The Last Phase of the Reprotonation Switch in Bacteriorhodopsin: The Transition between the M-Type and the N-Type Protein Conformation Depends on Hydration†

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ABSTRACT: In order to elucidate the mechanism of the reprotonation switch of bacteriorhodopsin, the protein conformation of the M intermediate of the D96N mutant was examined at various hydration conditions by X-ray diffraction and FTIR spectroscopy. We observed two distinct protein conformations at different levels of hydration. One is like in the N photointermediate, although in this case with an unprotonated Schiff base. It is stabilized in highly hydrated samples. The other is a protein conformation identical to that in the normal M intermediate of wild-type bacteriorhodopsin, which is stabilized in partially dehydrated samples. The hydration dependence of the structural transition between the M-type and the N-type conformations suggests that there is a change in the binding of water at the cytoplasmic surface. Thus, more water molecules bind in the N-type structure than in the M-type. This is consistent with the idea that the conformational change from the M-type to the N-type corresponds to the opening of the proton channel to the cytoplasmic surface by tilt of the cytoplasmic end of helix F, and that this is required for proton transfer from Asp-96 to the retinal Schiff base.

Bacteriorhodopsin (bR)[1] transports protons across the membrane against a chemical potential upon absorption of light by the retinylidene chromophore. The transport cycle consists of the interconversions of a sequence of intermediate states, J, K, L, M, N, and O (Mathies, 1991; Rothschild, 1991; Lanyi, 1993). In order to elucidate the mechanism of proton transport, we must clarify how the alternating connection of the active site with the two opposite sides of the membrane is regulated. This is the proposed universal element in ion pumps (Lanyi, 1995). The M intermediate of bR is produced when a proton is transferred from the retinal Schiff base (the active site) to Asp-85 located in the extracellular region, and a proton is then released to the extracellular surface from Glu-204 and probably bound water (Brown *et al.*, 1995a; Richter *et al.*, 1996). The reprotonation of the Schiff base from the cytoplasmic side represents the decay of the M state. Therefore, the switch of the access must consist of three phases: breaking the connection of the Schiff base to the extracellular proton channel (first phase), establishing a new one to the cytoplasmic channel (second phase), and lowering the high pK_a of Asp-96 so as to make it a proton donor (third phase).

Structural changes in the M intermediate have been revealed by neutron, X-ray, and electron diffraction experiments (Dencher *et al.*, 1989; Koch *et al.*, 1991; Nakasako *et al.*, 1991; Subramaniam *et al.*, 1993). More recently, those in the N intermediate were also detected by X-ray and electron diffraction (Kamikubo *et al.*, 1996; Vonck, 1996). The preliminary three-dimensional maps calculated from electron diffraction patterns obtained from tilted specimens suggested that the density changes occur in the vicinity of helices B, F, and G, and that they are localized to the cytoplasmic half of the protein. The changes include tilt of the cytoplasmic end of helix F away from the other helices (Subramaniam *et al.*, 1993; Vonck, 1996). We have shown that these structural changes are closely related to the elimination of electrostatic interaction between the protonated Schiff base and its counterion, Asp-85, upon the transfer of a proton from the former to the latter (Kataoka *et al.*, 1994; Brown *et al.*, 1997). In the unphotolyzed protein (conformation E), the proton channel is open to the extracellular side, and in the M intermediate it is open to the cytoplasmic side (conformation C) (Kataoka *et al.*, 1994). Thus, the proton transport mechanism is elegantly explained by an alternating protein conformation model in which the access change is initiated by the proton transfer at the active site (Kataoka *et al.*, 1994; Lanyi, 1995; Brown *et al.*, 1997).

The initial pK_a of Asp-96 is high, and we had suggested that it is lowered by increased hydration of the cytoplasmic region of the protein (Brown *et al.*, 1995b). Indeed, there is a small but significant difference in the structures of the M and the N [or MN in the D96N mutant (Sasaki *et al.*, 1992)] intermediates. The structural changes in the M-type structure are at helices B and G, and in the N-type structure they are at helices F and G (Kamikubo *et al.*, 1996). We suggested that these two structures correspond to two distinct phases in the reprotonation process (Kamikubo *et al.*, 1996). In our hypothesis, the cytoplasmic region (perhaps including the cytoplasmic proton channel) becomes more open in the MN intermediate but not in the early M intermediate, and

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared.

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remains open until N decays. That is to say, the proton channel is more hydrated in the MN and N intermediates than in M. To examine this hypothesis, we investigated the effect of hydration on the structures of these photointermediates by X-ray diffraction and FTIR.

During the M decay process, the deprotonated Schiff base can be reprotonated from Asp-96. In the Asp96 → Asn mutant of bR, in which its proton donor activity is lost, the lifetime of the M intermediate is prolonged extremely at alkaline pH (Butt *et al*., 1989; Cao *et al*., 1991). The previous FTIR experiment suggested that the M intermediate in this mutant had the N-type conformation although the chromophore and the Schiff base were the same as the M intermediate observed in wild type, so-called MN (Sasaki *et al*., 1992). Using this mutant, we report here that there seems to be a structural equilibrium between the M-type (M) and the N-type conformations (MN), and it depends on hydration. The N-type conformation is observed only in highly hydrated samples, while the M-type conformation is arrested at lower hydration. This result suggests a different binding affinity of water molecules to the cytoplasmic surface between the M-type and N-type structures, and supports our model of the reprotonation switch. A recent study examined this question with similar techniques, but reported on the hydration-dependent presence or absence of an M-type structure in D96N rather than detecting the stabilization of an N-like structure by X-ray diffraction (Sass *et al.*, 1997). In this paper, we clarify the difference in X-ray diffraction between the M state and the MN state, whose stabilization depended on the hydration.

Nomenclature. The photointermediates of bR were named from their spectra in the visible. Therefore, their names, K, L, M, N, etc., originally indicated differences in the chromophore environment. Recently, these intermediates can be recognized also by FTIR and diffraction, which distinguish changes in the protein moiety as well as in the retinal. This situation has brought confusion to the discussions of the proton pump. In this paper, we use the following terminology. The M intermediate represents the intermediate with an unprotonated Schiff base, whose absorption maximum is near 410 nm. The N intermediate represents the intermediate that follows the M intermediate, which has a reprotonated Schiff base and an absorption maximum of 565−570 nm. The M-type and N-type conformations represent the protein structures of the M and the N intermediates observed in the wild type protein, respectively. It is possible to produce, however, the N-type conformation with an unprotonated Schiff base (an M intermediate). This so-called MN intermediate (Sasaki *et al.*, 1992) is, thus, categorized as an M intermediate, although we will use the term MN intermediate occasionally, in its original meaning. The E and C conformations represent the protein structures whose proton access channel is open to the extracellular and the cytoplasmic side, respectively.

MATERIALS AND METHODS

Samples. The D96N mutant was constructed by inserting a nonintegrating vector with novobiocin resistance, and *Halobacterium salinarium* was transformed as described (Ni *et al.*, 1990; Needleman *et al.*, 1991). The mutated bR were purified as a purple membrane sheet according to a standard method (Oesterhelt *et al.*, 1974).

X-ray Diffraction Experiments. The X-ray diffraction experiments were carried out with the MUSCLE Diffractometer (Amemiya *et al.*, 1983) installed at BL-15A in the Photon Factory at Tsukuba, Japan, as described in an earlier publication (Nakasako *et al.*, 1991). The purple membranes were suspended in 10 mM borate, 5 mM NaCl (pH 10) up to 20 ODU at 570 nm. A 100-μL drop of the suspension was dried on a piece of Mylar sheet at room temperature. The moderately dehydrated samples were obtained by vapor exchange over saturated Na_2HPO_4 (95% relative humidity at 293 K), saturated $(NH_4)_2SO_4$ (81% relative humidity at 293 K), and saturated sodium acetate (76% relative humidity at 293 K). Two kinds of highly hydrated samples were prepared: by adding a 10-μL drop of the buffer onto a moderately dehydrated sample (81% relative humidity) and by soaking buffer into a highly hydrated sample for 1 h, producing an even better hydrated sample. After both procedures, the excess buffer was removed. In this way, we prepared five kinds of samples at different hydration levels.

X-ray diffraction profiles were recorded at the same position of the sample with and without continuous illumination light by a 1-kW slide projector, using a Y-50 cutoff filter ($\lambda > 500$ nm). The temperature (283 K) was controlled by circulating thermostated water through a sample holder. Exposure was for 180 s.

The integral intensity of each Bragg reflection was calculated by a profile fitting with an asymmetric function (Nakasako *et al.*, 1991). Phases and intensity ratios for overlapping reflections were derived from cryoelectron microscopy data in the calculation of the electron density map (Henderson *et al.*, 1990).

Comparison of the structural changes was carried out with X-ray diffraction intensity changes. Correlation coefficients between pairs of data sets were calculated as follows:

$$\text{correlation coefficient} = S(1,2)/\sqrt{S(1,1) \times S(2,2)}$$

where

$$S(i,j) = \Sigma(\Delta I_i \times \Delta I_j) - (\Sigma \Delta I_i) \times (\Sigma \Delta I_j)/N$$

and N is the total number of Bragg reflections.

We used the R-factor as a criterion of the overall relative change. The R-factor was calculated as follows:

$$R = \Sigma |I_{\text{photo}} - I_{\text{unphoto}}| / \Sigma I_{\text{unphoto}}$$

where I_{unphoto} and I_{photo} represent integrated intensities after Lorentz correction and background subtraction obtained from unphotolyzed and photolyzed bR, respectively.

FTIR Spectra. A 10-μL sample of D96N mutant bR suspended in 10 mM borate at pH 10 was placed on a BaF_2 window (10 mm diameter), and dried under a gentle stream of N_2 gas. The films were incubated under 81% relative humidity and sealed using a silicone rubber spacer and another BaF_2 window and set in the cell holder. For hydrated samples, 0.5 μL of H_2O was put inside the spacer before sealing. The sample cell holder was mounted in an optical cryostat (DN1204, Oxford) connected to a temperature controller (ITC4, Oxford). Spectral recordings were performed in a Horiba FT-210 Fourier transform infrared spectrophotometer equipped with an MCT detector. The M intermediate of hydrated and partially hydrated samples at

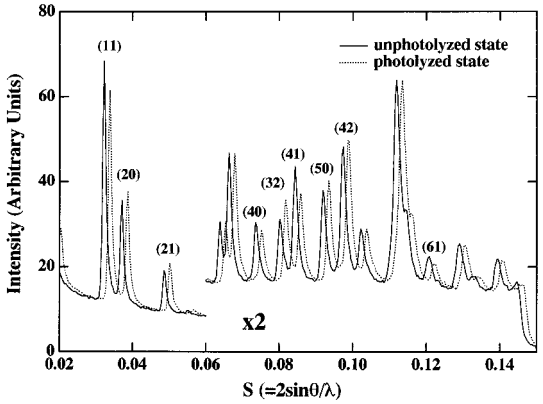

FIGURE 1: X-ray diffraction profiles for the D96N mutant of bR incubated under 95% relative humidity. The solid line indicates the diffraction profile in the dark, and the dashed line is the diffraction profile under continuous illumination (>500 nm). The dashed line is slightly shifted to the right for the sake of easy comparison. Intensities for $S > 0.06$ are multiplied by 2.

283 K was produced by irradiation of bR with >500-nm light for 30 s. Difference FTIR spectra were obtained by 8 independent recordings before and after irradiation, each of which was 16 scans (resolution = 2 cm^{-1}).

RESULTS

Figure 1 shows X-ray diffraction profiles obtained from oriented purple membrane films of D96N mutant bR at 283 K and 95% relative humidity, in the presence (solid line) and absence (dashed line) of continuous illumination. In the D96N mutant, the M intermediate, whose absorption maximum is about 410 nm, is stabilized at alkaline pH (Butt *et al.*, 1989), and little of the unphotolyzed protein is present under these conditions. Significant changes in some of Bragg reflections are clearly observed in Figure 1. The most prominent of these occur in the (11), (20), (40), (32), and (41) reflections, almost identical to the changes previously reported by Koch *et al.* (1991). The calculated lattice constant was increased by 0.1 Å in the photointermediate. It is obvious that this intermediate has a structure that is distinct from the unphotolyzed state.

Figure 2 shows light minus dark difference X-ray diffraction intensity profiles in the low-angle region that includes only the (11) and (20) reflections, obtained at different hydration levels. The profile was normalized to the change of the (11) reflection. It is clear that the increase of the (20) reflection becomes prominent at the lower hydration levels. For the fully hydrated film prepared by soaking, no intensity change in the (20) reflection was observed. We observed significant differences in the higher angle reflections (cf. Figure 1) as well. The differences strongly indicate that the structural change in the photointermediate is hydration-dependent. A decrease in the lattice constant under dry conditions was reported previously to be up to about 1 Å (Henderson, 1975). Such a large decrease in the lattice constant was not observed at the moderate dehydration in these measurements.

To characterize the difference in the structures obtained between relatively high and low hydration levels, we performed parallel FTIR measurements. The difference FTIR spectra of D96N mutant bR between the spectrum obtained before and after illumination are shown in Figure 3. The spectrum was measured at 283 K. Figure 3a and

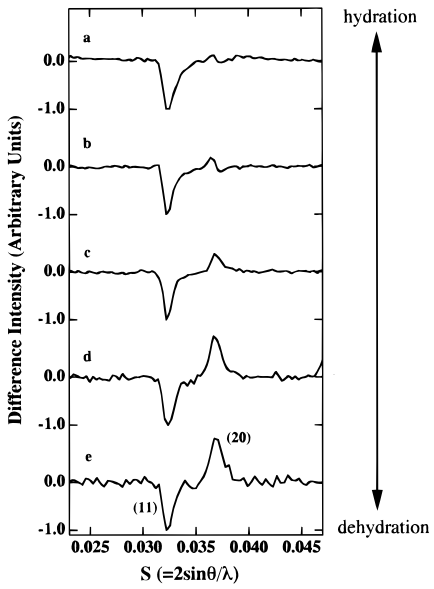

FIGURE 2: Difference X-ray diffraction profiles in the low-angle region that includes the (11) and (20) Bragg reflections. For comparison, the profiles are normalized by adjusting the amplitude of the (11) reflection to 1. Profiles a−e were obtained from samples of various hydration levels as follows: (a) fully hydrated sample obtained by soaking; (b) fully hydrated sample obtained by placing a drop of buffer solution for 1 h; (c) 95% relative humidity; (d) 81% relative humidity; and (e) 76% relative humidity.

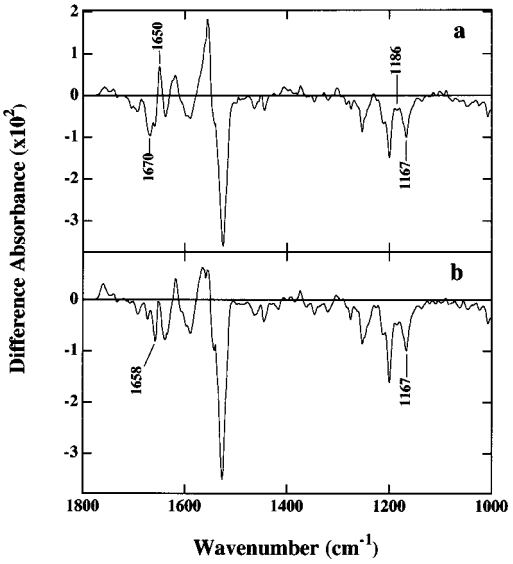

FIGURE 3: Difference FTIR spectrum of D96N bR at pH 10 in the 1800−1000 cm^{-1} region after irradiation (>500 nm) at 283 K for 30 s, for fully hydrated sample (a) and partially dehydrated sample with 81% relative humidity (b).

Figure 3b show the difference spectrum for a relatively high level and a relatively low level of hydration, respectively. Both samples were irradiated with >500 nm light for 30 s, and the spectrum of the photolyzed state was measured between 5 and 60 s after the illumination. The experimental condition is essentially the same as that for X-ray diffraction measurements, except for the continuous illumination for diffraction and the use of the much thinner film for FTIR. The two spectra in (a) and (b) are significantly different. In Figure 3a, there is a pair of positive and negative amide I bands at 1650 and 1670 cm^{-1}, respectively, which are characteristics of the N-type protein conformation (Pfefferlé *et al.*, 1991; Ludlam *et al.*, 1995). However, the large

positive band at 1186 cm^{-1}, due to the chromophore of the N intermediate with a protonated Schiff base, was not observed. Therefore, the photointermediate of hydrated D96N mutant bR is the MN intermediate with the N-type conformation despite its deprotonated Schiff base. It is the same photoproduct reported by Sasaki *et al.* (1992). In contrast, the amide I bands in Figure 3a are absent at a relatively low hydration. The difference spectrum under these conditions (Figure 3b) is essentially identical to the difference spectrum of the M intermediate obtained for wild-type bR at 230 K. Thus, the photointermediate of partially dehydrated D96N is the true M intermediate with an M-type conformation (by the FTIR criterion) and a deprotonated Schiff base.

The FTIR measurements with D96N bR strongly suggest that the difference in diffraction profile shown in Figure 2 is attributable to the M-type and the N-type conformations. We have already observed and reported the difference of the change in the relative difference intensity of the (20) reflection to that of (11). The increase of the (20) reflection at the M intermediate was clearly visible for arginine-treated wild-type bR (Nakasako *et al.*, 1991), while there was almost no change in the (20) reflection for the N intermediate (Kamikubo *et al.*, 1996). The M intermediate of D96N at alkaline pH, which is now designated as the MN intermediate, also exhibits little change in the (20) reflection (Kataoka *et al.*, 1994). Therefore, it seems that the change in the (20) reflection is an indication of whether the M-type or the N-type conformation is produced. Recently, Vonck (1996) and Sass *et al.* (1997) have claimed that the M intermediate accumulated by arginine treatment of the wild-type protein is the MN state. However, our FTIR measurements clearly indicate that the photointermediate of arginine-treated wild-type bR is a true M (manuscript in preparation). This discrepancy could be explained by the different levels of hydration, and by the fact that the arginine treatment can also stabilize the N intermediate.

Figure 4a,b shows difference electron density maps calculated from the average of the separated Bragg intensities in Figure 2b,c, and in Figure 2d,e, respectively, which correspond to the conditions in Figure 3a,b. The data of Figure 2a were not used for the calculation because of a broad reflection width caused by disorder in the membrane orientation. Many positive and negative peaks especially around helices B, F, and G in both maps are observable, which are very similar to the various difference maps for the M and N intermediates reported so far (Dencher *et al.*, 1989; Koch *et al.*, 1991; Nakasako *et al.*, 1991; Subramaniam *et al.*, 1993; Kamikubo *et al.*, 1996; Vonck, 1996). It should be noted that the small but essential differences reported before in the positive peaks around helices B and F between the M and the N structures are evident in these maps. The most prominent positive peak is near helix F in the hydrated sample (Figure 4a), while it is in the vicinity of helix B in the partially dehydrated sample (Figure 4b). This difference is the characteristic structural change between the M and N intermediates, as we reported previously (Nakasako *et al.*, 1989; Kamikubo *et al.*, 1996). Thus, the difference Fourier maps also suggest that under relatively lower levels of hydration the M-type conformation is accumulated, while at higher levels of hydration the N-type conformation is formed. Recently, a similar study of the hydration dependence of the conformation of the M states in the D96N mutant was published (Sass *et al.*, 1997), but it reports only on the presence and/or the absence of conformational change by X-ray diffraction at hydration levels between 100% and as low as 57%, and interprets these results in terms of the M1−M2 transition.

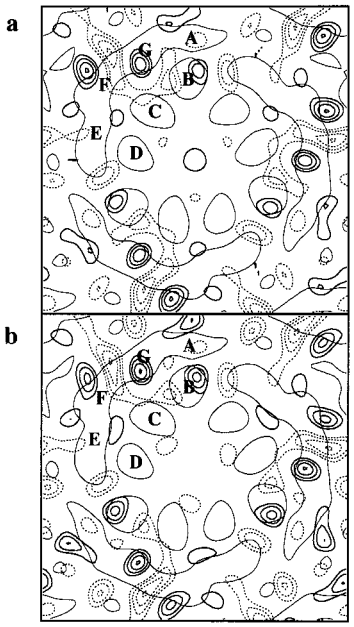

FIGURE 4: Two-dimensional difference Fourier maps projected to the membrane normal. Difference maps between the M intermediate and initial state at pH 10 in hydrated D96N bR (MN) (a) and in partially dehydrated D96N bR (M) (b). Two diffraction profiles corresponding to Figure 2b,c were combined and normalized to obtain map (a), and the diffraction profiles of Figure 2d,e were combined for map (b). Thick contour lines indicate positive density changes, where the electron density is increased in the M intermediate; dashed contours indicate negative density changes.

Table 1: Comparison of the Intensity Changes Observed for M, N, and MN in Previous X-ray Diffraction Studies with the Intensity Changes Observed for M of Hydrated and Dehydrated D96N in This Study

data (previous study)	data (this study)	correlation coefficient
N[a]	D96N (hydrated)	0.95
N[a]	D96N (dehydrated)	0.87
M[b]	D96N (hydrated)	0.84
M[b]	D96N (dehydrated)	0.90
MN[c]	D96N (hydrated)	0.92
MN[c]	D96N (dehydrated)	0.81

[a] Data are taken from Kamikubo *et al.* (1996). [b] Data are taken from Nakasako *et al.* (1991). [c] Data are taken from Koch *et al.* (1991).

The two sets of intensity changes, obtained at low and high hydration, in this study are compared in Table 1 with the changes obtained for the M intermediates of arginine-treated wild-type bR (Nakasako *et al.*, 1991), the D96N mutant (Koch *et al.*, 1991), and the N intermediate of the F171C mutant (Kamikubo *et al.*, 1996). The intensity changes in the hydrated samples are almost identical to those in the M intermediate of D96N (Koch *et al.*, 1991) and the N intermediate (correlation coefficients 0.95 and 0.92, respectively), consistent with the idea that the M intermediate accumulated in D96N mutant bR at alkaline condition has N-type conformation in spite of the unprotonated Schiff base. In contrast, the intensity changes in the partially dehydrated samples resemble those in the M intermediate of arginine-treated wild-type bR (correlation coefficient 0.90). The other

correlation coefficients are lower. The result again demonstrates that the M intermediate stabilized under partially dehydrated condition has the M-type conformation; i.e., it is the true M rather than the MN intermediate.

DISCUSSION

We observed two types of structural changes in the photointermediate with deprotonated Schiff base, stabilized at alkaline pH in the D96N mutant: the M-type structural changes in partially dehydrated samples and the N-type structural changes in well-hydrated samples. This observation suggests that there is an equilibrium between the M-type and the N-type structures, and this equilibrium is affected by hydration.

We had earlier clarified the structural changes in the M and the N intermediates, and suggested that the two structures are similar but there is a small difference between them that is significant (Kamikubo *et al.*, 1996). In these studies, the photointermediates were stabilized in different kinds of samples. In the case of the M intermediate, we used arginine-treated wild-type bR, and for the N intermediate the F171C mutant. Therefore, it could not be firmly stated that the proposed structural transition from the M-type to the N-type structure can occur in the same sample. Likewise, we have never been able to precisely compare the electron density changes in the N intermediate with those in the M intermediate and/or the MN intermediate.

In this study, we demonstrate that it is possible to produce the two distinct conformations in the photoreaction of the same protein. We can now compare the absolute values of the changes. The *R*-factors of the separated Bragg reflection intensity changes used in the calculation of the electron density maps (Figure 4a,b) are 6.5% for M of dehydrated D96N and 8.5% for MN of hydrated D96N, respectively. The *R*-factors reported before are 7% for M of arginine-treated wild-type bR and 9% for MN of D96N mutant bR (Koch *et al.*, 1991). The values are thus in good agreement with the previous results. The greater *R*-factor suggests that larger density changes occur in the N-type structure than in the M-type. In fact, the intervals of the contours in Figure 4a (N-type structure) are larger than those in Figure 4b (M-type structure) by a factor of about 1.6, suggesting that in the N state the structural changes have a larger amplitude than in the M state, rather than only a different shape. This could explain that the amide I bands in N and MN are quite large although the bands cannot be clearly seen in M (Figure 3). However, the amide bands do not necessarily accompany the structural changes detected by diffraction (Brown *et al.*, 1997).

Recently, Sass *et al.* (1997) also reported the hydration dependence of the conformational change of D96N bR with FTIR and X-ray diffraction. Although they examined a wider hydration range than in our measurements, they described only whether the structural change (which they refer to as the M1 to M2 transition) occurs or not. They report that it does not occur at 57% humidity, a hydration level lower than we employed. However, otherwise their results seem to be consistent with our observations. Their difference electron density map at 75% relative humidity [Figure 2 in Sass *et al.* (1997)], which they ascribed to M2, is rather close to our partially hydrated map (Figure 4b). The characteristic features that discriminate the M-type conformation from the N-type conformation are the large positive peak near helix B and the distribution of negative region around helix G. Their FTIR spectrum at 75% relative humidity contains more M-like properties than those at 100% relative humidity [Figures 4 and 5 in Sass *et al.* (1997)]. Therefore, what they observed at 75% relative humidity would be the M-type conformation. Apparently, their FTIR spectrum at 100% relative humidity indicated the N-type conformation. It is plausible that they would observe the difference electron density map at 100% relative humidity which is similar to our map with full hydration (Figure 4a).

How does hydration affect the structural equilibrium between M and N? One of the possible hypotheses is that it is the physical properties of unphotolyzed bR which are changed upon dehydration, for example, its static and/or dynamic structure. But there were no remarkable structural changes including changes in the lattice constant during the dehydration under our experimental conditions. Changes of protein internal dynamics in dry PM by inelastic neutron scattering were reported (Ferrand *et al.*, 1993). This study suggested that large-amplitude anharmonic atomic motions, which play a central role in the function of this protein, disappeared upon dehydration. They suggested that the structural transition from M1 to M2 is inhibited without the possibility of such anharmonic motion (Ferrand *et al.*, 1993). These experiments were performed under extremely dry conditions, in which structural changes in the photocycle could not be seen at all (Glaeser *et al.*, 1985). This should be what was observed by Sass *et al.* (1997).

The alternative possibility is a perturbation of an equilibrium between the two protein conformations, caused by differing stabilities of the M-type and the N-type conformations at different levels of hydration. The results indicate that in this case the transition between the two conformational states occurs only at a hydration of about 85% relative humidity or higher. A neutron diffraction study of the location of the water of hydration in purple membranes suggested that at 85% relative humidity only water molecules at the surface are withdrawn, while internal water remains unaffected (Zaccai, 1987; Papadopoulos *et al.*, 1990). Therefore, the stability of the N-type structure must be connected with water molecules at the surface. This could arise if the N-type structural changes include an increase of the binding of water molecules at the surface area of bR, while the M-type structural changes are more internal and less affected by surface-bound water. It was previously reported that the hydration levels influence the photocycle of bR. The M to N step is specifically inhibited in partially dehydrated samples at 85% relative humidity (Váró *et al.*, 1991; Pfefferlé *et al.*, 1991; Cao *et al.*, 1991). On the basis of our results, this inhibition would be caused by a higher barrier to the formation of the N-type structure. This mechanism is consistent also with the suggestion (Brown *et al.*, 1995b; Váró et al., 1996) that the functional role of the conformational shift is that increase of the hydration inside the channel upon structural changes during the M to N transition causes a decrease of the pK_a of the proton donor Asp-96.

Participation of water in the structure of the N state would extend the model we had suggested earlier (Kataoka *et al.*, 1994). In the model, bR has at least two distinct conformations. In conformation E, the proton channel between the

Schiff base and the extracellular surface is open, while in conformation C the channel between the Schiff base and the cytoplasmic surface is open. The interconversion between conformations E and C is an essential part in the switch of the reprotonation in the photocycle. It is not sufficient for the reprotonation process, however. Since both M and N intermediates have the C conformation, there must be an additional phase of the switch which corresponds to the M to N transition. Thus, the first phase is to break the connection between the Schiff base and Asp-85 located in the extracellular side, which facilitates transition to the M-type conformation. The second phase establishes the connection between the Schiff base and Asp-96, located in the cytoplasmic side. Although the structure is conformation C at this time, the pK_a of Asp-96 is still high and the cytoplasmic proton channel might not be open so as to block proton release from Asp-96 to the cytoplasmic aqueous phase. This state corresponds to the M (M2) intermediate. Asp-96 becomes a proton donor only when the third conformation, dependent on the binding of water and detected in the MN and N intermediates, is reached. Indeed, the formation of the M-type structure is shown in this study to not be affected by partial dehydration. If the structure of M were already open to the cytoplasmic surface and the surface hydrated, its formation would be hindered by partial dehydration. We find that it is the conformational transition that follows M, that involves the change at helix F and leads to the N-type structure, that is hindered upon dehydration. This is consistent with the reprotonation of the Schiff base being dependent on the decrease in the pK_a of Asp-96 by the binding of water molecules to the more open cytoplasmic surface.

We have some extent of experimental evidence, therefore, for the three proposed phases of the accessibility change in bR. One is the disconnection of the Schiff base from the extracellular side (Kataoka *et al.*, 1994). This process depends on the rise in the pK_a of Asp-85 upon release of a proton to the extracellular surface (Balashov *et al.*, 1996; Richter *et al.*, 1996). The other is the structural change of the N-type that involves hydration of a more open cytoplasmic surface and allows proton transfer from Asp-96 to the Schiff base, as demonstrated here. We suggest that together with the third component, the internal reorientation of the Schiff base from the extracellular to the cytoplasmic channel that may be associated with the structural change of the M-type, these steps account for the vectoriality of the proton transport in the photocycle.

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REFERENCES

Amemiya, Y., Wakabayashi, K., Hamanaka, T., Wakabayashi, T., Matsushita, T., & Hashizume H. (1983) *Nucl. Instrum. Methods 208*, 471−477.
Balashov, S. P., Imasheva, E. S., Govindjee, R., & Ebrey, T. G. (1996) *Biophys. J. 70*, 473−481.
Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., & Lanyi, J. K. (1995a) *J. Biol. Chem. 270*, 27122−27126.
Brown, L. S., Váró, G., Needleman, R., & Lanyi, J. K. (1995b) *Biophys. J. 69*, 2103−2111.
Brown, L. S., Kamikubo, H., Zimányi, L., Kataoka, M., Tokunaga, F., Verdegem, P., Lugtenburg, J., & Lanyi, J. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5040−5044.
Butt, H. J., Fendler, K., Bamberg, E., Tittor, J., & Oesterhelt, D. (1989) *EMBO J. 8*, 1657−1663.
Cao, Y., Váró, G., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1991) *Biochemistry 30*, 10972−10979.
Dencher, N. A., Dresselhaus, D., Zaccai, G., & Büldt, G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7876−7879.
Ferrand, M., Dianoux, A. J., Petry, W., & Zaccai, G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9668−9672.
Glaeser, R. M., Baldwin, J., Ceska, T. A., & Henderson, R. (1985) *Biophys. J. 50*, 913−920.
Henderson, R. (1975) *J. Mol. Biol. 33*, 123−138.
Henderson, R., Baldwin, J., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899−929.
Kamikubo, H., Kataoka, M., Váró, G., Oka, T., Tokunaga, F., Needleman, R., & Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1386−1390.
Kataoka, M., Kamikubo, H., Tokunaga, F., Brown, L. S., Yamazaki, Y., Maeda, A., Sheves, M., Needleman, R., & Lanyi, J. K. (1994) *J. Mol. Biol. 243*, 621−638.
Koch, M. H., Dencher, N. A., Oesterhelt, D., Plöhn, H.-J., Rapp, G., & Büldt, G. (1991) *EMBO J. 10*, 521−526.
Lanyi, J. K. (1993) *Biochim. Biophys. Acta 1183*, 241−261.
Lanyi, J. K. (1995) *Nature 375*, 461−463.
Ludlam, C. F. C., Sonar, S., Lee, C.-P., Coleman, M., Herzfeld, J., RajBhandary, U. L., & Rothschild, K. L. (1995) *Biochemistry 34*, 2−6.
Mathies, R. A., Lin, S. W., Ames, J. B., & Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 491−518.
Nakasako, M., Kataoka, M., Amemiya, Y., & Tokunaga, F. (1991) *FEBS Lett. 292*, 73−75.
Needleman, R., Chang, M., Ni, B., Váró, G., Fornes, J., White, S. H., & Lanyi, J. K. (1991) *J. Biol. Chem. 266*, 11478−11484.
Ni, B., Chang, M., Duschl, A., Lanyi, J. K., & Needleman, R. (1990) *Gene 90*, 169−172.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667−678.
Papadopoulos, G., Dencher, N. A., Zaccai, G., & Büldt, G. (1990) *J. Mol. Biol. 214*, 15−19.
Pfefferlé, J. M., Maeda, A., Sasaki, J., & Yoshizawa, T. (1991) *Biochemistry 30*, 6548−6556.
Richter, H. T., Brown, L. S., Needleman, R., & Lanyi, J. K. (1996) *Biochemistry 35*, 4054−4062.
Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.
Sasaki, J., Shichida, Y., Lanyi, J. K., & Maeda, A. (1992) *J. Biol. Chem. 267*, 20782−20786.
Sass, H. J., Schachowa, I. W., Rapp, G., Koch, M. H. J., Oesterhelt, D., Dencher, N. A., & Büldt, G. (1997) *EMBO J. 16*, 1484−1491.
Subramaniam, S., Gerstein, M., Oesterhelt, D., & Henderson, R. (1993) *EMBO J. 12*, 1−8.
Váró, G, & Lanyi, J. K. (1991) *Biophys. J. 59*, 313−322.
Vonck, J. (1996) *Biochemistry 35*, 5870−5878.
Zaccai, G. (1987) *J. Mol. Biol. 194*, 569−572.

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