FEATURE ARTICLE

# Thermal Properties of Bacteriorhodopsin

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In this Feature Article we review the effects of various parameters on the structure and thermal stability of one of the most widely studied membrane proteins, bacteriorhodopsin. This protein has recently been crystallized and its high-resolution structure elucidated to 1.55 Å. This information has proved invaluable in understanding its proton-pumping mechanism. However, the question of why bacteriorhodopsin is so stable over a wide range of conditions, and which factors contribute to this stability, is still largely unanswered. Spectroscopic and calorimetric experiments provide information on the thermodynamics, kinetics and structural changes upon unfolding and refolding the protein under various environmental perturbations. FT-IR spectroscopy has been particularly useful in determining the changes in secondary structure upon heating through its thermal transitions after changing pH and cations, removing and adding lipids and detergents, retinal reduction and removal, and site-directed mutagenesis. These experiments are reviewed, and the information that they have afforded have been brought together to try to understand how nature has controlled the conditions of bR to make it one of the most stable proteins known.

## 1. Introduction

For the past 30 years, the purple membrane of *Halobacterium salinarum* has been the focus for a large number of researchers in many scientific and engineering fields. This interdisciplinary effort is centered on the only protein present in the purple membrane of the archaebacterium, the G protein coupled receptor (GPCR) protein, bacteriorhodopsin (bR).[1,2] It has a specific function to utilize light energy to produce a proton motive force across the membrane by pumping a proton, unidirectionally, from the cytoplasmic side to the extracellular side.[3,4] This photoinduced process provides the organism with the necessary energy to undertake the energy storing phosphorylation reaction of ADP to ATP under low oxygen conditions and is a form of photosynthesis far simpler than the electron pumping mechanism of chlorophyll.[3−5] The light activation is made possible by the retinal chromophore buried in the interior of the protein, bound to the 216th residue, lysine, by means of a protonated Schiff base.[6,7] The environment in the protein interior causes the retinal polyene to have a broad visible absorption spectrum, centered at 568 nm, and renders the protein purple by means of the protein-induced opsin shift.[8−10] Upon photon absorption, the retinal is excited, which results in isomerization of the $C_{13}$═$C_{14}$ bond from all-trans to 13-cis.[11,12] This isomerization process is both very efficient and bond-specific in the protein-bound retinal, which is in contrast to retinal in solution and highlights the catalytic role of the protein environment.[13,14] The isomerization leads to a high-energy photoisomerized state, K, which relaxes along a thermal pathway of proton-transfer steps and conformational changes over the course of several milliseconds to result in the proton-pumping function according to the following scheme, where the subscripts refer to the visible absorption $\lambda_{max}$, and approximate transition lifetimes are shown above the arrows.[15−18]

$$\xrightarrow{\quad} bR_{568} \xrightarrow{h\nu} I_{480} \xrightarrow{0.5\ ps} J_{625} \xrightarrow{2\ ps} K_{610} \xrightarrow{2\ \mu s} L_{550} \xrightarrow{80\ \mu s} M_{412} \xrightarrow{10\ ms} N_{550} \xrightarrow{ms} O_{640} \xrightarrow{\quad}$$

However, FT-IR,[19−23] Raman[24−26] and kinetic analyses of visible spectroscopic experiments[27−31] have shown the existence of more physical processes than described above. This suggests that processes occur that do not cause retinal absorption changes. This is perfectly reasonable considering that the retinal is only a (physically) small part of the whole system. An important example is the observation of two M substates, $M_1$ and $M_2$, which are considered important for the proton switching mechanism to enable the unidirectionality of the proton pump.[27,29,30,32−34]

The ability of the protein to perform its function over a wide variety of conditions that would normally be denaturing to other proteins is a particularly interesting property of the system. An extraordinary number of papers have been published (>4000 and still rising according to the CAS and Medline databases), and a number of excellent reviews have appeared over the years on many aspects of this system. These reviews include discussing the ultrafast primary dynamics of retinal isomerization,[35] proton-transfer reactions and conformational changes of individual amino acid groups in the protein,[19] the effects of various environmental factors on the thermodynamics and kinetics of the ground state and intermediate species,[18,30,36−38] its atomic

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structure from diffraction studies,[36,37] and its uses in biomaterial and bioelectronic applications.[39−41] A number of patents have been granted worldwide for the potential biomaterial uses. A series of papers concentrating on the structure of bacteriorhodopsin was published in a recent, special issue of BBA in 2000.[38] One excellent review article was also published in 1999, which reviewed the molecular mechanistic details of the pumping.[42] The reader is directed to the many excellent reviews referenced above for specific details on different aspects of bacteriorhodopsin. The aim of this review is to summarize much of the work in our laboratory and others in determining the reasons for, and the factors that contribute to, the extremely high thermal stability of bR, and the unusual phase transitions occurring as it is heated to and through denaturation.

The folding and unfolding of proteins is such a huge and important focus in the scientific community that it has been given its own title—the protein folding problem. All proteins require their polypeptide chain to fold into a very specific three-dimensional structure for them to perform their function. This requires a huge decrease in the entropy of the system, which is offset by the gain in enthalpy from interactions between the amino acids. Remarkably, the protein molecule can find its way from the unfolded state to the folded state in a very short time scale, which has given rise to the protein funnel description of the folding energy landscape.[43] However, this is such a complex model that it is very difficult to elucidate experimentally. Furthermore, the energy landscape can be further directed by various factors such as ions,[44,45] coenzymes and chaperones,[44,46] cofactors and lipid−protein interactions.[44] The folding landscape of many water-soluble proteins have been studied by means of in vitro reversible experiments in which various thermodynamic and kinetic parameters have been followed upon exposure to denaturing and renaturing conditions. This is generally a good approach for such studies as it allows the factors that control the energy barriers separating the states to be found for different conditions and, in some cases, intermediate states to be trapped and the structures elucidated. However, a problem exists for membrane proteins with this method in that the folding is not reversible in vitro. This is because of the importance of the internal hydrophobic environment of the bilayer in controlling the pathway. This environment means that membrane proteins are generally much more stable than globular proteins. However, the exact role of the environment is not yet fully elecuidated. Thus, although membrane proteins are extremely important in nature, the factors that control their folding and final structure are generally not very well understood.[47−51] Folding experiments on membrane proteins such as the model bacteriorhodopsin have been attempted by reconstitution into lipid bilayers or into vesicles (see section 5). Another approach to elucidate the relationship between structure and function of membrane proteins is to follow the unfolding transitions of membrane proteins upon systematic perturbation of externally controlled conditions such as cation, detergent, pH, etc. and follow the temperatures at which the transitions occur, and the structural changes occurring during these transitions. Our lab, and others, have been using this approach, and the aim of this review is to summarize the progress in this area.

## 2. Atomic Structure of bR—What It Does Not Show

Over the last 5 years, enormous advances have been made in understanding, at the atomic level, this model photoactivated transport system from nature. One of the major sources of this unsurpassed rate of discovery has been the recent ability to crystallize the protein into a three-dimensional lattice.[52] The

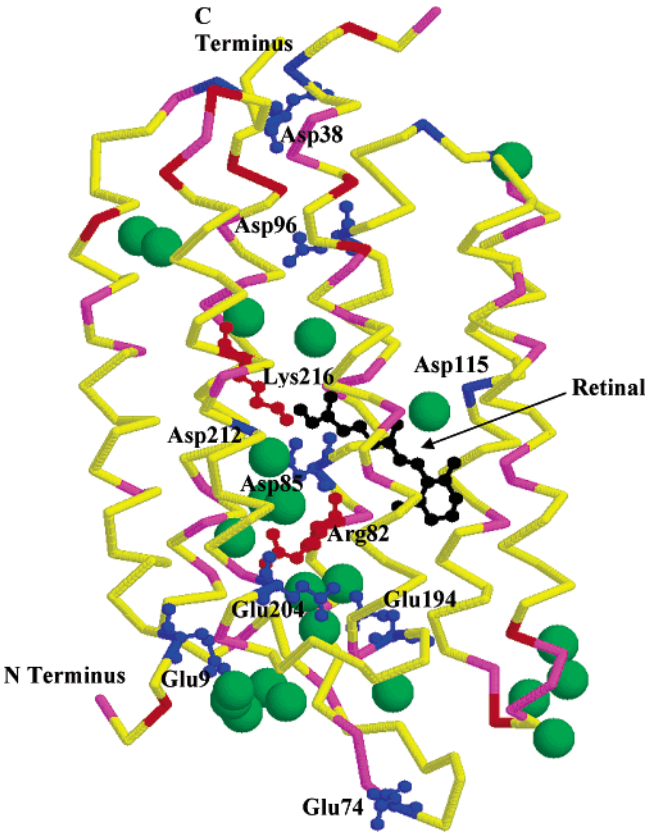


**Figure 1.** Backbone view of the bR monomer using the Rasmol program and the coordinates in the PDB under ref 1c3w.[53] Key: yellow, hydrophobic; magenta, uncharged hydrophilic; red, positively charged; blue, negatively charged; black, retinal; green spheres, water molecules. Various important charged residues are labeled for illustrative purposes, and some are shown in ball-and-stick mode for display of relative positions

focused efforts of a few groups of people over a number of years resulted in the discovery of a surprisingly simple method of producing well-diffracting crystals by changing the lipidic phase of the membrane. This resulted in the subsequent publication of the high-resolution 1.55 Å structure of the protein,[53] as well as a number of intermediates trapped by low temperature or mutation.[34,37,54] This 1.55 Å structure is located in the protein data bank, under the ref 1c3w,[53] and has recently been modified to 1.43 Å.[54] The structure is shown in Figure 1, which shows hydrophilic and hydrophobic areas and a number of important amino acid residues.

Although these atomic structures have provided many molecular details of the structure and function of bR and other transport proteins, the factors that contribute to the high stability observed by calorimetry and visible absorption[55] studies as well as the unusual helical structure observed by FT-IR,[56] circular dichroism (CD),[57,58] and Raman[57] have not been fully identified. This is partly due to the arrangement of the native lipids and the protein−lipid interactions not being identified to high resolution. In addition, none of these structures have determined the presence and location of cations in bR, with the exception of low-resolution diffraction studies of bound $Pb^{2+}$ in regenerated bR, which identified six helix bound $Pb^{2+}$ ions in the purple membrane.[59,60]

For the native purple membrane, two proposals have been put forward to explain the binding of cations to bR. The first suggests that the cations only interact electrostatically with the lipid bilayer, according to the Guoy−Chapman theory[61−64] which competes with proton binding at the surface, thereby

increasing surface pH and changing the protonation state of certain amino acid residues. A second proposal suggests that one (or more) is (are) strongly bound to the protein,[65−69] which changes the protonation state of amino acid residues more directly. More recently, results from nuclear magnetic resonance (NMR),[70] electron spin resonance (ESR),[71] and differential scanning calorimetry (DSC) of mutants[72] have suggested that up to two cations are bound directly to protein amino acid groups near the extracellular surface. It is known that removal of metal cations by ion exchange,[65,73] acidification,[74] or chelation[68] results in a shift of the $\lambda_{max}$ of the retinal from 568 to 603 nm (purple to blue) and a cessation of the proton pumping. This is reversed upon adding cations back to the deionized system.[75] It is widely agreed upon that the reason for the shift in color is due to the Asp-85 group, which is deprotonated in the native ground-state bR, becoming protonated upon cation removal. Asp85 is the residue that accepts the SB proton during M formation, and its protonation in the ground-state renders this step not possible, and thus the proton pumping ceases in blue bR. The assignments of the protonation state and the proton-accepting role of Asp-85 were based on studies of visible and IR spectroscopy as well as photocycle efficiency measurements upon mutating the single Asp85 group.[19,76−78] This assignment was confirmed by the electron[79] and X-ray diffraction structures[80] of the M intermediate, in which the proton has been transferred from the Schiff base to Asp-85. However, the complex titration of the purple-blue color transition and the Asp85 protonation state,[81−83] as well as the effects of different cations on the photocycle, color, stability and protein structure[63,67,69,75,84−88] have rendered the question of the cation location and involvement still open for debate. On the other hand, however, removal of the lipids and the lattice by detergent treatment causes a blue shift in the absorption spectrum,[61,89] while the proton pumping function is retained.[90,91] The kinetics and energetics of the photocycle are affected in the latter stages,[92] but the monomeric protein is functional without the native interprotein and protein−lipid interactions.

## 3. bR Thermal Unfolding

**3.1. Multiple Phase Transitions.** As native bR is heated from 20 to 100 °C, Jackson and Sturtevant observed that the DSC profiles show the presence of two thermal phase transitions.[55] There is a smaller (premelting) transition at ∼80 °C, which was shown to be reversible upon cooling back to 60 °C. Following the premelting transition, there is an irreversible transition at ∼97 °C that causes protein denaturation (melting). The enthalpies of these transitions were found to be ∼400 kJ/mol for the irreversible denaturation transition, and ∼30 kJ/mol for the reversible, premelting transition. The irreversible transition was shown to be concurrent with loss of the purple color, and thus the opsin-shifted bound retinal, as evidenced by the decrease in the absorption of the opsin-shifted band at 568 nm and a concurrent increase of the absorption band at 380 nm from the unbound retinal.

Although these types of calorimetric studies have allowed researchers to find the phase transition temperatures and the thermodynamics associated with them, a more rigorous quantitative treatment had been precluded. The primary obstacle in such a treatment is that, generally, membrane proteins are not in thermodynamic equilibrium during the unfolding process. Once unfolded, membrane proteins do not spontaneously fold back to the native structure, as is the case with the reversible folded ↔ unfolded equilibrium of soluble proteins. Therefore, it is necessary to use spectroscopic and diffraction methods to characterize the processes occurring during the thermal transitions in a more quantitative manner.

X-ray diffraction experiments on the purple membrane have shown that there is a gel-to-liquid transition of the hexagonal packed lattice during the premelting transition.[93,94] The X-ray diffraction pattern showed that the local order of the trimers is maintained but that the trimeric unit cells are no longer packed in the hexagonal lattice. This was found to be reversible, confirmed by re-forming of the original diffraction pattern upon cooling from above the premelting transition (but below the melting transition). Once heated through the main melting transition, bR was shown to lose all local order and this change was an irreversible process.[93,94] UV−CD spectroscopy showed that a change in the double-minima shape of the CD spectrum around 190−225 nm is consistent with loss of α-helical structure.[93] FT-IR spectroscopy further revealed that the main transition is accompanied by an increase in an absorption band at 1623 $cm^{-1}$, indicative of uncoiled random amide I C═O bonds (the so-called random coil), although the α-helix to random coil transition was not 100% completed.[95] This indicates that some α-helical secondary structure remains in the denatured protein.

**3.2. The Premelting Transition: Accompanied by an α-Helical Conformational Change?** A most interesting observation was made concerning the C═O amide I stretching frequency for native bR, published at about the same time as the thermal phase transition study of Jackson and Sturtevant. The stretching frequency was shown to be centered at 1665 $cm^{-1}$, more than 12 $cm^{-1}$ blue shifted from what would be expected for the normal α-helical frequency.[56] $\beta$-Sheet and random coil structures each have vibration frequencies *lower* in energy than α-helices, so larger contributions from these bands would only serve to lower the observed frequency, not increase it.[96] The exact origin of this anomously high frequency has, even yet, not been unambiguously elucidated. However, clues arose soon after this IR study[56] from normal mode calculations on the α-helical polypeptide, polyalanine.[97] It was postulated that α-helices could exist as two slightly different conformations. A decrease in the Φ dihedral angle and an increase in the Ψ dihedral angle by −15 and +15°, respectively, results in an α-helix with the same total energy as the normal α-helix, the same number of residues per turn and the same pitch (rise) but with different intrahelical H-bond lengths (N−H···O bond lengths of 2.84 Å in the normal α-helix and 3.00 Å in the newer, calculated one). This is shown in Figure 2. The increase in H-bond strength would serve to increase the vibrational frequency to the observed 1665 $cm^{-1}$. The two α-helix conformations were denoted as $\alpha_I$ and $\alpha_{II}$, having vibration frequencies at 1652 and 1665 $cm^{-1}$ respectively. Since then, other spectroscopic evidence has been shown to be inconsistent with sole $\alpha_I$ helix content. The shapes of the linear dichroism (LD)[98,99] and circular dichroism (CD)[58] spectrum are not as one would expect from a purely $\alpha_I$ helix. The UV−CD ellipticity for $\alpha_I$ and $\alpha_{II}$ were calculated to be at similar positions, but the intensities of the positive and negative lobes are lower for $\alpha_{II}$. The lower observed intensities of the bilobic shape in the bR UV−CD spectrum were considered to be the result of some $\alpha_{II}$ content, which would not show as much dichroism due to the change in angle of the amide I plane.[58] These authors also discussed the calculations of Krimm and Dwivedi[97] in terms of the previously reported data that poly (L-alanine) in hexafluoro-2-propanol[100] have dihedral angles between these two values, and this effect was possibly due to $\alpha_{II}$. IR linear dichroism (LD) was shown by Rothschild and Clark[98] and Aldeshev[99] to exhibit

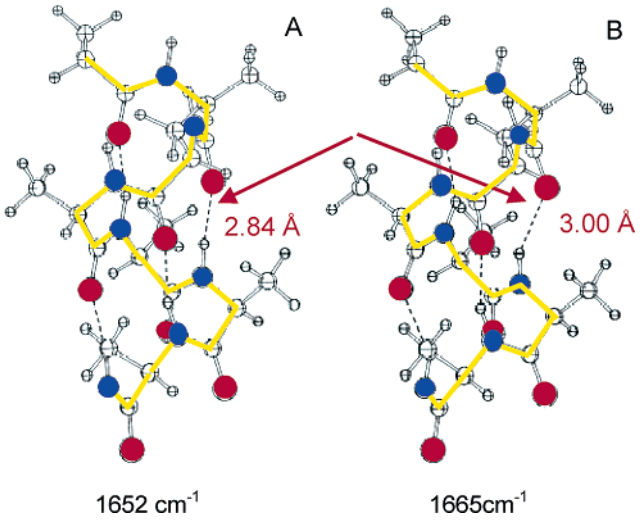


**Figure 2.** Schematic of (A) $\alpha_I$ and (B) $\alpha_{II}$ helices. The backbone is highlighted in yellow, the oxygen atoms are red, and the nitrogens are blue to aid the reader in following the structures. Modified from Krimm and Dwivedi.[97] Used with permission from the American Association for the Advancement of Science, copyright 1982.

tilts that are roughly in the membrane normal. It was found that better agreement with diffraction and CD data is obtained using a combination of $\alpha_I$ and $\alpha_{II}$ helix content.[101] The Raman spectrum of bR was analyzed by Vogel and Gartner[57] and was shown to consist of two amide I bands, the regular $\alpha_I$ band, and another band, which they attributed to $\alpha_{II}$. Solid state $^{13}C$ NMR has recently been interpreted to contain some chemical shifts due to the amide I bands higher than $\alpha_I$ (16.4 ppm compared to the normal 14.9 ppm for $\alpha_I$-helix), which is similar to poly(L-alanine) in hexafluoro2-propanol (considered to have some $\alpha_{II}$ content).[102−104]

However, despite these spectroscopic clues, the $\alpha_{II}$ helix dihedral angles expected have not been observed in the Ramachandran plots derived from electron diffraction studies,[105] and structures obtained from X-ray diffraction.[53] Thus, the proposal is not universally accepted. An alternate view of the high C═O stretching frequency was put forward by Hunt,[106] in which transition dipole moment coupling between localized vibrational modes of different α-helices was considered the source of the high frequency. It is also possible that reasons for the unobserved $\alpha_{II}$ helices in the diffraction data and unobserved cations could result from the method that the samples are treated by prior to measurement. Sample preparation could result in changing the native structure, favoring formation of $\alpha_I$ helices prior to diffraction measurement. At this time, the existence of $\alpha_{II}$ helices in native bR is neither confirmed nor negated.

It was discovered by Taneva et al. in 1995[95] that upon heating bR through its premelting transition, there is a shift in the amide I band of the FT-IR spectrum from the higher 1665 $cm^{-1}$ to the more usual 1652 $cm^{-1}$ frequency, which is then followed by amide II N−H deuteration in $D_2O$ solvent. The amide I transition occurred even in water, showing that the solvent effect from $D_2O$ is not the major contributor to the band shift. At about the same time, Torres et al.[107] followed the amide I band upon dissolving and fragmentation of bR in organic solvents. The high 1665 $cm^{-1}$ frequency was shifted to the normal α-helix 1652 $cm^{-1}$ frequency in the unfragmented bR and in all fragments when exposed to organic solvents. Each fragment showed slight differences in peak maximum, but none as blue shifted as the native bR in water. The first study[95] suggested that the unfolding pathway of native bR proceeds through the premelting transition, which involves a rearrangement of the helices from $\alpha_{II}$ to $\alpha_I$, and the second study[107] interpreted the presence of an $\alpha_{II} \leftrightarrow \alpha_I$ equilibrium, which is dependent on the conditions. It was further shown that a major criterion of the 1665 $cm^{-1}$ band is the trimeric structure.[107]

More recently, the time scale of the amide I C═O α-helical transition and amide II N−H deuteration in native bR were studied by Wang and El-Sayed using a laser T-Jump apparatus and time-resolved step-scan FT-IR.[108] It was found that upon excitation with a 10 ns laser pulse with the appropriate energy to be absorbed by the overtone of the $D_2O$ solvent at 1.9 $\mu$m, rapid heating occurred that raised the temperature of the sample to just above the premelting transition temperature. This caused the 1665 to 1652 $cm^{-1}$ transition to occur in <80 ns, followed by H−D exchange in 180 $\mu$s. The amide I band shift was shown to be reversible within 1.8 ms, consistent with cooling of the bR and reversibility of the transition within these time scales. This transition was interpreted as rapid rearrangement of the dihedral angles to form the $\alpha_I$ helix, which allows $D_2O$ penetration into the interior of the protein (as concluded from the exchange of $D_2O$ with amide II N−H bonds). This is consistent with the earlier, steady state study.[95] The penetration of water into the interior of the protein presumably causes "water damage" and is required for the irreversible melting of the protein as it eliminates the hydrophobic forces and breaks the H-bonds of the helical structure.[108]

**3.3. Kinetic or Thermodynamic Control of Unfolding?** The results of the above study[108] and the early DSC experiments by Jackson and Sturtevant[55] show that the premelting transition is reversible. However, it was later found that the DSC profiles were somewhat dependent on the scanning rate.[109,110] This indicates that kinetic control may be present at certain time scales under particular conditions. We have analyzed these time scales for up to 60 min using FT-IR and visible spectroscopic experiments.[111] It was found that the secondary structural changes, (as observed by the amide I peak in the FT-IR spectrum), and the retinal binding (as observed by visible spectroscopy) were reversible if the temperature was held above the premelting transition (but below the melting transition) for up to 10 min. However, if the temperature was held at this temperature for 30 min, there is a permanent shift in the amide I peak to 1652 $cm^{-1}$, and a slight increase in the melting peak at 1623 $cm^{-1}$. This increase in the melting peak becomes larger if it is held above the premelting temperature for 60 min. This was interpreted as a process in which stabilizing bonds begin to diffuse upon heating through the premelting transition but can still re-form for up to 10 min. If it is held above the premelting temperature too long, the native structure cannot re-form. One possible source of this diffusion was postulated to include cation diffusion away from their binding sites (see below). There is also likely to be involved residue side groups and water in this diffusion process.

## 4. Roles of pH, Cations, Lipids, Retinal, and Specific Amino Acids on the Secondary Structure and Thermal Transitions

As described above, the UV−visible spectrum shows that during the irreversible melting transition the bound opsin-shifted retinal is lost.[55] It was also found that, upon heating above 50 °C, there is a small increase in the intensity of the lower energy sideband of the absorption spectrum, and a decrease in the main band.[69,112,113] The difference spectrum showed an isosbestic point, which was considered the result of the purple membrane losing bound cations upon heating. This assignment is based on the observation of the isosbestic point if bR is deionized at

room temperature,[68,73,74] albeit much more extensively than that observed upon heating.

A number of studies have focused on the effects of pH, cations, native lipids, specific amino acid residues, and even on the retinal chromophore on the unfolding of bacteriorhodopsin. The effect of increasing pH of bR to between 7 and 13 on its thermal stability was studied by Brouillette et al. using DSC, CD, and protein fluorescence spectroscopy.[114] It was found that above neutral pH (in the presence of different buffers), the denaturation temperature decreased and secondary structure changes corresponding to the premelting transition occurred at about 80 °C. This study was extended to acid pH by Kresheck et al.[88] Using DSC, it was found that the denaturation temperature decreases with pH to a minimum of 65 °C at about pH 2.6 (corresponding to acid blue membrane). However, this DSC technique was not as sensitive to the smaller premelting transition as the earlier studies and, therefore, this transition was not followed. Surprisingly, the acid purple membrane at ~pH 0 showed a thermal stability almost as high as the purple membrane at neutral pH. In addition, this study investigated the denaturation temperature upon deionization and cation regeneration with $Mg^{2+}$ and $Mn^{2+}$. An earlier study had investigated the thermal stability of $Mn^{2+}$ and $Hg^{2+}$ regenerated bR using DSC.[115] Both found that the denaturation temperature of cation-regenerated bR is increased relative to the acid blue (deionized) bR, but not as much as in the native bR. The earlier study noticed that there is a premelting transition observed by DSC for $Mn^{2+}$ regenerated bR,[115] and the origin was discussed in reference to the earlier X-ray data on lattice disruption.[93] The FT-IR study on the shift in amide I frequency[95] (discussed above) during the premelting transition had not yet been published at the time of this DSC study, and hence the frequency shift of the amide I peak during this transition was not discussed. From these studies, it was evident that titration of the bR by acidification or alkination caused a decrease in the thermal stability and that cations had a role in regeneration of the thermal stability. Furthermore, and unsurprisingly, the highest stable species studied was the native bR at physiological pH. None of these studies gave a complete picture of the origins of the observations, because either the techniques were not sensitive enough, as in the studies of Kresheck et al.,[88] or all details on the helix structure were not known at the time of the publication, as in the study of Cladera et al.[115]

Our lab has been systematically studying the effects of cations, lipids, and changing the pH on the FT-IR spectrum as a function of temperature to determine the roles of these factors on both the melting and premelting transition. A typical temperature-dependent FT-IR spectrum is shown in Figure 3a. During the premelting transition, the amide I band shifts from 1665 $cm^{-1}$ to 1652 $cm^{-1}$, which is then followed by an increase in a band at 1623 $cm^{-1}$. During the transitions, the backbone becomes more exposed to the $D_2O$ solvent and there is an exchange of the amide II N−H for N−D, at 1545 and 1432 $cm^{-1}$, respectively. Figure 3b shows the difference spectrum from 20 °C with increasing temperature, which allows these changes to be more easily quantified. Figure 3c shows the determined transition temperatures as a function of pH. In contrast to the melting transition, which varies from 65 to 97 °C, depending on pH, the premelting transition of native bR shows no pH dependence.[85] It is always at the same 80 °C temperature. However, once all the cations are removed (below pH 3.3),[68,74,116] there is a shift in the room-temperature amide I band from 1665 to 1658 $cm^{-1}$, together with a broadening of the band (Figure 4). Correspondingly, the melting transition is

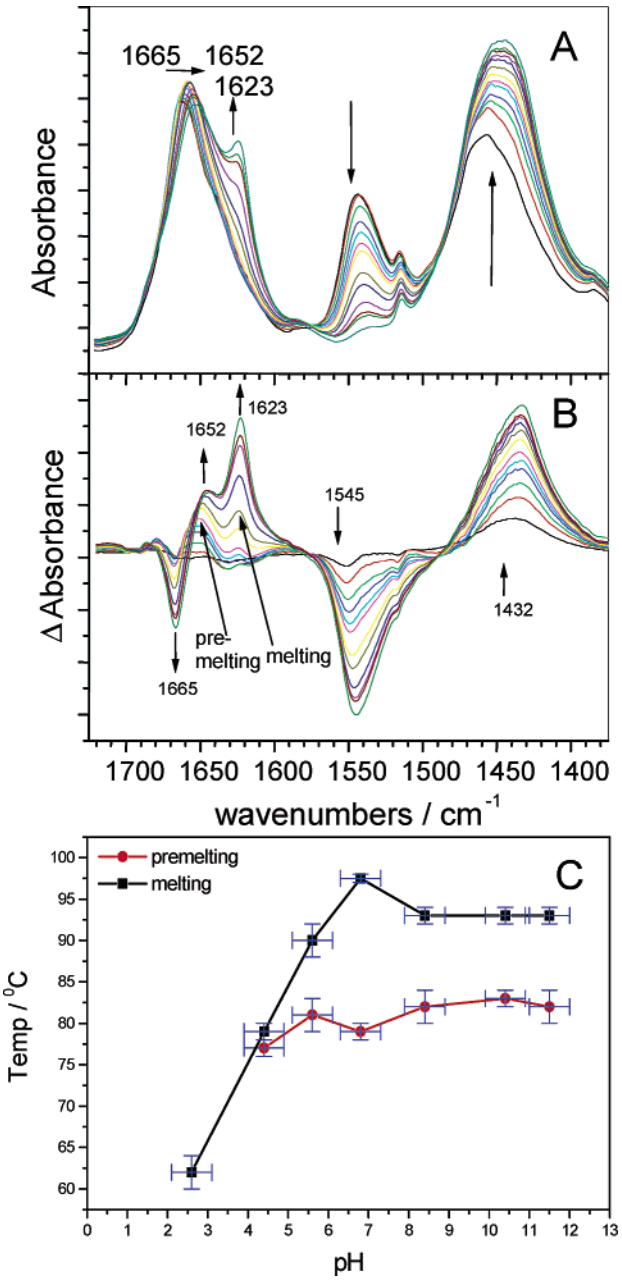


**Figure 3.** (A) Typical temperature-dependent FT-IR spectrum of bR in $D_2O$ from 20 to 98 °C. A shift in the amide I band from 1665 to 1652 $cm^{-1}$ occurs during the premelting transition, which is followed by an increase in the 1623 $cm^{-1}$ band during the melting transition. Correspondingly, the amide II N−H at 1545 $cm^{-1}$ decreases with increased backbone exposure to the solvent and the amide II N−D band at 1432 $cm^{-1}$ increases. This is shown more clearly in the difference spectrum from 20 °C in (B). (C) Dependence of the premelting and melting transition temperatures on pH (in the absence of buffers). From Heyes and El-Sayed.[85]

reduced to 65 °C and no premelting transition is observed (Table 1). Figure 4 shows that the amide I peak does not change with pH, until the p$K_a$ of the purple-blue transition at pH ~ 3. Thus, it was postulated that one or more bound cations are responsible for the higher amide I frequency and thus may play a role in stabilization of the $\alpha_{II}$ helices.[85] This was supported by the fact that the addition of native $Ca^{2+}$ and $Mg^{2+}$ cations reversed this frequency shift (Figure 4).[85] This study was then extended to look at other, non-native cations.[86] These included $Mn^{2+}$, $Hg^{2+}$, and a large, divalent bolaform cation.[117] Large organic cations have been shown to regenerate the color and function of deionized bR,[118,119] but little was known of their effect on the

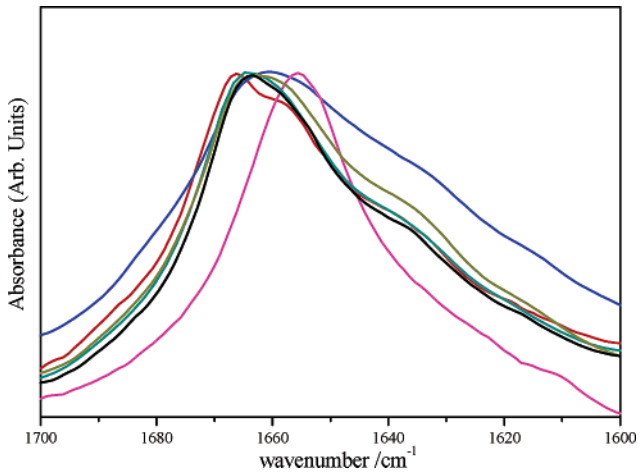


**Figure 4.** Influence of pH, cations, and lipid removal by detergents on the amide I (secondary structure) peak at 20 °C. Key: black, native at pH 8.4; cyan, native at pH 4.4; blue, pH 2.4 (acid blue); yellow-green, $Ca^{2+}$ regenerated bR; red, CHAPS treated (75% delipidated) bR; magenta, TRITON treated (monomerized) bR. From Heyes and El-Sayed.[85,111] Used with permission from the American Society for Biochemistry and Molecular Biology, copyright 2002.

**TABLE 1: Premelting and Melting Transition Temperatures of Deionized, Cation Regenerated, and Detergent Treated (Delipidated and Monomerized) bR Compared to Native bR at Neutral pH (Except Where Indicated)[a]**

| bR sample at neutral pH | premelting transition (°C) | melting transition (°C) |
|---|---|---|
| native | 78 ± 2 | 97 ± 1 |
| acid blue deionized (pH 2.6) | *b* | 65 ± 3 |
| $Ca^{2+}$ regenerated bR | 78 ± 2 | 84 ± 2 |
| $Mg^{2+}$ regenerated bR | 78 ± 2 | 82 ± 2 |
| $Hg^{2+}$ regenerated bR | 78 ± 1 | 80 ± 1 |
| $Mn^{2+}$ regenerated bR | 78 ± 1 | 88 ± 1 |
| organic$^{2+}$ cation regenerated bR (see text) | 80 ± 2 | 91 ± 1 |
| CHAPS treated (75% delipidated) bR (pH 5.5) | 91 ± 2 | 94 ± 2 |
| TRITON treated (monomerized) bR | *b* | 65 ± 2 |

[a] From refs 85, 86, and 111. [b] Not observed.

structure and the stability. It was found that all cations, even $Hg^{2+}$, which has no effect on the visible spectrum of deionized blue bR and on regenerating the proton pumping function,[75] did regenerate the amide I frequency shift to 1665 $cm^{-1}$. This further supports our hypothesis on cation involvement in the amide I frequency. All cations increased the thermal stability relative to deionized bR, but was lower than that of native bR (Table 1). Additionally, the actual melting temperature of the cation-regenerated bR depended strongly on the cation, supporting specific binding to the bR and not only due to an electrostatic double layer effect proposed by Szundi et al.[63] On the other hand, all cation-regenerated bR samples (even $Hg^{2+}$) showed a premelting transition and were at the same 80 °C temperature as the native. This suggests the requirement of a cation to be present for the premelting transition but does not depend on its identity. Thus, this transition is perhaps due to an electrostatic effect from the divalent cation.

To correlate the effect of cations on the structure and stability to the effect of removing the lipidic surface charges, and on monomerization of the protein, the effect of using CHAPS or TRITON X-100 to remove either 75% of the lipids (retaining the hcp lattice) or solubilizing the lattice altogether into bR monomers was studied.[111] Removal of 75% of the lipids, in which the hcp lattice was maintained, resulted in only a slight change in the amide I band (Figure 4), an increase in the premelting transition temperature to 91 °C, and a slight decrease in the melting transition (Table 1). On the other hand, monomerization resulted in a large shift of the amide I band to 1652 $cm^{-1}$ (Figure 4), lack of a premelting transition, and the thermal irreversible melting temperature reduced to 65 °C (Table 1). These results on the bR monomer were surprisingly similar to those of cation removal. Thus, it seems that the cation plays as important a role in the secondary structure and thermal stability as does the global lattice structure. The first 75% of the lipids, however, play little role in the helical structure and stability to melting but play a significant role in the premelting. This further supports the hypothesis that the premelting is related to the lipids and surface electrostatic effects.

A number of important amino acid residues have been identified over the years for their structural significance to the photocycle of bR.[19,37] Site-directed mutagenesis found that Asp85 was the proton acceptor from the Schiff base,[19,76−80] provided a catalytic role in retinal isomerization,[13,14] played an important role in the opsin shift and spectral tuning,[8−10] and was connected with the cation binding sites.[66] Recently, it has also been considered to play a role in the complex proton release pathway of the proton to the extracellular surface.[81−83] With all these important roles, it is not surprising that it also plays a key role in the secondary structure and thermal stability of bR. Taneva et al. used the D85T mutant to follow the effect of this amino acid on the thermal stability, as well as the secondary structure and electrooptic properties.[120] The melting temperature was reduced from 97 to 93.5 °C and the enthalpy of melting reduced by approximately 80 kJ/mol. The FT-IR spectrum of the mutant also showed a more red-shifted amide I peak relative to native, which the authors assign to a rearrangement of $\alpha_{II}$ helices and/or altered retinal−helix interactions.

The four extracellular Glu residues have also been investigated for their roles in color regulation, cation binding, and thermal stability.[72] These Glu residues were systematically replaced for Gln by single or multiple site-directed mutagenesis. Using DSC, it was found that of the single mutants, E74Q had almost no effect on both the melting and premelting transitions. Both the melting and premelting transition temperatures decrease for the rest of the single mutants in the order E194Q > E204Q > E9Q. Also, the intensities of the DSC peaks apparently decrease for both transitions with decreasing transition temperature, although this was not quantified. Upon multiple site-directed mutagenesis the premelting transition is no longer observed, and the thermal stability reduces slightly when compared to single mutants. The effect of removing these acid groups on the stability and on the titration of cations into the deionized mutants allowed the authors to propose a cation-binding complex involving Glu194, Glu204, and Glu9.[72] Even though it was found that the effects of removing and replacing the cations on the thermal stability[85,86] were larger than the effects of mutagenesis, the trend of destabilization upon removal of the biding sites is consistent. Additionally, once the samples are regenerated with other cations, as in the melting experiments,[85,86] the pH is ∼5, whereas the pH of the mutants are ∼7 and may be one source of the differences observed. One other source may be the differences in protein−lipid interactions upon residue replacement, and the differences observed in premelting transitions further support this possibility. Another study recently highlighted the importance of the lipids on the premelting transition.[111] The mutant study[72] adds further support for cation and specific residue (which are most likely coupled) involvement in the unusually high stability of bR.

Another approach has been taken by the Bowie group on the effects of amino acid residues on the stability of membrane proteins such as bacteriorhodopsin. Rather than mutating specific residues, which usually result in lower bR stability, to determine relative stabilizing effects of native bR residues, they have screened ways of mutating bR to make it *more* stable than the native state (such as adding disulfide bridges).[121,122] The goal of this type of study is to overcome the problem of membrane proteins being very unstable once removed from their bilayer. If they can be made stable outside of the bilayer, by specific mutations, then their industrial uses in areas such as biosensing may be taken advantage of.

The roles of retinal and the protonated Schiff base on the stability and structure of bR have also been studied.[48,123−126] Both the reduced (containing a deprotonated Schiff Base) and the bleached (retinal removed) membrane show very different thermal characteristics.[123,127] The melting transition of bleached bR was reduced to 82 °C and the enthalpy reduced to 110 kJ/mol. The premelting transition was difficult to resolve, but the DSC profiles of Figure 1 of Kahn et al.[127] show a shoulder at about 72 °C. It is also interesting to note that Kahn et al.[127] studied the effect of cleaving the BC loop, and the effect of this was small compared to the effect of retinal removal. The studies of Cladera et al.[123] show that the FT-IR spectral changes of reduced and bleached bR are similar to the native (i.e., the amide I premelting frequency shift and the melting peak), but that they were difficult to separate into two processes due to their wide temperature distribution. Thus, both the premelting and melting transitions (temperatures and enthalpies) are present and depend highly on the retinal condition, suggesting that the retinal has a very important stabilizing role and somewhat determines the pathway of unfolding. It has also been discovered that the refolding of the secondary structure following retinal addition to the bleached membrane has a strong pH dependence,[128] implicating the role of the side group protonation state and water H-bonding networks in determining the native folded structure.

## 5. Refolding of bR

Of course, unfolding tells only half the story. Although unfolding studies have revealed much about the factors that contribute to the high thermal stability of bR, further insights have been provided by a number of researchers investigating the re-folding process. Many of these experiments and interpretations have been discussed in detail in an excellent, recent review by Booth,[48] and the reader is referred to this paper for additional material. Membrane proteins in general, and bR in particular, are resistant to denaturation as discussed above. Furthermore, once they have been denatured, the refolding process is difficult to achieve. Chemical denaturation tends to destroy the native membrane environment, and heat denaturation causes the solvent exposed protein to aggregate. However, environments have been found in which some aspects of this re-folding process can be followed (see Huang et al.[129] and the Booth review[48]). These environments are generally micelles of detergent−lipid mixtures. One must consider this difference when comparing unfolding in native-like states to refolding in micellar states. Even so, these types of experiments have afforded a number of details on the mechanism of bR folding, as well as that of membrane proteins in general.[51]

Recently, the re-folding of heat-denatured bR from the premelting transition and from the melting transition in both the native environment, and in the micellar, monomeric bR environment has been investigated in our group.[130] This was followed in terms of the secondary structure formation, the retinal rebinding, and the proton-pumping function. Upon heating to a few degrees above the premolten state (up to 85 °C then immediately removing the heat), native bR refolds almost completely. This is monitored by the amide I band of the FT-IR spectrum as well as the UV−CD spectrum. At 85 °C, there is a broadening of the visible spectrum and a decrease in the intensity due to changes in the retinal chromophore binding, but upon cooling, ∼90% of the spectrum is recovered. This corresponds to a 90% recovery in the photocycle efficiency, monitored by the transient M intermediate formation following flash photolysis. The time scales of the secondary structure reformation and the visible spectrum recovery were monitored by time-resolved measurements, and it was found that the secondary structure re-forms before the retinal rebinding, in agreement with previous results that the native-like secondary structure can be formed in the absence of retinal.[48,131,132] However, upon thermal treatment of the native bR above its melting temperature, the changes in the secondary structure, the retinal re-rebinding, and the proton pumping efficiency only are found to recover to the extent of ∼15% of the original values. Heating the monomer above its melting transition (65 °C) results in almost no recovery in its secondary structure or in retinal rebinding. These experiments highlight the importance of the native lipids in controlling the refolding process of heat-denatured bR in terms of helical structure and functional importance.

## 6. Conclusions and Outlook

Many of the factors that control the structure and high stability of bacteriorhodopsin have been determined over the years using calorimetry and spectroscopic experiments on the native membrane, and on bR that has been systematically perturbed by changing pH, by removal or exchanging metal cations, by removing lipids, by adding detergents, by site directed mutagenesis, by Schiff base reduction, and or by retinal removal. It is now known that helix side chain interactions with protons, cations, lipids, and with each other alter the physiological secondary structure and the thermal stability significantly. Removal of the native lipids and, therefore, surface charges seems less important to the melting transition but does affect the premelting transition. This is true until the lattice structure is broken, in which case the structure and stability are significantly altered. These effects have been quantitatively assessed by systematically varying as few parameters as possible for a given experiment. Whether the reversible, premelting transition is accompanied by helical changes from $\alpha_{II}$ to $\alpha_{I}$ as well as by changing the lattice structure from gel to liquid phases has still not yet been completely answered, but indirect evidence supports the possibility of a helical transition, which could be affected by the lipids and cations. The absence of observed cations in the crystal structure of bR is still a cause for concern, because it is known that cations are certainly important to the structure, stability, and function. Until this is shown using diffraction experiments, the exact location and specificity of binding of such cations still relies on less direct calorimetric and spectroscopic evidence.

Finally, the majority of this review has concentrated on the unfolding of bR. The refolding of bR is much more difficult to study, but the efforts of a number of groups are making excellent progress in this area. The rest of the unfolding−refolding story must be detailed soon, and obtaining results relating to the native state is of paramount importance. Only then can we start to really understand the physical principles that biology has

developed over millions of years that provide the relationships between structure, function, and stability of membrane proteins.

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