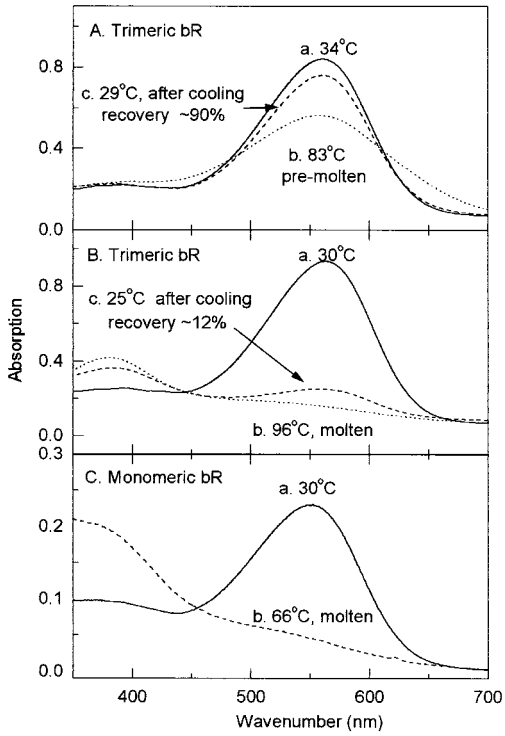

Figure 1. Visible absorption spectra (350−700 nm) of retinal in bR/H_2O at various temperatures. (A) Native bR in trimer form at 34 °C (curve a); predenatured state at 83 °C (curve b; just above the premelting transition temperature 76−78 °C); refolded state at 29 °C (curve c) after a slow cooling process, showing a recovery of ∼90% for the bound retinal. (B) Native bR in trimeric form at 30 °C (curve a); thermal denatured state at 96 °C (curve b; above the main transition temperature ∼96 °C); refolded state at 25 °C (curve c) after a slow cooling process, showing a recovery of ∼12% for the bound retinal. (C) Triton X-100 solubilized monomeric bR at 30 °C (curve a); thermal denatured state at 66 °C (curve b). The refolded state at 25 °C after a slow cooling process shows almost no bound retinal recovery.

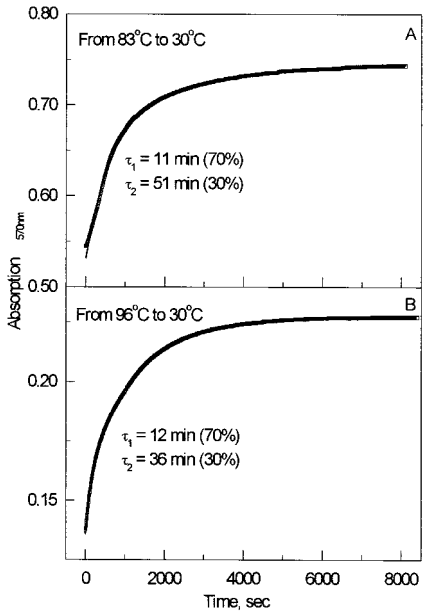

Figure 2. Kinetics of the thermal relaxation of bR/H_2O from the predenatured state and denatured state as shown in Figure 1. (A) Recovery of the predenatured state of bR as monitored at 570 nm, as the temperature decreases from 83 to 30 °C as a function of time. (B) Recovery of the denatured state of bR as monitored at 570 nm, as the temperature decreases from 96 to 30 °C. Curve fitting results are also shown in each case.

changes its configuration from all-trans to 13-cis. In this study, it appears that those changes in the premelting transition might be needed for the retinal final dissociation, so as to lead to a significant loss (∼88%) of bound chromophore during the irreversible main transition.

For the monomeric bR (Figure 1C), it is noticed that upon heating, a complete loss of the 552 nm band occurs at ∼66 °C, indicating a denatured bR monomer with full dissociation of bound retinal. Correspondingly, free retinal is shown to appear at 385 nm. It is also found that this process is also irreversible, and no retinal rebinding is observed following the thermal relaxation (data not shown).

Figure 2 shows kinetic measurements of the thermal relaxation of bR/H_2O from its predenatured state and denatured state. The rebinding of retinal is monitored at the change in peak intensity at 570 nm in the UV−vis spectrum. It can be seen that two exponential function components fit the relaxation process with 11 min (70%) and 51 min (30%), accounting for the near 90% recovery of bound retinal as bR refolds from its premolten state; whereas two components with 12 min (70%) and 36 min (30%) account for the ∼12% recovery of bound retinal as bR refolds from its molten state.

In the CD spectra for bR trimer in the visible region shown in Figure 3A, retinal shows an asymmetric bilobed CD band (curve a), consisting of a positive band at ∼530 nm and a negative band at ∼600 nm, which is an indication of trimer structure.[29] Heating the sample to the premolten state (85 °C) followed by cooling to room temperature has no effect on the bilobed CD band (curve b). A significant decrease of spectral intensity (without changing the spectral shape) is observed when cooling from 100 °C (curve c). Such a bilobic spectral shape disappears completely once bR is heated above 125 °C (curve d), indicating a total dissociation of bound retinal in bR. This is in agreement with the results observed in the visible absorption spectra shown in Figure 1. In the case of monomerized bR (Figure 3B), it is observed that a monophasic band is

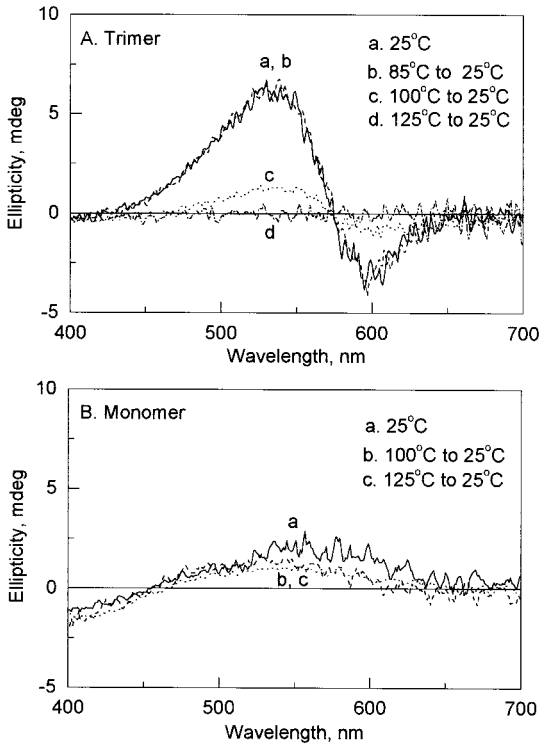

Figure 3. Visible CD spectra showing bound retinal. (A) Native trimeric state of bR at room temperature (curve a); refolded state of bR at 25 °C after being heated to its predenatured states ($T \geq 78$ °C; curve b); refolded state of bR at 25 °C after being heated to its main denatured states ($T \geq 96$ °C, curve c). Curve d represents the refolded protein states of bR at 25 °C after being heated to a higher temperature, e.g., 125 °C. (B) Triton X-100 solubilized monomeric state of bR at room temperature (curve a); refolded states of bR 25 °C (curves b, c) after being heated to 100 and 125 °C, respectively.

present in the retinal region at 30 °C, which is an indicator of losing retinal exciton coupling,[33] or changing the retinal heterogeneity[34,35] as a result of breaking the trimeric structure. Above 100 °C bR monomer still shows the monophasic retinal CD band with a decreased molar ellipticity.

3.2. Protein Conformation Change during Thermal Denaturing and Refolding of bR. Figure 4 shows the FTIR absorption spectra of bR in D_2O at various temperatures as bR undergoes its premelting process followed by its refolding. In the spectral region of 1900−1300 cm^{-1} shown in Figure 4A, a decrease in the absorption intensity is observed in both the amide I band (essentially the polypeptide C═O stretching mode) and the amide II band (mainly the N−H in plane bending mode) at the premolten state (86 °C). The thermal relaxation from this predenatured state re-forms almost all the amide I band; however, only a smaller amount of the amide II band (curve c). In the spectral region of 3700−3100 cm^{-1} shown in Figure 4B for the amide A band (a composite band that contains protein side chain O−H stretching and backbone N−H stretching), it is observed that almost a total recovery of the amide A band from the premolten state at 86 °C is observed after the temperature decreases back to 30 °C. The above data indicate that protein conformational structure from the premolten state can be almost completely recovered to its initial state.

Figure 5 shows the FTIR absorption spectra of bR in D_2O as it undergoes the thermal denaturation above the main melting transition temperature (96 °C) and then as it cools to 30 °C. In the spectral region of 1900−1300 cm^{-1} for the amide I and II bands, it is observed that neither amide I nor amide II band recovers completely. In fact, besides the appearance of the new

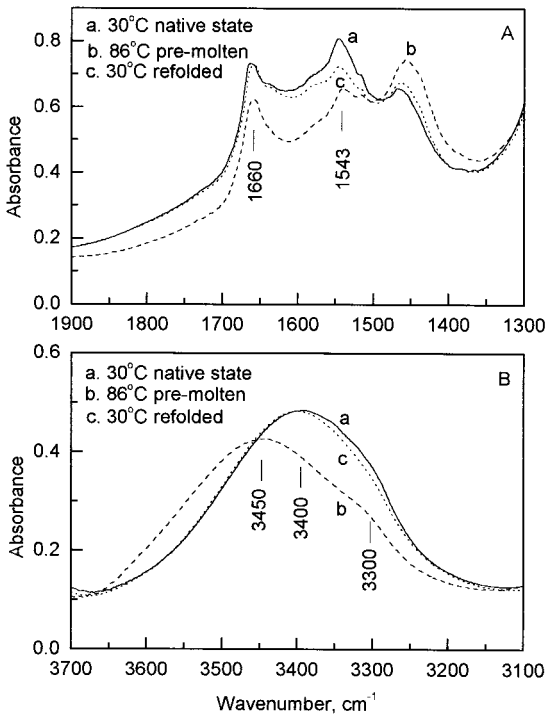

Figure 4. FTIR absorption spectra of native trimeric bR in D_2O at selected temperatures during which bR undergoes the predenaturing and refolding afterward. Spectral regions are (A) 1900−1300 cm^{-1} for the amide I and II bands and (B) 3700−3100 cm^{-1} for the amide A band. Spectra are labeled as (a) native state at 30 °C, (b) thermal predenatured state at 86 °C, and (c) refolded state at 30 °C after thermal relaxation from (b), respectively. Almost total recovery of amide I band and amide A and only partial amide II recovery are observed (c).

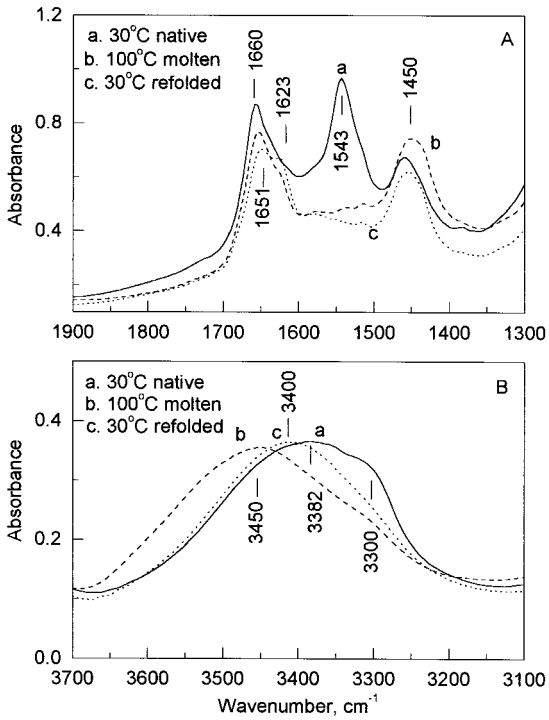

Figure 5. FTIR absorption spectra of native trimeric bR in D_2O undergoes the main melting transition and renaturing afterward. Spectral regions are (A) 1900−1300 cm^{-1} for the amide I−III bands and (B) 3700−3100 cm^{-1} for the amide A band. Spectra are labeled as (a) native state at 30 °C, (b) thermal denatured state at 100 °C, and (c) refolded state at 30 °C after thermal relaxation from (b), respectively. None of amide I or amide II has total recovery. Besides the unordered structure at 1623 cm^{-1}, partial recovery of the amide I band and almost a total lose of amide II are observed (c).

band at 1623 cm^{-1} in the refolded state, which is a signature band of the unordered secondary structure, only partial recovery of the amide I band and almost a total loss of amide II are observed (c).

In the spectral region of 3700−3100 cm^{-1} of the amide A band, it is shown that the disappearance of a band near 3300 cm^{-1} is associated with the formation of the denatured state at 100 °C. In the refolded state, a blue shift is observed in the absorption maximum (from 3382 to 3400 cm^{-1}). This can be explained as a result of protein side chain exposed to the aqueous medium, and the protein backbone N−H to N−D exchange is irreversible in the refolded species (N−D stretching frequency is below 2700 cm^{-1}, which is buried in the broad O−D stretching region, and cannot be resolved in our experiment).

Figure 6 shows the FTIR spectra of monomerized bR following its thermal denaturing and refolding processes. It can be seen from Figure 6A that in the molten state (98 °C, curve b), about 40% of the amide I band (with a maximum at ∼1660 cm^{-1} for α-helices, 25 °C, curve a) is converted into the unordered structure (giving rise to a band at ∼1623 cm^{-1}, curve c). Cooling the sample to room temperature does not fully refold bR monomer to its original α-helices, indicating an irreversible damage to the protein secondary structure. The amide II shows similar behavior as the trimeric bR in Figure 5A. Figure 6B gives the spectral change in the amide A region, in which an almost full recovery is observed from 98 to 25 °C, except the loss of the 3300 cm^{-1} band shoulder, which is assigned as an indicator of the N−H to N−D exchange. This is in good agreement with Figure 5B. In addition, as shown in Figures 4−6, spectral changes are also observed in the protein amide II band: decrease at ∼1543 cm^{-1} and increase at ∼1450 cm^{-1}, which is due to the exchange from the N−H to N−D in-plane bending mode as a result of heating in the D_2O solvent.

Figure 7 shows the absorption intensity change for bR trimer at 1663 and 1543 cm^{-1} as a function of time, during the thermal relaxation of bR/D_2O from the premolten state (86 °C, Figure 7A,B) and the molten state (100 °C, Figure 7C,D). The recovery of the protein amide I band at 1663 cm^{-1} from the premolten state (Figure 7A) can be fitted to two exponential functions, with their time constants and relative amplitudes as 7 min (40%) and 40 min (60%). On the other hand, from the premolten state, the recovery of the protein amide II band as monitored at 1543 cm^{-1} (Figure 7B) can also be fitted to two exponential functions, with their time constants and relative amplitudes as 7 min (40%) and 34 min (60%), very similar to those values for the recovery of the amide I band. The thermal relaxation kinetics from the molten state is somewhat different, as shown in Figure 7C,D. In Figure 7C, the amide I band shows a two exponential recovery process, with time constants and relative amplitudes of 1 min (60%) and 37 min (40%). Similarly, amide II shows a recovery process that can be fitted by two exponential relaxation processes: 1 min (55%) and 31 min (45%). As can be seen from Figure 7C,D, the amide I and amide II bands have quite similar lifetimes, but slightly different relative amplitudes. A relatively faster recovery process than the case in Figure 7A,B suggests a rapid recovery of protein conformation from the molten state. We believe this fast recovery phase is associated with a delayed protein conformation change that is due to the reverse process of the main transition, even though the sample temperature is already a few degrees below T_m.

Figure 8A shows the far-UV CD spectra for the native trimeric state at room temperature and those thermal denatured

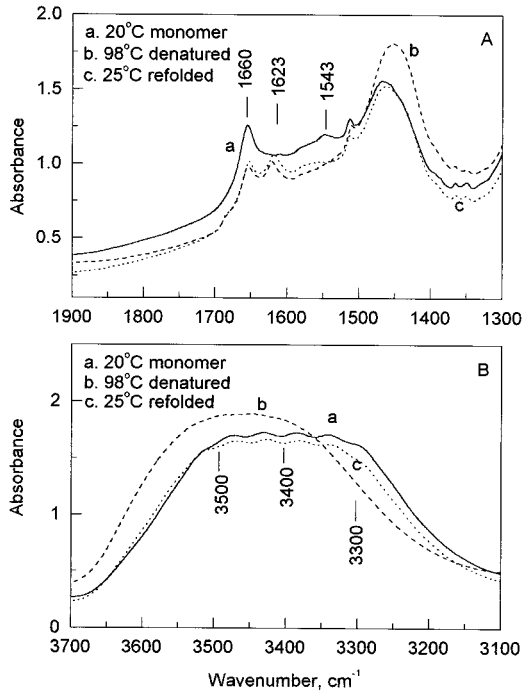

Figure 6. FTIR absorption spectra of Triton X-100 solubilized monomeric bR in D_2O undergoing the thermal unfolding and subsequent renaturing. Spectral regions are (A) 1900−1300 cm^{-1} for the amide I−III bands and (B) 3700−3100 cm^{-1} for the amide A band. Spectra are labeled as (a) 20 °C, (b) thermal denatured state at 98 °C, and (c) refolded state at 25 °C after thermal relaxation.

and refolded trimeric states of bR. In this region, they all show a double minimal spectral feature at ∼208 and 222 nm, which is the signature of the protein secondary structure in the form of α-helices.[36,37] A previous study [23] has shown that far-UV CD is sensitive to protein denaturation, the negative bands at 222 and 208 nm decreasing by about 27% at 86 °C.

Heating bR to the premolten state (85 °C) does not affect its secondary structure, in comparison to its native structure (curve a, b). This is in agreement with the FTIR results. Thermal treatment above the main transition temperature affects bR CD spectra (curves c and d). The molar ellipticity at 222 nm decreases with heating bR above 100 °C, and the relative ratio of the double minima changes. This indicates that bR does not refold its helical conformation from this molten state. Considering the results in Figure 8A, it is concluded that the refolded trimeric bR molecule is in a form of mixture possibly containing altered α-helical, original α-helical, and random coil, or even *β*-sheet conformation, only a small amount of them may have bound retinal chromophore. On the other hand, refolding from the premolten state restores the protein secondary structure near unity (90%, as shown in Figures 1A, 3Ab, and 4Ac).

In the case of monomeric bR, a double minimum CD band is shown (Figure 8B), similar to that of the trimeric bR. However, the peak intensity at 222 nm is significantly lower than that in the trimeric bR, indicating a lower amount of α-helix in the monomer at room temperature (curve a). Heating the monomer gives a similar featureless CD band as shown for the trimer, as a result of changing the original secondary structure. The similarity between the CD spectra at high temperatures of the monomer and the trimer (Figure 8Ad,Bc) probably indicates that similar conformational compositions are formed in these cases (similar amount of helix, sheet, and random coil), in agreement with a previous X-ray study[29] that bR trimeric structure is broken into monomerics as it denatures.

3.3. Photocycle and Proton Pumping Ability of Refolded bR. To confirm whether the refolded bR molecules have a photocycle and the proton pumping ability, we have compared the kinetics of the M intermediate. The results of these are shown in Figure 9. The formation and relaxation of the M intermediate probed at 412 nm for the native bR and thermally denatured bR (85 and 100 °C) have been compared. It is found that refolded bR does have its M intermediate formed (Figure 9A). In addition, all the rise phase of M can be fitted as two exponential components with their rise times slightly longer as the temperature increases. It is also noticed that the refolded species have a smaller amount of M formed compared to native bR (curve b, ∼90%, curve c, ∼15%), more or less in agreement with the corresponding recovery of the bound retinal shown in Figure 1. The kinetics of M decay also show a similar trend in the change of the amount of M intermediate. It should be mentioned that the refolded bR is a mixture of correctly folded bR molecules with and/or without retinal, or incorrectly folded molecules with and/or without retinal. Among these, those that completely lost their purple color do not have a bound retinal chromophore and therefore no photocycle and thus have no contribution to the kinetics of M. In other words, they are biologically inactive.

A previous study[21] has proposed that bR monomer solubilized in Triton X-100 has a similar photocycle as the trimer form, except for a faster M formation. Our laser flash photolysis measurement verifies fhe formation of M, which is accelerated at least 3−5 times in the monomeric bR than in the trimeric bR. However, the relaxation of M shows a rather slower component in the monomeric bR, indicating a slightly longer photocycle period. In addition, it is noticed that bR trimer refolded from 100 °C (even though only ∼12−15%), does not show significantly faster M formation. This indicates that those refolded bR molecules are still kept in the form of the trimer structure, whereas a majority of other types of bR molecules do not have photocycles as a result of losing the retinal (Figure 3C, forming "bacterioopsin (bO)"), and are probably in the form of altered or destroyed trimeric, or newly formed monomeric, state.

3.4. Protein Stability, Helical Conformation, Retinal Binding State. A previous study[29] showed that PM loses its hexagonal lattice during the premelting transition. Our recent results[30,38] have shown that along with the premelting transition, bR protein conformation changes from originally α_{II}- to α_{I}-helices. Considering these observations, it seems that the refolded state from the premolten state is a nativelike conformation with its retinal binding restored, and most importantly, it pumps protons. On the other hand, the majority of the refolded state after the main melting transition is most likely in a form of altered helical conformation and also random coils. They do not have bound retinal and therefore do not pump protons.

The question we asked earlier, during the thermal relaxation process of bR, does bR refold its protein structure first or does the retinal recombine first, may now be answered. As we have shown, thermally denatured bR has retinal dissociated on one hand and has protein secondary structure altered on the other. By comparing the fitted results in Figures 2 and 7, it is noticed that (1) from the premolten state to the refolded state, the recovery of protein color (from free to protein-bound retinal as shown in Figure 2A) has time constants of 11 min for the fast component and 51 min for the slow component, which are longer than the time constants of 7 and 39 min, respectively, for the recovery of the protein secondary structure (from melted to refolded helical conformation as shown in Figure 7A,B). This

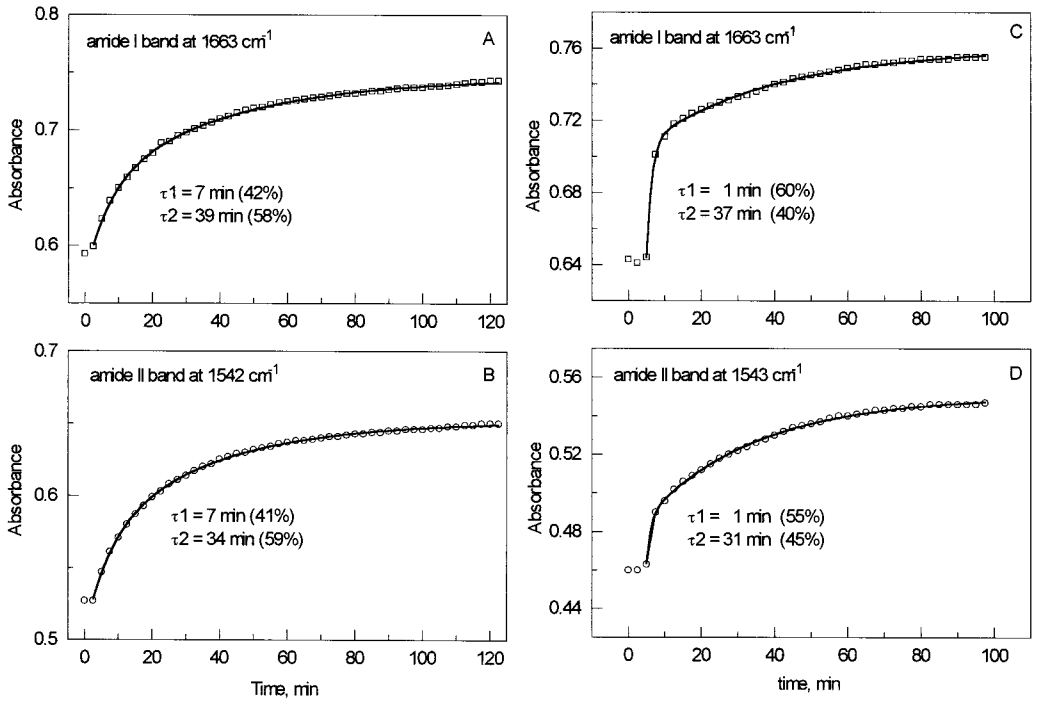

Figure 7. Kinetics of the thermal relaxation of bR/D_2O from the predenatured state (86 °C) and the denatured state (100 °C) as shown in Figures 3 and 4 (curve c's). (A) Recovery of the protein amide I band as monitored at 1663 cm^{-1}, and (B)recovery of the protein amide II band as monitored at 1543 cm^{-1}, as temperature decreases from 86 to 30 °C as a function of natural cooling. (C) Recovery of protein amide I band as monitored at 1663 cm^{-1}, and (D) recovery of the protein amide II band as monitored at 1543 cm^{-1}, as temperature decreases from 100 to 30 °C as a function of natural cooling. Curve fitting results are also shown in each case; see text for details.

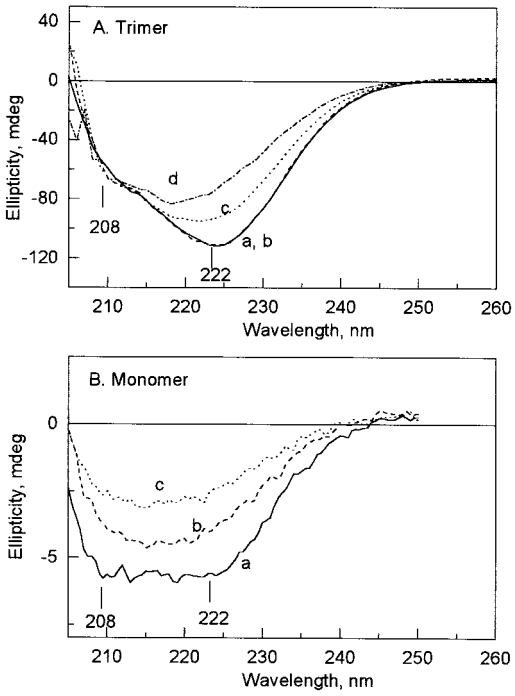

Figure 8. Far-UV CD spectra showing protein conformation signature. (A) Native trimeric bR at room temperature (curve a); refolded state of bR at 25 °C after being heated to its predenatured states ($T \geq 78$ °C; curve b); refolded state of bR at 25 °C after being heated to its main denatured states ($T \geq 96$ °C, curve c). Curve d represents the refolded protein states of bR at 25 °C after being heated to higher temperatures, e.g., 125 °C. (B) Triton X-100 solubilized monomeric bR at room temperature (curve a); refolded states at room temperature (curves b and c) after being heated to higher temperatures (100 and 125 °C, respectively).

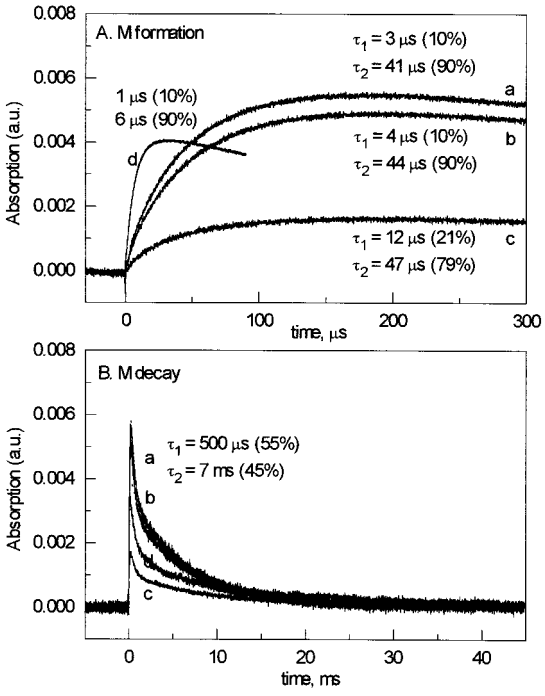

Figure 9. M formation (A) and M decay (B) kinetics of bR. Native trimeric bR at room temperature (curve a's); refolded states of bR from premolten states ($T > 78$ °C; curve b's); refolded states of bR from the main denatured states ($T > 96$ °C, curve c's). Data of Triton X-100 solubilized monomeric bR at room temperature are also shown (curve d's) in each case for comparison. Curve fitting of M formation/decay gives various exponential components, with their time constants and relative amplitudes shown.

means that protein secondary structure recovery occurs first followed by retinal recombination to the Schiff base into the folded structure, whenever bR refolds from the premolten state or molten state.

Our results can thus be summarized by the following conclusions. (1) The premelting transition does not irreversibly

affect the retinal binding site, nor does it irreversibly affect the protein conformation. (2) The native trimeric structure of bR gives inherent stability to the protein secondary structure of the protein of the individual bR molecules, assuming that Triton has no destabilizing affect directly on the protein. It is certainly possible that as the Triton-bR complex is heated, the detergent−protein interaction changes, which renders the protein less stable. (3) Retinal rebinds after the re-formation of the secondary structure, providing the proper retinal binding pocket. This is consistent with the fact that retinal can be removed easily by hydroxylamine treatment,[35,39,40] and the regenerated bR can be re-formed by addition of free retinal to the bO in which the active binding site remains.[41,42]

Knowledge of the forces stabilizing soluble proteins comes principally from thermodynamic and theoretical studies of the folding/unfolding process.[43] Combined with the literature data, our results can help to understand the more complicated system of membrane proteins. A four-step thermodynamic cycle has been proposed for membrane protein folding,[43,44] including peptides partitioning, secondary structure folding, insertion, and association. A theoretical model has been proposed to explain the folding of bR.[45] In that model, retinal binding is among the factors that may drive transmembrane helix association, other factors include external constraints associated with helix-connecting loops, helix-packing and helix−lipid interactions.[45] The retinal−protein interaction is important to protein stability, removal of retinal reduces the enthalpy of bR denaturation by 60−100 kcal/mol.[46] The significance of our results is that the folding of the protein seems to be independent of the retinal binding. This is in agreement with previous studies,[42,47] in which it was shown that bR can be refolded from a denatured state to a state with native secondary structure in the absence of retinal, implying that retinal does not initiate protein folding. Another study[40] has shown that retinal and bO interaction is a second-order reaction that occurs after a rate-limiting step of protein folding. However, these results, together with the data presented here, suggest that the binding of retinal to the protein is involved in the driving forces of the correct and complete refolding of the bR from its premolten or molten state, which is also crucial to its biological functions.

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