Evidence for a Controlling Role of Water in Producing the Native Bacteriorhodopsin Structure

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ABSTRACT The experiments reported in this paper, based on reconstitution of bacteriorhodopsin (bR) from apomembrane at varying environmental conditions, demonstrate that the presence of water is a controlling factor in generating a native wild-type bR conformation. If water is lacking during this reconstitution process, then a non-native bR structure is formed that exhibits altered M formation and decay kinetics, as well as different behavior following extensive dehydration. It is shown that mutants affecting the ability of bR to form appropriate structures of water in specific protein cavities also affect the ability to generate a native bR conformation. The results suggest that aspartic acid 96 plays a major role in anchoring the appropriate water structure conformation associated with bR. It is also demonstrated that the glutamic acid 204 residue is pivotal in controlling the protein/water affinity. This water affinity can be further controlled by modifying the charge environment of the protein with altered pH. These data, based on kinetic absorption spectroscopy and Fourier transform infrared spectroscopy, highlight the central role of water in this protein.

INTRODUCTION

Bacteriorhodopsin (bR) is the integral protein of the purple membrane of *Halobacterium salinarium* (Oesterhelt and Stoeckenius, 1971). It is composed of seven transmembrane helical segments enclosing the binding pocket for the all-*trans* retinal chromophore. The latter is bound to Lys-216 via a protonated Schiff base linkage. The pigment absorbs light at 570 nm in its light-adapted form, due to specific chromophore-protein interactions that red-shift the absorption maximum relative to the absorption of the retinal-protonated Schiff base model compound in methanol solution (440 nm). Light absorption initiates a multistep reaction cycle with several distinct spectroscopic intermediates that are designated J_{625}, K_{590}, L_{550}, M_{412}, N_{520}, and O_{640}. K_{590} is characterized by a retinal chromophore that has isomerized to a 13-*cis* isomer. In the M_{412} state, the protonated Schiff base undergoes deprotonation, which is accompanied by protonation of Asp-85. For a recent series of reviews on bR, see *Isr. J. Chem.* Vol. 35, Nos. 3–4, 1995.

Linkage of the retinal chromophore to the protein (bacterioopsin) and subsequent formation of the protonated Schiff base linkage is an important process that is not fully understood. Oesterhelt and co-workers have shown that after addition of an all-*trans* retinal to a suspension of bacterioopsin, a complex that absorbs at 430/460 nm is formed (Schreckenbach et al., 1977, 1978). This complex is converted to bR, which absorbs at 570 nm. It was suggested that the initial complex was composed of a retinal chromophore rather than a Schiff base or a protonated Schiff base. A similar complex was obtained when a retinal chromophore was added to a mutant protein in which lysine 216 was substituted with alanine, supporting the suggestion that the 430/460-nm complex is composed of a retinal chromophore (Schweiger et al., 1994).

We have recently studied the binding of the retinal chromophore to bacterioopsin at low humidity (Rousso et al., 1995a, 1996). We showed that under these conditions, a complex composed of a retinal chromophore absorbing at 470 nm is formed. The dry apomembrane lacks the ability to couple the retinal via a Schiff base to the amino group of the lysine. The pigment absorbing at 570 nm is formed only following addition of water. In our study (Rousso et al., 1995a) the retinal binding process was followed by Fourier transform infrared (FTIR) spectroscopy. More recently, similar FTIR studies were carried out by Rüdiger et al. (1997). The importance of water in bR formation reaction highlights the crucial role that water plays in the conformational states of bR.

The first studies indicating the role water plays in the function of bR were carried out by Korenstein and Hess (1977), who demonstrated that the decay rate of the M photocycle intermediate is affected by the level of the membrane hydration. Varo and Keszthelyi (1983) showed that the photoelectric signals of bR are affected by the amount of water present.

The presence of water in the binding site and its role in stabilizing the protonated Schiff base has been suggested by several authors (Dupuis et al., 1980; Hildebrand and Stockburger, 1984). de Groot et al. (1989) suggested the presence of water on the basis of ^{15}N nuclear magnetic resonance studies, and the presence of strongly bound water even in highly dried membranes was suggested by neutron diffraction (Papadopoulos et al., 1990). A defined geometrical structure that includes the protonated Schiff base linkage, bound water and Asp-85 was suggested to stabilize the ion pair in the binding site (Gat and Sheves, 1993; Rousso et al.,

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1995b). This role of water in the bR binding site was further supported by theoretical calculations (Sampogna and Honig, 1994) and by molecular dynamic simulations (Humphrey et al., 1994). FTIR studies further indicated interaction of Asp-85 with a water molecule and the protonated Schiff base in the L intermediate of the photocycle (Maeda et al., 1994).

To shed further light on the critical role water plays in the structure and formation of bR, we studied the 570-nm species, obtained following irradiation of the bacterioopsin-9-*cis* retinal complex at low humidity. We found that although the absorption maximum of this species is similar to native bR, its structure and reaction to light are quite different. This different behavior originated from an altered bound water structure. Our data indicate that water is a crucial component in controlling the protein structure and its function.

MATERIALS AND METHODS

Wild-type (Oesterhelt and Stoeckenius, 1974), E204Q, and D96N (Brown et al.,, 1995) apomembranes were prepared by illuminating suspensions of the corresponding pigments with a wavelength >550 nm, in the presence of 1 M NH_2OH at neutral pH, 25°C. The suspension was centrifuged and washed five times. The membranes were resuspended in distilled water and the apomembrane suspensions were incubated with a 9-*cis* retinal at room temperature for 1 h under a dim red light in the presence of the appropriate buffer. The complexes were dried on glass slides using mild vacuum (~15 mm Hg). Variable degrees of film hydration were obtained by equilibrating the samples with different relative air humidities produced by saturated salt solutions, whereas the high-vacuum films were prepared using 1 mm Hg. The samples were irradiated using a Schott (KL1500 electronic) halogen lamp. Absorption spectra were taken on a Hewlett Packard 8452A diode array spectrophotometer. FTIR spectra were recorded on a Bruker IFS113V FTIR spectrometer, as previously described (Rousso et al., 1995a). Flash photolysis experiments were carried out using a JK Nd:YAG laser that is doubled to produce 532 nm emission.

RESULTS

Recently it was shown (Rousso et al., 1995a) that the complexation reaction of all-*trans* retinal chromophore (obtained by irradiation of 9-*cis* retinal) to bacterioopsin produces, at reduced humidity, a mixture of species absorbing at 470 and 570 nm. The ratio between these two species was found to be highly hydration-dependent. It was suggested that the formation of the bR pigment, absorbing at 570 nm, occurs through the following transitions:

$$390\ \text{nm} \xrightarrow{h\nu} 390'\ \text{nm} \rightleftarrows 470\ \text{nm} \rightleftarrows 470'\ \text{nm} \rightarrow 570\ \text{nm}$$

To further check the nature of the 570-nm species and its possible identity to bR, we have examined its character at various humidities. As will be shown below, it was found that below 86% humidity, the 570-nm species produced (defined as bR′) is not identical to bR.

To study the correlation between the formation of this bR′ and the external humidity, we used the above described light-induced complexation reaction of retinal to bacterioopsin. The amount of the 570-nm pigment was maximized when the apomembrane-9-*cis* retinal complex (AbR) was irradiated at 100% humidity. The reconstituted bR obtained by this procedure was converted into a species absorbing light at 530 nm following dehydration at high vacuum (1 mm Hg). This result is identical to that obtained with a film of W.T bR (Hildebrand and Stockburger, 1984).

Fig. 1, *trace a*, shows the difference absorption spectra of W.T bR (570 nm) to 490 nm transition following dehydration using high vacuum. A similar experiment of bR′ (570 nm) species formed at 57% humidity is presented in Fig. 1, *trace c*. The difference spectrum is considerably different from that of native bR, exhibiting a 440-nm positive band

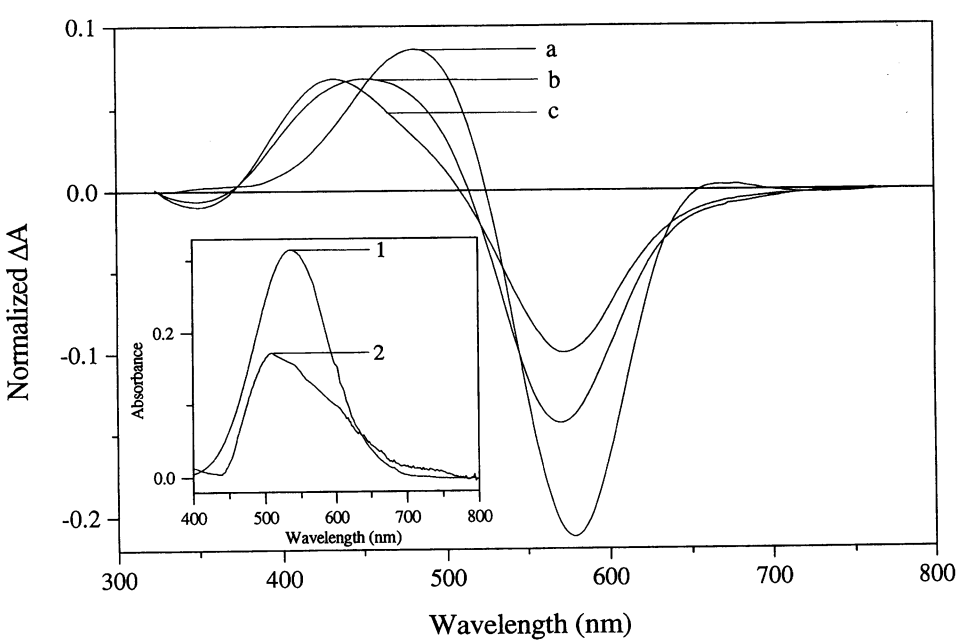

FIGURE 1 Difference absorption spectra of 570 nm to 490 nm or 440 nm transition following dehydration. The dehydration was carried out by applying high vacuum (1 mm Hg). (*a*) native bacteriorhodopsin; (*b*) reconstituted 570-nm species under 86% humidity; (*c*) reconstituted 570-nm species at 51% humidity. *Inset:* (*1*) Absorption of native bacteriorhodopsin following dehydration. (*2*) Absorption of reconstituted 570-nm species at 51% humidity following dehydration.

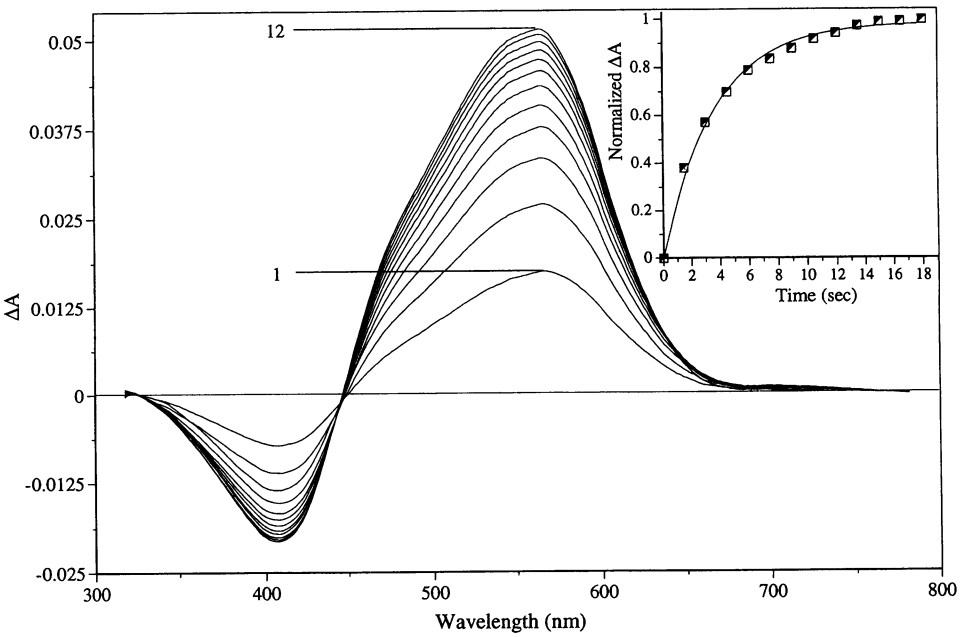

FIGURE 2 Difference absorption of 410-nm species (M′) thermal transition to the 570-nm species (bR′) following light absorption by bR′. Spectra were taken every 1.5 s, *12* after 18 s. *Inset:* The best fit (*solid line*) for the kinetic of bR′ reformation from M′ based on a monophasic model.

versus 490 nm in bR. This result reappeared after dehydration of the 570-nm species produced at 67% and 75% humidities (data not shown). Desiccation of the 570-nm species, formed under 86% humidity, produced a mixture of 440/490-nm species (Fig. 1, *trace b*).

Upon steady-state light illumination (using a 570-nm cutoff filter) the 570-nm species was transformed into a blue-shifted photoproduct absorbing at 410 nm (resembling the M of native bR). The decay of this M′ photo intermediate back to 570 nm at 67% humidity is shown in Fig. 2. The regeneration of bR′ from M′ was analyzed using a kinetic scheme based on a monophasic model (Fig. 2, *inset*). The lifetime of the M′ intermediate was found to be ~20 s. The decay of M′ was also studied in D_2O and was found to be very similar to that of H_2O (data not shown) and did not show a significant deuterium effect.

It is known that the lifetime of the M intermediate of bR is highly dependent on the external humidity (Korenstein and Hess, 1977). In contrast, the lifetime of the bR′ photoproduct (M′) was unchanged when the illumination was carried out in the range of 57–86% humidities (data not presented). In order to rule out the possibility that the M′ photointermediate originated from a secondary photointermediate irradiation, we measured the kinetics of this intermediate using flash photolysis. In Fig. 3 we show the absorption change at 412 nm following absorption of a laser pulse by bR′ formed at 67% humidity. As shown in Fig. 3 *A*, the accumulation of the M′ intermediate was completed after ~20 μs. The lifetime of this intermediate is much longer than our measurement limitations, and it was detectable in a significant amount two seconds following the laser pulse. However, irradiation with a flash lamp produced an M′ intermediate characterized by ~20 s lifetime (Fig. 3 *B*), identical to that formed following steady-state illumination, indicating that the latter procedure does not introduce secondary photochemical processes.

In addition to the crucial role played by water molecules controlling the binding reaction, the formation of the 570-nm pigment is regulated by additional factors. In a previous study (Rousso et al., 1995a) we showed that the amount of 570-nm species increased as the pH was elevated. This transition was characterized by a pK_a of 8.9. Therefore, 9-*cis* retinal-apo mixture (AbR) at pH = 9.5 at 10% humidity formed, following light absorption, a 470/570-nm mixture. Thus, even at such low humidity, the high pH resulted in a smaller 470/570 nm ratio. The 570-nm species produced in the process exhibited, after dehydration, a difference spectrum identical to that obtained after dehydration of bR′ (a positive band at 440 nm). Fig. 4 shows the formation of the 570-nm species following irradiation of 9-*cis* apobR complex (AbR) at pH = 5.5 at 51% humidity. The dehydrated form of this 570-nm species (dehydrated using 1 mm of Hg) exhibits, in its difference spectrum, a 490-nm positive band similar to native bR (data not shown). It should be noted that at neutral pH at similar humidity (51%) the formation of the 570-nm species is accompanied by a 470-nm species.

A further interesting observation is associated with M′ lifetime. As mentioned above, the decay of M′ is not dependent on external humidity, in contrast to M in native bR. It appears that the lifetime of M′ is also not pH-dependent. The results show a monophasic decay with a lifetime of ~20 s, regardless of humidity and pH.

The 570-nm (bR′) pigment can also be formed upon illumination of the 470-nm species. When the 470-nm species is irradiated using a 470-nm cutoff filter (at steady-state conditions and 15% humidity) it is converted to a 390-nm species (Rousso et al., 1995a). The newly formed 390-nm

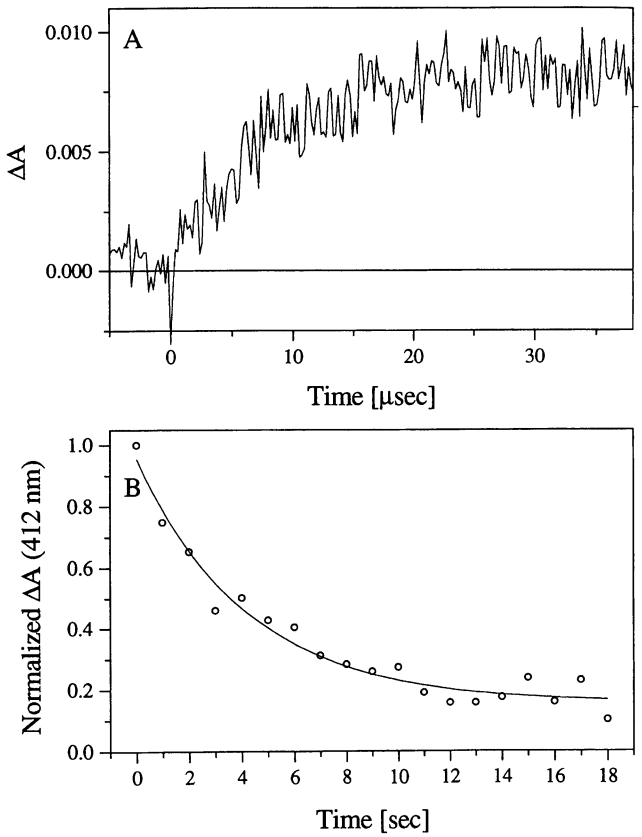

FIGURE 3 Formation and decay kinetics of M′ at 67% humidity. (*A*) Formation of M′ detected at 412 nm following irradiation with 532-nm NdYag laser. (*B*) Decay of M′ after excitation with a flash lamp.

species thermally regenerates the 470-nm species. During this thermal reaction a small amount (~10%) of a species absorbing at 570 nm is formed (Fig. 5). The relative amount of the 570-nm species can be increased by repetitive illuminations of the 470-nm species. This 570-nm species following light absorption generates a blue-shifted photointermediate with a lifetime of ~20 s, and after dehydration, the difference spectrum indicates a positive band at 440 nm. The above results were also observed when the 470-nm species was irradiated using mild vacuum (~20 mm Hg).

We have previously shown that Asp-96 (D96) plays an important role in controlling water absorption by the protein during the binding reaction of the retinal to bacterioopsin (Rousso et al., 1995a). When its binding reaction was carried out at 100% humidity, using an apo-protein of a mutant in which the aspartic acid 96 was replaced by an asparagine (D96N), a mixture of 470- and 570-nm species was formed. The ratio of 470 to 570 nm in this mutant favors the 470-nm species relative to the wild type. Therefore, Asp-96 residue may play a role during the formation of bR′. Interestingly, in contrast to membrane fragments suspensions, films of this mutant at 100% humidity showed similar behavior to that of bR′. Namely, a flash photolysis experiment (at 67% humidity) indicated that excitation of D96N mutant film is characterized by an intermediate detected at 410 nm, whose rise time was equivalent to that obtained for bR′ (~30 μs—Fig. 6 *A*). Excitation with a flash lamp indicated that the 410-nm species is characterized by a lifetime of ~30 s (Fig. 6 *B*). In addition, the removal of water from the mutant film by high vacuum resulted in a difference spectrum similar to that obtained with bR′ (data not shown). Moreover, the decay rate of M was not affected by pH similarly to M′.

Formation of bR′ may also be affected by a point mutation of E204. The complex of 9-*cis* retinal/E204Q apoprotein, following light illumination at 51% humidity, was transformed completely to 570 nm in contrast to wild-type, in which a mixture of 470/570-nm species was obtained. Interestingly, this 570-nm species, upon dehydration, gave a similar species to that obtained from bR.

To further characterize M′ species we studied its structure using FTIR. The spectra are shown in Fig. 7. The figure shows a difference FTIR spectrum of the bR′ (obtained at 67% humidity) to M′ transition (Fig. 7 *A*). After this measurement, the bR′ sample was warmed to 295 K and was equilibrated with 100% humidity for four hours, and a difference spectrum of the bR to M transition was taken again (Fig. 7 *B*). Both measurements were carried out at 250 K.

DISCUSSION

Characterization of bR′

It has been shown that upon dehydration, bR absorption is blue-shifted from 570 to 530 nm (Hildebrand and Stockburger, 1984). This transformation is presented in Fig. 1, *trace a*, where the absorption maximum in the difference spectra is shifted from 570 to 490 nm. Furthermore, the decay of the M photointermediate in bR is well studied, and its rate was found to be highly hydration-dependent. The relaxation of the M intermediate is characterized by a multiexponential decay (for a review, see Lanyi and Varo, 1995). In this paper we have used the decay of the M photoproduct and the absorption shift following dehydration as a probe to distinguish between bR and a new species, denoted as bR′.

The complexation reaction of 9-*cis* retinal to bacterioopsin, when carried out at reduced humidities, produces a mixture of 470- and 570-nm species. Thus, by applying the above analyses, we can determine whether the product of this complexation reaction, which absorbs at 570 nm, is bR or another species. The results obtained clearly show that when the complexation reaction is carried out at humidity levels lower than 86% the pigment which is formed is not a native bR, although it absorbs similarly to bR (570 nm). However, upon dehydration using high vacuum, the absorption difference spectrum is characterized by a positive band at 440 nm, rather than 490 nm in bR. Moreover, the decay of its photoproduct (M′) is characterized by ~20 s lifetime and is not hydration-dependent. The characteristics of this M′ will be discussed in more detail further on. Recently, we have shown that there are at least three binding sites that

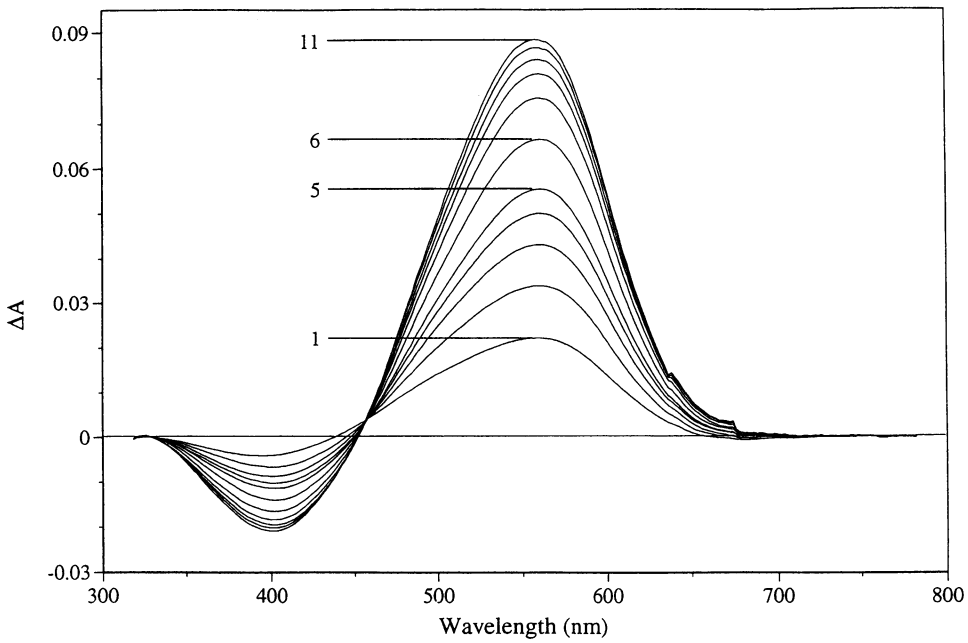

FIGURE 4 Formation of 570-nm species from AbR complex under 51% humidity at pH = 5.5. Difference spectra between 570-nm species and 390-nm species following irradiation of the AbR complex. *1–5* spectra were taken every 30 s, *6* after 240 s, *11* after 14 min (6–11 every 120 s).

have to be filled with water molecules allowing the protein to adopt a structure, which leads to formation of the 570-nm species (Rousso et al., 1995a). Since bR′ is formed at low humidities, we conclude that bR′ is less hydrated than bR. This deficiency in structured water molecules may induce a conformational change in the protein structure in comparison to bR. The altered structure causes the observed differences, namely, the blue shift following dehydration and the formation of a M′ photointermediate, which is hydration- and pH-independent.

The similarity of the D96N mutant at 100% humidity to bR′ suggests that the proposed deficiency in the water molecules is associated with the cytoplasmic side of the protein. More specifically, it is tempting to suggest that the water molecules network between the protonated Schiff base and D96, which was proposed to be present in bR (Cao et al., 1991; Humphrey et al., 1994), is absent or altered in bR′. It should be noted that the transformation of bR′ to bR is irreversible. Hydration of bR′ by raising the external humidity leads to bR formation. This transformation is irreversible and it is impossible to obtain bR′ following dehydration of bR.

An interesting observation is associated with the absorption maximum of bR′. The absorption maximum of bR′ is very similar to that of bR (570 nm). It is currently assumed that the absorption maximum of bR is determined by spe-

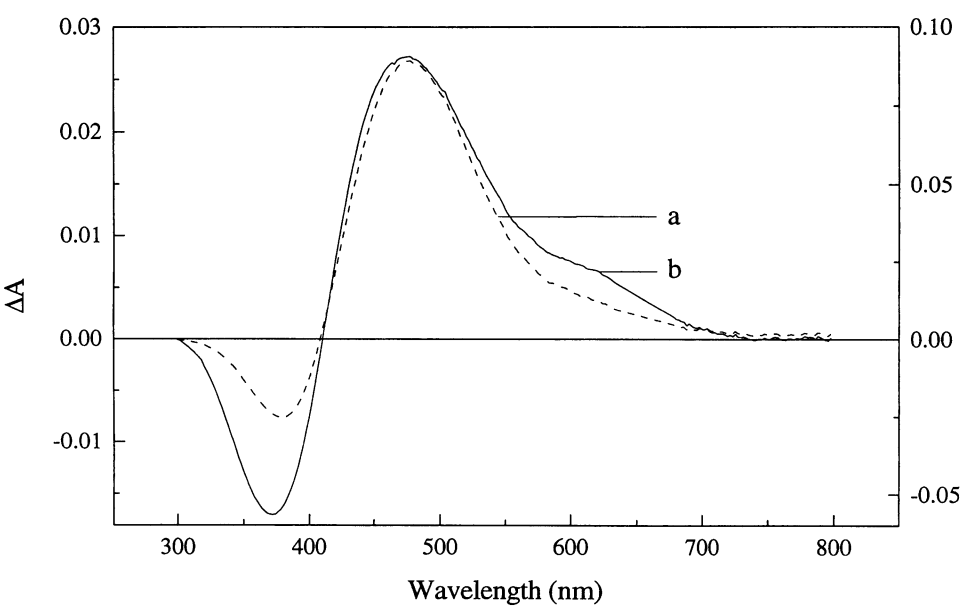

FIGURE 5 Formation of the 570-nm species at low humidity. (*a*) Difference spectrum between 470 and 390-nm species, following irradiation of 390 species and thermal process for 15 min (right *y*-axis). (*b*) Difference spectrum after irradiation of the 470-nm species with 470 nm cutoff filter and thermal process for 15 min (left *y*-axis).

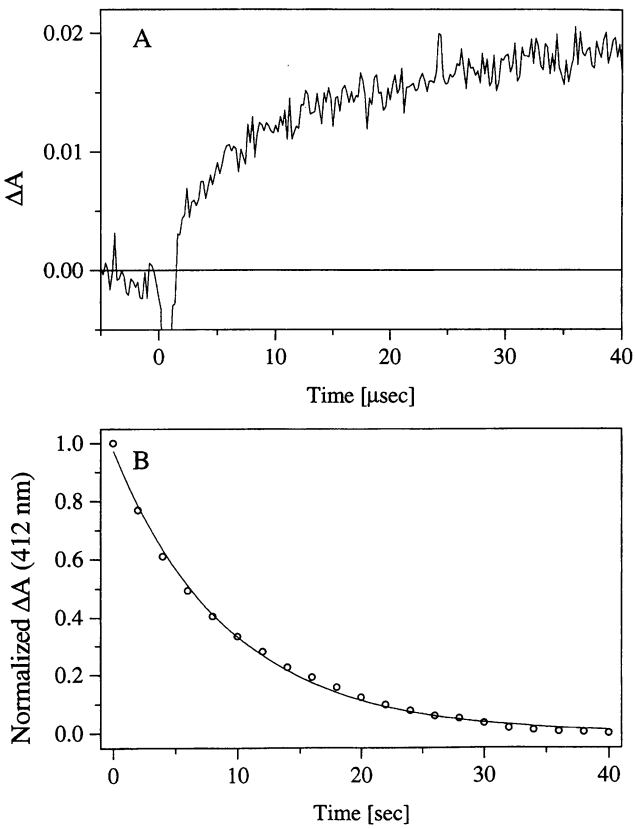

FIGURE 6 Formation and decay kinetics of M′ of the D96N mutant at 67% humidity. (*A*) Formation of M′ detected at 412 nm after irradiation with 532-nm NdYag laser. (*B*) Decay of M′ after excitation with a flash lamp.

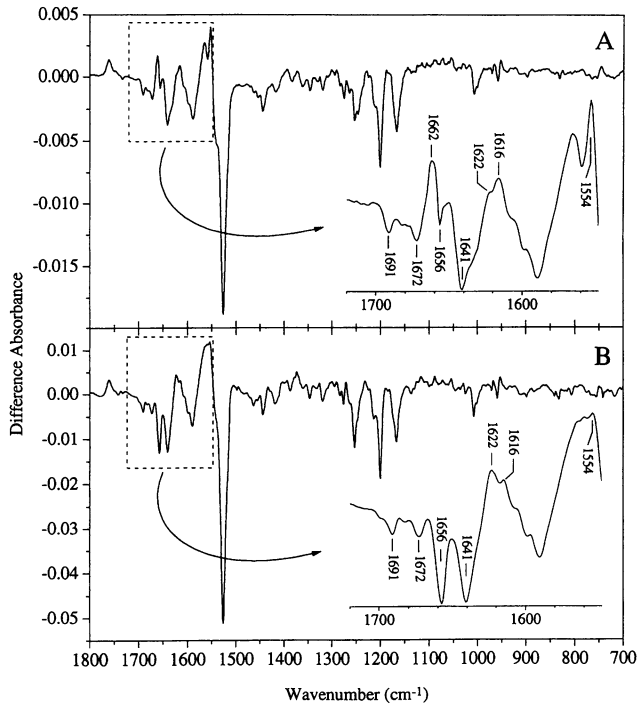

FIGURE 7 Difference FTIR spectra of M′/bR′ spectra transition. (*A*) M′/bR′ at 67% humidity; (*B*) M/bR difference spectrum of the same sample obtained after an increase in the humidity to 100%. Positive peaks are due to M′ or M species and negative peaks are due to bR and bR′. Spectra were taken at 250 K. The sample was obtained by illumination of 9-*cis* retinal-bacterioopsin complex (AbR) under 67% humidity, followed by thermal conversion under the same humidity.

cific interactions of the protonated Schiff base with its neighboring negatively charged aspartic acids (Asp-85 and Asp-212) (Harbison et al., 1983; Sheves et al., 1985; Spudich et al., 1986; Baasov and Sheves, 1985; Albeck et al., 1992; Hu et al., 1994) and by an s-*trans* ring chain conformation (Schreckenbach et al., 1978; Harbison et al., 1985; Albeck et al., 1992). Therefore, the similarity of the absorption between bR′ and bR indicates that the structure around the Schiff base and the retinal ring is similar in the two species and the alteration in the vicinity of the D96-Schiff base moieties does not affect this structure. Specifically, the structural change induced at the Schiff base by lack of water or D96 mutant at 100% humidity is not of a character that affects the absorption spectrum.

The role of aspartic acid 96 in bR′

It is well established that D96 plays a crucial role in the latter steps of the bR photocycle. Accumulated evidences indicate that the M→N transition is associated with a proton transfer from D96 to the Schiff base (Gerwert et al., 1989; Butt et al., 1984; Stern et al., 1989; Holz et al., 1989; Tittor et al., 1989; Otto et al., 1989). In addition, there have been several studies in which it was suggested that water molecules are present between D96 and the Schiff base. Cao et al. (1991), who studied bR suspensions in the presence of osmotically active solutes, have shown that water molecules are needed for proton transfer from D96 to the Schiff base. It was shown, in native bR, that the M→N transition is inhibited by withdrawing water from the protein. In contrast, in suspensions of D96N mutant, this transition was less affected by dehydration. On the basis of their results the authors suggested that water is required inside the protein for the above protonation reaction. In addition, molecular dynamic simulations indicated the feasibility of the presence of water between the D96 and the Schiff base (Humphrey et al., 1994). Recent electron diffraction studies have provided evidence for several cavities in the model structure, with most of them located in the region of the Schiff base. One of those cavities is found between D96 and the Schiff base. These authors proposed that such cavities could be filled with water molecules in bR (Grigorieff et al., 1996).

As described in the previous section, a D96N film produced from D96N water suspension, even at 100% humidity, has similar characteristics to bR′. Unlike D96N in solution, which is characterized by an M intermediate with a short lifetime that depends significantly on pH, the M produced at 100% humidity is identified as M′. This result indicates that the transformation of bR′ (D96N) to bR

(D96N), following hydration, is reversible, unlike wild-type bR. These results highlight the crucial role played by D96 in binding water molecules inside the protein. It appears that the D96 residue acts as an "anchor" that is involved in tight binding of the water molecules found in the cytoplasmic side. When the D96 residue is present, it is not possible to withdraw the water from this binding site, thus the transformation from bR′ to bR is irreversible. However, upon removal of this residue, the water can be easily removed.

The characteristics of M′ photointermediate

The decay of M intermediate was extensively studied during the last decade. It has been shown that the regeneration of bR from M occurs through a multiphasic transition. There have been attempts to elucidate this behavior from two perspectives. One is based on the existence of parallel photocycles while the other concept is based on a decay composed of a series of thermal reactions (see Lanyi and Varo, 1995,for a recent review). Surprisingly, M′ decays back to bR′ in a monophasic step. The regeneration of bR′ is accompanied by at least two processes: thermal isomerization of the retinal chromophore and protonation of the Schiff base. We can probably rule out the possibility that the Schiff base protonation is the rate-limiting process by the lack of deuterium isotope effect on the decay of M′. All previously recorded cases of thermal isomerization in retinal proteins have involved a protonated Schiff base species. This seems to indicate that the thermal isomerization barrier for Schiff base double bond isomerization is relatively high. In view of this assumption, it is reasonable that isomerization will not occur until Schiff base reprotonation. Thus, the regeneration of bR′ must be controlled by a yet undefined molecular process. Therefore, it is plausible that a protein conformational change and/or structural water alteration allow for proton transfer to the Schiff base and control the rate of M′ decay.

It has been suggested that the pK_a of protein residues and the transfer of a proton from a carboxyl group to the Schiff base is controlled by a specific angle between the two groups, which allow bound water to stabilize the ion pair (Gat and Sheves, 1993; Rousso et al., 1995b). Therefore, a defined structure between the Schiff base and the proton donor should be obtained in order to achieve a proton transfer to the Schiff base. From a molecular point of view, this could be the rate-controlling step in M′ decay. In support of this idea, it has been suggested that the formation of the M intermediate in native bR involves a protein conformational alteration (Sheves et al., 1986). This alteration probably adjusts the ΔpK_a value of the donor and acceptor groups that allow for proton transfer.

During the formation of M the proton is transferred from the protonated Schiff base to Asp-85 located in the extracellular part of the protein. To ensure the proton pumping activity, the Schiff base should accept the proton from the intracellular side, thus changing its direction of exposure. This process is defined as the "switch mechanism" (for a recent review, see Honig et al., 1995).

The switch mechanism was attributed to a change in the retinal conformation and/or to protein conformational alteration. Recent molecular dynamic simulations suggested that the Schiff base linkage changes its exposure during the M intermediate, due to reorientation of structured water in the cytoplasmic side of the protein, as a result of protein alteration (Xu et al., 1995). The role of D96 in the proton transfer mechanism to the Schiff base was demonstrated by studies on the D96N mutant. In addition, it was shown that water is important for proton transfer from D96 to the Schiff base linkage. Furthermore, below ~50% humidity, it was demonstrated that proton pumping does not take place and the Schiff base regains its proton from the extracellular side (Varo and Keszthelyi, 1983). Thus, it is tempting to propose that in bR′ a defective water structure in the vicinity of D96 does not allow the switch process to take place in M′, and the proton is regained from the extracellular side (possibly from D85). This proposal is supported by the fact that M′ decay is not sensitive to D96 mutation, nor to environmental humidity. The lack of the switch event in M′ is in keeping with the suggestion that structured water plays an important role in the switch mechanism. It is quite possible that the presence of such structured water would underlie the required retinal conformational alterations that allow Schiff base reprotonation form D96 and subsequent isomerization.

Unlike M of bR in solution at pH = 7, which is formed in ~200 μs, the formation of M′ is completed in 20 μs (in 67% humidity). A fast rate of formation of M was observed also at high pH (~10) in W.T bR, at neutral pH in mutants E204Q (Brown et al., 1995), and R82A (Balashov et al., 1993). The formation of M was interpreted as an equilibrium between L and M, which shifted to M only when the substate of M (denoted at M_1) is shifted to another M substate (M_2) (Lanyi and Varo, 1995). Titration of a group at high pH (~10) shifts the equilibrium from L to M, due to stabilization of M. Our present studies demonstrate that also in the fast formation process of M′, the equilibrium is shifted from L′ to M′. It was shown above that the water network in bR′ is different from that of bR. The hydrogen bonding network established by structured water in the protein is probably involved in controlling the ΔpK_a between the donor (protonated Schiff base) and the acceptor group (Asp-85). In order to transfer a proton between these two groups, the ion pair should be destabilized, probably by perturbing the water hydrogen bonding network and, thus, produce an M species more rapidly. Our results indicate that there are several ways to perturb this network. These include: 1) limiting the water in the protein leading to bR′ formation, 2) increasing the pH in the native protein, or 3) mutating groups such as E204 and R82.

The difference in bR and bR′ structures is further reflected in the FTIR M′/bR′ difference spectrum. It is clearly evident that the region around 1656 cm^{-1} is altered in M′/bR′ relative to M/bR. The negative peak at 1656 cm^{-1} in native bR (Fig. 7 *B*) diminished in M′/bR′, probably due

to the appearance of a positive band corresponding to M′ at 1662 cm^{-1} (Fig. 7 *A*). This band, which is probably due to amide I, points to a different structure in M′ relative to M. An additional change is observed in the 1620 cm^{-1} region, which includes several vibrations: the C=N stretch of the retinal linkage, the carbonyl stretching vibration of proline (Gerwert et al., 1990), and unusual amide I lysine backbone frequency (Takei et al., 1994). Furthermore, there is an additional change in the bR′/M′ difference spectrum at around 1550 cm^{-1}, which could correspond to the amide II vibrational mode. These alterations further point to the different structure of M′ relative to M. The FTIR spectra also clearly support our finding that bR′ is transformed into bR following humidity elevation from 67 to 100%.

At lower humidities, the amount of bR can be maximized by lowering the pH or by mutating the protein replacing E204 with glutamine. In both of these cases the protein can absorb more water molecules into its internal store at lower humidity. The involvement of E204 in the reconstitution process in order to obtain the appropriate charge environment has been further highlighted by the recent results of Rüdiger et al. (1997). These workers noted a change in the E204 mutant for proton uptake and release depending on pH.

Our results further indicate that formation of a 570-nm species at low humidity and high pH produces bR′ instead of W.T bR. Thus, high pH enhances protonated Schiff base formation at low humidity but prevents the achievement of a protein structure associated with bR.

In conclusion, the data in this paper emphasize the fact that the absorption at 570 nm is not diagnostic of whether a protein structure exists, which can generate the native photocycle of bR. Depending on the humidity and a complex of factors, which include the surrounding pH, a protein structure can be achieved that is different from native bR. A crucial component in this difference is the water present around specific protein groups. The presence of this water appears to be a controlling factor in producing what is recognized as native bR. Humidity and pH have substantial effects on the native protein of the 570-nm structural state that is achieved. As noted above, at high humidity, the 470-nm species is converted to the protonated state. At 100% humidity, this 570-nm state is W.T bR.

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