The Role of Water in the Extracellular Half Channel of Bacteriorhodopsin

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ABSTRACT The changes in the photocycle of the wild type and several mutant bacteriorhodopsin (D96N, E204Q, and D212N) were studied on dried samples, at relative humidities of 100% and 50%. Samples were prepared from suspensions at pH ~5 and at pH ~9. Intermediate M with unprotonated Schiff base was observed at the lower humidity, even in the case where the photocycle in suspension did not contain this intermediate (mutant D212N, high pH). The photocycle of the dried sample stopped at intermediate M_1 in the extracellular conformation; conformation change, switching the accessibility of the Schiff base to the cytoplasmic side, and proton transport did not occur. The photocycle decayed slowly by dissipating the absorbed energy of the photon, and the protein returned to its initial bacteriorhodopsin state, through several M_1-like substates. These substates presumably reflect different paths of the proton back to the Schiff base, as a consequence of the bacteriorhodopsin adopting different conformations by stiffening on dehydration. All intermediates requiring conformational change were hindered in the dried form. The concentration of intermediate L, which appears after isomerization of the retinal from all-*trans* to 13-*cis*, during local relaxation of the protein, was unusually low in dried samples. The lack of intermediates N and O demonstrated that the M state did not undergo a change from the extracellular to the cytoplasmic conformation (M_1 to M_2 transition), as already indicated by Fourier transform infrared spectroscopy, quasielastic incoherent neutron scattering, and electric signal measurements described in the literature.

INTRODUCTION

The purple membranes (PMs) seen as patches in the cell membrane of *Halobacterium salinarum* represent a highly ordered two-dimensional lattice of bacteriorhodopsin (BR) molecules embedded in lipids. BR, an integral membrane protein, belongs to the family of retinal proteins; it converts light energy into electrochemical energy, creating a proton gradient across the membrane (Stoeckenius et al., 1979). Despite the large number of experimental data that have been collected over more than two decades, which have permitted a detailed characterization of its structure and function (Oesterhelt et al., 1992; Krebs and Khorana, 1993; Lanyi, 1993; Lanyi and Váró, 1995), the mechanism of proton pumping in BR is not yet fully elucidated. The photocycle of BR is described by several spectrally distinct intermediates: after absorbing a photon, the excited state relaxes to the intermediate K and decays thermally back to its initial form BR, through subsequent intermediates: L, M, N, and O (Lozier et al., 1975). Almost all of the intermediates are split into spectroscopically identical substates (Váró and Lanyi, 1990; Cao et al., 1993; Gergely et al., 1993; Zimányi et al., 1993; Chizhov et al., 1996), which implies that the actual transport process is much more complex. The major functional steps are as follows: photoisomerization of the retinal from the all-*trans* to the 13-*cis* conformation; transfer of the proton to the internal proton acceptor from the Schiff base (SB) linking the chromophore and the protein, with subsequent proton release to the extracellular side; reprotonation of the SB from the cytoplasmic site, via the proton donor; and finally, the 13-*cis* to all-*trans* reisomerization of the retinal, bringing the protein back to its initial form, BR. A three-dimensional structural model of the protein (Henderson et al., 1990; Grigorieff et al., 1996) helps in the construction of the proton pathway from the cytoplasmic to the extracellular side. This indicates that the retinal SB separates the conducting pathway into two domains: the extracellular and the cytoplasmic half-channels, which contain the main amino acid residues that are presumably involved in the transport mechanism.

A powerful tool for clarification of the main steps involved in the proton transfers became available with the introduction of site-directed mutagenesis, which allowed identification of amino acid D85 in the extracellular half-channel as the proton acceptor from the SB, and amino acid D96 in the cytoplasmic half-channel as the proton donor (Mogi et al., 1988; Butt et al., 1989; Thorgeirsson et al., 1991).

Studies have revealed that water has an important role in the normal functioning of BR. The kinetics of the photocycle is modified as the humidity of the sample changes (Korenstein and Hess, 1977a; Váró and Keszthelyi, 1983). In BR films at humidities below 90%, intermediate O was no longer observable, the M rise accelerated, the M decay slowed down, and there was less proton transfer across the protein (Korenstein and Hess, 1977a; Váró and Keszthelyi, 1983; Kovács and Váró, 1988). The thermal *cis-trans* isomerization equilibrium was also modified (Korenstein and Hess, 1977b). As the sample was dried, the ratio of the forward and reverse photocycle reactions changed signifi-

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cantly, indicating that the differences in free energy of the intermediates altered with dehydration (Váró and Lanyi, 1991). For vacuum-dried samples, the spectrum of BR was shifted to 530 nm, and the photocycle stopped before intermediate M (Hildebrandt and Stockburger, 1984; Kovács and Váró, 1988). Dehydration led to changes in the surface water content of the PM, associated with a closer packing of the lipid headgroups (Zaccai and Gilmore, 1979; Rogan and Zaccai, 1981; Jaffe and Glaeser, 1987; Zaccai, 1987). Consequently, the protein motions are inhibited by a more closely packed environment (Zaccai, 1987). Fourier transform infrared (FTIR) studies (Váró and Eisenstein, 1987) and quasielastic incoherent neutron scattering (Fitter et al., 1996) demonstrated that a decrease in the hydration state results in a decrease in internal molecular flexibility of the protein.

A number of investigations in recent years, on wild-type and mutated BR samples, have provided substantial evidence concerning the involvement of water in the main stages of the proton translocation process. A neutron diffraction study revealed the presence of four tightly bound water molecules close to the chromophore-binding site of BR (Papadopoulos et al., 1990). Hydrogen/deuterium exchange followed by a resonance Raman technique suggested the presence of structural water molecules at the retinal binding site (Deng et al., 1994). From FTIR difference spectroscopy on mutant BR, it was concluded that at least one water molecule undergoes an increase in H bonding during the BR-K photoreaction, and this water is located near the active site (Fischer et al., 1994). Its position is altered for intermediates L and M of the photocycle, and an additional water molecule may undergo a decrease in H-bonding during the K-L and L-M transitions. FTIR studies on mutants T46V and D96N showed that water from the cytoplasmic D96-T46 domain undergoes structural changes in intermediate L, and these changes are transmitted to the extracellular domain, affecting the interaction of the SB with Asp at position 85 (Maeda et al., 1992; Yamazaki et al., 1995).

Not only experimental studies, but also theoretical modeling based on molecular dynamic calculations (Humphrey et al., 1994, 1997; Logunov et al., 1995) indicate that the normal function of BR requires the presence of several water molecules in the proton-conducting channel. The structural model (Henderson et al., 1990) allows the presence of water in both extracellular and cytoplasmic half-channels.

To contribute to the elucidation of the role of the water in different steps of the photocycle, we set out to study the effects of partial dehydration on wild-type and several mutant BRs: D96N, E204Q, and D212N.

MATERIALS AND METHODS

PM suspensions of the BR mutants D96N, E204Q, and D212N, expressed in *H. salinarum* strain L-33, were supplied by J. K. Lanyi. The suspensions were centrifuged and the pellets were resuspended in tridistilled water to give an optical density of ~20. The pH of the suspension was ~5. The suspension was divided into two parts, and in one part the pH was raised, by adding concentrated NaOH, to ~9. Samples were prepared by drying a droplet from each suspension on a clean glass surface at room temperature. Although we refer to the samples as low- and high-pH samples, no information is available as to their actual pH; only their absorption kinetic properties provide a hint about this, as described in the results, with the 100% humidity samples behaving like the suspensions they were prepared from.

The dried films, with an optical density of ~1, were sealed in a sample holder, in which the humidity was controlled with saturated salt solution as described previously (Pethig, 1979; Váró and Eisenstein, 1987). Two values were chosen: 50% (referred to as the dry sample) and 100% relative humidity (wet sample). For 50% relative humidity, the samples were kept overnight at room temperature in the vapor of a saturated $Ca(NO_3)_2$ solution, and for 100% relative humidity, they were kept in the vapors of distilled water. The measurements were performed at 20°C with a set-up described earlier (Váró and Keszthelyi, 1983). Optical absorption kinetic data at five wavelengths (410, 500, 570, 610, and 650 nm) were recorded after laser excitation on a logarithmic time scale covering the interval from 100 ns to 10 s.

Published data (Needleman et al., 1991; Zimányi and Lanyi, 1993; Gergely et al., manuscript submitted for publication) and our own unpublished measurements were used to estimate the extinction coefficients of the intermediates at the measured wavelengths. With the aid of the program RATE, a model was fit to the data, and the rate constants and the relative concentrations of the intermediates were calculated as described elsewhere (Gergely et al., 1993; Váró et al., 1995).

RESULTS

The kinetics of the wild-type BR in the suspension and in the wet sample were almost the same (Fig. 1, *A* and *B*) (the 610-nm trace is not shown). When the sample was dried to 50% humidity, characteristic changes were observed in the kinetics (Fig. 1 *C*). The rising part became faster, whereas the decay was much slower and strongly multiphasic. Intermediate O disappeared, whereas the signal measured at 650 nm showed that intermediate K existed for a longer time, indicating an equilibrium between the early and late parts of the photocycle, even in the sample prepared from the suspension at high pH (Fig. 1 *D*). The fit for the model (Váró and Lanyi, 1990; Lanyi and Váró, 1995)

$$K \Leftrightarrow L \Leftrightarrow M_1 \Leftrightarrow M_2 \Leftrightarrow N \Leftrightarrow O \Rightarrow BR \qquad (m1)$$

gave rather similar concentrations for the suspension (Fig. 2 *A*) and the wet sample at low pH (Fig. 2 *B*). The fits were so good that the measured and fitted lines could hardly be distinguished (not shown), and the goodness of the fit characterized by the standard deviation was under 10^{-3}. For the dried samples, another model was considered (Váró and Keszthelyi, 1983, 1985; Váró and Lanyi, 1991), which did not contain intermediates after M_1. Of the several possible models initially contemplated, the one finally accepted was

$$K \Leftrightarrow L \Leftrightarrow M_1 \Leftrightarrow M_1' \Rightarrow BR \text{ and } M_1 \Leftrightarrow M_1'' \Rightarrow BR \qquad (m2)$$

The fit here was not as good as for the wet samples (Fig. 3 *A*, *dotted line*), but this was expected from the changes in the spectrum of BR and possibly in the spectra of the intermediates too, when the humidity was lowered (Lazarev and Terpugov, 1980). The exact extinction of the interme-

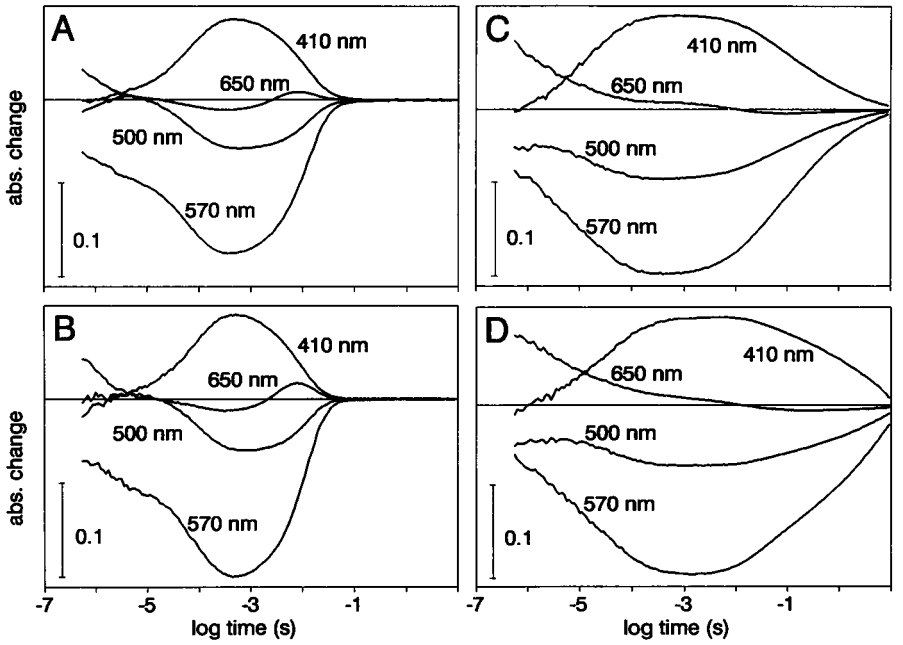

FIGURE 1 Optical absorption changes in wild-type bacteriorhodopsin measured at different wavelengths. (*A*) Suspension at pH ~5 in distilled water. (*B*) Dried sample prepared from the suspension at pH ~5, at 100% relative humidity. (*C*) Same as *B*, but at 50% relative humidity. (*D*) Dried sample prepared from the suspension at pH ~9, at 50% relative humidity.

diates in the dried samples was not determined, as this was not the aim of the present study, but was taken from the studies made on suspension. Even so, the fit was acceptable and described the phenomenon under investigation. The use of three spectrally indistinguishable intermediates M_1 in the model will be discussed later. M_1 contains deprotonated SB, without switch-like conformational change in the protein (Váró and Lanyi, 1990). The absence of the late intermediates (N and O) and the absence of the conformational change, measured by FTIR and neutron scattering (Váró and Eisenstein, 1987; Fitter et al., 1996), indicate that the photocycle stops at intermediate M_1.

The properties of mutant samples prepared at either low or high pH were similar in the suspension and the wet sample; only slight changes in reaction rates were observed. A very interesting feature appeared with the change to lower humidity: although some of the mutants had strongly different photocycles, relative to that of the wild-type in the suspension, all of them displayed very similar characteris-

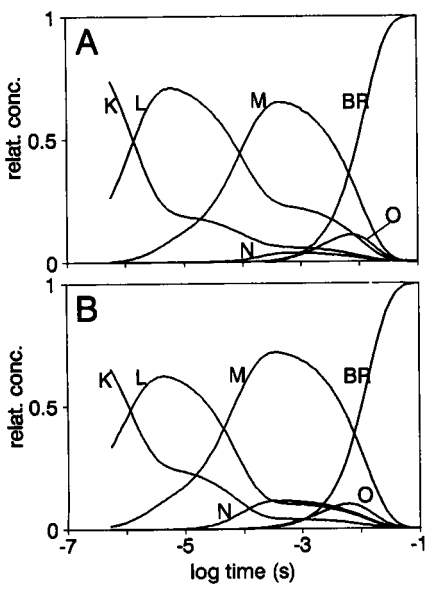

FIGURE 2 Concentration changes of the intermediates in wild-type bacteriorhodopsin. In addition to the measurements shown in *A* and *B*, the 610-nm signal was included in the fit. The relative concentration 1 means the amount of bacteriorhodopsin excited by the laser flash.

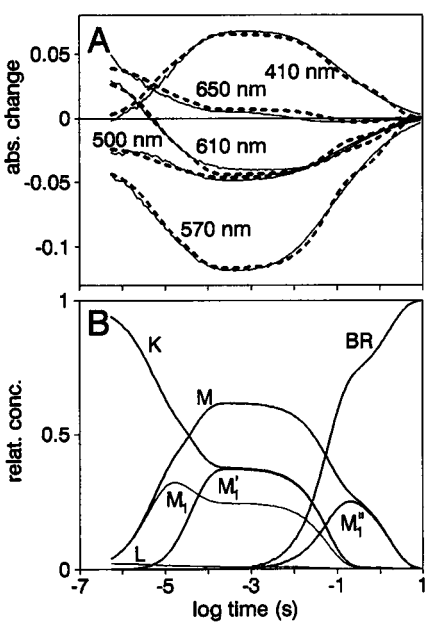

FIGURE 3 (*A*) The fit to all five wavelengths and (*B*) the concentrations of the intermediates of the dried sample of the measurement presented in Fig. 1 *C*.

TABLE 1 Time constants of the photocycle reactions of dried samples at 50% relative humidity for wild-type and mutant bacteriorhodopsin

Reaction	WT low pH	WT high pH	D96N low pH	D96N high pH	E204Q low pH	D212N low pH	D212N high pH
K → L (μs)	3.16	3.2	3.21	3.24	3.1	6.35	5.0
L → K (μs)	0.78	0.34	1.65	1.02	0.96	5.9	2.55
L → M_1 (μs)	3.98	3.7	3.66	3.72	3.9	2.44	0.33
M_1 → L (μs)	16.2	27.9	3.36	12.6	26.5	0.16	0.0013
M_1 → M_1' (μs)	153	200	29.9	119	177	6.6	0.027
M_1' → M_1 (μs)	91	200	83.6	123	209	49	34.7
M_1 → M_1'' (ms)	67.8	73	38.1	116	17.5	0.017	0.0023
M_1'' → M_1 (ms)	1100	2650	1590	3810	520	0.18	0.13
M_1' → BR (ms)	16	48	43	95	780	130	0.54
M_1'' → BR (ms)	∞	∞	∞	∞	8.5	12	∞

tics at 50% humidity. All of them could be fitted with the same model (m 2), that applied for the wild-type low-humidity sample. The fits were similar in quality to that for the wild-type dried sample, for the same reason as described above. The rate constants of the transitions between the intermediates of the dried samples are listed in Table 1.

The photocycle of mutant D96N in the wet state was much slower at high pH than at low pH (compare curves W,H and W,L in Fig. 4), as is known from the literature (Tittor et al., 1989; Cao et al., 1991; Zimányi and Lanyi, 1993). Both cases gave a good fit to the model from the literature:

$$K \Leftrightarrow L \Leftrightarrow M_1 \Leftrightarrow M_2 \Rightarrow BR \qquad (m3)$$

In the dried state, both the rise and the decay of the photocycle were almost indistinguishable from the corresponding wild-type kinetics (compare curves D,L and D,H in Fig. 4 with the analogous curves in Fig. 1, *C* and *D*). It is interesting that, when the high pH sample was dried, the decay of the photocycle apparently became faster (compare curves W,H and D,H in Fig. 4).

The rising part of the photocycle of mutant E204Q was not strongly influenced by drying (Fig. 5); even in the wet form, this behaved in the same way as the dried sample of the wild type. The decay part of the photocycle for the wet sample revealed a high content of O intermediate (Fig. 5 *A*), as in the suspension (Brown et al., 1995), and the model applied to the wild type gave a good fit (not shown). The photocycle of the dried sample was slower than that for the wild type (Fig. 5 *B*), but no O was observed either.

The kinetics for mutant D212N in suspension was markedly different from that for the wild type (Fig. 6). There was no intermediate M at high pH, or only a small part of the photocycle passed through M at low pH (Needleman et al., 1991). A strong equilibrium was observed between the L- and N-like intermediates. The samples of this mutant in the wet state were not stable. After a longer period of excitation, a small absorption appeared at 410 nm (Fig. 6 *A*), but the kinetics was very similar to that for the suspension. When the sample was dried to lower humidity and reequilibrated to 100%, the absorption change at 410 nm increased. To avoid such changes, we used freshly prepared samples for every measurement. The model used for the wet samples was that from the literature:

$$K \Leftrightarrow L \Leftrightarrow N \Rightarrow BR \qquad (m4)$$

When either low- or high-pH samples were dried, the M form appeared (Fig. 6, *B* and *C*), and, although the photo-

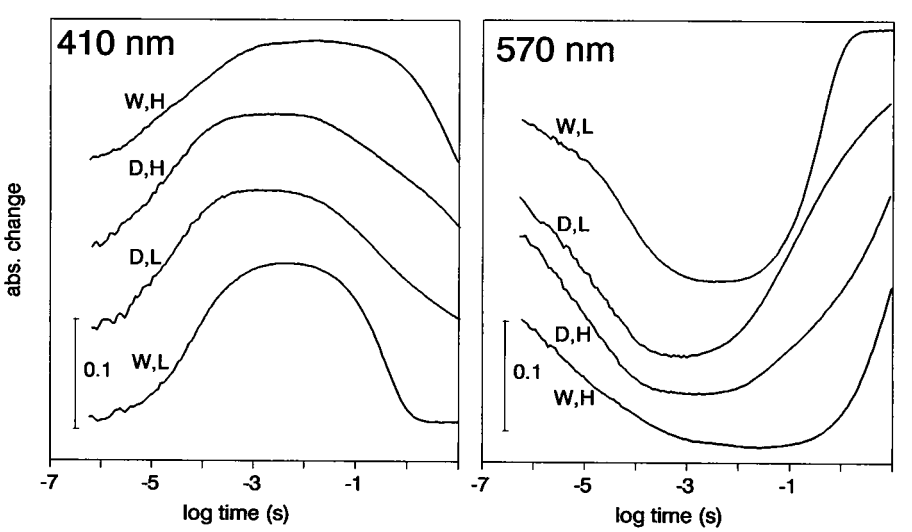

FIGURE 4 Optical absorption changes for mutant D96N at 410 nm (*A*) and 570 nm (*B*). W,L, Sample prepared from the suspension at approximately pH 5 and measured at 100% relative humidity. D,L, Same sample, but at 50% relative humidity. W,H, Sample prepared from the suspension at pH ~9 and measured at 100% relative humidity. D,H, Same sample, but at 50% relative humidity.

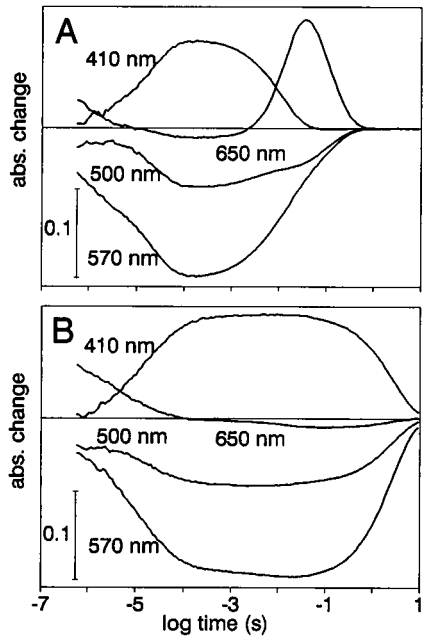

FIGURE 5 Optical absorption changes for mutant E204Q. (*A*) Sample prepared from the suspension at pH ~5 and measured at 100% relative humidity. (*B*) Same sample, but at 50% relative humidity.

cycle was rather fast, it had the same features as those of the wild type and fitted the model (m 2) used for dried samples (see Table 1). As the photocycle was of a totally different character, after the sample had been dried, we were interested to learn whether this was only a hydration effect or whether the dehydration produces some other kind of alteration in the protein. Another way to induce mild dehydration is to use an osmotically active agent such as glycerol, which withdraws solute-inaccessible water from BR (Cao et al., 1991). Fig. 6 *C* depicts the kinetics for mutant D212N measured in 70% glycerol. It was very reassuring that the same qualitative changes were observed as when the sample was dried, although they differed quantitatively.

DISCUSSION

As pointed out in the Results, the model used to fit the data for the wild type and all mutant samples at 100% humidity was that taken from the literature. For the dried samples, we searched for a unique model for the wild type and the mutants, as the kinetics exhibited common features in all cases. Different models were considered, involving several M intermediates, based on the literature finding, mainly relating to wild-type BR, that there are no intermediates after M and its decay is strongly multiphasic (Korenstein and Hess, 1977a; Váró and Keszthelyi, 1983). Another argument in favor of not considering intermediates N and O in the second part of the photocycle for the dried samples is the time dependence of the ratio of the absorbance changes at 410 and 570 nm (Fig. 7). At 410 nm only the M intermediate displays a considerable positive absorption; in contrast, at 570 nm all of the intermediates exhibit a negative relative absorption. For the wild-type wet sample there is an increase in the ratio in the second part of the photocycle, which correlates with the appearance of intermediates N and O. This feature is totally missing at 50% humidity (see Fig. 7, *top*). The monotonous decrease in the ratio for almost all of the dried samples (Fig. 7, *bottom*) means that the content of intermediate M is constantly increasing in the second part of the photocycle, and no other intermediate is accumulating. This increase in the M intermediate can be seen in the fit for the wild-type dried sample (Fig. 3 *B*). Only mutant E204Q furnished a constant ratio, showing that no change occurs in the intermediate composition in this time interval, i.e., the intermediates are in equilibrium. The following two models, with equal numbers of independent parameters, gave fits of the same quality:

$$K \Leftrightarrow L \Leftrightarrow M_1 \Leftrightarrow M_1' \Rightarrow BR \text{ and } M_1 \Leftrightarrow M_1'' \Rightarrow BR \qquad \text{(m2)}$$

$$K \Leftrightarrow L \Leftrightarrow M_1 \Leftrightarrow M_1' \Leftrightarrow M_1'' \Rightarrow BR \qquad \text{(m2a)}$$

These models were better than other models with the same number of parameters. We accepted model m 2 because the multiplicity of intermediates M_1 originates from the fact that the dried protein has a reduced flexibility and is fixed in different conformations (Váró and Eisenstein, 1987; Fitter et al., 1996), so that the sample becomes inhomogeneous. The proton can move back to its original position via several different pathways, which leads to multiplicity of the decaying pathways, described here by two parallel M_1 intermediates. When more parallel M_1 substates were introduced, the quality of the fit could be improved, but without any real gain in information. In our opinion model m 2 is a first approximation of a distributed kinetics model, where great numbers of conformations exist, and every one of them obeys the same model, with slightly different kinetic constants.

A general feature of the fits is the relatively low concentration of L in almost all of the samples. It can be seen from Table 1 that this is a result of the fact that the reactions outgoing from intermediate L are faster than those leading to L. The only exception was mutant D212N. Its photocycle for the suspension or wet sample revealed mostly L-like (L and N) intermediates after the K form. In intermediate L the Schiff base N-H undergoes strong interactions with D85, D212, and several water molecules (Maeda et al., 1991, 1992; Kandori et al., 1995). These interactions naturally cease on deprotonation of the SB in intermediate M. The appearance of intermediate L is also related to a conformational relaxation of the retinal and its surroundings. On drying, the motion of the molecules in the sample is hindered and the possibility of conformational changes decreases, and this results in a very low L concentration.

The change from Asp to Asn at position 96 has a strong effect on the kinetics for the suspension and wet samples (see the 100% humidity traces in Fig. 4). It is generally accepted that D96 is the proton donor for the SB, after the protein switched from the extracellular to the cytoplasmic

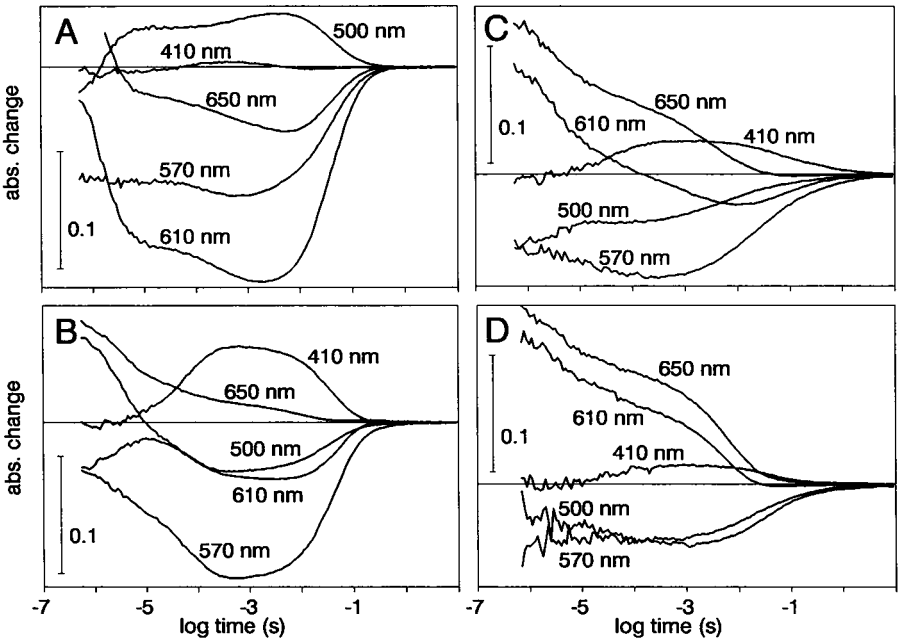

FIGURE 6 Optical absorption changes for mutant D212N. (*A*) Sample prepared from the suspension at pH ~5 and measured at 100% relative humidity. (*B*) Same sample, but at 50% relative humidity. (*C*) Sample prepared from the suspension at pH ~9 and measured at 50% relative humidity. (*D*) Sample prepared in 70% glycerol.

conformation (Mogi et al., 1988; Butt et al., 1989; Thorgeirsson et al., 1991). At high pH in wet sample, intermediate M decays much later than in the dried sample, although in all the other cases the drying slows M decay. The M decay in a suspension of the mutant D96N is known to be strongly pH dependent (Miller and Oesterhelt, 1990). The acceleration and the multiphasic form of this step after the sample has been dried show that a totally different process leads to the reprotonation in this case. The strong similarity between the kinetics at low humidity for the wild-type and mutant D96N (compare Fig. 1, *C* and *D*, and the corresponding traces in Fig. 4) also indicates that the photocycle stops at intermediate M_1 and that the SB is reprotonated from somewhere in the extracellular half-channel. D96 is linked to the SB via several intervening water molecules (Cao et al., 1991; Le Coutre et al., 1995), and the drying of the sample could remove these, hindering the reprotonation from the intracellular part of the membrane.

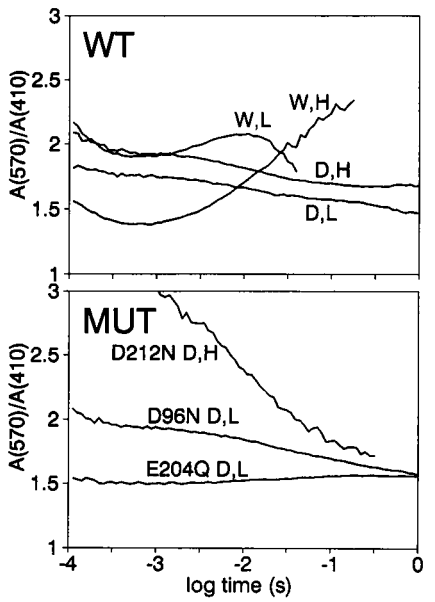

FIGURE 7 Time dependence of ratio of absorption changes measured at 570 and 410 nm for wild-type (WT) and several mutant (MUT) forms of bacteriorhodopsin. W, 100% relative humidity. D, 50% relative humidity. L, Sample prepared from the suspension at pH ~5. H, Sample prepared from suspension at pH ~9.

Amino acids D85, D212, and R82 with several coordinated water molecules constitute a diffuse counterion to the SB (De Groot et al., 1989, 1990; Dér et al., 1991). E204 seems to be connected to the SB region by a chain of H-bonded water (Brown et al., 1995). When Glu is replaced by Gln, the water structure is disturbed, and the H-bond chain is broken, which influences the kinetics of the M rise in a way that is similar to the effect of drying the wild-type sample (Fig. 5 *A*) or adding to the suspension some water-removing agent such as glycerol (Cao et al., 1991). The reduced charge at the surface of the extracellular half-channel results in the penetration of fewer water molecules, and their stabilization effect around the charges inside the protein is not so effective. The conformation of the protein is also disturbed, as reflected by the appearance of a much higher concentration of O intermediate in the second part of the photocycle of the wet sample and in suspension. When the sample is dried, the rising part does not change, but the decay of intermediate M is much slower, showing that the conformation change due to the mutation causes the reprotonation of the SB from the extracellular side to become even slower. The communication pathways between D85 and SB are hindered.

The photocycle for mutant D212N in the suspension or wet sample at high pH reveals only L (N)-like intermedi-

ates, and at low pH only a small fraction of M intermediate (Needleman et al., 1991). This shows that, when the above-mentioned counterion is disturbed, the SB cannot transfer its proton to D85, because there is an increase in the pK_a difference between the proton donor and acceptor. This change could be caused by rearrangement of the water molecules. It is known that Asp can coordinate six water molecules, but Asn only two (Franks, 1975). By losing a charge in the extracellular half-channel through changing D212 to Asn, more water molecules are made available to stabilize the charge on D85 and SB, increasing their pK_a difference. This impedes the appearance of intermediate M in the photocycle. When water is removed from the half-channel in the dried sample, or a water-removing agent like glycerol in suspension is added, intermediate M does appear (Fig. 6). The properties of the water near D85 are detected in the L and M states by FTIR (Brown et al., 1995). In the wild-type unphotolyzed protein, it is weakly H bonded (O-H stretch at 3643 cm^{-1}), but becomes somewhat more strongly bonded (shift to 3636–3638 cm^{-1}) when either D212 (Kandori et al., 1995) or E204 is replaced, which lends support to the above idea.

Water molecules bound in BR exert different effects: 1) they shield charges through solvation; 2) they participate in proton transfer reactions (Váró and Lanyi, 1991); and 3) by intercalating in the H-bond system of the amino acids of the protein, they provide flexibility for the conformational changes. With increasing degree of dehydration, the configurational motions of the retinal are accommodated less by the appropriate conformational motions of the protein, because of the restricted motion after dehydration (Zaccai, 1987). This qualitative picture provides a ready explanation for the appearance of the proposed shunt pathways for the reprotonation of the SB, which would be allowed by condensed protein conformations not highly populated at higher degrees of hydration.

The changes in the O-H stretch mode of water in the IR spectrum when D212 is replaced by N (Kandori et al., 1995) suggest that D212 is an important participant in the H bonding and is required for the proton transfer. Therefore it seems likely that water is held in a H-bonded network by R82, D212, E204, and D85, and that this network in the unphotolyzed state is characterized by a balance of opposing H bonds (Kandori et al., 1995; Brown et al., 1995). After photoexcitation, in intermediate K, the water is more strongly bound to D212 and creates the conditions in the L state for D85 to act as a proton acceptor for the SB in the L-to-M reaction and for the subsequent release of the proton to the surface. R82 and E204 have functions different from those of D85 and D212 in this network. When D85 or D212 is replaced, the complex in L becomes nonfunctional for proton transfer and the SB remains protonated. The replacement of R82 or E204 redistributes the H bonds of the bound water to favor the remaining residues and accelerates rather than abolishes the proton transfer from SB to D85.

Although the photocycle kinetics of the studied wild-type and mutant BR were different in suspension and wet sample, they become similar at 50% dehydration. This is attributed to a hindering of the conformational changes, leading to an alternative pathway, with the decay of the M_1 directly back to BR intermediate, without the late intermediates and without proton release and uptake.

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