Effects of Substitution of Tyrosine 57 with Asparagine and Phenylalanine on the Properties of Bacteriorhodopsin†

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ABSTRACT: Tyrosine 57 is one of the residues present in the retinal binding pocket and is conserved in all the halophilic retinal proteins. We have studied mutants of bacteriorhodopsin, expressed in *Halobacterium salinarium*, in which tyrosine 57 is replaced by an asparagine (Y57N) or phenylalanine (Y57F). In Y57N the photocycle proceeds only up to the L intermediate; no M is formed at neutral pH. The lifetime of L intermediate is extremely long, ca. 500 ms. Proton release is severely affected in both the mutants which suggests that Y57 is associated with the proton release pathway. By comparing the pH-induced absorption changes in the UV in Y57N and Y57F with those in the wild-type (WT), we determined that the pK_a of Y57 is 10.2. In Y57F, which shows M formation, the rate constant of the L → M transition is pH dependent (pK_a 8.7) suggesting that Y57 is probably not the residue that normally controls the transition into the alkaline photocycle. Y57 is either part of the counterion complex or in close proximity to D85 since its mutation influences the pK_a of Asp85. In Y57F the pK_a of D85 is ~4.9 (compared to ~2.9 in the WT). The Y57N mutant shows two pK_a's in the purple to blue transition, ~3.8 and <1. In the presence of hydroxylamine, at neutral pH, Y57N is stable in the dark but bleaches very rapidly upon illumination compared to the WT. Since the lifetime of L intermediate is long in Y57N, we suggest that the Schiff base becomes accessible to hydroxylamine in this state.

Purple membrane from *Halobacterium salinarium* contains a single protein, bacteriorhodopsin (bR), which consists of a retinal molecule bound to the apoprotein via a Schiff base linkage to lysine 216. bR contains 11 tyrosine and 8 tryptophan residues, and several of these are part of the retinal binding pocket including Y57 which is conserved in all the halophilic retinal proteins. The roles of many of the amino acid residues in the retinal binding pocket and the proton channel have been elucidated using site-specific mutants. Replacement of Asp85 or Asp96 with neutral asparagine greatly alters the proton-pumping activity of bR. Asp85 has been proposed to be part of a complex counterion to the protonated Schiff base and acts as a primary proton acceptor upon deprotonation of the Schiff base during the L to M transition in the photocycle. The purple to blue membrane conversion upon lowering the pH is due to its protonation. D96 is the proton donor in the reprotonation of the Schiff base during the M to N transition in the photocycle [see reviews: Mathies et al. (1991), Oesterhelt et al. (1992), Rothschild (1992), Ebrey (1993), Lanyi (1993)]. The role of the aromatic residues in the functioning of bR is controversial. Observations on the light-induced changes in fluorescence and absorption in the near-ultraviolet region were ascribed to the deprotonation of a tyrosine residue(s) and/or the charge perturbation in the vicinity of tryptophans during the bR photocycle (Bogomolni et al., 1978; Fukumoto et al., 1981, 1984; Aldashev & Efremov, 1983; Maeda et al., 1986, 1988; Balashov et al., 1991; Wu & El-Sayed, 1991). M formation and decay kinetics and proton pumping were observed to be affected in bR in which the tyrosine residues were chemically modified (Konishi & Packer, 1978; Lemke et al., 1982; Rosenbach et al., 1982; Scherrer & Stoeckenius, 1984, 1985). FTIR experiments suggested the presence of a tyrosinate in light-adapted bR (Dollinger et al., 1986; Rothschild et al., 1986, 1992).

Balashov et al. (1991) have shown that at high pH the visible absorption band of bR shifts slightly to longer wavelengths and there is an increase in optical density around 240 and 296−300 nm; upon raising the pH to about 12, approximately three tyrosine residues deprotonate per bR. However, resonance Raman experiments have not supported the formation of a tyrosinate during the bR photocycle (Ames et al., 1990). Also using NMR, Herzfeld et al. (1990) did not detect any tyrosinates in bR or any of the photocycle intermediates up to about pH 11 (McDermott et al., 1991). Using site specific mutants of bR (expressed in *Escherichia coli*), Mogi et al. (1987) suggested that tyrosine residues are not essential for bR function since the substitution of each of the tyrosine residues in bR with phenylalanine did not affect the proton pumping. Surprisingly Soppa et al. (1989) found that a mutant of *Halobacterium* sp GRB in which Tyr57 was replaced with an asparagine (Y57N) did not show any proton pumping or M photointermediate (Govindjee et al., 1992, 1994). Also, Y57N shows altered light/dark adaptation so that even in the light-adapted state it has 25%

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13-cis isomer present compared to almost none in the wild-type (WT).

In order to clarify the role of Y57 in bR we have studied *Halobacterium* mutants in which Y57 was changed to either N or F. We find that the phenotypes of Y57 mutants depend on the actual substitution. Y57F maintains several important features of the native bR which are drastically altered in Y57N mutant. We find that the pK_a of Y57 is approximately 10.2. Y57 influences the pK_a of D85 and is probably part of a proton release complex, since its mutation affects the proton release/uptake process.

MATERIALS AND METHODS

Purple membrane from *Halobacterium* sp GRB and the Y57N mutant (Soppa et al., 1989) were a gift from Dr. D. Oesterhelt. Y57F membranes were a gift from Drs. J. Lanyi and R. Needleman. The two mutants come from different wild-type strains, Y57N from GRB and Y57F from S-9.

Acid and alkaline titrations of the absorption spectrum were carried out using an Aviv 14DS spectrophotometer (Aviv Assoc., Lakewood NJ). All data manipulations were done with PV-WAVE visual data analysis software (Precision Visuals Inc.) customized to our needs. Acid titrations were done in the dark on dark-adapted aqueous suspensions of purple membrane in the presence of 25% glycerol using H_2SO_4 (i.e., without Cl^-) to avoid complications due to the formation of the acid purple membrane. For alkaline titrations the purple membrane suspension (in 0.166 M KCl) was light adapted at pH 7; the pH was then raised with NaOH in the dark and the absorption spectrum measured. The sample cuvette was thermostated at 20 °C.

Flash-induced absorbance changes of the photocycle intermediates and proton release and uptake were measured on a home-built kinetic spectrophotometer as described previously (Govindjee et al., 1990). Actinic excitation was provided from a Nd-YAG laser, 532 nm, 7 ns pulse (Quanta Ray DCR-11; Spectra Physics, Mountain View, CA). For proton release/uptake measurements absorbance changes of the pH sensitive dye, pyranine, were measured at 460 nm. All measurements were carried out in a thermostated cuvette under magic angle conditions.

Bleaching of Y57N was carried out under illumination with orange light (500 W projector plus Corning CS3-72 filter) in the presence of 1 M hydroxylamine and 0.5 M NaCl at pH 7, 25 °C. 13-Desmethylretinal was prepared according to Gartner et al. (1980). 13-Desmethyl-Y57N pigment was reconstituted by incubating 13-desmethylretinal with Y57N apomembrane at pH 7.

RESULTS

Light/Dark Adaptation

Wild-Type. Bacteriorhodopsin from the WT sp GRB is identical with the WT strain S-9 as far as the absorption properties and light/dark adaptation are concerned. The absorption maximum is at 558 nm (dark adapted) and 568 nm (light adapted), and the half-time of dark adaptation is approximately 120 min at 20 °C, pH 7.

Y57N. The absorption maximum of Y57N is blue shifted compared to that in the WT (DA λ_{max} = 556 nm) (Soppa et al., 1989). The extent of light adaptation in Y57N is dependent upon the wavelength of illumination. In orange light the red shift upon light adaptation is small, only a few nanometers (from 556 to 559 nm), and there is only a very small increase in extinction. On the other hand, blue-green light (400−550 nm) causes a bigger red shift, ~5 nm, and a 7% increase in extinction. The simplest explanation of the wavelength dependence of light adaptation is that light induces photoisomerization in both directions, not only cis to trans but also trans to cis (Casadio et al., 1980; Imasheva et al. Personal communication). Due to the photoreversibility of the process, 13-cis pigment is present in the light-adapted state, as reported by Soppa et al. (1989). The rate of dark adaptation is very slow, the half-time being ~8 h at 20 °C, pH 6.6.

Y57F. The absorption maximum of Y57F is also considerably blue shifted compared to bR. The λ_{max} of the light-adapted pigment is at 558 nm, and that of the dark-adapted pigment is at 548 nm. Light adaptation in Y57F suspensions is similar to that in the WT. There is a 10 nm red shift in the absorption maximum and an approximately 20% increase in extinction upon light adaptation with 460−560 nm light. Unlike Y57N, the rate of dark adaptation in Y57F is very fast, with a half-time of approximately 7 min at 20 °C, pH 6.6.

Alkaline Titration of the Ultraviolet and Visible Absorption Spectrum

As reported earlier (Govindjee et al., 1992), alkalinization of the WT strain GRB shows absorption changes similar to those observed in the WT strain S-9 (Balashov et al., 1991). Upon alkalinization, between pH 7 and 11, the optical density decreases around 560 nm and increases around 460 and 630 nm. The increase at 630 nm is from the red shift of the absorption due to the formation of $bR_{alkaline}$, and the increase in optical density in the 460−470 nm region is due to the formation of P480 (hereafter called $P480_{alkaline}$ to distinguish it from $P480_{acid}$, see later). $P480_{alkaline}$ is a mixture of two species with different pK_a's. The lower pK_a species can be photoconverted into a species with a broad band around 360−380 nm in the difference spectrum, P380 (Balashov et al., 1991). The second species has a pK_a similar to that of the Schiff base. In the near-ultraviolet region, between pH 7 and 12, absorbance increases around 240 and 296−300 nm and a shoulder at about 288 nm are observed. As seen in Figure 2 a total of three tyrosines deprotonate per bR between pH 7 and 12, with pK_a's at approximately 9.4, 10.2, and 11.9 in 166 mM KCl. The pK_a of the Schiff base is around 13.

Y57N. Upon alkalinization, there is a decrease in absorbance at 560 nm and an increase around 460−470 nm with a cross-over point at 510 nm. The absorbance increase around 460−470 nm and the absorbance decrease at 560 nm, presumably representing the titration of the Schiff base, have the same pK_a of about 12.1 (Govindjee et al., 1992). In contrast to the WT no red shift of the visible absorption band is seen and no P380 is observed. In the ultraviolet region, an absorbance increase is observed around 240 and 296−300 nm with a shoulder at about 288 nm. A plot of the number of tyrosinates per bR vs pH is shown in Figure 2 (top panel). Over the same pH range, only two tyrosines deprotonate per bR in Y57N as compared to three in the WT. Subtracting this titration curve from that of the native bR results in a single titration with a pK_a of 10.2, suggesting

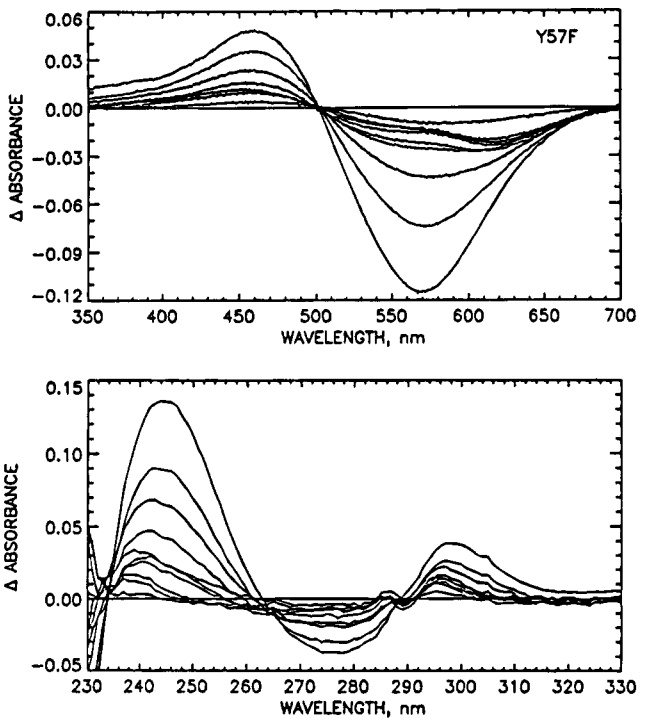

FIGURE 1: Alkaline titration of light-adapted bR from Y57F in 0.15 M KCl. Samples were light adapted at pH 7, and then the pH was changed in the dark. Difference absorption spectra at pH_i − pH 7, where pH_i inside to outside is 8.7, 9.3, 9.7, 10.1, 10.5, 10.9, 11.23, 11.67, and 11.9.

that the pK_a of Y57 is ~10.2 in 166 mM KCl (Figure 2, bottom panel).

Y57F. Upon alkalinization, the Y57F mutant shows absorbance changes similar to those observed in Y57N. In the visible region there is a decrease in absorbance at 560 nm and an increase in the 460−470 nm region (Figure 1, top panel). The pK_a of the Schiff base is approximately 12.4. No red shift of the visible absorption band is observed. In the near-ultraviolet region, absorbance increases around 240 and 296−300 nm and a shoulder around 288 nm are observed (Figure 1, bottom panel). Y57F also shows only two tyrosines per bR deprotonating between pH 6 and 12 (Figure 2). Again no tyrosine deprotonation with a pK_a around 10.2 was observed.

Acid Titration of the Absorption Spectrum: The Purple to Blue Transition

Y57N. In bR, acidification causes the conversion of purple membrane to blue membrane and the absorption maximum shifts to longer wavelengths due to the protonation of D85. By measuring the pK_a of this spectral transition, one can determine the pK_a of D85. In the WT the purple to blue transition has a single pK_a which ranges from 2 to 5 depending upon the ionic strength (Kimura et al., 1984; Jonas & Ebrey, 1991). In the Y57N mutant acidification of an aqueous suspension of dark-adapted membranes exhibits more complex spectral changes. Between pH 7 and 0.3 three different transitions are seen; formation of blue membrane in two steps with different pK_a's (hereafter referred to as blue membrane I and blue membrane II) and a new species, $P480_{acid}$. When the pH is lowered from around neutral to 2, the absorption at the maximum decreases while increasing around 620 and 480 nm. The difference spectra (pH_i − pH

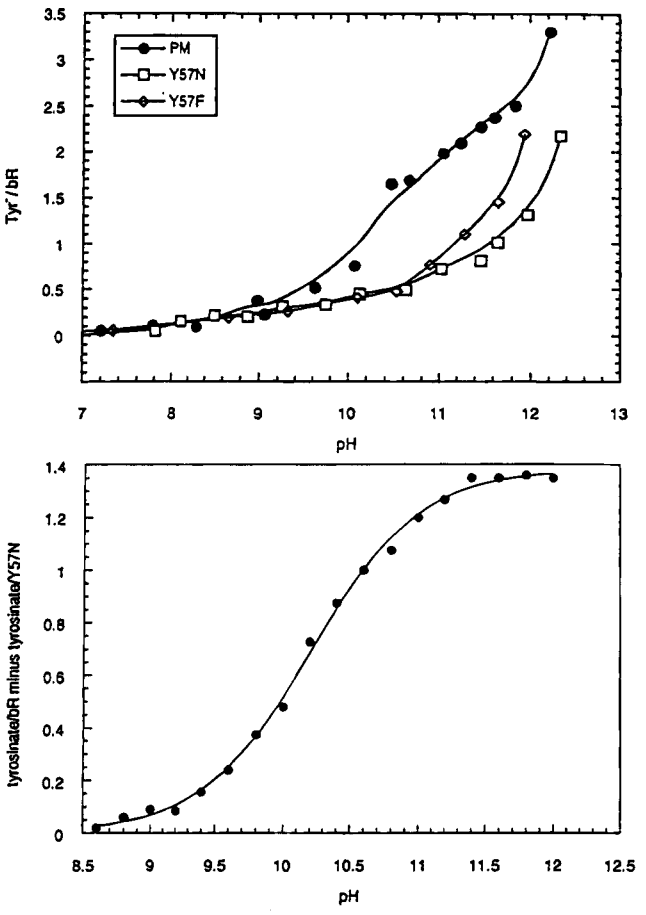

FIGURE 2: Top panel, tyrosinates per bR in WT, Y57N, and Y57F as a function of pH [WT and Y57N data taken from Govindjee et al. (1992)]. The number of tyrosinates per bR was calculated using a differential extinction coefficient of 11 000 at 240 nm as discussed in Balashov et al. (1991). Bottom panel, difference between the WT and Y57N titration curves in the top panel.

7) show a minimum around 560 nm and maxima around 460−470 and 620−630 nm with cross-over points at 505−515 and 580−585 nm (Figure 3, panels A and B). Thus, in addition to the increase in absorbance on the long wavelength side of the main absorption band due to the formation of blue membrane I, there is an absorbance increase on the short wavelength side also which we assign to the formation of another species, $P480_{acid}$. As the pH is lowered below ca. 2, the absorption maximum shifts to longer wavelengths (from 556 nm at pH 7 to ~580 nm at pH 0.2); between pH 2 and 0.2 the difference spectra show a decrease at 480 nm and an increase at 605−610 nm with a cross-over point around 545 nm (Figure 3C,D) which can be ascribed to the transition of $P_{480acid}$ to blue membrane II. By taking advantage of the shift in the cross-over point, we were able to choose the appropriate wavelengths for distinguishing between the different transitions and calculate their pK_a values.

Figure 4 (top panel) is a plot of ΔA_{630} vs pH showing the formation of blue membranes I and II. As the pH is lowered, the absorbance at 630 nm increases until pH 3 due to the formation of blue membrane I, and then it levels off or even decreases a little bit and finally increases again due to the formation of blue membrane II. The pK_a's of blue membranes I and II are approximately 3.8 and <1, respectively. At 475 nm the optical density increases between pH 5 and 2 and then decreases at lower pH's (Figure 4, bottom panel).

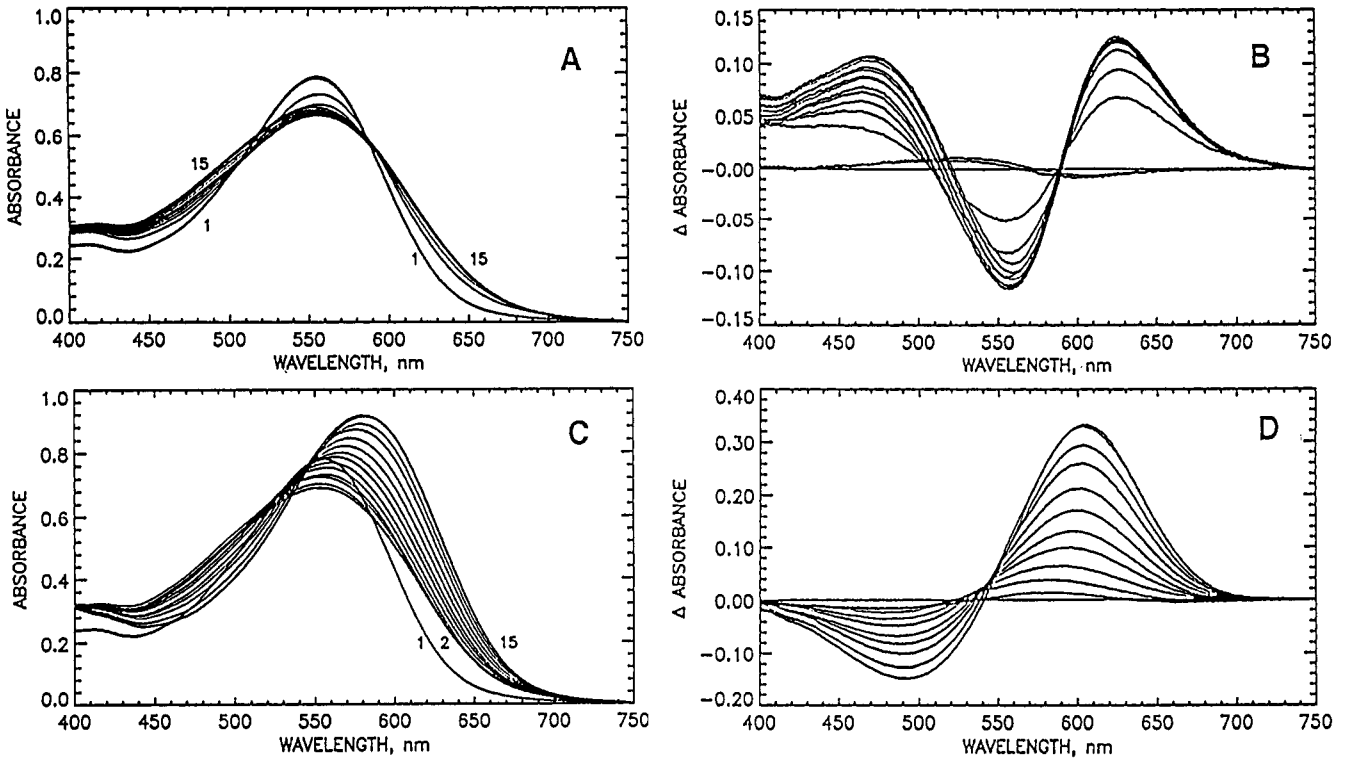

FIGURE 3: Acid titration of Y57N. (A) Absorption spectra from pH 6.84 (1) to 2.34 (15). (B) Corresponding difference absorption spectra (pH_i − pH 6.84) where pH_i (inside to outside) is 6.6, 6.1, 3.86, 3.49, 3.26, 3.12, 3.02, 2.86, 2.8, 2.73, 2.66, 2.58, 2.47, and 2.34. (C) Absorption spectra from pH 2.2 (2) to pH 0.34 (15), also shown is the spectrum at pH 6.84 (1). (D) For the sake of clarity, only some of the corresponding difference spectra (pH_i − pH 1.8) are shown; pH_i (inside to outside) is 1.61, 1.48, 1.33, 1.17, 1.05, 0.91, 0.8, 0.66, 0.51, 0.41, and 0.34. Dark-adapted sample in 25% glycerol was titrated with H_2SO_4 at 20 °C.

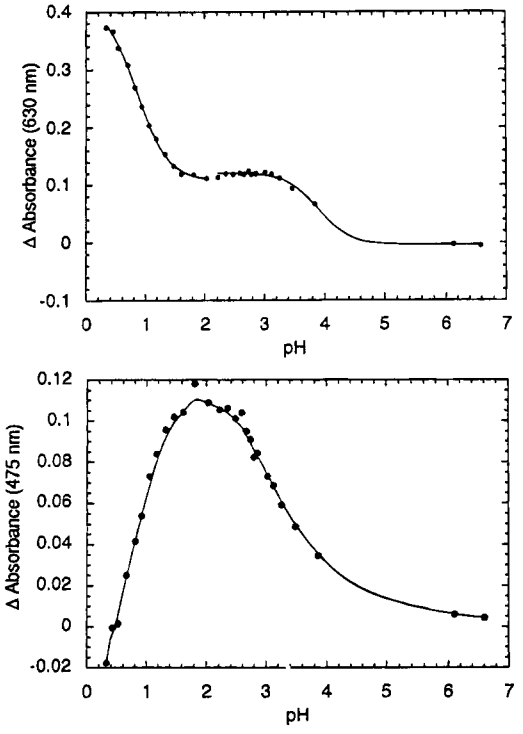

FIGURE 4: Absorption changes of Y57N at 630 nm (top) and 475 nm (bottom) as a function of pH (data taken from Figure 3). Top panel, ΔA_{630} shows the two-step purple to blue transition with pK_a's around 3.8 and <1. Bottom panel, ΔA_{475} vs pH showing the formation of $P480_{acid}$. The pK_a's of the formation and disappearance of $P480_{acid}$ are ~3.2 and <1, respectively.

The increase represents the formation of $P480_{acid}$ with pK_a ~ 3.2. The pK_a of the decrease in $P480_{acid}$ is the same as for the formation of blue membrane II, <1, suggesting that blue membrane II is formed from $P480_{acid}$. This behavior is in contrast to the WT where formation of $P480_{acid}$ has not been observed.

Y57F. While the alkaline titration of Y57F is similar to that of Y57N, the acid titration is markedly different. Acid titration of Y57F is similar to that of the WT in that no $P480_{acid}$ could be observed. Upon lowering the pH of an aqueous suspension of Y57F, there is a decrease in the main absorption band and an increase around 620−630 nm due to the formation of the blue membrane (Figure 5A,B). The pK_a of purple to blue transition is around 4.9 (Figure 5C), which is almost 2 pH units more than in the WT under similar conditions. Formation of $P480_{acid}$ could not be detected; however, a small amount of blue membrane with pK_a < 1 was observed at lower pH's (Figure 5C).

13-Desmethyl-Y57N. Since Y57N differs from Y57F in the extent of light/dark adaptation and the number of phases in the purple to blue transition, we suspected that the two phases seen in Y57N represent the formation of blue membrane from cis and trans isomers. In order to better understand the origin of the different transitions in the acid titration of Y57N, we titrated an analogue pigment, 13-desmethyl-Y57N (reconstituted by incubating 13-desmethylretinal and Y57N apomembrane). 13-Desmethyl-bR has almost 85% 13-cis and only 15% all-trans isomers in both the light- and dark-adapted states and does not show the usual absorption changes seen upon light adaptation in bR (Gartner et al., 1983). While it should be confirmed by future studies, it seems reasonable to assume that 13-desmethyl-Y57N also has a similar isomeric composition, since it does not show any absorbance changes upon light adaptation and shows a large flash-induced absorption change at 660 nm due to the

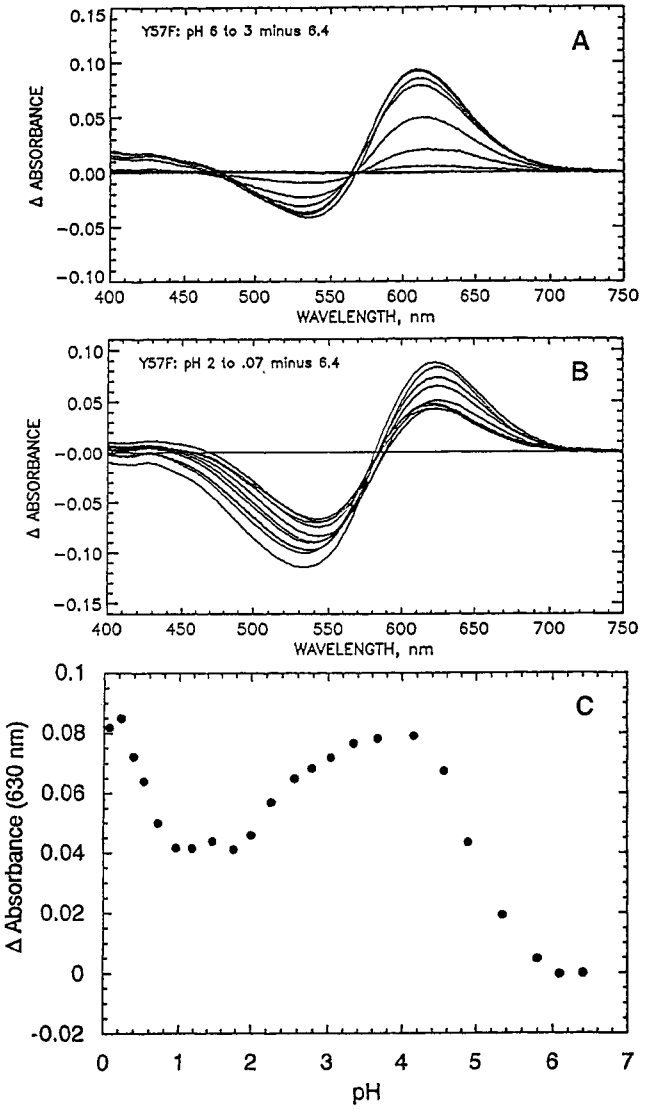

FIGURE 5: Acid titration of dark-adapted Y57F in 25% glycerol. (A) Difference absorption spectra at pH_i − pH 6.4, where pH_i (inside to outside) is 6.09, 5.85, 5.35, 4.89, 4.56, 4.15, 3.67, 3.03. (B) pH_i − pH 6.4, where pH_i (inside to outside) is 1.77, 1.47, 1.18, 0.96, 0.72, 0.53, 0.39, 0.23, and 0.07. (C) Absorption changes at 630 nm as a function of pH. The blue membrane is formed predominantly with a pK_a of 4.9 with a minor second phase with $pK_a < 1$. Conditions were the same as in Figure 3.

primary photoproduct of the 13-cis photocycle (data not shown). Thus, the titration of 13-desmethyl-Y57N should allow us to determine if the high- and low-pK_a blue membranes are formed, respectively, from trans and cis isomers of the pigment.

Acidification of 13-desmethyl-Y57N shows the same basic features as those seen in the native Y57N (i.e., two phases in the purple to blue transition and the formation of $P480_{acid}$). Initially, when the pH is lowered, there is a decrease in absorbance around 550 nm, an increase around 630 nm (blue membrane I), and a very small increase around 460−470 nm ($P480_{acid}$) (Figure 6A). While the absorbance increase at 630 nm reaches a maximum around pH 4.7, the absorbance at 475 nm continues to increase until pH 2.5 (Figures 6B and 7B). This suggests that blue membrane I and $P480_{acid}$ represent two separate transitions and their pK_a's are different and better separated in 13-desmethyl-Y57N (Figure 7A,B). Thus, part of the pigment converts into blue membrane I with a pK_a of approximately 5.4 (Figure 7A), which is

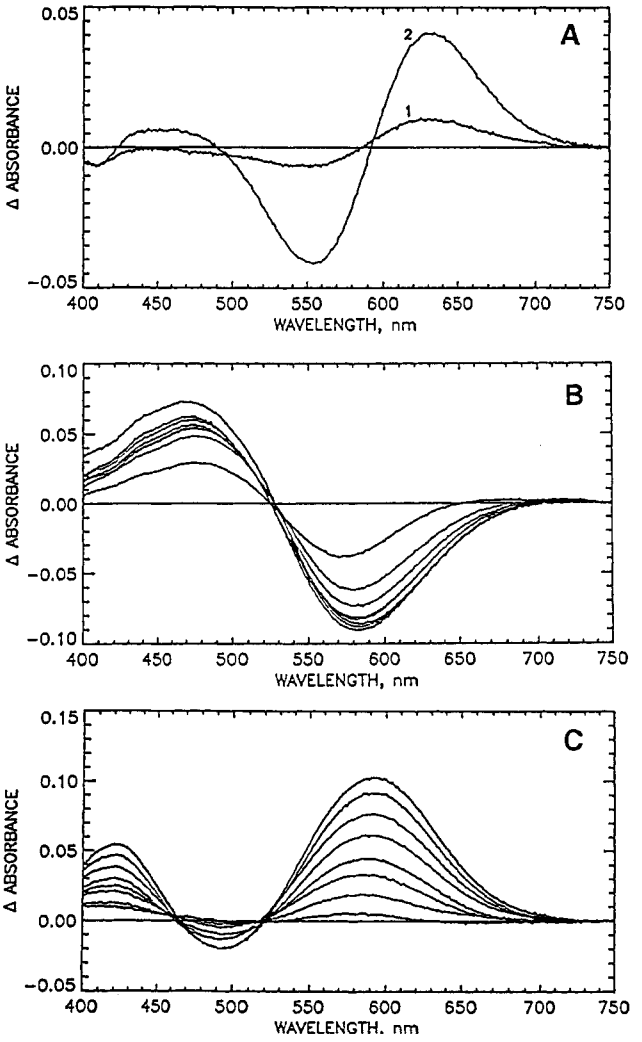

FIGURE 6: Acid titration of dark-adapted 13-desmethyl-Y57N in 25% glycerol. (A) Difference absorption spectra showing the first phase of purple to blue membrane transition. pH 5.75 − 6.73 (1) and pH 4.67 − 6.73 (2). (B) Formation of $P480_{acid}$. pH_i − pH 4.67, where pH_i is (inside to outside) 3.97, 3.45, 3.2, 2.94, 2.77, 2.52, 2.33, and 2.01. (C) Second phase of purple to blue membrane transition: pH_i − pH 2, with pH_i (inside to outside) being 1.58, 1.36, 1.11, 0.95, 0.78, 0.64, 0.45, and 0.33.

somewhat higher than in the native Y57N, and the rest of the pigment converts to $P480_{acid}$ with a pK_a of about 3.8 (3.2 for Y57N) (Figure 7B). When the pH is decreased below 2, there is a decrease in absorbance around 490−500 nm, an increase at 600 nm, and a small increase at 425 nm (Figure 6C). The pK_a of blue membrane II is quite low, <0.7 (Figure 7C), as in Y57N.

Since the amount of blue membrane I is smaller, and the amount of trans isomer in 13-desmethyl pigment is also smaller, we propose that blue membrane I is formed from the trans pigment. The amount of blue membrane II is larger as is the amount of 13-cis isomer; we assign it to the cis pigment. However, it seems that the 13-cis pigment does not convert into blue membrane II directly but first goes to $P480_{acid}$ and then the latter converts to the blue membrane at low pH.

In summary, there are three acid-induced transitions in Y57N: conversion of all-trans pigment to blue membrane I, formation of $P480_{acid}$ probably from the 13-cis pigment, and conversion of $P480_{acid}$ to blue membrane II (Figure 12).

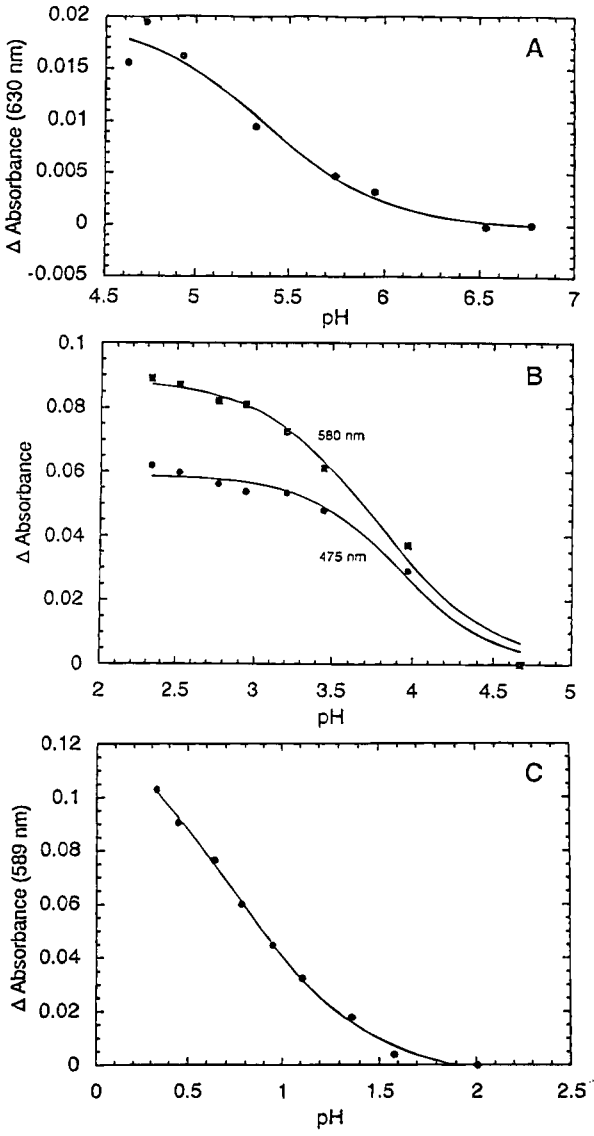

FIGURE 7: 13-Desmethyl-Y57N. (A) ΔA_{630} vs pH showing the first phase in the purple to blue membrane transition, pK_a 5.4. (B) ΔA_{475} and ΔA_{580} (multiplied by -1) vs pH showing that the formation of P480$_{acid}$ and the depletion of the pigment at 580 nm have similar pK_a's, 3.9. (C) ΔA_{589} showing the second phase in the purple to blue membrane transition, pK_a < 1. Data taken from Figure 6.

Light-Induced Transitions at Acid pH

In order to further sort out the origin of P480$_{acid}$ and blue membranes I and II, we studied light-induced blue membrane formation at low pH's.

Y57N. As discussed earlier, upon lowering the pH of dark-adapted Y57N from 6.5 to 3.4, blue membrane I and P480$_{acid}$ are formed. The difference spectrum (pH 3.4−6.5) shows an absorbance decrease around 550 nm and absorbance increases around 620−630 and 460−480 nm (P480$_{acid}$) (Figure 8A, curve 1). If the sample (at pH 3.4) is illuminatetd with 550 nm light, more blue membrane is formed with a further increase in absorbance around 620−630 nm and a decrease at 550 nm; a small increase around 460−480 nm also takes place (see Figure 8A, curve 2, and Figure 8B, curve 1). Light-induced blue membrane formation results from the photoisomerization of 13-cis pigment to all-trans pigment, which at pH 3.4 converts to blue membrane. Illumination with 470 nm light drives P480$_{acid}$ back to the

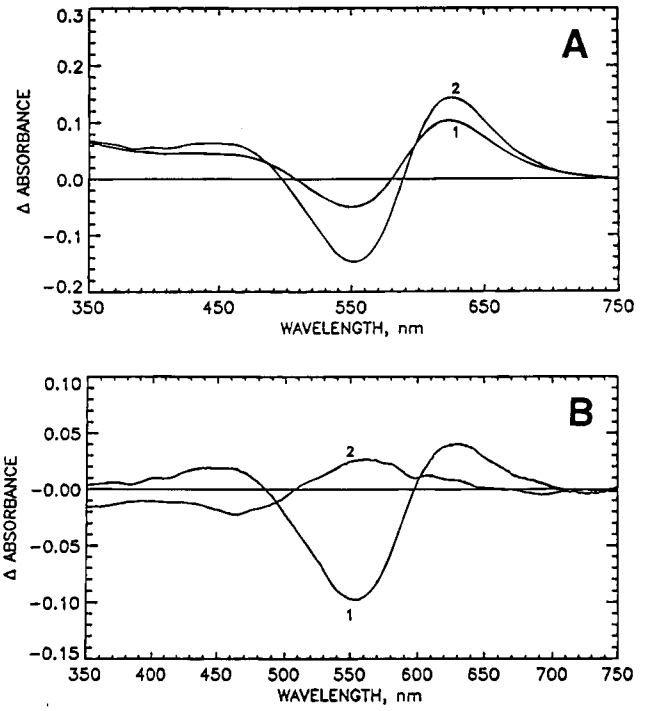

FIGURE 8: Light-induced transitions in Y57N at pH 3.4. (A) Curve 1, pH 3.4 − 6.5 in the dark; curve 2, pH 3.4 − 6.5 but after 20 min illumination with 550 nm light at pH 3.4 showing the light-induced formation of blue membrane. (B) Curve 1, the difference of curves 2 and 1 in panel A; curve 2, the conversion of P480$_{acid}$ to the pigment upon illumination with 470 nm light at pH 3.4.

parent pigment causing a decrease around 460 nm and an increase around 550 nm (Figure 8B, curve 2). This again suggests that the origin of blue membrane I and P480$_{acid}$ is different: possibly *trans*-Y57N converts to blue membrane I, while P480$_{acid}$ is formed from 13-*cis*-Y57N (see, below, transitions in 13-desmethyl-Y57N). If, after illumination with 550 nm light, the sample is left in the dark, blue membrane I and P480$_{acid}$ slowly decay back to the original pigment. Some denaturation of the pigment also occurs if the sample is left at this pH.

13-Desmethyl-Y57N. Unlike Y57N, illumination of 13-desmethyl-Y57N at pH 4.7 with 550 nm light does not produce more blue membrane. At pH 4.7 the first phase of purple to blue transition is almost complete (Figure 9A, curve 1), and since 13-desmethyl-Y57N does not show cis to trans isomerization upon light adaptation, there should be no light-induced purple to blue transition. Indeed, illumination with 550 nm light causes a decrease in absorbance around 560 nm and an increase around 450 nm (due to the formation of P480$_{acid}$), with no additional increase at longer wavelengths (Figure 9A, curve 2, and Figure 9B, curve 1). P480$_{acid}$ can be reversed to the pigment with 450 nm illumination resulting in a decrease around 450 nm and an increase at 570 nm (Figure 9A, curve 3, and Figure 9B, curve 2). This observation lends further support to the idea that blue membrane I is formed from the pigment (probably the trans isomer) and P480$_{acid}$ (which at pH's below 2 converts to blue membrane II) is also formed from the parent pigment (probably the 13-cis isomer).

Photochemical Cycle and Proton Transfer

Y57N. The flash-induced difference absorption spectrum of Y57N, recorded 1 ms after the laser pulse, shows an

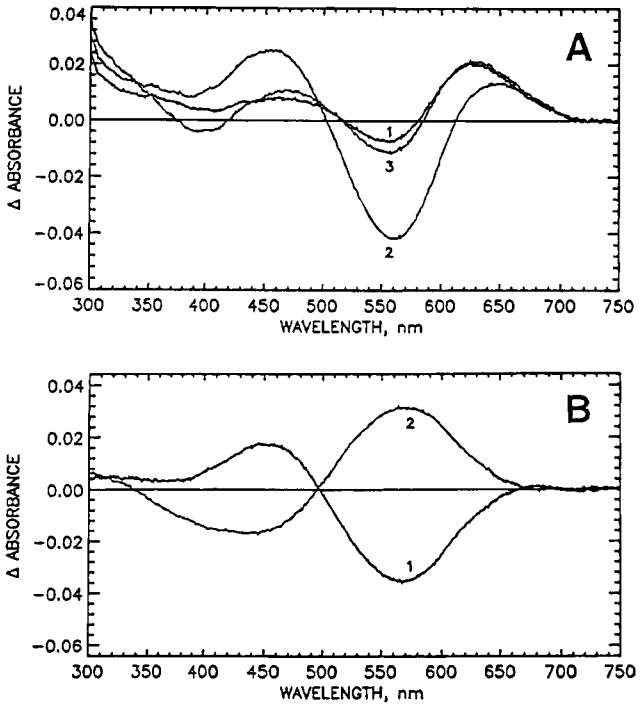

FIGURE 9: Light-induced transitions in 13-desmethyl-Y57N at pH 4.7. (A) Curve 1, pH 4.7 − 6.7; curve 2, pH 4.7 − 6.7 but after 10 min illumination with 550 nm light at pH 4.7; curve 3, pH 4.7 − 6.7 but the sample was further illuminated with 450 nm light at pH 4.7. (B) Curve 1, the difference between curves 2 and 1 in A; curve 2, the difference between curves 3 and 2 in A.

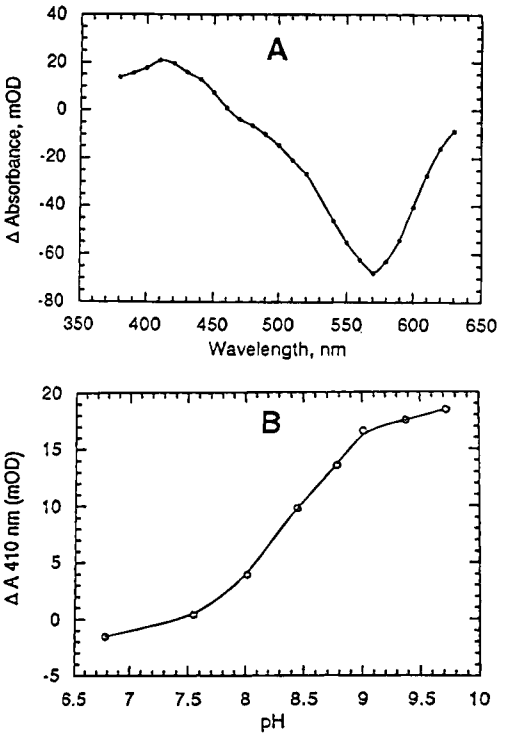

FIGURE 10: (A) Flash-induced difference spectrum of Y57N, 1 ms after the laser flash, at pH 9.3. (B) pH dependence of the amplitude of the flash-induced absorption changes at 410 nm. The sample was suspended in 50% glycerol and 2 M NaCl.

absorbance decrease at 560 nm and increases around 660 and 460−480 nm (Govindjee et al., 1992). The absorbance increase at 660 nm is due to the bathoproduct of the 13-cis pigment [in Y57N approximately 25% of the 13-cis isomer is present in the light-adapted state (Soppa et al., 1989)], whereas the 460−480 nm increase is probably due to the L photointermediate which has an unusually long lifetime, ~500 ms (Govindjee et al., 1992). Thus, at neutral pH the photocycle of Y57N proceeds only up to the L intermediate, with the L to M transition being blocked. However, at higher pH and high-salt concentrations, a flash-induced absorbance increase at 410 nm due to the M photointermediate is observed (Figure 10A) but the total amount of M formed is only about 25−30% of that in the WT. Figure 10B shows the pH dependence of M yield has a midpoint of ~8.5 (in 2 M NaCl). The rate of M formation is slow compared to that of the WT (Govindjee et al., 1992). No proton release/uptake was observed in Y57N, as measured with the pH sensitive dye pyranine at pH 7 (Soppa et al., 1989; Govindjee et al., 1992). In addition, pH dye (thymol blue) measurements at pH 9 and photocurrent measurements at either neutral or high pH show no evidence for proton release (data not shown). When there is no M intermediate forming, there is likewise no substantial positive photocurrent component; when M forms at alkaline pH, there is an analogous photocurrent component, but this appears to be due to only internal charge movement and not proton release because it is insensitive to salts and buffers as WT bR is (Liu, 1990; Liu et al., 1990, 1991).

Accumulation of L and M Intermediates in Y57N at Low Temperature. Formation of the L intermediate with a characteristic L/bR difference spectrum was observed at −70 °C after illumination of the sample at 610 nm plus subsequent illumination at 645 nm to convert the residual bathoproduct back to the initial pigment. In the WT bR, almost all the pigment (about 98%) can be easily converted into the M intermediate by illuminating the sample with >500 nm light at high pH during cooling to −70 °C (Becher & Ebrey, 1977; Litvin & Balashov, 1977). In Y57N, a similar procedure resulted in the accumulation of mainly L intermediate (since N does not form at −70 °C) with only a minor amount of M (approximately 4% of the total pigment). The absorption maximum of the M intermediate at −70 °C is at 415 nm with a shoulder at 442 nm which is close to the spectrum of M in the WT. Illumination with blue light causes the phototransformation of M to the pigment at 565 nm, which is apparently *trans*-Y57N.

Y57F. The photocycle of light-adapted Y57F, at pH 7, shows flash-induced absorbance changes similar to that of the WT at 410, 520, and 610 nm (Figure 11A). At low or neutral pH a large amount of O (610 nm) is seen, and at higher pH's N accumulates. The rate of M (410 nm) formation is pH dependent, but in contrast to the WT and Y57N it is very fast, ~15 μs at neutral pH and ~2 μs at pH 10 (Figure 11C). Despite the fact that flash-induced M is formed in Y57F, proton release and uptake are not normal. As measured with pyranine, the flash-induced proton changes are very small and the proton uptake precedes proton release (Figure 11B) in contrast to the native pigment.

Sensitivity to Hydroxylamine. The Y57N pigment can be bleached to the apoprotein plus retinaloxime very rapidly upon illumination in the presence of 1 M hydroxylamine and 0.5 M NaCl at neutral pH. Complete bleaching occurs within 15 min. On the other hand, only a small fraction of Y57F or the WT is bleached under similar conditions. Thus, the Schiff base in Y57N is much more accessible to hydroxylamine during illumination at neutral pH.

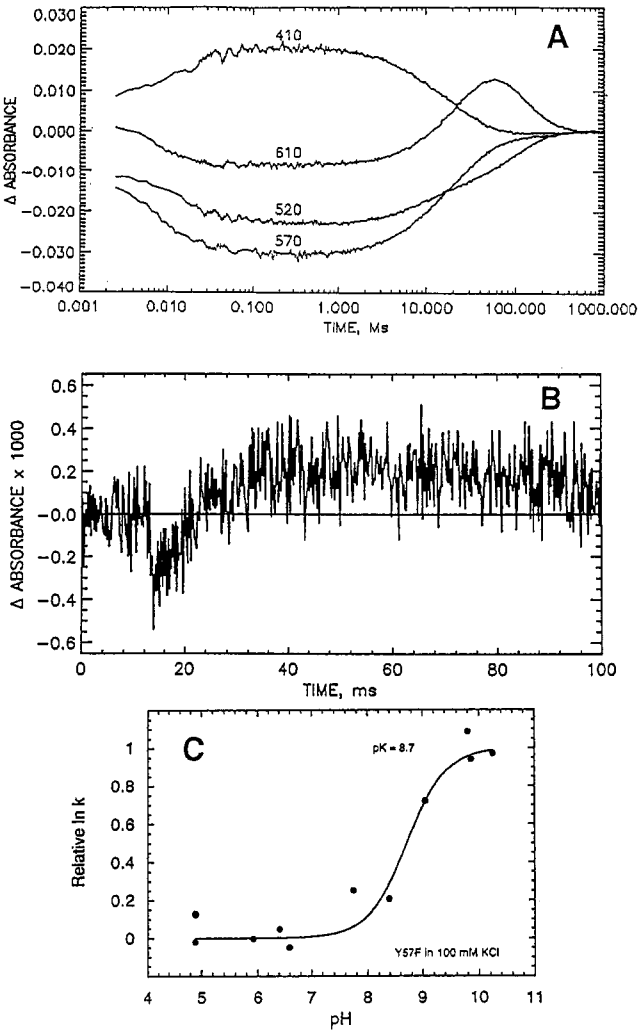

FIGURE 11: (A) Flash-induced absorption changes of Y57F at wavelengths as marked in 1 M NaCl, pH 6.98. (B) Light-induced absorption changes of the pH sensitive dye pyranine. (C) Relative rate of M formation as a function of pH.

DISCUSSION

Y57 is conserved in all the halophilic retinal proteins which have been sequenced—six bacteriorhodopsins and related proton pump pigments, as well as halorhodopsins and sensory rhodopsins I and II (Henderson et al., 1990; Mukohata et al., 1991; Otomo et al., 1992a,b; Soppa et al., 1993)—which suggests that it has a significant role in these pigments. Y57 is one of the residues in the retinal binding pocket and is suggested to be part of the retinal Schiff base binding complex (Henderson et al., 1990; Jonas & Ebrey, 1991; Humphrey et al., 1994; Govindjee et al., 1994). Humphrey et al. (1994) have suggested that Y57 is hydrogen bonded to R82 and D212 via a water molecule.

In this paper we have presented evidence from the *Halobacterium* mutants Y57N and Y57F which suggests that Y57 contributes to or influences several properties of bR. There are several questions that we have addressed in this paper: (1) the pK_a of Y57 and its role in the alkaline transitions, (2) the influence of Y57 in light/dark adaptation (thermal isomerization) of bR, (3) the influence of Y57 on the pK_a of D85, the purple to blue membrane transition, and (4) the effect of Y57 substitution on the photocycle and proton release and uptake.

(1) Alkaline Transitions

pK_a of Y57. According to Balashov et al. (1991), in bR the absorbance changes in the near-ultraviolet region around 235−240 nm, upon raising the pH from 7 to 12, represent the deprotonation of approximately three tyrosine residues. These have pK_a values of ~9, 10.3, and 11.6 in 166 mM KCl. The rest of the tyrosines in bR must have even higher pK_a values. bR from *Halobacterium* sp GRB also shows the deprotonation of three tyrosine residues in this pH range with similar pK_a values. The titration of Y57N and Y57F on the other hand shows only two tyrosines deprotonating in this same pH range (Figure 2). By subtracting the mutant titration curve from that of the native bR, we find that the missing group has a pK_a of 10.2. The most straightforward interpretation is that Y57 has a pK_a of 10.2. It is conceivable, however, that the Y57 mutation has altered the pK_a of some nearby tyrosine having pK_a 10.2 in the WT, and it is this shift that we are observing.

$P480_{alkaline}$. The nature of $P480_{alkaline}$ is not clear; probably the deprotonation of some residue destabilizes the Schiff base causing the absorption maximum to shift to shorter wavelengths. The Schiff base must, however, remain protonated in $P480_{alkaline}$ since its illumination produces a short wavelength photoproduct, P380, in the WT GRB, similar to that reported by Balashov et al. (1991). In Y57N illumination of $P480_{alkaline}$ converts it to the parent pigment, and its pK_a is similar to that of the Schiff base. The pK_a of the protonated Schiff base of the WT is ca. 13, while it is lowered to 12.1 for Y57N. Earlier, we had presented evidence that, upon illumination of Y57N to form the L intermediate, the pK_a of the Schiff base does not go as low as for the WT (Govindjee et al., 1994). Thus both experiments indicate that Y57N mutation changes the pK_a of the protonated Schiff base, but differently in the pigment and its L intermediate.

(2) Light and Dark Adaptation

Replacement of Y57 with either asparagine or phenylalanine alters the light and dark adaptation behavior of the pigment. The rates of dark adaptation for the two Y57 mutants differ greatly not only from the WT but also from each other. Y57N dark adapts very slowly (8 h at neutral pH), but Y57F dark adapts quickly (7 min) under the same conditions. These changes are presumably due to changes in the hydrogen-bonding interactions of groups that comprise the counterion complex to the Schiff base. The hydrogen-bonding network within the counterion complex should be altered by the substitution, and asparagine and phenylalanine will affect this structure differently since the former is capable of H-bonding whereas the latter is not. The photoreversibility of light adaptation of the Y57N mutant might be due to altered water structure within the protein, since such behavior has been observed in native bR when dehydrated or solubilized in detergents (Korenstein & Hess, 1977; Casadio et al., 1980; Kouyama et al., 1985; Balashov et al., 1988; Milder et al., 1991).

(3) Acid Transitions

Acidification of the purple membrane leads to the formation of blue membrane. D85 is protonated in the blue species (Subramaniam et al., 1990; Jonas & Ebrey, 1991; Metz et al., 1992); thus the pK_a of the purple to blue transition

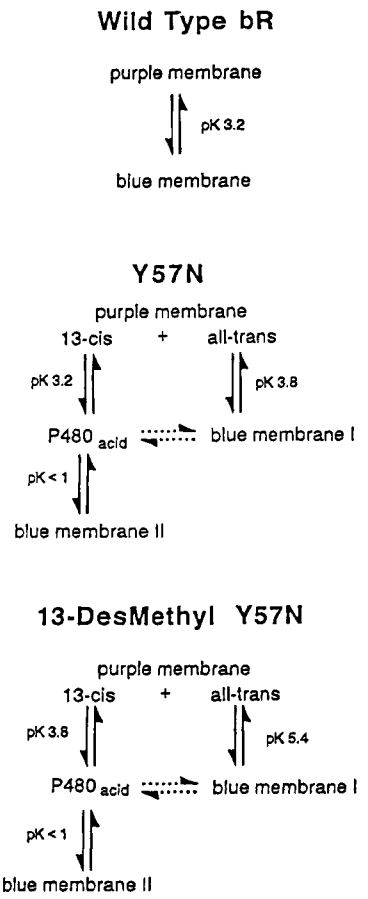

FIGURE 12: Schematic representation of the acid-induced transitions in WT bR, Y57N, and 13-desmethyl-Y57N. Interconversion of $P480_{acid}$ and blue membrane I is not certain and, hence, is indicated with dotted arrows.

represents the pK_a of D85. The pK_a of D85 is influenced by other residues in the protein. For example, the replacement of the positively charged R82 by neutral alanine (in the R82A mutant) has a profound effect on the pK_a of D85, shifting it from 2.8 in bR to 7.2 in R82A in 0.15 M KCl (Subramaniam et al., 1990; Balashov et al., 1993).

There are three different but related transitions in Y57N at acid pH, two phases in the purple to blue transition with pK_a's ~3.8 and <1 and the formation of $P480_{acid}$ (Figures 3 and 4). The questions that we have addressed here are as follows. What is the origin of the two phases in the purple to blue transition? Are they due to the pK_a of D85 being different in the different pigment isomers or to the titration of two different residues? And what is the nature of $P480_{acid}$?

Purple to Blue Transition. The data can be most easily discussed by reference to Figure 12. In dark-adapted bR, even though there are roughly equal amounts of cis and trans isomers present, the purple to blue transition shows a single pK_a ranging from ~2 in 4 M salt to ~5 in aqueous suspension. This single pK_a could be either due to the rapid cis/trans equilibration in the dark or that the pK_a's of the purple to blue transition (i.e., of D85) for the cis and trans pigments are very close to each other (Balashov et al. Personal communication). Y57N shows two pK_a's in the purple to blue transition, 3.8 and <1 (Figure 4, top panel); a simple interpretation is that somehow the cis/trans equilibration in the dark is affected and the pK_a of D85 is different in the different isomers of the pigment. There are two separate observations that support the idea that the pK_a of D85 may be different in different isomers of the Y57N pigment: First is the light-induced purple to blue transition seen in Y57N but absent in 13-desmethyl-Y57N (see Figures 8 and 9). Illumination of the pigment at a pH close to the pK_a of blue membrane I leads to the formation of more blue membrane in Y57N but not in 13-desmethyl-Y57N. As pointed out in Results, this is because light causes cis to trans isomerization in Y57N and the trans pigment converts into blue membrane at this pH. In 13-desmethyl-Y57N, since no light adaptation occurs, no further blue membrane is formed upon illumination. Second, the acid titration of 13-desmethyl-Y57N pigment, which presumably contains more 13-cis than all-trans isomer, shows two unequal size phases in the acid titration. The first phase, blue membrane I, is small (as is the amount of the trans pigment) and has a pK_a of 5.4, but the second and major phase, blue membrane II, has a pK_a of <1 (Figure 7A,C). However, after blue membrane I formation is almost complete (or maybe even simultaneously), the pigment converts to $P480_{acid}$ with a pK_a of 3.8 (3.2 in Y57N), and at lower pH's $P480_{acid}$ decreases and blue membrane II increases (see Figures 6 and 7). Since the decrease in $P480_{acid}$ and the formation of blue membrane II have the same pK_a, <1 (see Figure 4), it is reasonable to suggest that blue membrane II is formed from $P480_{acid}$; the latter is formed from the 13-cis pigment (see, below, The Nature of $P480_{acid}$).

The Nature of $P480_{acid}$. On the surface $P480_{acid}$ appears to be similar to BR_{acid} [a species formed during the purple to blue membrane transition (Varo & Lanyi, 1989)]; at neutral pH both the species have absorption maximum slightly blue shifted with respect to the pigment. However, $P480_{acid}$ differs from BR_{acid} in that it is formed in parallel with the blue membrane (the first phase or blue membrane I) rather than before it as is the case with BR_{acid}. $P480_{acid}$ does, however, lead to the formation of the second phase of the blue membrane. It is possible that $P480_{acid}$ might be a 9-cis pigment similar to the species reported by Maeda et al. (1980) and Fischer et al. (1981).

The source of $P480_{acid}$ seems to be the 13-cis pigment and not blue membrane I. Firstly, in Y57N the pK_a's of the formation of $P480_{acid}$ and blue membrane I, although very close, are clearly different at 3.2 and 3.8, respectively. In 13-desmethyl-Y57N (see Figure 7A,B), the pK_a of $P480_{acid}$ (3.8) is distinctly lower and much better separated from that of blue membrane I (5.4). In addition there is a close correspondence between the absorbance increase at 470 nm (due to the formation of $P480_{acid}$) and the decrease in absorbance of the pigment at 580 nm (Figure 7B) suggesting that $P480_{acid}$ is formed from the pigment itself. $P480_{acid}$ can also be produced by illuminating the pigment with 550 nm light (Figures 8 and 9); upon illumination with 480 nm light, it photoreverses to the original pigment, suggesting that $P480_{acid}$ is formed from the pigment. Secondly, since the fraction of the 13-cis pigment is higher in 13-desmethyl-Y57N as is the fraction of $P480_{acid}$ and consequently blue membrane II, we suggest that they are formed from the 13-cis pigment.

It is worth mentioning that the Y57N pigment is not very stable around pH 3 in the $P480_{acid}$ state. If left at this pH for a prolonged period, the pigment denatures and absorbance increases around 370 nm. WT pigment is much more stable under similar conditions.

Acid titration of Y57F shows a single pK_a (4.9) for purple to blue transition similar to that of bR. Thus the pK_a of D85 is almost 2 units higher than in the WT. This can easily be explained if we consider that Y57 is part of the counterion complex and replacing Y57 with a more hydrophobic residue in Y57F increases the pK_a of D85.

(4) Photocycle of Y57N and Y57F

Substitution of Y57 with asparagine has major effects on the photocycle and proton pumping (Soppa et al., 1989). The photocycle of *trans*-Y57N proceeds only up to the L intermediate; L then decays back to the pigment (Govindjee et al., 1992). No absorbance increase around 412 nm due to the M photointermediate was observed at neutral pH. The absence of M in Y57N, at neutral pH, is probably due an improper ΔpK between the Schiff base and D85. Probably due to structural and hydrogen-bonding changes, the Schiff base pK does not lower sufficiently in light, and thus it is unable to deprotonate. Recently, we have shown that M can be restored at neutral pH if the pK_a of the Schiff base is changed from 12.1 in Y57N to 9 in Y57N reconstituted with 14-F-retinal (Govindjee et al., 1994).

Y57N does show the light-induced formation of an M intermediate at high pH and high ionic strength. A plot of the M vs pH has a pK_a of 8.5 in 2 M NaCl (Figure 10B), suggesting the presence of a residue with a pK_a of 8.5 in 2 M NaCl which probably interacts with D85; upon deprotonation, at high pH, its interaction with D85 decreases, and the latter can act as a proton acceptor allowing M formation to occur. The identity of this residue is not certain, but it could be arginine 82. However, the amount of M observed is small, and its rate of formation is much slower than that of the WT under similar conditions. Both these observations and the pK_a of M are similar to those reported for the M-type intermediate formed from the 13-*cis*-bR (Kaulen et al., 1992; Drachev et al., 1993). Since in Y57N the 13-cis isomer is present in the light-adapted state, it is possible that the M (or at least some fraction of it) is formed from the 13-cis pigment.

Substitution of Y57 with phenylalanine results in a quite different phenotype. The Y57F mutant shows all the photocycle intermediates (Figure 11A), but the rate of M formation is faster than in the WT, the lifetime being 15 μs at neutral pH. The rate of M formation is pH dependent, the lifetime decreasing to about 2 μs at high pH, and thus it is unlikely that the deprotonation of Y57 is responsible for the pH dependence of the M rise as suggested earlier (Govindjee et al., 1993). Maybe the absence of H-bonding due to the absence of the hydroxyl group in Y57F affects the pK_a of the group that is responsible for determining the rate of M formation; arginine 82 is a good candidate (Balashov et al., 1993).

Proton Release and Uptake. Y57 substitution affects the proton release and uptake in bR. No proton release/uptake is seen in Y57N mutant at neutral pH. In Y57F the rate of proton release is slow relative to the proton uptake (Figure 12B). The half-times of proton release and uptake are 50 and 9 ms, respectively. In this respect it is similar to the R82A mutant which also shows faster proton uptake than release (Balashov et al., 1993). It is possible that Y57 is part of a group of residues which together form a proton release complex.

Accessibility of the Schiff Base to Hydroxylamine in L. Since Y57N does not have the M intermediate at neutral pH and the lifetime of the L intermediate is long, the rapid bleaching of the pigment clearly suggests that the hydroxylamine must attack the protonated Schiff base (in the L intermediate) rather than the deprotonated Schiff base (in the M state). In the dark the pigment is stable to hydroxylamine action. This suggests that light-induced conformation changes must occur upon the formation of the L intermediate which make the Schiff base more accessible to hydroxylamine, as suggested by Subramaniam et al. (1991).

In conclusion Y57 mutation affects several photochemical and ground state properties of bR and shares some similarities with other mutants. If the results from site-directed mutants are considered individually, it is tempting to assign specific functions to the particular residues that are mutated. But, increasingly it is observed that similar results are produced upon the mutation of different residues, particularly those in the retinal binding pocket and the proton conduction pathway. For example: Y57N resembles R82K in that both show photoreversibility in light adaptation (Balashov et al. Personal communication). Y57F and R82K mutants show accelerated rates of dark adaptation and have accelerated rates of M formation (Balashov et al., 1994). Altered proton release and uptake have been observed in R82A, Y57F, and Y57N mutants. One possibility is that Y57 and R82 are both part of a complex and perturbation of the interactions between them, upon mutation of either residue, results in somewhat similar phenotypes. Alternatively, it is possible that these mutations affect some common parameter which is eventually responsible for the effects observed. For instance, these mutations could alter the water structure, the role of which has not been explored in most of the studies involving mutants. There are several reports suggesting the presence and role of structured water in the integrity and functioning of bR (Dencher et al., 1992; Zhou et al., 1993; Humphrey et al., 1994). One of the differences between Y57N and Y57F mutants could be in their hydrogen-bonding capability; thus it is possible that the mutation of Y57 causes alterations in hydrogen bonding and water structure leading to conformational changes which are responsible for the observed results.

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