Connectivity of the Retinal Schiff Base to Asp^{85} and Asp^{96} during the Bacteriorhodopsin Photocycle: The Local-Access Model

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ABSTRACT In the recently proposed local-access model for proton transfers in the bacteriorhodopsin transport cycle (Brown et al. 1998. *Biochemistry.* 37:3982–3993), connection between the retinal Schiff base and Asp^{85} (in the extracellular direction) and Asp^{96} (in the cytoplasmic direction) is maintained as long as the retinal is in its photoisomerized state. The directionality of the proton translocation is determined by influences in the protein that make Asp^{85} a proton acceptor and, subsequently, Asp^{96} a proton donor. The idea of concurrent local access of the Schiff base in the two directions is now put to a test in the photocycle of the D115N/D96N mutant. The kinetics had suggested that there is a single sequence of intermediates, $L \leftrightarrow M_1 \leftrightarrow M_2 \leftrightarrow N$, and the $M_2 \rightarrow M_1$ reaction depends on whether a proton is released to the extracellular surface. This is now confirmed. We find that at pH 5, where proton release does not occur, but not at higher pH, the photostationary state created by illumination with yellow light contains not only the M_1 and M_2 states, but also the L and the N intermediates. Because the L and M_1 states decay rapidly, they can be present only if they are in equilibrium with later intermediates of the photocycle. Perturbation of this mixture with a blue flash caused depletion of the M intermediate, followed by its partial recovery at the expense of the L state. The changes in the amplitude of the C═O stretch band at 1759 cm^{-1} demonstrated protonation of Asp^{85} in this process. Thus, during the reequilibration the Schiff base lost its proton to Asp^{85}. Because the N state, also present in the mixture, arises by protonation of the Schiff base from the cytoplasmic surface, these results fulfill the expectation that under the conditions tested the extracellular access of the Schiff base would not be lost at the time when there is access in the cytoplasmic direction. Instead, the connectivity of the Schiff base flickers rapidly (with the time constant of the $M_1 \leftrightarrow M_2$ equilibration) between the two directions during the entire L-to-N segment of the photocycle.

INTRODUCTION

In the light-driven pump bacteriorhodopsin, the pathway of the transported proton from the cytoplasmic (CP) to the extracellular (EC) membrane surface is known in considerable molecular detail. The first proton transfer after photoisomerization of the all-*trans* retinal to 13-*cis*,15-*anti* is from the retinal Schiff base to the anionic Asp^{85} (Braiman et al., 1988; Butt et al., 1989; Thorgeirsson et al., 1991). Protonation of Asp^{85} causes dissociation of a proton from a site that is either Glu^{204} or depends on Glu^{204} (Brown et al., 1995; Richter et al., 1996; Balashov et al., 1996; Rammelsberg et al., 1998), through mediation by Arg^{82} (Balashov et al., 1993; Brown et al., 1994b; Cao et al., 1995), and this proton is transferred to the anionic Glu^{194} (Balashov et al., 1997; Dioumaev et al., 1998), from which it is released to the EC surface. The Schiff base is then reprotonated from the CP side by the initially protonated Asp^{96} (Braiman et al., 1988; Butt et al., 1989; Gerwert et al., 1989; Otto et al., 1989). This occurs upon a large-scale protein conformation change in the CP region (Subramaniam et al., 1993; Kataoka et al., 1994; Kamikubo et al., 1996; Vonck, 1996; Thorgeirsson et al., 1997). Reprotonation of Asp^{96} from the CP surface (Zimányi et al., 1993) and reisomerization of the retinal to all-*trans* (Smith et al., 1983) follow. The proton transfer events in this "photocycle," as well as the changes in the configuration of the retinal and the conformation of the protein, are detected as interconversions of the intermediate states J, K, L, M, N, and O. The change in access of the Schiff base from the EC to the CP direction requires two consecutive M states, designated as M_1 and M_2 (Váró and Lanyi, 1991a,b). The existence of substates of M is supported by a large variety of experimental evidence (Váró and Lanyi, 1991a; Druckmann et al., 1992; Brown et al., 1994a; Dickopf and Heyn, 1997; Hessling et al., 1997; Nagel et al., 1998), which suggests that M_1 is in equilibrium with the L intermediate and M_2 with N, and therefore that they correspond to proton transfers between the retinal Schiff base and Asp^{85} and Asp^{96}, respectively.

Although there is a degree of consensus about the path of the transported proton, the principles that regulate the changing direction of the access of the buried Schiff base to the EC and CP surfaces are still debated. In the recently proposed IST model (Haupts et al., 1997; Tittor et al., 1997) the photoisomerization from all-*trans* to 13-*cis*,15-*anti* (I*) initiates a switch event (S) and a proton transfer (T) that are independent of one another. The various modes of proton transport observed in the wild type, and in Asp^{85} mutants where the photocycle of the unprotonated Schiff base results in CP-to-EC transport but a two-photon reaction causes transport in the reverse direction (Tittor et al., 1994; Ganea et al., 1998), are explained by "kinetic competition" be-

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tween S and T. Because they are not dependent on one another, either S or T may occur first. Thus the switch is conceptualized as a single and distinct, although mechanistically undefined, event that determines the EC or CP connectivity of the Schiff base. In this model it is a step common to wild-type bacteriorhodopsin and the Asp^{85} mutants, as well as to halorhodopsin and the sensory rhodopsins, related retinal proteins with transport activity.

The local-access model (Brown et al., 1998) explains these various transport modes, as well as new data on the associated photochemical reactions, with a different mechanism. The connectivity of the Schiff base to the entrances of the EC and CP half-channels is distinguished from the proton conductivities of the half-channels to the EC and CP surfaces. The local access of the Schiff base is determined by its geometry relative to the immediate proton acceptor and donor, whereas the proton conductivities of the half-channels depend on the pK_a's of groups that are in the pathway of the protons to and from the surfaces and their proximities that determine the rates of the proton transfers. In the photointermediates containing photoisomerized metastable retinal, the local access of the Schiff base flickers rapidly, with the time constant of the $M_1 \leftrightarrow M_2$ equilibrium ($\sim$30 μs) between the EC and CP directions. Therefore, in all intermediates where the retinal configuration is 13-*cis*,15-*anti*, the Schiff base is connected locally to both EC and CP half-channels. In the wild-type protein, the direction of the flow of protons is determined by the changing pK_a's of the acceptor Asp^{85} and the donor Asp^{96}, i.e., by the conduction of protons to and from the EC and CP surfaces, respectively. In the D85N/D96N mutant the situation is different, because it lacks Asp^{85} and Asp^{96}, and the proton conductivities of the half-channels are thereby fixed. (It should be noted that they are several orders of magnitude less than in the wild type.) Whereas the local access in the photointermediates is maintained in both directions as in the wild type, the proton transfer after photoisomerization happens to be more rapid in the CP than in the EC direction. Transport can occur because unlike in the photoisomerized state, the access in the thermally stable initial all-*trans* isomeric state is fixed entirely in the EC direction (Brown et al., 1998). Thus, when in the photocycle of the initially unprotonated Schiff base the retinal reisomerizes to all-*trans*, access is shifted to the slower, but in the all-*trans* form obligatory, EC pathway. This ensures that although protonation is from the CP direction, the deprotonation will be in the EC direction. The local-access model excludes kinetic competition between S and T. First, the molecular events that constitute S are not the same in the wild type and in the Asp^{85} mutants. Second, S and T are not independent of one another. Both kinds of switch events are direct consequences of a proton transfer that precedes them. The pK_a change for Asp^{85} in the wild type is caused by proton release to the EC surface (Richter et al., 1996; Balashov et al., 1996). The pK_a change for Asp^{96} is caused by a large-scale protein conformation change upon deprotonation of the Schiff base (Kataoka et al., 1994; Brown et al., 1997) that was termed "conformational shuttling" (Spudich and Lanyi, 1996). Finally, the reisomerization of the retinal to all-*trans* in the photocycles of Asp^{85} mutants, that functions in this case as the switch, is made possible by protonation of the Schiff base (Orlandi and Schulten, 1979; Tavan et al., 1985).

The unique and central feature of the local-access model (Brown et al., 1998) is that unless further changes in the protein intervene, the retinal Schiff base retains its connection to both Asp^{85} and Asp^{96} throughout the photocycle, and thus the potential to exchange protons with either residue. There is, in fact, much indirect evidence that the local access of the Schiff base is inherently bidirectional. The kinetics in the visible region suggested (Zimányi et al., 1992) that although transport is normal at $pH < 6$, the sequence of reactions is reversible, i.e., $L \leftrightarrow M_1 \leftrightarrow M_2 \leftrightarrow N$, where in $L \leftrightarrow M_1$ the Schiff base is in protonation equilibrium with Asp^{85}, and in $M_2 \leftrightarrow N$ it is in equilibrium with Asp^{96}. The back-reaction from M_2 to M_1 was found to be more rapid at lower pH, and in the kinetic model its rate depended on $[H^+]$ in such a way as to suggest its association with proton uptake. Thus the $M_1 \rightarrow M_2$ forward reaction, in turn, could be associated with proton release, and the proton release that occurs above pH 6 (the pK_a for the release to the EC surface) would therefore shift the $M_1 \leftrightarrow M_2$ equilibrium more toward M_2. This shift toward M_2 is now understood to be caused by an increase of the pK_a of Asp^{85} through its coupling to the proton release chain (Balashov et al., 1996; Richter et al., 1996). According to this mechanism, the two substates of M with different local access are not separated by a large free energy difference. Where the unidirectionality of the $M_1 \rightarrow M_2$ reaction implies a large negative ΔG, as at $pH \geq 7$ (Váró and Lanyi, 1991b), it originates not from a change of accessibility, which the local-access model assumes to be as it is at pH 5, but from the dissipation of free energy when the pH is higher than the pK_a for proton release to the EC surface.

Recent experiments on the effects of an imposed external electric field on the photocycle (Nagel et al., 1998) indicated that the $M_1 \leftrightarrow M_2$ equilibrium can be forced back toward M_1, consistent with the reversibility of the M_1-to-M_2 reaction and its dependence on charge translocation. The existence of a pH-dependent equilibrium between L and an M state was demonstrated by two-photon perturbation experiments on the wild-type photocycle (Althaus and Stockburger, 1998). Importantly, it is implied by such kinetics that because at $pH < 6$ both L and M_1 persist until the recovery of the initial state through reprotonation of the Schiff base from the CP side (Zimányi et al., 1992), access of the Schiff base to Asp^{85} is retained, even though access to Asp^{96} exists.

As a critical test of this kinetic model, we report here studies of the intermediates of the photocycle that accumulate in a suitable mutant, D115N/D96N, during sustained illumination with yellow light. Consistent with the earlier proposed kinetics and the pK_a of 6 for proton release (Zimányi et al., 1992), at pH 5 the photostationary mixture

contains what appears to be the L intermediate together with M (mostly M_2) and N. After depletion of M by a blue flash, M was found to partially recover at the expense of L, and the deprotonation of the Schiff base in this reequilibration resulted in protonation of Asp^{85}. Thus, under these conditions the accumulated L state is a genuine L, and the $L \leftrightarrow M_1$ and $M_1 \leftrightarrow M_2$ equilibria are both reversible. Because the $M_2 \rightarrow N$ reaction represents reprotonation of the Schiff base from the CP surface, the connection of the Schiff base is to both Asp^{85} in the EC direction and the CP surface. On the other hand, at pH 8, where proton release to the EC surface raises the pK_a of Asp^{85} during the $M_1 \rightarrow M_2$ reaction, neither the L state nor the refilling of M after its depletion could be observed. This is as predicted, because the higher pK_a of Asp^{85} under these conditions makes the $M_1 \rightarrow M_2$ reaction unidirectional. The presence of a sustained protonation equilibrium between the Schiff base and Asp^{85} at a pH below but not above the pK_a for proton release, as proposed earlier (Zimányi et al., 1992), provides support for the local-access model.

MATERIALS AND METHODS

The bacteriorhodopsin mutants D115N/D96N and D96N were described previously (Zimányi et al., 1992). After their expression in *Halobacterium salinarium*, the proteins were purified as purple membrane patches (Oesterhelt and Stoeckenius, 1974). All spectroscopy was done at 5°C.

Transient absorbance changes at single wavelengths were measured on polyacrylamide gel-encased samples as described earlier (e.g., Brown and Lanyi, 1996), except that the data were acquired with a 30-MHz analog-to-digital converter (Gage Compuscope 6012/PCI-4M card; Gage Applied Sciences, Montreal, Canada), utilizing custom software. The 4×10^6 data points collected were reductively averaged to provide 250 points on a logarithmic time scale. Photoexcitation was with either a 532-nm ("green") or a 420-nm ("blue") laser flash. The former was from a Continuum Surelite I frequency-doubled Nd-YAG laser (Santa Clara, CA), the latter was generated with a home-built dye laser (1 mM stilbene in methanol) pumped by a Continuum Surelite II frequency-tripled Nd-YAG laser. Illumination to produce photostationary states was provided by a 175-W Cermax xenon lamp (ILC Technology, Sunnyvale, CA) through a 500-nm cutoff filter ("yellow" light) and a 5-mm-diameter, 6-ft liquid light guide. One hundred percent intensity refers to 300 mW/cm^2. For the determinations of transient absorbance changes after pulse perturbation of the photostationary mixture without illumination during the measurements, a mechanical shutter was constructed to block the yellow light, and the laser pulse (blue or green) was provided 7.5 ms after the shutter closed. Spectra of photostationary states were determined with an optical multichannel analyzer (Zimányi et al., 1989) based on a model TRY700S/B diode array from Princeton Instruments (Trenton, NJ) while the samples were illuminated.

Time-resolved Fourier transform infrared (FTIR) spectra were measured on a Bruker IFS-66/S spectrometer in the rapid-scan mode for the millisecond time range and in the step-scan mode for the microsecond range, at 8 cm^{-1} resolution. As described elsewhere (Dioumaev et al., 1998), the samples were prepared by drying purple membrane suspensions in distilled water (15–20 nmol of bacteriorhodopsin), at about neutral pH, on a CaF_2 window (Harrick, Ossining, NY). The semidry films, ~15 mm in diameter, were equilibrated with an excess of buffer solution at the desired pH for at least 20 min. They were then partly dried and rehydrated with 3.5 μl of the same buffer, and a second window was placed on the sample without a spacer. Background illumination that produced a photostationary state was provided with the same lamp/light-guide as in the measurements in the visible, but at one-third the intensity. Because of technical difficulties of delivering a suitable intensity of yellow light to these samples with a shutter inserted for automated on/off switching, in the FTIR measurements the actinic light was left on throughout. The yellow light had no detectable effect on the kinetics of absorbance changes after the blue flash in the microsecond to millisecond time range, as expected, because the recovery of the initial state had a time constant of 140 ms. Control experiments in the visible confirmed this.

RESULTS

Photocycle of the D115N/D96N bacteriorhodopsin mutant

Fig. 1 shows absorbance changes at 410 and 570 nm after photoexcitation of D115N/D96N with a green flash, at pH 5 (Fig. 1 *A*) and 8 (Fig. 1 *B*). The considerably slower decay of the M state, measured at 410 nm, at the higher pH is as reported before. At the higher pH, the ratio of absorbance change $\Delta A_{570}/\Delta A_{410}$ becomes constant with time (ratio = 1.35) after the formation of M, because the changes reflect the accumulation of M as the only intermediate present (Zimányi and Lanyi, 1993; Nagle et al., 1995). There is no evidence for the accumulation of N at the end of the photocycle. At the lower pH, however, $\Delta A_{570}/\Delta A_{410}$ is higher (ratio = 2.35) after M accumulates, and becomes even higher during the decay of M. This indicates the presence of additional intermediates that do not absorb at 410 nm, i.e., which have protonated Schiff base. Earlier we had reported the coexistence of L with M throughout this kind of photocycle (Zimányi et al., 1992), and that would account for

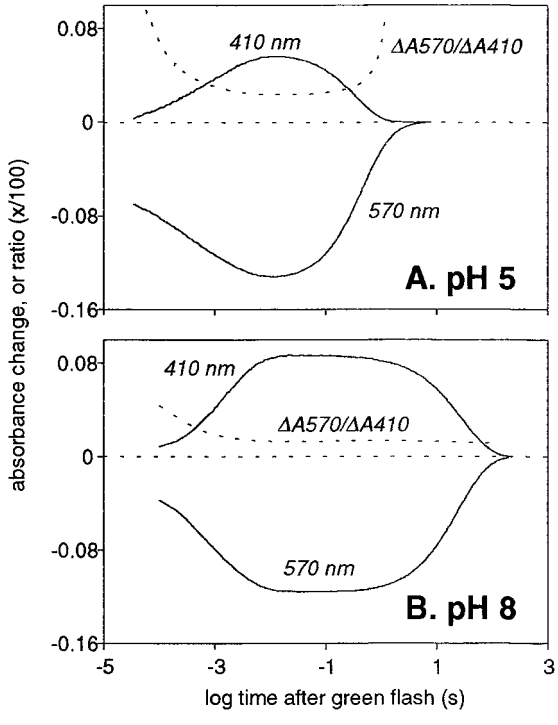

FIGURE 1 Transient absorbance changes in D115N/D96N bacteriorhodopsin after a green flash. (*A*) pH 5. (*B*) pH 8. Traces at 410 nm, 570 nm, and their ratios (×1/100) are shown. Conditions: 8 μM bacteriorhodopsin, 100 mM NaCl, (*A*) 50 M succinate, pH 5; (*B*) 50 mM phosphate, pH 8.

the higher ratio in the millisecond time range. In the second time range, the further increase in the ratio could originate only from the formation of the N state, because no changes occurred in the red region that would suggest the presence of the O state (not shown). Because the amplitude at 410 nm reaches zero well before the amplitude at 570 nm does (Fig. 1 *A*), at pH 5 there is no obvious equilibrium between M and N. This is unlike in the wild-type photocycle (Zimányi et al., 1993; Druckmann et al., 1993), where this equilibrium depends on the pK_a difference between Asp^{96} and the Schiff base. It is as expected for the photocycle of D96N, however, where the protonation of the Schiff base is from the bulk medium, and the pH is well below the pK_a of the Schiff base (the pH is 5 and the pK_a is 8.3, as determined by Brown and Lanyi, 1996), strongly favoring the M-to-N transition.

The slow decay of the M state in Asp^{96} mutants is rationalized on the basis of the removal of the aspartate that is the internal proton donor to the Schiff base (Holz et al., 1989; Miller and Oesterhelt, 1990; Cao et al., 1991), but the rate of the decay of N is also slower than in the wild type, and the reasons for this are not as clear. In an earlier study we had found (Brown and Lanyi, 1996) that unlike the decay of M, the decay of N in D96N is not pH dependent. It seems likely that the decay of N reflects the reisomerization of the retinal to all-*trans*, and this process is affected by the difference between the side chains of an aspartate and an asparagine at position 96. Whatever the reasons are, it is fortunate for this study that N can accumulate in the photocycle of D115N/D96N, because in the photostationary state at pH 5 the connection between the Schiff base and the CP surface is thereby established, and we can test the prediction that the connection with the EC surface is retained.

Photostationary states of D115N/D96N

Constant illumination of D115N/D96N with yellow light will cause M to accumulate, together with any intermediate that has a similar (or slower) decay rate or earlier intermediates that are in equilibrium with M. Fig. 2 shows spectra measured during illumination at various pH values between 5 and 8. Given the decay times of seconds to minutes in this pH range at the low temperature employed, the intensity of the actinic light was sufficient to produce full depletion of the unphotolyzed chromophore in the photostationary state. At pH 8 virtually all of the absorption band of the protonated Schiff base at 570 nm (*dashed trace*) is shifted to the band of the unprotonated Schiff base at ~410 nm. Below pH 8, however, an absorption band at 550 nm remains, with greater amplitude at lower pH. The species with protonated Schiff base that persists is not residual unphotoconverted bacteriorhodopsin. Absorbance changes after a green flash indicated that unphotolyzed bacteriorhodopsin was not present at any of the pH values listed in Fig. 2 (cf. below). This will be a great advantage in interpreting the effects of pulse perturbation, because unlike in two-pulse perturbation experiments (Druckmann et al., 1993; Zimányi et al., 1993; Dickopf et al., 1997; Hessling et al., 1997; Althaus and Stockburger, 1998), corrections for the photoexcitation of unphotolyzed chromophore need not be made.

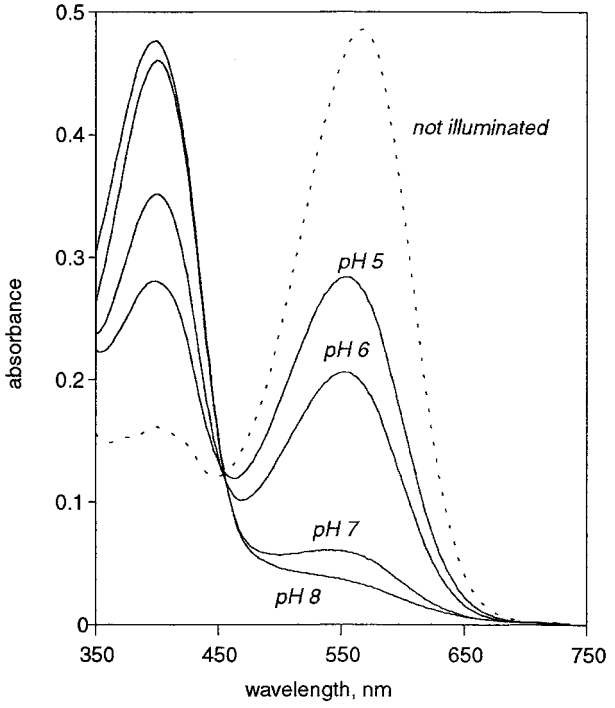

FIGURE 2 Spectra of the photostationary state of D115N/D96N bacteriorhodopsin during illumination with yellow light at various pH values. Conditions are as in Fig. 1, but 25 mM 1,3-bis[[tris(hydroxymethyl)methyl] amino] propane (Bis(Tris)-propane) for pH 7 and 8.

Fig. 3 shows light minus dark difference FTIR spectra at pH 5 (*solid line*) and 8 (*dashed line*), for continuous yellow illumination as in Fig. 2. At pH 8 the spectrum is typical for M minus BR spectra described before in the photocycle (Gerwert et al., 1990; Braiman et al., 1991; Zscherp and Heberle, 1997), except that there is a pair of positive and negative bands in the amide I region, at 1650 and 1670 cm^{-1}, that would normally indicate the presence of N, but which here originate from the M_N state (Sasaki et al., 1992) that in D96N replaces the usual M (or M_2) state in the wild type. However, for the purposes of this report we need not distinguish between M_N and M_2, as they are both "late" M states. Little or no contribution from L or N is present, as evident from the fingerprint region between 1100 and 1305 cm^{-1}, where all C-C stretch bands are below the zero line. In contrast, at pH 5 the presence N is evident from the increased amplitudes of the amide bands, the increased amplitude at 1186 cm^{-1}, as well as at 1302 and 1400 cm^{-1}, characteristic of N (Pfefferlé et al., 1991; Zscherp and Heberle, 1997). The presence of L at pH 5 is indicated by the significantly lower amplitude of the band of the protonated Asp^{85} at 1759 cm^{-1}. The FTIR difference spectra confirm therefore that in the photostationary state the M state coexists with the L and N states at the lower, but not at the higher pH, as expected from the photocycle kinetics (Fig. 1).

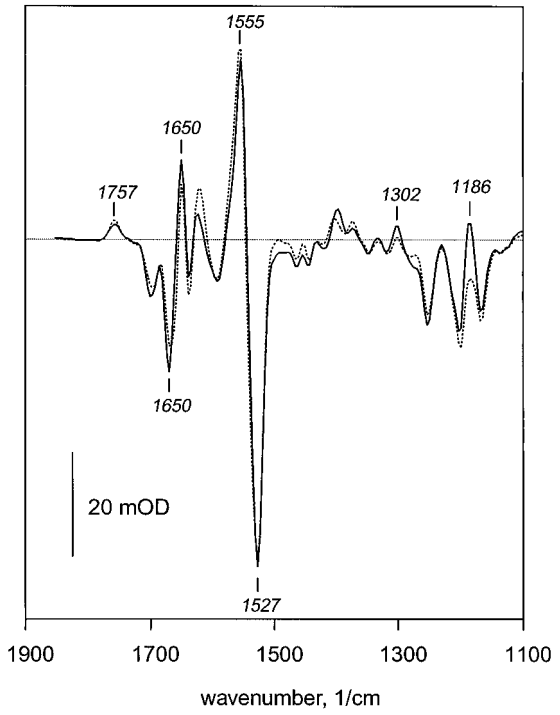

FIGURE 3 FTIR spectra of the photostationary state of D115N/D96N bacteriorhodopsin during illumination with yellow light at pH 5 (——) and pH 8 (– – –). Conditions: 100 mM NaCl, 40 mM 2-[morpholino]ethanesulfonic acid for pH 5 and 50 mM Bis(Tris)-propane for pH 8.

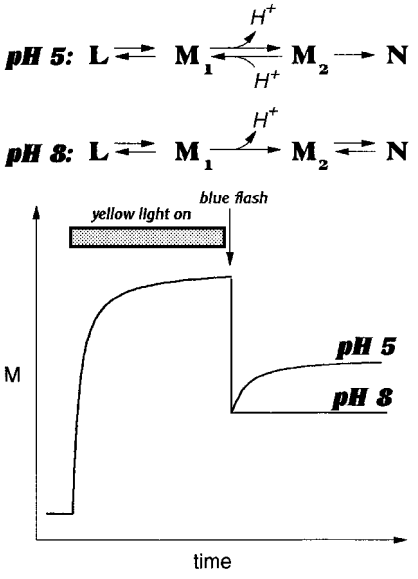

FIGURE 4 Schematic description of the experimental strategy for testing the Schiff base-Asp[85] protonation equilibrium in D115N/D96N. The sample is illuminated with yellow light to create a photostationary state that contains the M intermediate (and other states, depending on the conditions). The actinic light is turned off, and 7.5 ms later a blue flash depletes M. The absorption changes after this are followed on the millisecond time scale. The kinetics (Zimányi et al., 1992) predict that at pH 8 the unidirectionality of the M_1-to-M_2 reaction will allow accumulation of only M_2, and thus refilling of M can be only from N, which is very slow (τ = 30 s). At pH 5, however, both M_1 and M_2 should accumulate and rapid refilling of M from L (within a few milliseconds, from the rise kinetics of M in the photocycle) should be possible, whereas refilling from N is precluded by the unidirectionality of the M-to-N reaction at this pH (Brown and Lanyi, 1996).

Pulse perturbation of the photostationary state: measurements in the visible

The experimental strategy employed is sketched in Fig. 4. Our goal was to confirm the scheme suggested by earlier kinetic measurements (Zimányi et al., 1992), in which L is in equilibrium with M_1, and proton release to the EC surface is linked to the $M_1 \rightarrow M_2$ reaction. Thus, at pH lower than the pK_a for release (~6 for D96N; Zimányi et al., 1992), the M_1 and M_2 states should remain in equilibrium throughout, but at higher pH the L and M_1 states should decay fully with the appearance of M_2 (Fig. 4). The photostationary state, therefore, will contain L, M_1 in addition to M_2 (and N) at lower pH, but almost pure M_2 at higher pH, as indeed is found (Figs. 2 and 3). The photostationary mixture was then perturbed by a blue flash, so as to deplete the M state(s). Because the decay of M in the D115N/D96N mutant is very slow (τ = 0.35 s at pH 5, and even slower at higher pH; cf. Fig. 1), the photostationary state will remain nearly unchanged for many milliseconds after the yellow light is turned off. The blue flash was provided 7.5 ms after the end of illumination, in the way shown in Fig. 4. Because the $L \leftrightarrow M_1 \leftrightarrow M_2$ equilibration occurs on a time scale as rapid as a few milliseconds, whereas the $M_2 \rightarrow N$ reaction is slow and virtually unidirectional at the lower pH (cf. above), the M and L states should reequilibrate so as to partly refill the M state that was depleted (Fig. 4), but no refilling will occur from N. The objective was to test the model by measuring this predicted refilling of M at low but not high pH.

Flash artifacts were avoided by following the disappearance of the protonated Schiff base during the refilling rather than the appearance of the unprotonated Schiff base. Fig. 5 *A* shows the time course of absorbance change at 500 nm after a blue flash, at different pH values. The initial rise that corresponds to protonation of the Schiff base in the "back-photoreaction" of M within a few microseconds (Kalisky et al., 1981; Druckmann et al., 1992; Dickopf and Heyn 1997; Hessling et al., 1997) is not resolved on this time scale. The decrease in absorbance that follows it at lower pH is as expected. It occurs with two time constants, 270 μs and 1.1 ms, with relative amplitudes of 0.4 and 0.6 (at pH 5). These kinetic parameters agree with those measured for the rise of the M state in a direct photocycle measurement for D115N/D96N (e.g., in Fig. 1 *A*). A small increase evident at pH 7.5 in Fig. 5 *A* was unexpected, but did not significantly affect the observation of the recovery of the deprotonated Schiff base. Fig. 5 *B* shows the amplitude of the total absorbance change, normalized to 1 at the lowest pH measured (pH 4.5). The apparent pK_a for the refilling is 6.3. This is similar to the pK_a of ~5.9 measured earlier for proton release in the wild type and for the reversibility of the M_1-to-M_2 reaction in D96N (Zimányi et al., 1992), as well as in the wild type (Dickopf and Heyn, 1997), or the L-to-M_1 reaction (Althaus and Stockburger, 1998).

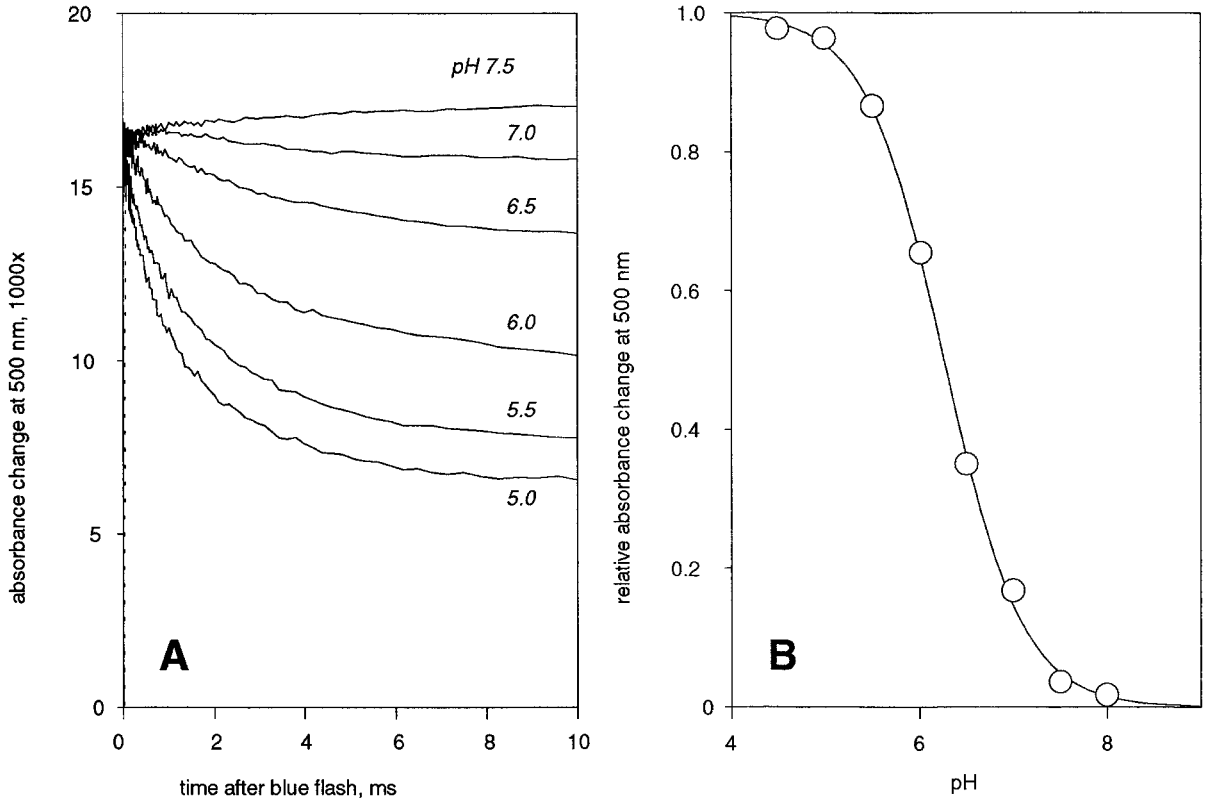

FIGURE 5 Absorbance changes after depletion of M in the photostationary state with a blue flash. Zero time refers to the time of the flash; the initial increase of absorbance by 17 mOD (– – –) refers to the protonation of the Schiff base that corresponds to depletion of the M species in the photostationary state. (*A*) Traces of absorbance change at 500 nm at various pH values between 5 and 7.5. Traces measured at pH 4.5 and pH 8 (not shown) were very similar to the traces at pH 5 and pH 7.5, respectively. (*B*) Amplitudes of the time-dependent changes in *A* as a function of pH. The data are described by an apparent pK_a of 6.3. Conditions: 100 mM NaCl, 25 mM Bis(Tris)-propane, 25 mM succinate.

For the unambiguous interpretation of these experiments, controls were needed to establish that no unphotoconverted BR remained during illumination with yellow light, because its photoreaction upon blue flash would yield absorbance changes similar to the refilling of M. With green rather than blue flash, BR will be photoexcited but not M, because the latter does not absorb at 532 nm. Although the green flash used was about two times more effective in these experiments than the blue in photoexciting BR, there was no evidence for the formation of M (at 410 and 570 nm) with green flash that would have originated from unphotoconverted BR. At 500 nm, on a millisecond time scale, photoreaction of N or L was noted. However, the absorbance change was small (<15% of the effect measured under conditions similar to those in Fig. 5 *A* at pH 5; not shown), and it was an increase rather than a decrease. It demonstrated that the absorption decrease in Fig. 5 *A* could not have originated from photoreaction of the N or the L states in the mixture.

If the observed absorption decrease is refilling of M, as it now appears, and it occurs at the expense of the L intermediate, as we expect, the dependence of the changes in Fig. 5 *A* on the wavelength of the measurement should produce an amplitude spectrum equivalent to the absorption spectrum of the L state (or, more accurately, an M minus L difference spectrum that will be equivalent to the negative of the L spectrum at wavelengths greater than 480 nm). Fig. 6 shows the total amplitudes of change at pH 5 (Fig. 6 *A*) and 8 (Fig. 6 *B*) for both blue-flash-induced initial reaction (*open circles*) and the subsequent redistribution reaction on the millisecond time scale (*filled circles*). The spectra for the former, with maxima at 570 nm at both pH values, indicate that the initial reaction converts M to bacteriorhodopsin (the first photoproduct is BR′; cf. Kalisky et al., 1981; Druckmann et al., 1992). The spectrum for the slower reaction, with a maximum at ~530 nm at pH 5, is consistent with the spectrum of L (Zimányi and Lanyi, 1993) and thus with depletion of the L state in the refilling. It is inconsistent with conversion of either BR or N to M, because the maxima of these species are at 570 and 560 nm, respectively. At pH 8, the shape of the corresponding spectrum (*filled circles*) is different. It probably originates from a shift of the maximum of BR′ toward a shorter wavelength (Balashov and Litvin, 1981) and thus refers to the slower step in the photoreconversion of the M state to BR, designated as the BR′-to-BR reaction (Kalisky et al., 1981; Druckmann et al., 1992). It is most obvious at pH 8, where the refilling is virtually absent, but may contribute to the changes at all pH.

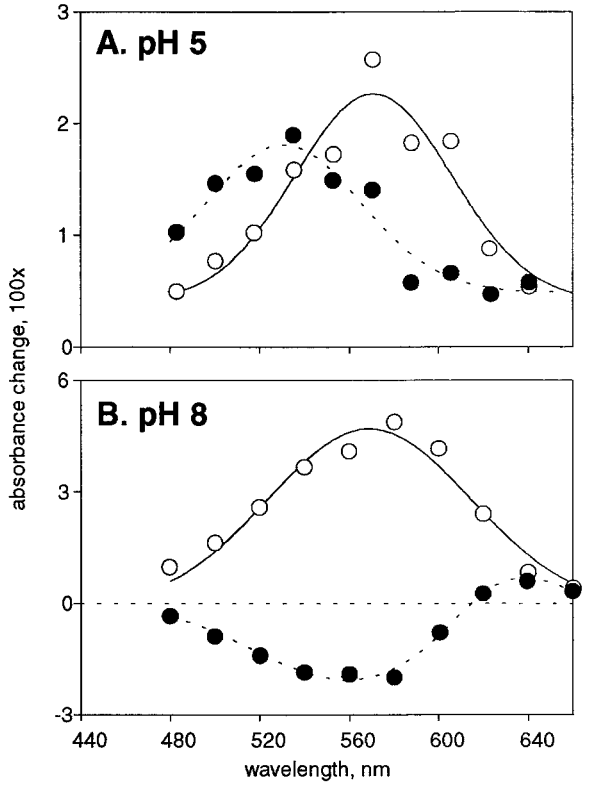

FIGURE 6 Amplitude spectra for absorption changes after depletion of M in the photostationary state with a blue flash. (*A*) pH 5. (*B*) pH 8. ○, Absorbance changes immediately after the flash, not resolved in time. ●, Absorbance changes that occur within a few milliseconds, inverted in sign (cf. Fig. 5 *A*). Lines represent the best fits of sums of Gaussian curves. Conditions are as in Fig. 2.

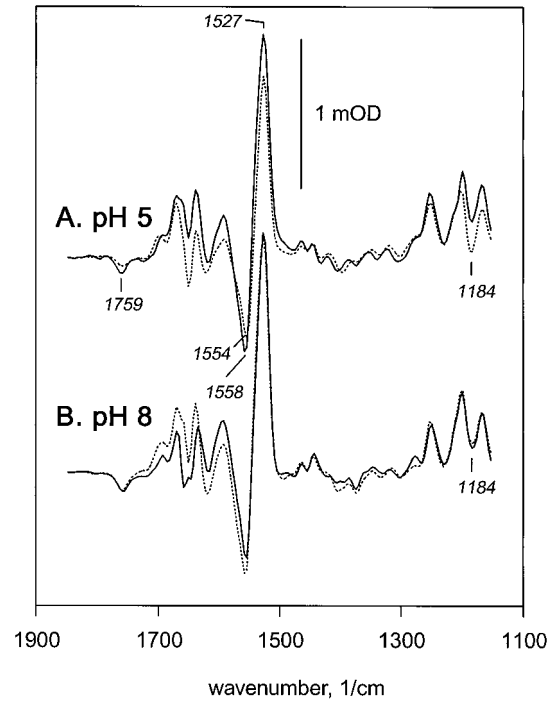

FIGURE 7 FTIR difference spectra after depletion of M in the photostationary state with a blue flash. ——, Spectra averaged between 15 and 105 μs. – – –, Spectra averaged between 1 and 2 ms, after the blue flash. Conditions are as in Fig. 3.

Pulse perturbation of the photostationary state: measurements in the infrared

The experiments in Figs. 5 and 6 were repeated, but with the absorption measured in the infrared. Fig. 7 shows FTIR difference spectra at <0.1 ms after the blue flash and a few milliseconds later, at pH 5 (Fig. 7 *A*) and pH 8 (Fig. 7 *B*). The spectral changes that occur first are qualitatively the same at both pH values. They are characteristic of what photoconversion of an M-like initial state (in the photostationary mixture) into a BR-like state would produce. When turned upside down, these spectra are virtually indistinguishable from M minus BR FTIR spectra (Gerwert et al., 1990; Braiman et al., 1991; Takei et al., 1992; Zscherp and Heberle, 1997). The only exception is the amide I region (1630–1690 cm^{-1}), in which the bands otherwise characteristic of N originate from the M_N state (Sasaki et al., 1992). In the millisecond time range there are specific changes that occur at pH 5 but not at pH 8. They are at the C═O stretch frequency of the protonated Asp^{85} at 1759 cm^{-1} (cf. below), in the ethylenic stretch band at 1527 cm^{-1}, and the C-C stretch bands, particularly in the negative band at 1184 cm^{-1}. When turned upside down, these appear to correspond to an increased contribution from the L state. In this case they reflect the L-to-M conversion detected in the visible (Figs. 5 and 6). In contrast, the changes in the amide I region occur at both pH 5 and 8, and we attribute them to conformation change during the transition from the initial photoproduct of M, i.e., BR′ to BR.

The decrease in the depletion band at 1759 cm^{-1} at pH 5 suggests that after Asp^{85} is deprotonated by the blue-flash photoreaction, it becomes partly reprotonated. As described before (Hessling et al., 1997), the deprotonation follows the photoisomerization by the blue flash within 1 μs. The reprotonation that follows is slower. Fig. 8, *A* and *B*, shows this frequency region at selected times after the blue flash, at pH 5 and 8. The spectra indicate that at pH 8 no further change occurs within a few milliseconds, but at pH 5 the negative amplitude becomes significantly less with time. The time course of these effects is shown in Fig. 9. The partial recovery of the protonated form of Asp^{85} is observed at pH 5 but not at pH 8. It occurs with an overall time constant of 480 μs (kinetic components, if any, unresolved), which is consistent with the overall rise kinetics of the M state in the photocycle. Its amplitude is about half of the depletion, roughly consistent with the $L \leftrightarrow M_1$ equilibrium (Zimányi et al., 1992) and the analogous observations in the visible (Fig. 5 *A*). Control experiments with green flash at pH 5 (conducted in a manner similar to that of the controls described for the experiments in the visible; cf. above) indicated that photoreaction of unphotolyzed BR with blue flash would produce an absorption increase of $\leq 7\%$ of the initial depletion amplitude in Fig. 9. It does not contribute

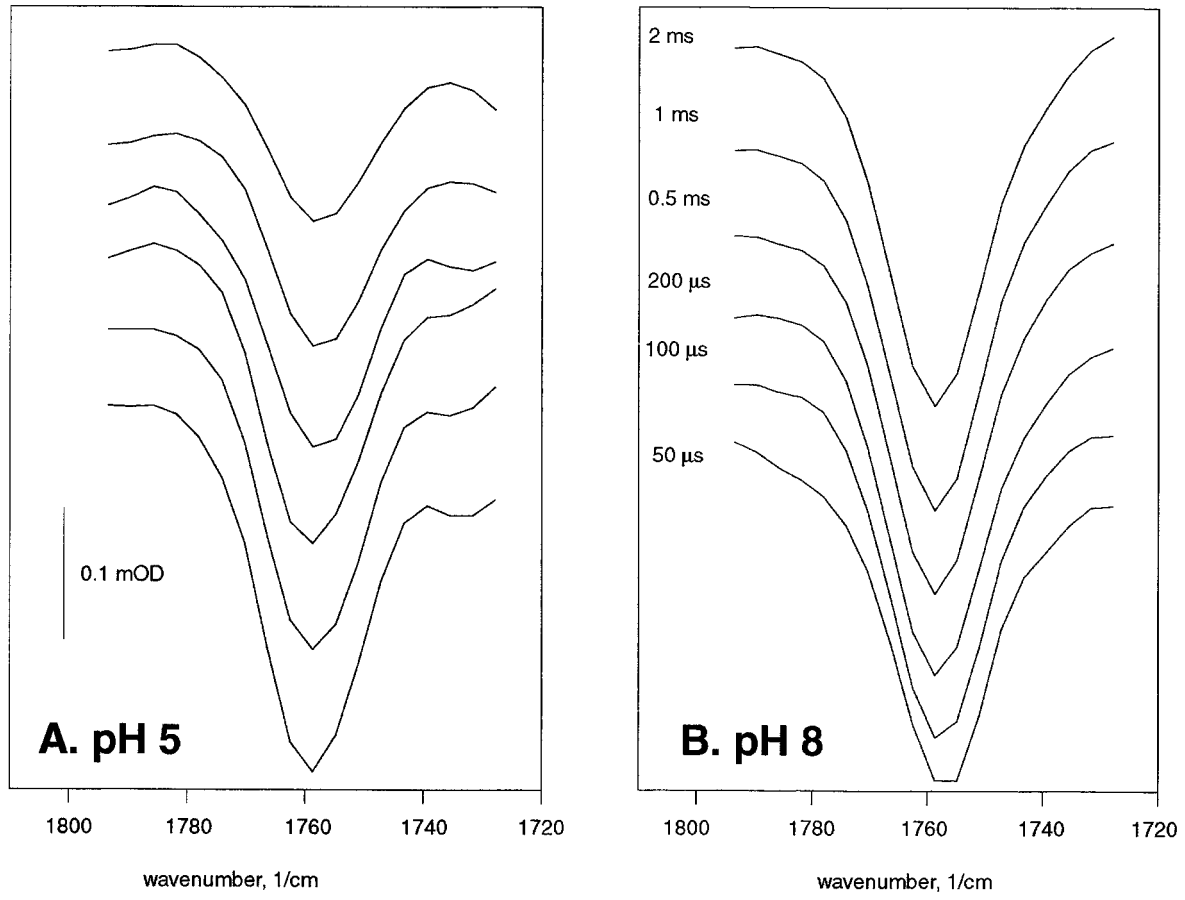

FIGURE 8 FTIR difference spectra after depletion of M in the photostationary state with a blue flash, region of the C═O stretch of the protonated Asp^{85}. The negative band is from the deprotonation of Asp^{85}. (*A*) At pH 5 the amplitude becomes smaller within 2 ms. (*B*) At pH 8 the amplitude remains constant. Conditions are as in Fig. 7, but with a denser bacteriorhodopsin film, to increase the size of the signal in this frequency region.

sufficiently to alter our interpretation of the increase as the recovery of M.

DISCUSSION

There are two possible mechanisms for the protonation switch in the bacteriorhodopsin transport cycle. In the first, the protonation switch resides in the geometry that connects the N-H bond of the retinal Schiff base to the EC or CP half-channel. In this case the local connectivity of the Schiff base is either to Asp^{85} or to Asp^{96}, but never to both. Because such a reorientation of the Schiff base must occur after its deprotonation but before its reprotonation, in kinetic terms this means that in the L-M_1-M_2-N sequence once the $M_2 \rightarrow N$ reaction becomes possible, the equilibrium mixture of the L and M_1 states will have decayed. Because the species that accumulate in a photostationary state are those intermediates with the longest decay time constants, and the decay of L and M_1 is at least two orders of magnitude faster that the decay of M, in this model the photostationary mixture will include the M_2 and N states, but never L and M_1. In the second mechanism, the local-access model (Brown et al., 1998), the protonation switch is provided by the changing proton conductivities of the EC and CP half-channels during the lifetime of the M state. As long as the retinal is photoisomerized, the Schiff base is locally connected to both Asp^{85} and Asp^{96}, and it is the proton affinities and occupancies of these aspartate residues that determine whether the proton transfer is in the EC or the CP direction. Unlike for the first model, the photostationary state in this case, under some conditions, e.g., when the pK_a of Asp^{85} is not modulated by proton release, may include not only N but also the L intermediate of the photocycle, and this L will be in a protonation equilibrium with M.

In this report we provide evidence from photostationary states that there are conditions that allow the connections of the Schiff base to both Asp^{85} and Asp^{96} to be retained throughout the photocycle, as suggested before from less direct experiments (Brown et al., 1998). The method of choice for proving the existence of an equilibrium is to demonstrate reequilibration after a perturbation pulse. We created a photostationary state in which the postulated desired species were present, and measured the reactions that ensued after depletion of the M state(s) by a blue flash. The intent was not just to demonstrate the reversibility of the

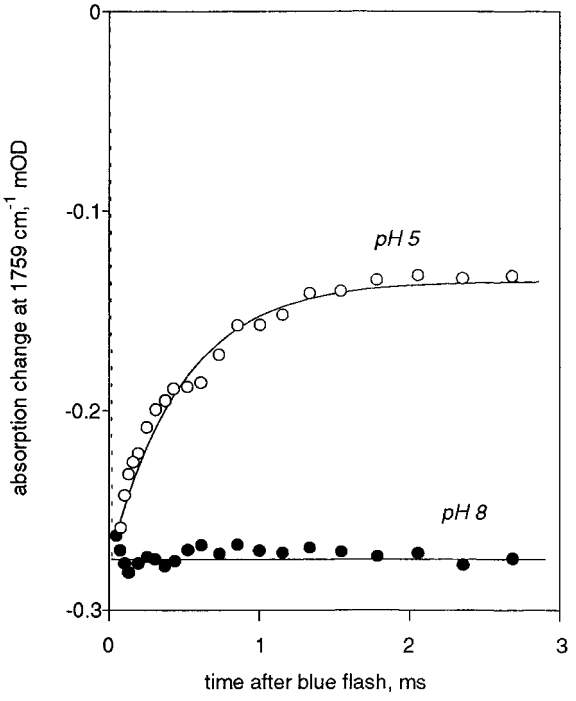

FIGURE 9 Time course of absorbance change at 1759 cm^{-1} after depletion of M in the photostationary state with a blue flash. The initial absorbance decrease after the flash, not resolved in time, is indicated with a dashed line. Data from experiments are as in Fig. 8. The soild line for pH 5 represents a single exponential with a time constant of 480 μs.

L $\leftrightarrow$ M_1 reaction during the photocycle (Althaus and Stockburger, 1998), but to show that an authentic L intermediate, in which access of the Schiff base is to the EC side, can coexist with the N intermediate that arose from M_2 through proton access to the CP side, as implicit in the kinetic scheme L $\leftrightarrow$ M_1 $\leftrightarrow$ M_2 $\leftrightarrow$ N (Zimányi et al., 1992). The rates of the photocycle reactions in the system suitable for such an experiment had to ensure that 1) the recovery of the initial state is slow enough to allow accumulation of the photoproducts at the available light intensity, and 2) the equilibrium created for L, M_1, M_2, and N contains a sufficient concentration of L to allow measurement of the expected refilling of the depleted M state at the current signal/noise ratio. These conditions were fulfilled in the D115N/D96N mutant (Zimányi et al., 1992). Because of the D96N mutation, the final step in the photocycle is slow enough for establishing a photostationary state in which essentially only photointermediates are present. This made the interpretation of the results simpler, because no corrections had to be made for the photoreaction of unphotolyzed BR. Although the reason for it is not yet clear, the D115N mutation shifts the M_1 $\leftrightarrow$ M_2 equilibrium about three times toward M_1, and thus L should be present in a concentration that is about three times greater in the photoequilibrium mixture than in D96N (Zimányi et al., 1992). Indeed, the refilling of M measured in D115N/D96N was about three times greater than in D96N (not shown). Inasmuch as the photocycle in D115N/D96N is similar to that of the wild type up to the M_2-to-N reaction (Zimányi et al., 1992), and the N state is produced by protonation of the Schiff base from the CP surface even if not from Asp^{96}, the general conclusions made will be valid for the wild type as well.

According to the earlier proposed kinetic model for the photocycles of the D96N and D115N/D96N mutants (Zimányi et al., 1992), when a proton is not released to the EC surface, e.g., at pH less than 6, the M_1 $\leftrightarrow$ M_2 equilibrium should maintain the L intermediate, in equilibrium with M_1 and together with the states that follow it in the L $\leftrightarrow$ M_1 $\leftrightarrow$ M_2 $\rightarrow$ N sequence, in the photostationary state. Thus at pH 5 access of the Schiff base to Asp^{85} should be maintained up to M_2. Access should not be maintained at pH 8, however, because the M_1 $\leftrightarrow$ M_2 equilibrium is shifted strongly toward M_2 by proton release to the EC surface, which raises the pK_a of Asp^{85} (Richter et al., 1996; Balashov et al., 1996). This turned out to be the case. In the photostationary state the Schiff base and Asp^{85} remain in protonation equilibrium until the end of the photocycle at pH 5, but not at pH 8. The idea of concurrent local access of the Schiff base to the EC and CP directions, and modulation of the proton conductivity of the EC and CP half-channels by influences in the protein such as proton release, i.e., the local-access model (Brown et al., 1998), is therefore feasible.

The results reported here thus demonstrate that at pH 5, in the absence of proton release to the EC surface, the Schiff base has access to both the EC and CP sides. As discussed in the Introduction, under physiological conditions (pH $>$7), the pK_a's of Asp^{85} and Asp^{96} are modulated to ensure the CP-to-EC flow of protons despite this. However, transport is possible also at lower pH, where the pK_a of Asp^{85} is not raised because protons are not released to the EC surface (Richter et al., 1996; Balashov et al., 1996), and in D96N, where Asp^{96} is not present. At pH less than 6, proton release at the EC surface is delayed until the recovery of the BR state (Zimányi et al., 1992). The direction of the initial proton transfer to the EC side is established in this case by the fact that the protonation equilibrium that develops within a millisecond after photoisomerization must be between the Schiff base and Asp^{85}, because only they can form an effective donor-acceptor pair. Deprotonation in the CP direction is either impossible or negligibly slow in the wild type, because Asp^{96} has an initially very high pK_a (Metz et al., 1992; Száraz et al., 1994). Asp^{96} is absent in D96N, but for other reasons proton transfer between the Schiff base and the CP surface is very slow, on the order of seconds to minutes, depending on the pH (Holz et al., 1989; Miller and Oesterhelt, 1990; Cao et al., 1991). Unidirectional CP-to-EC transport under these conditions is ensured by the fact (so far unexplained mechanistically) that recovery of the initial state cannot occur (or occurs very slowly) from the L intermediate. Transport against a transmembrane electrochemical proton gradient under these conditions is made possible by the unidirectional recovery of the initial state after reprotonation of the Schiff base from the CP side.

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