# The Complex Extracellular Domain Regulates the Deprotonation and Reprotonation of the Retinal Schiff Base during the Bacteriorhodopsin Photocycle†

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ABSTRACT: During the L → M reaction of the bacteriorhodopsin photocycle the proton of the retinal Schiff base is transferred to the anionic D85. This step, together with the subsequent reprotonation of the Schiff base from D96 in the M → N reaction, results in the translocation of a proton across the membrane. The first of these critical proton transfers occurs in an extended hydrogen-bonded complex containing two negatively charged residues (D85 and D212), two positively charged groups (the Schiff base and R82), and coordinated water. We simplified this region by replacing D212 and R82 with neutral residues, leaving only the proton donor and acceptor as charged groups. The D212N/R82Q mutant shows essentially normal proton transport, but in the photocycle neither of this protein nor of the D212N/R82Q/D96N triple mutant does a deprotonated Schiff base (the M intermediate) accumulate. Instead, the photocycle contains only the K, L, and N intermediates. Infrared difference spectra of D212N/R82Q and D212N/R82Q/D96N demonstrate that although D96 becomes deprotonated in N, D85 remains unprotonated. On the other hand, M is produced at pH >8, where according to independent evidence the L ⇌ M equilibrium should shift toward M. Likewise, M is restored in the photocycle when the retinal is replaced with the 14-fluoro analogue that lowers the p$K_a$ of the protonated Schiff base, and now D85 becomes protonated as in the wild type. We conclude from these results that the proton transfers to and from the Schiff base probably both occur after photoexcitation of D212N/R82Q, but the L ⇌ M and M ⇌ N equilibria are shifted away from M, and, untypically, D85 does not retain the proton it had gained. The mechanism of proton transport is not greatly changed when D85 is the only charged component of the Schiff base counterion, but the protonation equilibria in the proton transfer pathway across the protein are drastically altered.

Bacteriorhodopsin is the light-driven proton pump of halobacteria. Photoisomerization of the retinal chromophore sets into motion a sequence of thermal reactions (the "photocycle") that results in proton transfers inside the protein and between the protein and the aqueous phase, and culminates in the net translocation of a proton [reviewed in Mathies et al. (1991), Ebrey (1993), and Lanyi (1993)]. The intermediates of the photocycle have distinct spectra in the visible and the infrared, and define the sequence, K,[1] L, M, N, and O (Lozier et al., 1975). Several of these intermediates have been resolved into substates (Váró & Lanyi, 1991a; Váró et al., 1992; Sasaki et al., 1992; Zimányi et al., 1992a, 1993; Druckmann et al., 1992; Perkins et al., 1992). The key intermediate M refers to the deprotonated retinal Schiff base. Its formation from L is the equilibration of the Schiff base proton with the initially anionic D85, a residue located toward the extracellular surface. Its decay to N is the equilibration of the proton of D96, a residue located toward the cytoplasmic side, with the unprotonated Schiff base. The change of access from the acceptor to the donor (the "reprotonation switch") between the two proton transfers ensures that the transport will be unidirectional, and take place from the cytoplasmic side toward the extracellular side (Nagle & Mille, 1981; Fodor et al., 1988; Váró & Lanyi, 1991b; Henderson et al., 1990; Kataoka et al., 1994). Although conceptually this is a simple model, the proton transfers in the extracellular and cytoplasmic domains are in fact complex and involve numerous interacting residues and bound water (De Groot et al., 1989, 1990; Henderson et al., 1990; Dér et al., 1991; Brown et al., 1994a; Fischer et al., 1994; Kandori et al., 1995).

In this report, we focus on the domain on the extracellular side of the Schiff base. According to the structure of the protein (Henderson et al., 1990), this region contains four charged groups, the anionic D212 and D85, and the positively charged Schiff base and R82, as well as two tyrosines, Y57 and Y185. The resolution available at this time does not

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[1] Abbreviations: BR, bacteriorhodopsin; K, L, M, N, and O, photointermediates of the bacteriorhodopsin photocycle; site-directed mutants are described with the wild-type and mutated residues separated by the residue number, *e.g.*, D212N/R82Q; 14-F, 14-fluororetinal; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate.

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reveal the exact location of the hydrogen bonds that connect some, or more probably all, of these groups. The anomalous NMR shift of the retinal nitrogen had suggested (De Groot et al., 1989, 1990) that the counterion to the Schiff base is not a point charge but a diffuse complex formed by the interaction of the three charged residues in this vicinity and bound water. Such a structure would delocalize the negative counterion charge in the BR state and distribute the transferred proton over a hydrogen-bonded network in the M state. Comparison of this region with its equivalent in halorhodopsin, a chloride pump, where the residue corresponding to D85 is missing but a chloride ion may be bound, suggested that the Schiff base, D85, R82, D212, and a centrally coordinated water molecule form a *quadrupole* with zero net charge (Dér et al., 1991). Indeed, the O—H stretch region of FTIR spectra of the L and M photointermediates of various mutants (Maeda et al., 1992b, 1994; Brown et al., 1994a; Fischer et al., 1994; Kandori et al., 1995) has indicated that water is bound in this region. This water becomes first more strongly hydrogen bonded (in L), and then more weakly bonded (in M). Molecular dynamics calculations of the inclusion of water into this structure have produced a model with a network of hydrogen-bonded water molecules that link the proposed components of the Schiff base counterion together (Humphrey et al., 1994).

D85 remains protonated in the photocycle until recovery of the initial state (Bousché et al., 1992; Souvignier & Gerwert, 1992; Hessling et al., 1993), and the identity of the group that supplies the proton released to the surface at about the time the Schiff base deprotonates is uncertain. It must have a high $pK_a$ in the unphotolyzed protein (Kono et al., 1993; Balashov et al., 1993), and lowered to about 6 so as to release the proton (Zimányi et al., 1992b). At pH <6, proton release to the extracellular surface does not occur immediately after deprotonation of the Schiff base. Instead, the release is at the recovery of the initial state in the photocycle, *i.e.*, *after* proton uptake at the cytoplasmic surface (Zimányi et al., 1992b; Cao et al., 1993a). Because the vectoriality of these steps is preserved, the direction of the net transport is unchanged in spite of the reversal of the temporal sequence of release and uptake. A similar delay of the release was observed in R82 mutants and Y57F, leading to the suggestion that these residues constitute, or at least play an important part in, the normal proton release machinery (Balashov et al., 1993; Cao et al., 1993a; Brown et al., 1994b; Govindjee et al., 1995).

Given the complex structure of the Schiff base counterion, it should not be surprising that the replacement of R82 and Y57, or indeed any of the other residues in it, yields complicated phenotypes. In the D85N mutant, the normal proton acceptor is missing, and upon photoexcitation the Schiff base deprotonates poorly and to the cytoplasmic side only (Tittor et al., 1994; Kataoka et al., 1994). Similar behavior is found in D212N at pH >7 (Cao et al., 1993b), apparently because the $pK_a$ difference between donor and acceptor is unfavorable for proton transfer (Needleman et al., 1991), although at lower pH in the presence of chloride the Schiff base will deprotonate as in wild type, and the protein transports protons. Presumably, under the latter condition a bound $Cl^-$ replaces the charge of D212, as in many such mutants (Marti et al., 1992). Not only in R82Q (or R82A) but also in Y57F (Cao et al., 1993a; Govindjee et al., 1995) the deprotonation of the Schiff base is more rapid than in the wild-type photocycle, and proton release is delayed until the end of the photocycle as in the wild type at pH <6. The $pK_a$ of D85 is raised more than 4 units upon replacement of R82, suggesting specific interaction of these residues (Subramaniam et al., 1990; Otto et al., 1990; Thorgeirsson et al., 1991; Brown et al., 1993). The pH-dependent properties of the Y185F mutant suggested, in turn, specific interaction of D212 and Y185 (Rath et al., 1993). Several other residue replacements in this region also affect proton release. The Schiff base deprotonates in a normal manner in F208R and D212N/D96N (at pH <6, in the presence of chloride), but proton release is considerably delayed although it occurs before the proton uptake on the cytoplasmic side (Cao et al., 1995).

It seems likely that in any of these mutants the observed phenotype reflects the altered behavior of the remaining residues in the counterion complex, rather than simply the missing function of the replaced residue. This will be particularly so in mutants where the *net charge* of the domain is changed. Dissection of the functional roles of the various residues that make up the complex is therefore difficult, if not impossible. We have attempted to simplify the problem by replacing *both* D212 and R82 so as to convert the quadrupole to a dipole that consists only of the proton donor Schiff base and the acceptor D85. Thus, the net charge of the complex is unchanged, and the consequences of having removed much of what makes this region complicated can be explored. The results indeed show a simpler behavior than in single mutants. The protein is active in transport, proton release and uptake occur in the photocycle, and the photocycle is simplified in that the deprotonated Schiff base does not accumulate. The reason appears to be that the various protonation equilibria that drive the proton across the membrane during the photocycle are different from the wild type.

## MATERIALS AND METHODS

The site-specific mutations D212N/R82Q and D212N/R82Q/D96N were introduced into the *bop* gene, the changed gene was inserted into a newly developed nonintegrating vector to be described elsewhere, and *Halobacterium salinarium* was transformed as before (Needleman et al., 1991; Ni et al., 1990). The mutated and wild-type proteins were purified from *H. salinarium* as purple membrane sheets according to a standard method (Oesterhelt & Stoeckenius, 1974). All spectra in the visible, and all kinetic measurements were with the purple membrane samples encased in polyacrylamide gels (Cao et al., 1993b). Unless otherwise mentioned, the experiments were at 22 °C.

The 14-fluororetinal was synthesized and incorporated into bleached, washed bacterioopsin, as described before (Tierno et al., 1990).

Transient spectroscopy at single wavelengths and with a gated multichannel analyzer was as before (Cao et al., 1993b; Zimányi et al., 1989). Determination of pH changes during the photocycle was with and without pyranine (Grzesiek & Dencher, 1986; Heberle & Dencher, 1992; Brown et al., 1994b; Cao et al., 1995). Proton transport was determined by following pH changes during illumination of cell envelope vesicles (Needleman et al., 1991).

The samples for FTIR spectroscopy were films made by drying a 50 $\mu$L aliquot of the purple membrane suspension in either 2 mM phosphate, pH 7.0, or 5 mM borate, pH 9.0,

on a $BaF_2$ window ($d$ = 16 mm). They were humidified by placing 1 $\mu$L of water on the edge of the window before sealing it with an O-ring and a second window. The sample cell was then mounted on an Oxford DN1754 vacuum cryostat connected to an Oxford ITC-4 temperature controller. Light adaptation was at 274 K by irradiating with >500 nm light for 2 min. The light-adapted state will be denoted as BR. FTIR spectra were recorded in a BioRad FTS60A/896 spectrometer, with a 2 $cm^{-1}$ resolution. The L *minus* BR difference spectrum was obtained from 2 sets of 128 interferograms, recorded after and before irradiation with >600 nm light at 170 K. Difference spectra at 220 and 230 K were obtained in the same way, except that the irradiation was with >500 nm light. Difference spectra at 260 K were obtained from 32 interferograms because of rapid decay of the photoproducts at this temperature. In all cases, the recordings were repeated 3−4 times, and the results were averaged. Base-line distortion was corrected by subtracting corresponding difference spectra measured in the dark. The spectra are shown after adjusting the intensities of the negative bands in the fingerprint region.

## RESULTS

*Spectra and Photocycles of D212N/R82Q and D212N/R82Q/D96N.* Replacement of D212 and R82 by asparagine and glutamine, respectively, caused only minor changes in the absorption spectrum of the chromophore in the visible, as did the additional replacement of D96 with asparagine (not shown). Unlike in D212N (Needleman et al., 1991), the absorption maxima of the double and triple mutants were nearly unaffected by pH (or chloride ions up to 2 M) between pH 3 and 10. Formation of blue membrane, that reflects the protonation of D85 in the wild-type protein at acid pH (Subramaniam et al., 1990), was not observed at a pH as low as 1, in either 1 M sodium sulfate or 2 M NaCl (not shown). Thus, the p$K_a$ of D85 is not raised to 7 as in the R82Q single mutant, and may be lower than in the wild type. Figure 1 shows time-resolved difference spectra for D212N/R82Q after pulse photoexcitation, between 100 ns and 100 ms. A relatively normal K intermediate that absorbs in the red is followed by the L state with a difference maximum at about 470 nm, and an N state with a similar spectrum as L but with a somewhat red-shifted difference maximum. The difference spectra for D212N/R82Q/D96N were similar to these (not shown). At neutral pH or below, an M intermediate, that would absorb near 410 nm, was virtually absent in either of these photocycles.

Figure 2A,B shows absorption changes measured at selected single wavelengths in D212N/R82Q and D212N/R82Q/D96N, and absorbance changes of pyranine at 457 nm that measure the transient appearance and disappearance of protons in the medium. The overall turnover time of the photocycle is not very much changed from the wild type. If the exceedingly small absorbance changes observed at 410 nm originate from the M intermediate, *i.e.*, from Schiff base deprotonation, the time constants of its formation and final decay would be 10 $\mu$s and 50 ms. The decay of this possible M state is slowed, by a factor of about 10 at pH 7, when the internal proton donor is removed, as in the single mutant D96N (Holz et al., 1989; Miller & Oesterhelt, 1990; Cao et al., 1991). If the accumulation of M in these samples was low because of its slow rise or rapid decay, the additional replacement of D96 would increase it. However, in D212N/R82Q/D96N the absorbance change at 410 nm was not greater than in the double mutant. In spite of the lack of accumulation of the M intermediate after photoexcitation of D212N/R82Q and D212N/R82Q/D96N, protons were released into the bulk and subsequently taken up like in the wild type, and in comparable amounts. The release and

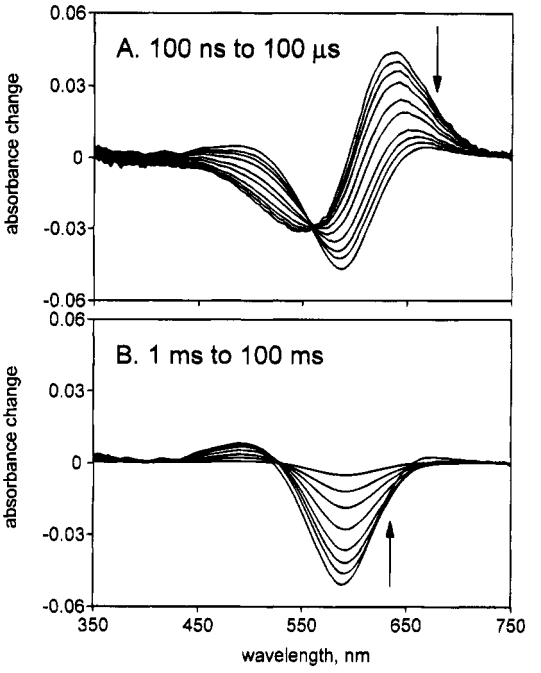


FIGURE 1: Time-resolved difference spectra measured after pulse photoexcitation of D212N/R82Q bacteriorhodopsin. Delay times, in the direction of absorbance change indicated with arrows, in (A): 100 ns, 250 ns, 400 ns, 600 ns, 1 $\mu$s, 1.5 $\mu$s, 2.5 $\mu$s, 4 $\mu$s, 10 $\mu$s, and 100 $\mu$s; in (B): 1 ms, 6 ms, 10 ms, 15 ms, 25 ms, 40 ms, 60 ms, and 100 ms. Conditions: 2 M NaCl, 50 mM phosphate, pH 7, 10 $\mu$M bacteriorhodopsin.

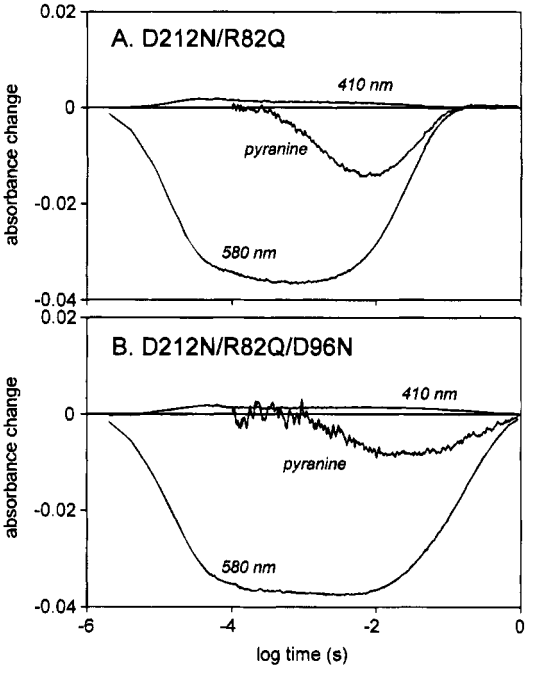


FIGURE 2: Absorbance changes at single wavelengths after pulse photoexcitation of D212N/R82Q (A) and D212N/R82Q/D96N (B) bacteriorhodopsins. Absorbance changes at 410 and 580 nm are plotted *vs* the log of time after pulse photoexcitation, as well as the net signal from pyranine that indicates a pH change in the aqueous phase (downward change is proton release from the protein). Conditions: 2 M NaCl with and without 50 $\mu$M pyranine, pH 6.6 for (A) and pH 6.7 for (B).

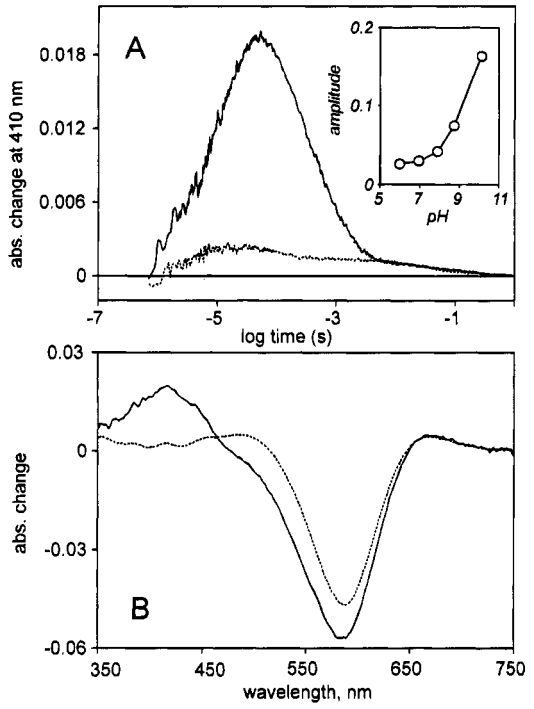


FIGURE 3: Appearance of the M intermediate in the photocycle of D212N/R82Q bacteriorhodopsin at pH >8. (A): Absorbance change at 410 nm *vs* the log of time after pulse photoexcitation at pH 7 (interrupted line) and pH 9.5 (solid line). Inset: pH dependence of the maximal absorbance change. (B): Time-resolved spectra at 100 $\mu$s: dotted line, pH 7; solid line, pH 9.5. Conditions same as in Figure 1.

uptake were somewhat slower in the triple mutant (Figure 2B).

Another possible reason for the lack of accumulation of M would be that it cannot be observed because the deprotonation and protonation equilibria are shifted away from M and toward L and N, respectively. This alternative was tested by performing the experiments in Figures 1 and 2A at pH above 8, where in the wild type the rise of M becomes more rapid (Balashov et al., 1991). We had found earlier that under these conditions (Zimányi et al., 1992a), and also in several mutants where M formation is rapid at any pH (Brown et al., 1994a; Cao et al., 1995), the formation of M appeared to be faster because the amplitude of the faster phase of the formation that corresponds to the L ⇔ M equilibration reaction became dominant. This suggested that the L ⇔ M equilibrium was shifted toward M. Figure 3A shows absorbance change after photoexcitation of D212N/R82Q at 410 nm at pH 7 and 9.5. The amplitude of the absorbance change dramatically increases with pH, and the increase begins above pH 8 (inset). This occurs without a significant change of the rates of rise and decay. Figure 3B shows difference spectra at 100 $\mu$s at pH 7 and 9.5. The shape of the spectrum at pH 9.5 indicates that the absorbance rise in Figure 3A is indeed due to M. At the higher pH, about half of the wild-type amount of M is observed, an amplitude that is lower than the amount actually produced partly because the decay is considerably faster than normal. The results in Figure 3 thus indicate that when the deprotonation equilibrium for the Schiff base is made to favor loss of the proton the M intermediate is restored in the photocycle. They strongly support the idea that M cannot be observed near neutral pH because the Schiff base−D85 proton transfer equilibrium is shifted to favor the protonated Schiff base.

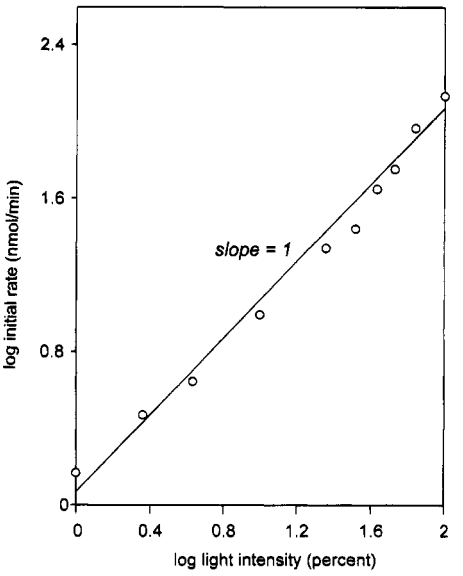


FIGURE 4: Light-driven proton transport in cell envelope vesicles containing D212N/R82Q bacteriorhodopsin. The log of the initial rate of proton extrusion (nmol/min) is plotted *vs* the log of light intensity (percent of maximal). The observed slope of 1 indicates that the transport is caused by a single-photon photoreaction. Conditions: 3 M KCl, pH 5, 21 °C. Total amount of bacteriorhodopsin in the assay, 1 nmol.

*Proton Transport by D212N/R82Q.* The observation of proton release and uptake in Figure 2 suggests that the light-dependent transport might not be eliminated by replacement of D212 and R82. It does not prove it because such measurements do not reveal whether the protons are released and taken up on opposite membrane surfaces. However, when cell envelope vesicles prepared from cells containing D212N/R82Q were illuminated with yellow light (>530 nm), large and rapid pH decreases demonstrated proton extrusion and authentic transport due to multiple turnovers of the chromophore (not shown). The initial rate of proton export from the vesicles was within a factor of 2 of those measured with envelope vesicles containing wild-type bacteriorhodopsin, *i.e.*, within the normal variability of the preparations. Since a sustained photocurrent had been observed with a similarly low accumulation of the M state in D85 mutants via a two-photon process (Tittor et al., 1994), the possibility of transport due to secondary photoexcitation of an intermediate of the photocycle was tested. Figure 4 shows that in this case the initial rate of proton translocation is linear with light intensity over more than 2 orders of magnitude, eliminating this possibility.

*Photocycles of the 14-Fluororetinal Analogues of D212N/R82Q and D212N/R82Q/D96N.* The results at high pH (Figure 3) suggest that the reason for the lack of accumulation of the M intermediate might be that the p$K_a$ of the protonated Schiff base relative to the p$K_a$ of D85 is inappropriate in the L state for proton transfer, as found in some other mutant proteins (Govindjee et al., 1994; Brown et al., 1994a), as well as in 13-*cis*-bacteriorhodopsin (Drachev et al., 1993; Steinberg et al., 1994). In those cases, M was restored in the photocycle upon lowering the p$K_a$ of the protonated Schiff base through replacing the retinal with its 14-fluoro or 13-trifluoromethyl analogue. This possibility was explored here also.

Figure 5 shows time-resolved difference spectra for D212N/R82Q containing 14-fluororetinal between 100 ns and 60 ms after photoexcitation. Two differences from the same

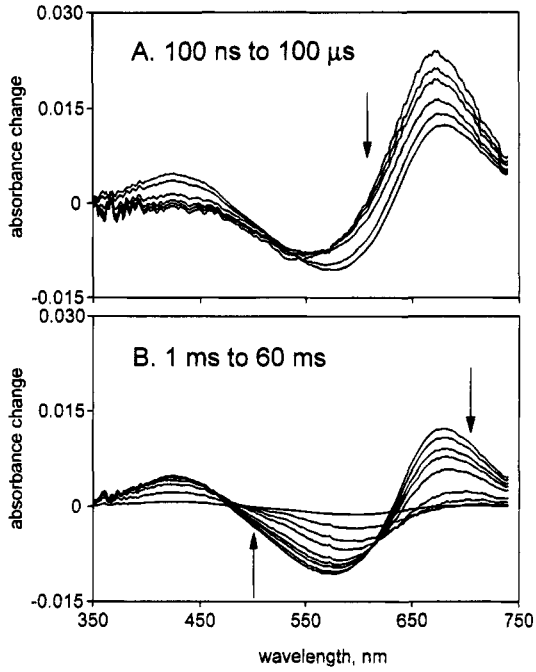


FIGURE 5: Time-resolved difference spectra measured after pulse photoexcitation of the 14-fluororetinal analogue of D212N/R82Q bacteriorhodopsin. Delay times in the direction of absorbance change indicated with arrows, (A): 100 ns, 400 ns, 1 μs, 2.5 μs, 10 μs, and 100 μs; (B): 100 μs, 400 μs, 1 ms, 1.5 ms, 2.5 ms, 4 ms, 6 ms, 10 ms, 25 ms, and 60 ms. Conditions: 2 M NaCl, 10 mM phosphate, pH 7.0. Absorbance of the sample (at 590 nm, its maximum) was 0.35.

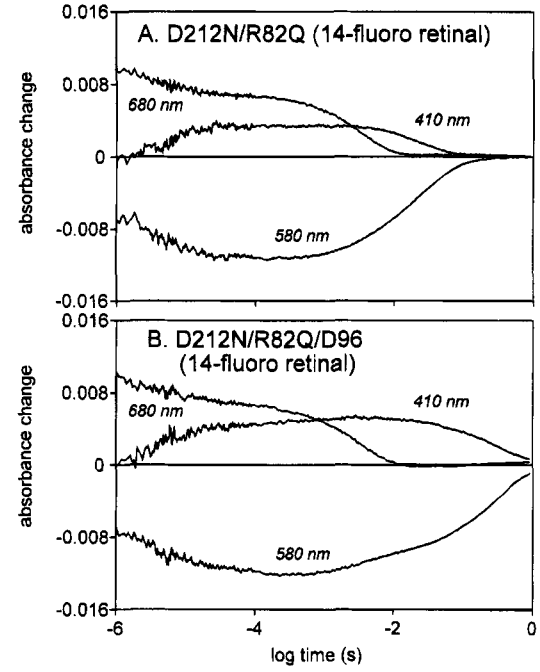


FIGURE 6: Absorbance changes at single wavelengths after pulse photoexcitation of D212N/R82Q (A) and D212N/R82Q/D96N (B) bacteriorhodopsins containing 14-fluororetinal. Absorbance changes at 410, 580, and 680 nm are plotted *vs* the log of time after pulse photoexcitation. Conditions as in Figure 5.

kind of sample but containing authentic retinal (Figure 1) are evident. First, the initial, red-shifted intermediate has a considerably longer lifetime. This was observed with the 14-fluororetinal analogue of wild-type bacteriorhodopsin also (Tierno et al., 1990), and attributed to the photocycle of the large 13-*cis* content in this artificial pigment. Second, an absorption increase near 410 nm and the lack of increase near 500 nm suggest the appearance of the M state and the production of considerably less L. These differences are shown more clearly in single-wavelength measurements in Figure 6. The longer lifetime of the absorbance change at 410 nm than at 680 nm, particularly in the triple mutant, strongly suggests that the red-shifted photoproduct is not O which would be normally produced upon decay of M, but originates from a separate photocycle (*i.e.*, from the 13-*cis* content). The considerably longer lifetime of the species that absorbs at 410 nm in the triple mutant is as expected if it is the M state, because the internal proton donor to the Schiff base is absent.

Figure 7 shows an average of difference spectra in the 15−100 ms time range of D212N/R82Q/D96N containing 14-fluororetinal, where the red-shifted species has completely decayed, as well as a difference spectrum for D212N/R82Q from Figure 1 where lack of M is evident, and an authentic difference spectrum for M *minus* BR (Zimányi & Lanyi, 1993; Nagle et al., 1995). Other than the expected red-shifted depletion band in the artificial pigment (Tierno et al., 1990) and a somewhat changed ratio of absorbance increase and decrease, the difference spectrum is like the wild-type M *minus* BR spectrum. The lower amplitude ratio suggests that it includes some amount of L or N *minus* BR as well. We conclude that lowering the $pK_a$ of the protonated Schiff base with the 14-fluororetinal substitution causes the reappearance of the M intermediate in the photocycle.

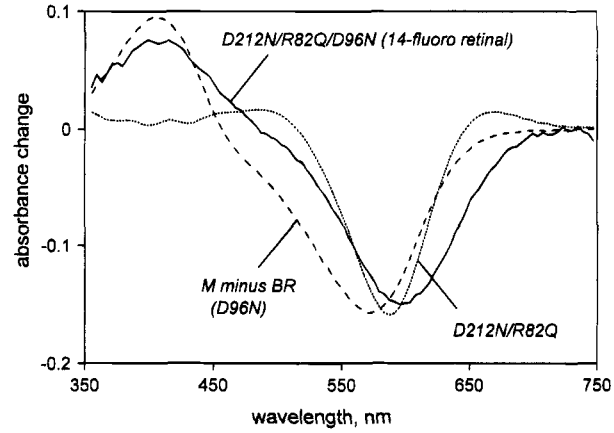


FIGURE 7: Appearance of the M intermediate in the photocycle of the 14-fluororetinal analogue of D212N/R82Q/D96N bacteriorhodopsin. Time-resolved difference spectra for the artificial D212N/R82Q/D96N pigment (solid line, average of measurements at 15 and 100 ms, measured as in Figure 6B) and for D212N/R82Q (dotted line, from Figure 1, 100 μs) are compared with a genuine M *minus* BR spectrum [dashed line, from D96N in Zimányi and Lanyi (1993) and Nagle et al. (1995)]. The spectra are scaled to approximately the same amplitude for the sake of the comparison.

*FTIR Spectra of the L and N Photointermediates of D212N/R82Q and D212N/R82Q/D96N.* Figure 8 shows difference FTIR spectra between 800 and 1800 $cm^{-1}$ after illumination of D212N/R82Q at various temperatures where in the wild-type protein L, M, and N would be produced. At 170 K, illumination results in many infrared bands characteristic of L *minus* BR. The identification of the photoproduct as L is confirmed also by a large positive band at 3486 $cm^{-1}$ assigned to the N−H stretch of W182 (not shown), diagnostic of the L state (Maeda et al., 1992a; Yamazaki et al., 1995b). At increasing temperatures up to 230 K, most of the bands do not exhibit any change. Although the difference spectra decay over several minutes during the measurement, there is no evidence in these spectra for the

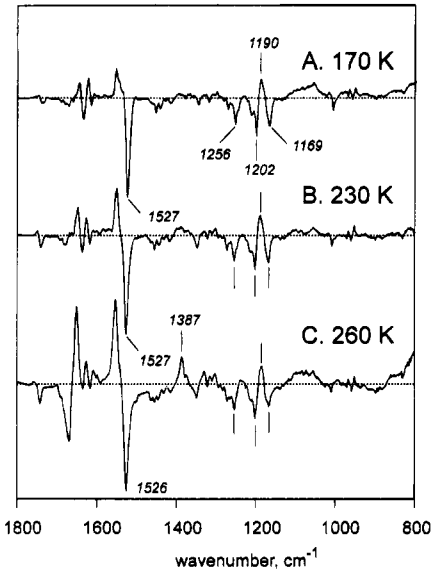


FIGURE 8: FTIR difference spectra for D212N/R82Q at different cryogenic temperatures, at pH 7 (170 K, 230 K, and 260 K, as indicated in spectra A–C). Full height of the ordinate (in absorbance) corresponds to 0.020, 0.019, and 0.017 for spectra A, B, and C, respectively.

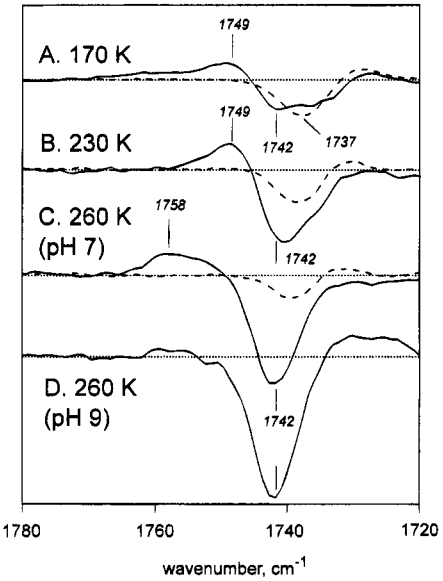


FIGURE 9: FTIR difference spectra for D212N/R82Q and D212N/R82Q/D96N at different cryogenic temperatures (170 K, 230 K, and 260 K, as indicated in spectra A–D) in the C=O stretch frequency region. D212N/R82Q, solid lines; D212N/R82Q/D96N, dashed lines. (A–C) pH 7; (D) pH 9. Full height of the ordinate corresponds to 0.010–0.014.

presence of M, as would be the case for wild type. At 260 K, however, the large changes in the amide I and II regions (between 1550 and 1700 $cm^{-1}$) indicate that the protein conformation observed in the N (Braiman et al., 1991; Souvignier & Gerwert, 1992) and $M_N$ intermediates (Sasaki et al., 1992; Perkins et al., 1992) occurs. According to the large positive C–C stretch band at 1190 $cm^{-1}$, the Schiff base is protonated [as discussed, for example, in Gerwert and Siebert (1986)]. On the basis of these features, as well as the C=O stretch frequencies (see below), we identify the spectrum at 260 K as originating from the N state.

The C=O stretch region shows characteristic changes upon illumination between 170 and 260 K. Figure 9 contains difference spectra for D212N/R82Q (solid lines) and D212N/R82Q/D96N (dashed lines) between 1720 and 1780 $cm^{-1}$.

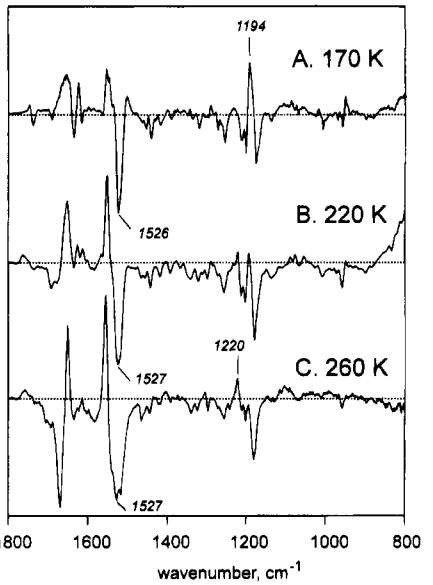


FIGURE 10: FTIR difference spectra for the 14-fluororetinal analogue of D212N/R82Q at different cryogenic temperatures (170 K, 220 K, and 260 K, as indicated in spectra A–C). Full height of the ordinate corresponds to 0.007 for each. (A and B) pH 7; (C) pH 9.

Below about 1700 $cm^{-1}$, the spectra from the double and triple mutants were superimposable. The negative band normally centered at 1740 $cm^{-1}$, that originates from perturbation of D96 and D115 in the L state at 170 K (Braiman et al., 1988; Maeda et al., 1992c), is broader than usual in the double mutant (Figure 9A), but the positive bands at the lower and higher frequency sides appear just as in L of the wild-type protein. In the triple mutant, the negative band moves to 1737 $cm^{-1}$, as expected from removal of the changes due to D96 (Sasaki et al., 1994).

Illumination at 230 K, where M is predominant in wild-type bacteriorhodopsin, yielded only the bands due to L *minus* BR. This is consistent with a shift of the L ⇔ M equilibrium toward L. At 260 K, the spectrum of D212N/R82Q resembles, superficially, that of N of the wild type which would exhibit a positive band at 1755 $cm^{-1}$ due to the protonated D85, and a negative band at 1740 $cm^{-1}$ due to the deprotonated D96. However, in the triple mutant, that lacks D96, not only is the negative band greatly reduced in amplitude but also the positive band completely disappears, indicating that the positive feature had originated from perturbation of D96 causing a shift to higher frequency. Deprotonation of D96 would have occurred additionally to this perturbation because the negative band at 1742 $cm^{-1}$ is much larger than the positive band. This is clearly shown by the N *minus* BR spectrum obtained with D212N/R82Q at pH 9 (Figure 9D). The negative 1742 $cm^{-1}$ band is not greatly increased in amplitude, but the small positive band is virtually absent. We conclude that in the N state D96 became at least partly deprotonated as in the wild type, but D85 remained unprotonated. The large positive band at 1387 $cm^{-1}$ (Figure 8C) might have originated from the C=O symmetrical stretch of the carboxylate produced, but its origin is more likely $C_{15}$–H bending because it is also present in the triple mutant (not shown).

*FTIR Spectra of the Photointermediates of the 14-Fluororetinal Analogues of D212N/R82Q and D212N/R82Q/D96N.* At 170 K, the spectrum in the 800–1800 $cm^{-1}$ region for D212N/R82Q with the retinal analogue (Figure 10A) is similar to that with authentic retinal (Figure 8A), and

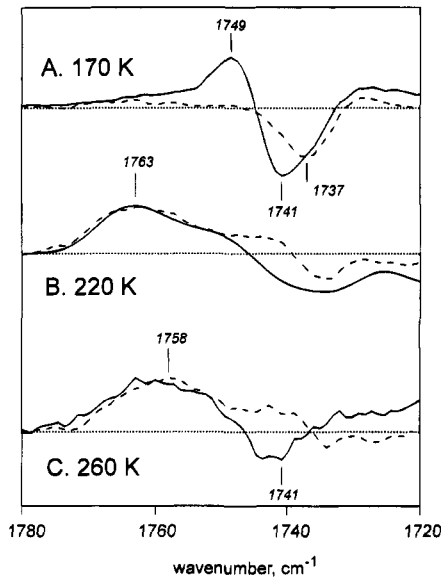


FIGURE 11: FTIR difference spectra for the 14-fluororetinal analogues of D212N/R82Q and D212N/R82Q/D96N at different cryogenic temperatures (170 K, 220 K, and 260 K, as indicated in spectra A–C) in the C=O stretch frequency region. D212N/R82Q, solid lines; D212N/R82Q/D96N, dashed lines. (A and B) pH 7; (C) pH 9. Full height of the ordinate corresponds to 0.003 for spectra A and B, and 0.0015 for the solid line and 0.0025 for the dashed line in spectrum C.

originates from L. Close inspection of the bands in the 1720–1780 $cm^{-1}$ region (Figure 11A) also indicates that at 170 K a normal L intermediate is produced. The C=O stretching bands in Figure 11B show that the spectrum at 220 K contains M with a small amount of L. The positive band at 1763 $cm^{-1}$ in D212N/R82Q was preserved in D212N/R82Q/D96N, but the negative band near 1741 $cm^{-1}$ disappeared. Thus, the negative band originates from the deprotonation of D96, as in the wild type, while the positive band is independent of this residue, consistent with its origin as the protonation of D85. We conclude that when the retinal is replaced with 14-fluororetinal in these proteins, the normal proton transfers involving D85 and D96 are restored.

The difference spectrum of D212N/R82Q/D96N at 260 K (Figure 11C, dashed line) exhibits a positive band at 1758 $cm^{-1}$ due to N. Because we did not detect N in the double mutant at pH 7 at this temperature (not shown), we measured the corresponding spectrum of D212N/R82Q at pH 9, which exhibits features characteristic of the N *minus* BR spectrum (Figures 10C and 11C). Although the complex ethylenic stretch bands besides at 1527 $cm^{-1}$ might have arisen from a complex photostationary state involving the 13-*cis* species, it is obvious that D85 is protonated and D96 is deprotonated.

*Assignment of C=O Stretch Frequencies in D212N/R82Q.* Table 1 shows the C=O frequencies of protonated D85, D96, and D115 derived from the FTIR spectra in Figures 9 and 11. Importantly, where these frequencies can be identified in the mutants, the aspartates do not experience environments greatly different from the wild type. An exception is the frequency of D96 in N, which is 1758 $cm^{-1}$. Such an unusually high frequency for D96 (1755 $cm^{-1}$) has been observed in the L intermediate of the T46V mutant (Yamazaki et al., 1995a). We note that the 1729 $cm^{-1}$ band of D115 of L is hardly shifted in the N intermediate of D212N/R82Q/D96N, at 1731 $cm^{-1}$, in contrast to the considerable upshift of this frequency from 1729 $cm^{-1}$ in L to 1742 $cm^{-1}$ in M and 1740 $cm^{-1}$ in N of the wild-type protein (Sasaki et al., 1994). The normal shift is restored upon replacement of the retinal with the 14-fluoro analogue, from 1728 $cm^{-1}$ in L to 1743 $cm^{-1}$ in M, and 1741 $cm^{-1}$ in N (Table 1).

Table 1: C=O Stretch Frequencies (in $cm^{-1}$) of the Three Protonated Aspartate Residues in Wild-Type and D212N/R82Q Bacteriorhodopsins, in the Unphotolyzed BR State, and Various Photointermediates[a]

| sample | residue | BR | L | M | N |
|---|---|---|---|---|---|
| wild type | D85 | $COO^-$ | $COO^-$ | 1762 | 1756 |
| | D96 | 1742 | 1748 | 1736 | $COO^-$ |
| | D115 | 1734 | 1729 | 1742 | 1740 |
| D212N/R82Q | D85 | $COO^-$ | $COO^-$ | – | $COO^-$ |
| | D96 | 1742 | 1749 | – | 1758, $COO^-$ |
| | D115 | 1738 | 1729 | – | 1731 |
| 14-F D212N/R82Q | D85 | $COO^-$ | $COO^-$ | 1763 | 1758 |
| | D96 | 1741 | 1749 | ? | $COO^-$ |
| | D115 | 1734 | 1728 | 1743 | 1741 |

[a] Assignments for wild type from Sasaki et al. (1994) and for the mutant with authentic retinal and the 14-fluoro analogue from Figures 9 and 11, respectively. The frequencies of D115 were determined from spectra for the D212N/R82Q/D96N triple mutant, and are considered to be essentially unaffected by the third mutation as in the single D96N mutant (Sasaki et al., 1994). Where $COO^-$ is shown, the aspartate in question is unprotonated, or partly unprotonated.

## DISCUSSION

The transfer of the Schiff base proton to D85 in the L → M reaction is the single critical proton transfer step in the bacteriorhodopsin photocycle (*e.g.*, Kataoka et al., 1994). In a simple view, therefore, whether the protein transports protons or not should depend on the Schiff base and D85, and their interaction, and not on other residues even if they are located close enough to be possible additional participants in the proton transfer. We find that this is indeed the case. Transport is normal when the two charged residues, D212 and R82, that are part of the complex counterion to the Schiff base, are replaced with asparagine and glutamine, respectively. Although as single mutations these two residue changes confer unusual behavior, such as chloride and pH dependencies of the absorption maximum and transport in D212N (Needleman et al., 1991; Moltke et al., 1992), and greatly delayed proton release in the transport cycle (Cao et al., 1995), as well as greatly elevated $pK_a$ for D85, in R82Q or R82A (Subramaniam et al., 1990; Otto et al., 1990; Thorgeirsson et al., 1991; Balashov et al., 1992; Brown et al., 1993), these effects disappear in the D212N/R82Q double mutant, suggesting that they are related more to the net charge near the Schiff base than to any specific influences of D212 and R82Q. We must conclude that neither D212 nor R82 plays an essential role in proton transport.

However, D212 and R82 are part of a suggested quadrupole (Dér et al., 1991) that comprises also the Schiff base and D85, and would be expected to strongly influence the configuration of coordinated water and the pattern of hydrogen bonding in this region. Indeed, the photochemistry is altered when both of these two residues are replaced, and in ways that raise interesting questions. Deprotonation of the Schiff base is not observed. The protonation states of putative acceptor D85 and donor D96 in the N intermediate will be important clues in deciding if the Schiff base had undergone any proton transfer reactions. Although in the blue form of D212N a proton was released to the cytoplasmic surface from D96, without deprotonation of the Schiff base and without transport (Cao et al., 1993b), in D212N/R82Q there is net transport of a proton from the cytoplasmic to the extracellular side. Thus, in this protein a deprotonated D96 in the N state would imply prior deprotonation of the

Schiff base, and it is in fact observed (Figure 9C). A protonated D85 would be additional evidence, but it does not appear. However, other reasons suggest that a protonated D85 should not be observed in this protein. If the Schiff base deprotonates, the proton transfer might be directly to the aqueous phase, without protonation of D85. More probably, D85 is the proton acceptor, but the protonation equilibria of the Schiff base, D85, and other group(s) involved in proton release are shifted against the accumulation of a protonated D85. This would come about because in the R82Q single mutant proton release is delayed until the recovery of BR, *i.e.*, the normal proton release machinery is not functional, and the observed proton release in D212N/R82Q and D212N/R82Q/D96N (Figure 2) might have to be directly from D85.

The fact that the unprotonated Schiff base (the M intermediate) does not accumulate in the photocycle is very unusual, considering that this protein transports protons. It could mean either that the Schiff base does deprotonate but the M state cannot be observed because the kinetics are poised against its accumulation, or that the Schiff base does not deprotonate and the transport is based on a completely different mechanism from the wild type. There are two possible kinetic reasons that would prevent the accumulation of M. The rate of formation for M could be slower than its decay, or the equilibria for L and M, and for M and N, could be both shifted away from M. Since introducing the D96N change as a third mutation does not cause accumulation of M in the photocycle (Figure 2) in spite of the fact that M decay should be considerably slowed (Holz et al., 1989; Miller & Oesterhelt, 1990; Cao et al., 1991), a slow rate of formation is not an adequate explanation. On the other hand, change in the equilibria is reasonable because (a) at pH >8, where the deprotonation equilibrium L ⇔ M is shifted toward M, the M state reappears (Figure 3); (b) when the p$K_a$ of the protonated Schiff base is made lower, by exchanging the retinal for 14-fluororetinal (Tierno et al., 1990), the normal photocycle is to a large extent restored; (c) a proton is released in the photocycle (Figure 2); and (d) finding D96 deprotonated in N (Figure 9C) suggests (cf. above) that proton transfer to an unprotonated Schiff base could have taken place. With the retinal analogue in place, not only does the deprotonated Schiff base appear in the photocycle (Figures 5−7, 10) but also the protonation of D85 as well as the deprotonation of D96 proceeds as in the wild type (Figure 11). Thus, no changes have been introduced into the protein by the D212N and R82Q mutations that would cause the proton acceptor and donor functions to be inherently different from wild type. Instead, the consequence of the two mutations appears to be that the p$K_a$'s that regulate the deprotonation and reprotonation equilibria for the Schiff base in the L, M, and N states are altered. It is not clear which groups will have changed p$K_a$'s. Although lowering the p$K_a$ of the protonated Schiff base will restore the wild-type phenotype, it does not necessarily follow that the mutant phenotype was caused by elevation of this p$K_a$. On the other hand, if it were the p$K_a$ of the protonated Schiff base that is increased by the D212N/ R82Q residue replacements, the two protonation equilibria would be shifted through more rapid M → L and M → N reactions, in the way required. In this alternative, the protonated state of D85 in the N intermediate of the 14-fluororetinal analogue (Figure 11B,C) would have to be assured by a changed Schiff base−D85 interaction.

In the other alternative, the Schiff base remains protonated throughout, and the transport is based on the light-dependent transient p$K_a$ change of another group. Such a group would have to be influenced by the photoisomerization of the retinal like normally the Schiff base, and it would have to be in position both to release a proton to the extracellular surface and to then be reprotonated from the cytoplasmic side. There is no evidence for or against this kind of a mechanism at this time; however, Sonar et al. (1994) suggested that it may occur in the mutant Y57D (see next paragraph).

Transport without deprotonation of the Schiff base has been observed in only a few mutant bacteriorhodopsins. We had recently described a case, where the formation of the M state in the V49A/D115N mutant was less favorable because the p$K_a$ difference between the protonated Schiff base and D85 was increased, and under some conditions M did not accumulate in measurable amounts because it was formed too slowly (Brown et al., 1994a). In that case, the possibility of deprotonation of the Schiff base in the photocycle could be demonstrated by changing the pH or introducing other mutations. These conditions caused wide variations in the amount of the M intermediate, from near-wild-type amounts to near-zero. The other cases where M was not produced are the Y57N and Y57D mutants. In Y57N, there was lack of both M and proton release in the photocycle, but replacement of the retinal with the 14-fluoro analogue restored both (Govindjee et al., 1994). In Y57D, deprotonation of the Schiff base could not be observed although the protein was partly functional in transport (Sonar et al., 1994). The introduced D57 residue deprotonated transiently during the photocycle. Although D85 did not become protonated, the deprotonation of D96 was normal. A novel transport mechanism was suggested for Y57D, unlike the one in the wild type, that does not involve Schiff base deprotonation. However, the possibility of a kinetic hindrance to observing M was not considered, and would provide an alternative explanation of their results.

It appears from the results we report here that neither proton transport nor the turnover time constant of the photocycle is greatly affected when the complex counterion to the Schiff base is simplified to remove two of the charges that constitute the quadrupole, as long as the net charge in the unphotolyzed state is unchanged. What is then the evolutionary rationale for this complex structure? One possibility is that it ensures the accumulation of the deprotonated Schiff base in the photocycle, that is, the signaling state in the related retinal protein, sensory rhodopsin I (Spudich & Bogomolni, 1992). However, convincing arguments have been made that bacteriorhodopsin is the evolutionary precursor of sensory rhodopsin I, rather than *vice versa* (Spudich, 1994). Another possibility is that the need is for D212 (and therefore R82 to compensate its charge) rather than the M state, and it arose in the other related retinal protein, halorhodopsin, that lacks an anionic residue equivalent to D85, and bacteriorhodopsin has retained this feature during its evolution from halorhodopsin.

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