Static and Time-Resolved Absorption Spectroscopy of the Bacteriorhodopsin Mutant Tyr-185→Phe: Evidence for an Equilibrium between bR_{570} and an O-like Species†

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ABSTRACT: The light–dark adaptation, photocycle kinetics, and acid-induced blue formation of the bacteriorhodopsin (bR) mutant Tyr-185→Phe (Y185F) expressed in *Halobacterium halobium* have been investigated by both static and time-resolved visible absorption spectroscopy. Evidence is presented that a pH-dependent equilibrium exists between a bR_{570}-like form (bR_{570}^{Y185F}) and a red-shifted species in the light-adapted form of Y185F. In two related papers, we show that this species has vibrational features similar to the O intermediate. Key findings are that light adaptation causes formation of a purple species similar to bR_{570} and a second long-lived red-shifted species with a λ_{max} near 630 nm, well above the pH for the acid-induced blue transition. The concentration of the red-shifted species is pH- and salt-dependent, decreasing reversibly at high pH and high ionic strength. The dark-adapted state of Y185F also contains a small amount of the red-shifted species which is reversibly titratable. Dark adaptation is much slower than wild-type bR and causes a parallel decay of light-adapted bR and the red-shifted species. Time-resolved visible absorption spectroscopy reveals that the purple and the red-shifted species undergo separate photocycles. The purple species exhibits a relatively normal photocycle except for an increased rate of M formation kinetics. The red-shifted species has a photocycle involving a red-shifted K intermediate and a second longer lived intermediate possibly similar to N. The apparent absence of an O intermediate in the late photocycle of Y185F is attributed to cancellation by depletion bands due to the photoreacting red-shifted species. These results are discussed in terms of active-site interactions including the effect of eliminating a Tyr-185/Asp-212 hydrogen bond on the pK_a of Asp-85 and Asp-212.

Bacteriorhodopsin (bR)[1] is the retinal-containing protein found in the purple membrane of *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982). Upon light adaptation, bR functions as a light-driven proton pump, producing a transmembrane electrochemical potential. Photon absorption by light-adapted bR (bR_{570}) triggers a photocycle consisting of a series of intermediates, each having a different visible absorption maximum:

$$bR_{570} \rightarrow K_{630} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{550} \rightarrow O_{640}$$

Major events occurring during the photocycle include isomerization of the chromophore from an all-trans→13-cis structure during the primary $bR_{570} \rightarrow K_{630}$ transition (Braiman & Mathies, 1982; Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntle, 1983; Smith et al., 1984), deprotonation of the Schiff base in the $L_{550} \rightarrow M_{412}$ step (Aton et al., 1977), reprotonation of the Schiff base in the $M_{412} \rightarrow N_{550}$ step (Fodor et al., 1988), and reisomerization of the chromophore back to an all-trans form in the $N_{550} \rightarrow O_{640}$ step (Smith et al., 1983; Ames & Mathies, 1990). The photocycle is normally completed in the $O_{640} \rightarrow bR_{570}$ transition, although a branched reaction of $N_{550} \rightarrow bR_{570}$ is also postulated to exist (Váró & Lanyi, 1990; Chernavskii et al., 1989).

In order to investigate the protein conformational changes which occur during the light→dark adaptation and during the bR photocycle, we have utilized a combination of site-directed mutagenesis (Khorana, 1988), UV/visible difference spectroscopy (Ahl et al., 1988, 1989; Stern et al., 1989; Duñach et al., 1990a,b), and vibrational spectroscopy (Braiman et al., 1988a,b; Rothschild et al., 1989b, 1990; Braiman & Rothschild, 1988; Rothschild, 1992). This combined approach is designed to provide information about how specific substitutions alter the bR "phenotype" (e.g., changes in proton pumping efficiency, UV, and visible absorption of bR and its photointermediates, alterations in photocycle kinetics) as well as to determine the structure of specific residues during the photocycle.

This approach was used, for example, to investigate the effects of replacing Tyr-185, a residue located in the retinal binding pocket of bR (Rothschild et al., 1989a; Henderson et al., 1990), with Phe (Ahl et al., 1988, 1989; Braiman et al., 1988b; Jang et al., 1990; Duñach et al., 1990a,b). The studies, all performed on bR expressed in *Escherichia coli* and reconstituted in halobacterial lipids, detected the existence of a red-shifted species which was present along with a second

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[1] Abbreviations: PM, purple membrane; bR, bacteriorhodopsin; FTIR, Fourier transform infrared; λ_{max}, wavelength of maximum absorption in the visible; BM^c and BM^T, 13-cis and all-trans components of the acid-induced blue form of bacteriorhodopsin.

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purple form of Y185F[2] [see, for example, Ahl et al. (1988, 1989) and Jang et al. (1990)]. The red-shifted species was attributed to an increase in the pK_a for the acid-induced purple to blue transition as compared to wild-type bR (Jang et al., 1990; Duñach et al., 1990a). However, a second red-shifted species has also been detected which forms upon light adaptation, and is stable over a period of hours in the dark (Dunach et al., 1990b).

In this and three related papers (Rath et al., 1993; He et al., 1993; Bousché et al., 1992), we have focused on further characterizing the effects of the substitution, Tyr-185→Phe (Y185F), on the bR photocycle, light–dark adaptation, and acid-induced blue formation. In addition, we sought to determine whether the unusual properties of Y185F observed in reconstituted membranes reflect intrinsic properties of the protein or are due to possible nonnative properties of the lipid environment of the protein. For this reason, we have utilized the mutant Y185F expressed in the native *H. halobium* in the form of intact purple membrane patches.

We present evidence that a red-shifted species exists in a pH- and salt-dependent equilibrium with a purple species corresponding to the all-trans component of Y185F (bR_{570}^{Y185F}). As discussed in the accompanying papers (He et al., 1993; Rath et al., 1993), this red-shifted species has a chromophore structure very similar to that of the O intermediate. Only above pH 9 or at very high salt concentrations is this red-shifted species not observed in both static and time-resolved visible absorption spectra. We also find that its decay occurs in parallel with dark adaptation which is much slower than in wild-type bR. These defects, along with the previously observed increase in M formation rate and increase in pK_a of the acid-induced blue transition, are discussed in terms of a model in which Tyr-185 and Asp-212 form a polarizable hydrogen bond (Duñach et al., 1990a; Rothschild et al., 1990) which acts to stabilize the configuration of the active site in light-adapted bR. It is postulated that the absence of Tyr-185/Asp-212 interaction in Y185F allows the O intermediate, the last step in the bR photocycle, to exist in equilibrium with bR_{570}^{Y185F}.

MATERIALS AND METHODS

Expression and Purification of Y185F. The mutant protein was expressed from an *H. halobium* strain bearing a chromosomal *bop* gene containing the Y185F mutation. To generate a plasmid carrying the mutation, the 0.7 kbp *Nhe*I–*Not*I fragment containing the *bop* gene from pPLBO6-Y185F (Hackett et al., 1987) was combined with the 5.4 kbp *Nhe*I–*Not*I fragment of pMPK39 (Krebs et al., 1991) to yield pMPK47. The 1.1 kbp *Bam*HI–*Not*I fragment of pMPK47, the 2.4 kbp *Not*I–*Hin*dIII fragment of pMPK39, and the 6.7 kbp *Hin*dIII–*Bam*HI fragment of pMPK54 (Krebs et al., 1992) were combined to yield pMPK70. *H. halobium* MPK40, a *bop* deletion strain (Krebs et al., 1993), was transformed with pMPK70, and recombinants containing a single copy of the *bop* gene were isolated as described (Krebs et al., 1993). The membrane form of Y185F was purified from recombinants as reported previously (Krebs et al., 1991). Wild-type purple membrane was purified from MPK1 (Krebs et al., 1991).

Static Visible Absorption Measurements. Visible absorption spectra were measured with a UV–visible Shimadzu 2101 absorption spectrometer (Shimadzu Corp., Kyoto, Japan) equipped with a 60-mm integrating sphere which reduced absorption loses due to light scattering of the sample. Measurements on dark-adapted samples were made by keeping the sample in the dark at room temperature for at least 72 h prior to recording a spectrum. Light adaptation of samples was accomplished using continuous illumination for 15 min from a 150-W tungsten light source filtered with 505-nm long-pass and 600-nm short-pass glass filters (Corion Corp., Holliston, MA).

Determination of the pK_a for the Purple to Acid-Induced Blue Transition. Titrations of completely dark-adapted wild-type bR and Y185F were performed in polyacrylamide gel slices (2-mm thickness) as described (Fischer & Oesterhelt, 1979), in 1 mM NaP_i and 100 mM NaCl, where the pH was adjusted with HCl and NaOH. The pK_a values for the acid-induced blue transition were calculated with a four-parameter fit to the absorbance measurements (at 627 nm for bR and at 607 nm for Y185F) using the program SigmaPlot (version 4.0, Jandel Scientific, Corte Madera, CA).

Time-Resolved Visible Difference Spectroscopy. Time-resolved difference spectra were measured using a gated optical multichannel analyzer (1420 UV-enhanced optical multichannel analyzer, Model 1460 controller; Princeton Applied Research, Princeton, NJ) and 532-nm pulsed excitation from a frequency-doubled Nd:YAG laser (DCR-11, Spectra Physics, Mountain View, CA) as described previously (Duñach et al., 1990a). The data acquisition time after each laser excitation pulse was varied from 100 ns to 100 μs in order to optimize the signal-to-noise ratio. The delay times given in the figure captions refer to the time between the laser flash and half the data acquisition period. The laser was operated at approximately 40 mJ/pulse, and the pulse width was 7 ns. The photoexcitation cycle was repeated at 0.3 Hz for Y185F and wild-type bR. In order to check for possible long-term photobleaching of the sample, several absorption spectra were recorded during the experiment. The spectral data shown in Figures 4–6 were obtained after Savitzsky–Golay smoothing using a fifth order polynomial over 25 points. However, all the kinetic constants were derived from unsmoothed data. We did not detect greater than 5% reduction in the absolute intensity of the final absorption spectrum of the sample at the end of the measurement.

RESULTS

pK_a for the Acid-Induced Purple to Blue Transition of Y185F. Our results indicate that the acid-induced conversion from a purple to a blue species in native purple membrane (bR) in the dark has a pK_a of 2.7, in close agreement with values previously reported (Kimura et al., 1984; Mowery et al., 1979). In contrast, the pK_a of this transition for Y185F is shifted to near pH 3.7. A similar, although larger, increase in pK_a due to the mutation Tyr-185→Phe has also been observed for samples expressed in *E. coli* (ebR and eY185F, respectively) and reconstituted in native halobacterial lipids (Duñach et al., 1990a). This overall increase in the pK_a's for the *E. coli* produced samples, which are reconstituted in halobacterial lipids, is most likely due to differences in their lipid composition and protein:lipid ratio compared to wild-type bR expressed directly in the form of purple membrane (Popot et al., 1987; Szundi & Stoeckenius, 1987, 1989).

[2] bR mutants are designated by the wild-type amino acid residue (standard one-letter code) and its position number followed by the substituted amino acid residue. Thus, "Y185F" signifies the mutant in which the tyrosine at position 185 has been replaced by phenylalanine. In this paper, Y185F corresponds to mutant bR expressed in native *Halobacterium halobium*; ebR and eY185F signify bacteriorhodopsin and the mutant Y185F expressed in *Escherichia coli* and reconstituted in halobacterial lipids.

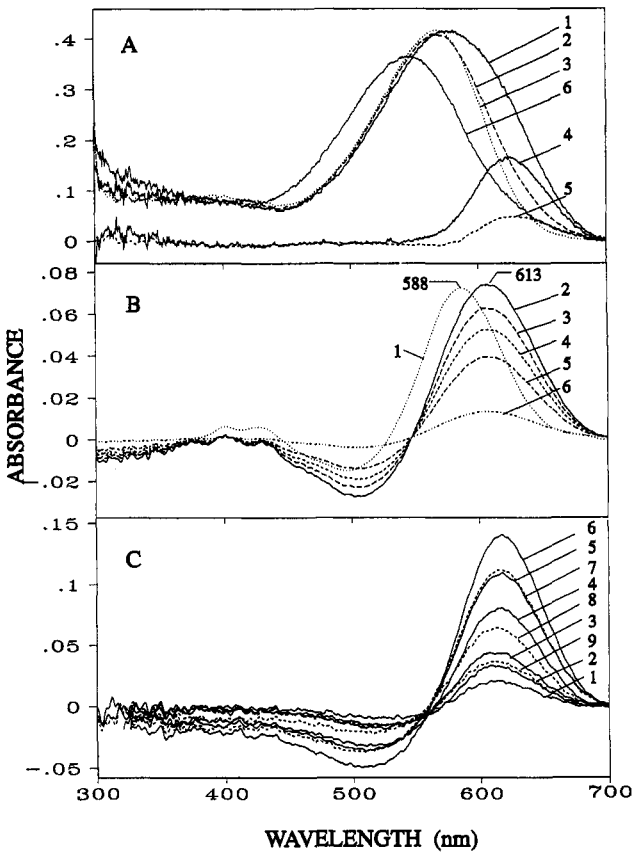

FIGURE 1: Formation of the red-shifted photoproduct of Y185F. (Panel A) Absorption spectra of light-adapted Y185F at pH 6.0 (curve 1) and at pH 9.1 (curve 2); light-adapted wild-type bR at pH 6.0 (curve 3). Curves 4 and 5 are difference spectra obtained by subtracting the absorption of light-adapted wild-type bR (curve 3) from light-adapted Y185F at pH 6.0 (curve 1) and at pH 9.1 (curve 2), respectively. See text for details. Curve 6 corresponds to the absorption spectrum of dark-adapted Y185F. (Panel B) The difference spectra of the light-adapted minus dark-adapted states at pH 6.0 are shown for wild-type bR (curve 1) and Y185F (curve 2). Curves 3–6 show sequential difference spectra obtained during dark adaptation of light-adapted Y185F after 3.5 (3), 7.0 (4), 11.5 (5), and 22 h (6). (Panel C) Difference spectra obtained by subtracting absorption spectra obtained at increasing pH values from the absorption spectrum of light-adapted Y185F at pH 6.0. Thus, the curves correspond to pH 6.31 (1), 6.86 (2), 7.70 (3), 8.05 (4), 8.55 (5), and 10.10 (6). Difference spectra obtained after reverse titration of the same sample to pH 8.48 (7), 7.85 (8), and 7.28 (3) show that the titration is reversible.

Properties of the Red-Shifted Species Present in Light-Adapted Y185F. (A) Visible Absorption Spectrum of Light-Adapted Y185F. In an earlier study, we found that in contrast to ebR, light adaptation of eY185F resulted in formation of a red-shifted photoproduct which slowly decayed in the dark with a half-life of 15 h to a stable purple form of eY185F (Dunach et al., 1990b). However, this red-shifted photoproduct is distinct from the acid-induced blue membrane since it does not form a pink photoproduct upon prolonged red illumination as normally observed for acid-induced blue membrane (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Muccio & Cassim, 1979).

The present study demonstrates that light adaptation of Y185F expressed in *H. halobium* also results in formation of a red-shifted species. In particular, the visible absorption spectrum of light-adapted Y185F at pH 6 (Figure 1A, curve 1) is very similar to light-adapted wild-type bR (curve 2) except for the existence of a distinct red-shifted shoulder, indicating the existence of a red-shifted species which is not present in light-adapted wild-type bR [see also Ahl et al. (1988) for similar results at low temperature on eY185F]. Subtraction of the wild-type light-adapted bR spectrum from the Y185F spectrum reveals that the λ_{max} of this species is close to 630 nm (Figure 1A, curve 4). The actual λ_{max} of this species, however, should be considered approximate since there may be a small downshift in wavelength of the purple form (He et al., 1993) of Y185F. Curve fitting of the absorption of light-adapted Y185F also results in two bands, one with a λ_{max} near 570 nm and a second near 630 nm (data not shown).

(B) Light–Dark Adaptation of Y185F. The dark→light (light minus dark) difference spectrum of Y185F at pH 6.0, i.e., the subtraction of the absorption of the dark-adapted state of Y185F from that obtained after 15 min of light adaptation (Figure 1B, curve 2), also reflects the formation of both normal light-adapted bR and this red-shifted photoproduct. In particular, the positive band in this difference spectrum appears at 613 nm relative to 588 nm in the corresponding difference spectrum for wild-type bR (Figure 1B, curve 1).

Figure 1B also shows the absorption differences measured at different times after light adaptation of Y185F (i.e., during dark adaptation) (curves 3–6). In comparison to light-adapted wild-type bR, which has a $\tau_{1/2}$ (half-life) of approximately 15 min, Y185F does not fully dark-adapt even after 22 h (curve 6). Because of the single isosbestic point in these differences, both light-adapted species must dark-adapt with identical rate constants (i.e., if the ratio of the two species remains fixed during the decay, the absorbance difference spectra will be indistinguishable from a single species decay). A similar behavior was found at pHs ranging from 6 to 8.5 (data not shown). The $\tau_{1/2}$ of the decay was determined by a single-exponential fit of the data to be approximately 13 h at pH 6–8 and decreases to 11 h at pH 8.5.

In the case of dark-adapted Y185F (Figure 1A, curve 6), the absorption has a λ_{max} at 548 nm, well below that of dark-adapted wild-type bR which is at 560 nm (Scherrer et al., 1989). This can be accounted for by two factors. First, there is an intrinsic shift in the λ_{max} of the 13-cis chromophore-containing species in dark-adapted Y185F (bR_{555}^{Y185F}) from 555 nm to near 548 nm as shown in a related paper[3] (Rath et al., 1993). Second, the 13-cis component is increased in Y185F over the 67:33 13-cis:all-trans ratio determined for wild-type bR (Scherrer et al., 1989). Both factors could cause a blue shift in the λ_{max} of dark-adapted Y185F relative to dark-adapted wild-type bR.

Effect of pH and Ionic Strength on Formation of the Red-Shifted Species. The extent of formation of the red-shifted species was studied as a function of pH and ionic strength. Figure 1A (curve 5), computed by subtracting a wild-type spectrum from a Y185F spectrum, both measured at pH 9.1, shows that the amount of the red-shifted species formed upon light adaptation is significantly decreased compared to at pH 6 (curve 4). The relative amount of the red-shifted species formed upon light adaptation at different concentrations of KCl and at different pHs is shown in Figure 3. At each combination of pH and KCl concentration, a sample was light-adapted, and the fractional change of the absorbance after light adaptation was calculated (i.e., ratio of change in absorbance to initial absorbance at 630 nm). All data points were then normalized to 1. At each concentration of KCl measured, the extent of photoproduct formation decreases

[3] For simplicity, we refer to the 13-cis component of dark-adapted Y185F as bR_{555}^{Y185F}, although it actually has a lower absorption maximum.

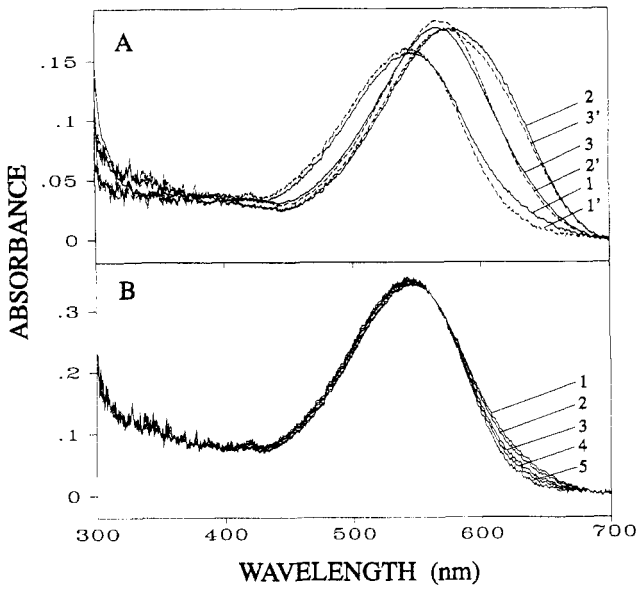

FIGURE 2: Effect of pH on light- and dark-adapted Y185F. (Panel A) Samples of dark-adapted Y185F at pH 6.3 and 9.6 (curves 1 and 1′, respectively) were light-adapted (curves 2 and 2′). The final pH of each sample was adjusted in the dark to match the initial pH of other sample; i.e., the pH of the light-adapted sample at pH 6.3 was adjusted to 9.5 (curve 3), and that of the light-adapted sample at pH 9.6 was lowered to 6.28 (curve 3′). (Panel B) Absorption spectra of dark-adapted Y185F at pH 6.0 (curve 1), 7.42 (2), 8.8 (3), 9.1 (4), and 9.6 (5). The pH-induced changes in the absorption spectra are also found to be completely reversible upon lowering of the pH (data not shown).

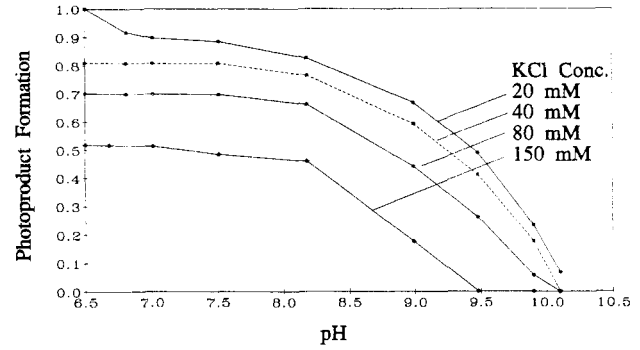

FIGURE 3: Effect of pH and ionic strength on the formation of the red-shifted photoproduct of Y185F. Extent of photoproduct formation determined as a function of pH and ionic strength (20, 40, 80, and 150 mM KCl).

with increasing pH, with almost no detectable formation of red-shifted species above pH range 10. However, for all concentrations of KCl used, significant levels of the red-shifted species are formed below pH 9 well above the pK_a (3.3) for the acid-induced blue transition of dark-adapted Y185F.

The long lifetime of the red species formed upon light adaptation allowed us to study its dependence on pH in the dark but prior to dark adaptation. As seen in Figure 1C, raising the pH gradually from 6.0 to 10.2 produced difference spectra (curves 1–6) which reflect a disappearance of the red-shifted species (positive band at 624 nm) and formation of a purple species (negative band at 513 nm). (Note that due to spectral overlap, these bands do not reflect the true λ_{max} of these species.) This alkaline titration is completely reversible as seen in the set of difference spectra (curves 7–9) obtained by lowering the pH back to 6.0. In a related study using resonance Raman spectroscopy (Rath et al., 1993), we were also able to demonstrate that the titration converts bR_{570}^{Y185F} and an O-like species (see below) at low pH into a single species similar to bR_{570}^{Y185F} (and not a second purple species such as the N intermediate) at high pH.

In a different titration experiment (Figure 2A), two samples of dark-adapted Y185F at the same concentrations were adjusted to pH 6.3 and 9.6 (curves 1 and 1′, respectively) and light-adapted (curves 2 and 2′, respectively), and the pH of each sample was readjusted in the dark to 9.5 and 6.28 (curves 3 and 3′). The close similarities between curves 2 and 3′, and between curves 3 and 2′, provide strong evidence that the final state of Y185F, and the concentration ratio of the two species formed upon light adaptation, is strictly a function of the final pH of the sample. For example, at high pH, Y185F forms little or no red-shifted species upon light adaptation. However, when the pH of the sample is lowered, the amount of red-shifted species formed is the same as a sample which is light-adapted at the lower pH. *This reversibility of the Y185F titration is therefore consistent with the existence of a pH-dependent equilibrium between the two species formed upon light adaptation, with the red-shifted component increasing at lower pH.*

Effects of pH on Dark-Adapted Y185F. The presence of a red-shifted species was also detected in completely dark-adapted samples but at a much lower concentration than in the light-adapted case. As shown in Figure 2B, at pH 6.0 (curve 1) the absorption spectrum of dark-adapted Y185F shows a shoulder in the long-wavelength region which upon titration of the sample disappears gradually and is not discernible above pH 9.6 (curve 5). Significantly, this pH titration is fully reversible as in the case of the light-adapted Y185F. Differences between these absorption spectra of dark-adapted Y185F at different pHs (data not shown) are very similar to those obtained for the light-adapted samples (Figure 1C). However, the relative size of the maximum difference over the pH range was approximately 20% of those obtained during the light-adapted Y185F difference titrations. Thus, we can conclude that this dark titration involves changes in the concentration of the same species (i.e., a red-shifted species and a bR_{570}^{Y185F} species) that occurs in the light. As discussed later, these results are consistent with the existence of an equilibrium between the bR_{570}^{Y185F} component present in dark-adapted Y185F and the red-shifted species.

Time-Resolved Visible Absorption Spectroscopy. Time-resolved absorption difference spectra of the photocycle of wild-type bR and Y185F at pH 6, 8, and 10 are shown in Figures 4, 5, and 6, respectively. Difference spectra were recorded at delay times ranging from 100 ns to 500 ms after photoexcitation of the sample. In contrast to wild-type bR, Y185F exhibits two distinct photocycling species at pH 6 and 8: one with a normal λ_{max} near that of wild type at 570 nm (Figure 4C) and a second above 600 nm, as seen from the corresponding depletion bands at these wavelengths (Figure 5A–C). The presence of an additional depletion band near 630 nm has been previously reported (Duñach et al., 1990a) for eY185F and attributed to the photoreaction of an acid-induced blue membrane which produces a 480-nm species at pH 6 (Heyn et al., 1989). However, no such 480-nm species is detected at pH 6 or 8 in Y185F. Furthermore, Y185F, which has a much lower pK_a for the purple to blue transition than eY185F, should not contain significant amounts of acid-induced blue membrane at pH 6 or higher. *Thus, we conclude that the depletion near 630 nm reflects the photocycle of the second red-shifted species formed during light adaptation.*

In addition to the two negative depletion bands, many of the other differences between the wild-type bR and the Y185F time-resolved visible difference spectra can be attributed to

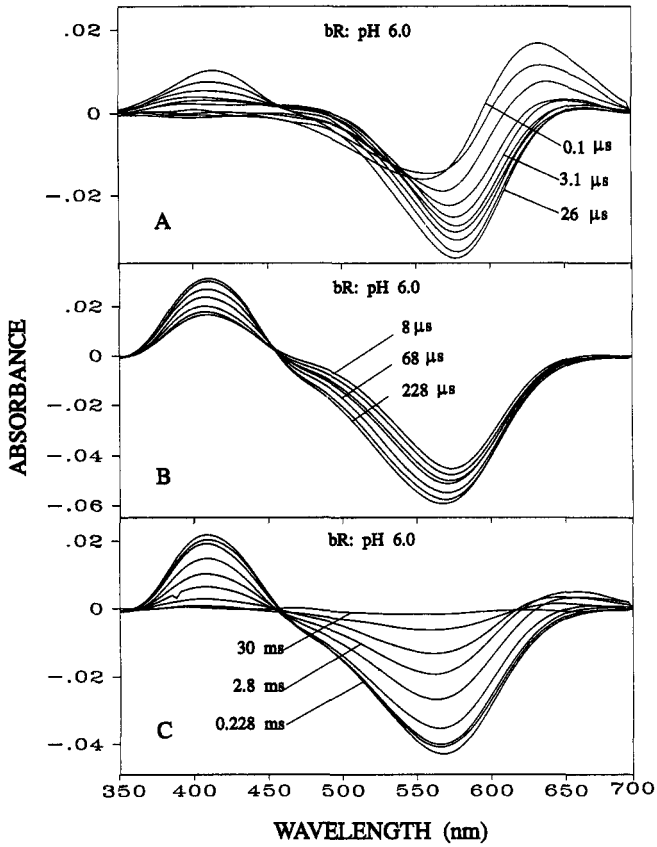

FIGURE 4: Flash-induced time-resolved difference spectra of wild-type bR. All spectra were recorded at pH 6.0 in 0.1 μs and 30 ms. (Panel A) K decay: difference spectra are obtained at 0.1, 0.33, 0.67, 1.1, 3.1, 4.8, 7.5, 11.4, and 26.0 μs (100-ns acquisition window). (Panel B) M rise: 8, 12.5, 29.3, 43.5, 68, 157, and 228 μs (10-μs acquisition window). (Panel C) M decay: 0.23, 0.35, 0.58, 1.6, 2.8, 4.5, 7.5, 12.5, and 30 ms.

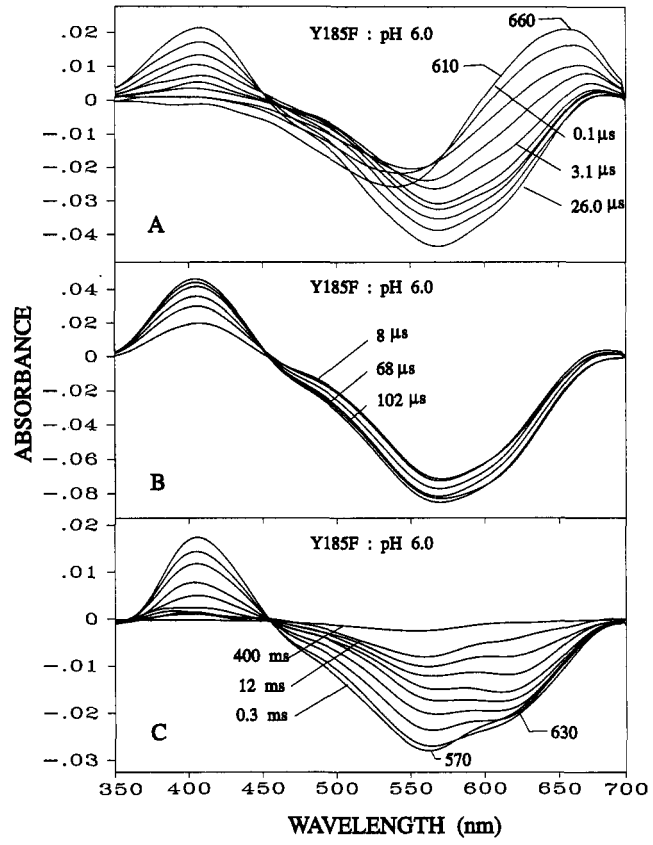

FIGURE 5: Flash-induced time-resolved difference spectra of Y185F at pH 6.0. (Panel A) K decay: difference spectra are obtained at 0.1, 0.33, 0.67, 1.1, 3.1, 4.8, 7.5, 11.4, and 26.0 μs (100-ns acquisition window). (Panel B) M rise: 8, 12.5, 29.3, 43.5, 68, and 102 μs (10-μs acquisition window). (Panel C) M decay at pH 6: 0.3, 0.6, 1.6, 2.7, 4.5, 7.5, 12.5, 20, 100, and 400 ms.

the existence of two separate photocycles. For example, in the early photocycle of wild-type bR (Figure 4A), a K intermediate is detected which absorbs at 610 nm and decays with a relaxation constant (τ) of approximately 0.5 μs. In contrast, Y185F forms two K-like species, one similar to that observed in wild-type bR and a second one at 660 nm (τ = 2.8 μs) (Figure 5A), in agreement with results obtained from eY185F (Duñach et al., 1990a). The slower decay of the 660-nm form accounts for the shift of the positive band to longer wavelength during the early photocycle. It is likely that this second red-shifted form of K originates from the photocycle of the red-shifted species produced on light adaptation.

Parallel to the faster decay of the 610-nm-absorbing species, there is an increase in intensity near 530 nm. This corresponds to formation of the L intermediate. Subsequently, L decays (Figure 5B) to form an M intermediate with a λ_{max} = 410 nm and rise time of τ = 16 μs which is approximately 2 times faster than for wild-type bR.

In the late photocycle of Y185F, the M intermediate exhibits two-exponential decay constants (τ_1 = 800 μs and τ_2 = 16 ms) compared to τ_1 = 6 ms for wild-type bR. The slower M decay constant has previously been related to the back-reaction of N→M (Otto et al., 1989; Holz et al., 1989) and may reflect a slower rate of N decay.

Finally, in the late photocycle of Y185F, an O-like intermediate absorbing above 600 nm is not observed below pH 9, in agreement with results obtained from eY185F (Duñach et al., 1990a). Instead, two negative bands near 570 and 630 nm are observed to decay with the intensity of 630-nm band decreasing relative to that at 570 nm (Figure 5C). In contrast, at pH 10, a positive band at 610 nm appears during M decay which subsequently shifts to longer wavelength (619 nm) and then decays with a τ_1 = 35 ms (Figures 6B,C). We have shown recently using time-resolved FTIR difference spectroscopy on hydrated films of Y185F containing high salt concentration that this red-shifted species has an all-trans chromophore structure very similar to the O intermediate in the native bR photocycle (Bousché et al., 1992).

DISCUSSION

Properties of the Mutant Y185F. Previous studies of Y185F (Ahl et al., 1988, 1989; Braiman et al., 1988b; Duñach et al., 1990a,b) were made on samples isolated from an *E. coli* expression system (eY185F) regenerated in DMPC/CHAPS/SDS micelles and reconstituted in halobacterial lipids. The absence of a native purple membrane lattice and the possible presence of nonnative lipids raise the possibility that many of the unusual features of Y185F including the formation of a red-shifted species upon light adaptation might be due to changes in the environment of the protein in the reconstituted membrane. The present study on Y185F expressed in *H. halobium* and isolated as intact purple membrane fragments offers an opportunity to compare the properties of Y185F derived using the two methods. Our results and those from three related studies (Bousché et al., 1992; He et al., 1993; Rath et al., 1993) show that all of the properties of eY185F are retained in the samples produced from the halobacterial

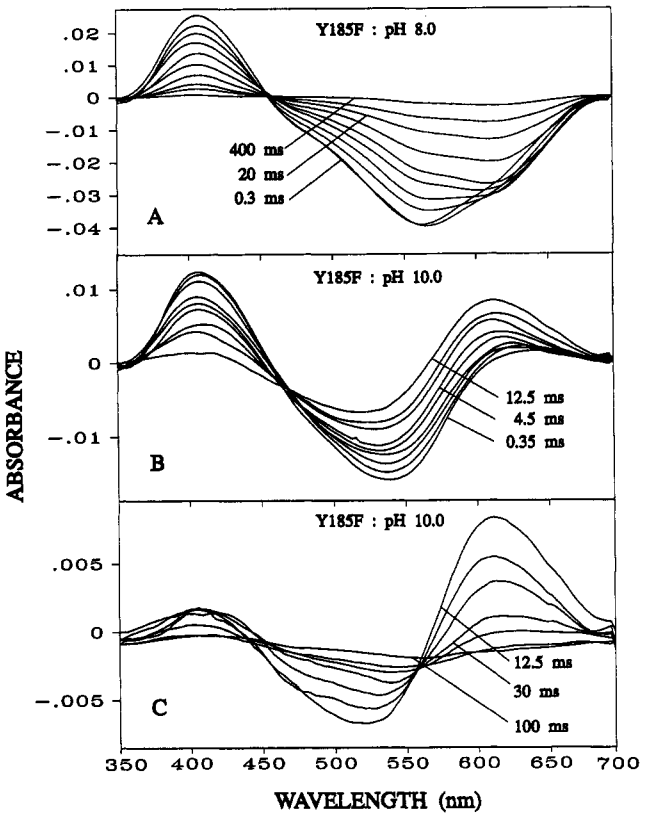

FIGURE 6: Flash-induced time-resolved difference spectra for the late photocycle of Y185F. (Panel A) M decay at pH 8: 0.3, 0.6, 1.6, 2.7, 4.5, 7.5, 12.5, 20, 100, and 400 ms. (Panel B) M decay at pH 10: 0.3, 0.58, 0.98, 1.6, 2.7, 4.5, 7.5, 9.3, and 12.5 ms. (Panel C) O decay at pH 10: 12.5, 21.6, 30, 45, 70, and 100 ms. Data acquisition window for all these measurements = 100 μs.

expression system and thus reflect intrinsic changes in the properties of the bacteriorhodopsin and not the reconstitution system. These properties are summarized below: (1) Y185F has an increased pK_a for the acid-induced purple to blue transition as compared to its wild-type counterpart. (2) Light adaptation of Y185F results in formation of a long-lived, red-shifted product which absorbs near 630 nm along with a normal bR_{570}^{Y185F} state. (3) The rate of dark adaptation of Y185F is significantly slower than wild-type bR and results in an increased content of bR_{555}^{Y185F}.[3] (4) The purple form of Y185F exhibits an increased rate for M formation. (5) All intermediates in the wild-type photocycle are also observed in Y185F except for an O-like intermediate. (6) Y185F exhibits two distinct photocycles below pH 9: one due to a purple form and another due to a red-shifted species absorbing above 600 nm. (7) Y185F exhibits a slowly decaying O-like intermediate in its photocycle above pH 9 which involves only the purple species.

Equilibrium between bR_{570}^{Y185F} and an O-like Species in Light-Adapted Y185F. In two related investigations of Y185F using vibrational spectroscopy, we have shown that an O-like species (O^{Y185F}) is formed along with bR_{570}^{Y185F} during light adaptation of Y185F (He et al., 1993; Rath et al., 1993). *In the context of the present work, we can therefore conclude that many of the altered properties of Y185F including formation, decay, and photocycling of a red-shifted species are due to this O-like species.*

An important question is why the substitution, Tyr-185→Phe, produces a stable O-like species in light-adapted Y185F. *We postulate that at room temperature there exists a pH-dependent equilibrium between the O intermediate and bR_{570}^{Y185F}.* Importantly, this hypothesis explains several of the key features of Y185F: (i) Light adaptation should lead to both the normal bR_{555}^{Y185F}→bR_{570}^{Y185F} conversion and formation of the O-like species. (ii) Due to the postulated equilibrium between bR_{570}^{Y185F} and the O^{Y185F} species, dark-adapted Y185F, which contains a small amount of an all-trans bR_{570}^{Y185F} component, should also contain a small level of the O-like species. (iii) O^{Y185F} decay should occur in parallel with the dark adaptation of bR_{570}^{Y185F}, since both bR_{570}^{Y185F} and O^{Y185F} remain in equilibrium during this slow decay process.[4] (iv) The relative concentrations of O^{Y185F} and bR_{570}^{Y185F} should be reversibly titratable by pH and ionic strength and not dependent on the initial conditions of illumination used for light adaptation. (v) Depletion of the O-like species due to photoexcitation should cancel bands due to the production of the O intermediate from the bR_{570}^{Y185F}. Note that while all of the above features of Y185F are explained by an equilibrium model, the alternate possibility that the O^{Y185F} species is formed by a block in the O to bR step of the Y185F photocycle is not consistent with features ii–iv (Sonar et al., 1992).

Since the O intermediate is the last step in the bR photocycle, the equilibrium model only requires simple thermodynamic reversibility in the photocycle for bR_{570} and O^{Y185F} to exist in equilibrium. Indeed, several other steps in the photocycle have already been found to involve a quasi-equilibrium between different photointermediates including the N and M intermediates (Lozier et al., 1992; Nagle, 1991). Although the O intermediate has not yet been detected at room temperature in native light-adapted bR, thermodynamic considerations require that this be due to a large difference in the free energy between the two states. In support of this, at higher temperature evidence has been found for formation of an O-like species in wild-type bR (Fukuda & Kouyama, 1992).

A key question in the context of the equilibrium model is what factors play a role in shifting the equilibrium of Y185F toward the O^{Y185F} intermediate (O-like species) relative to wild-type bR. On a molecular level, this is likely to be related to changes in the effective pK_a of Asp-85 and Asp-212 in the active site of bR. FTIR studies have established that in bR_{570}, these residues exist in an ionized form and are postulated to interact with the protonated Schiff base along with positively charged Arg-82 (Braiman et al., 1988a; Rothschild, 1992). FTIR evidence also indicates that in M and N intermediates of the bR_{570} photocycle, and also in the M, N, and O intermediates of the Y185F photocycle, Asp-85 is protonated and Asp-212 is partially protonated (Braiman et al., 1988a; Bousché et al., 1992; Rath et al., 1993; He et al., 1993). It should be noted that while recent solid-state NMR studies (Metz et al., 1992; Engelhard et al., 1989) confirm the FTIR conclusions about the ionized states of Asp-85 and Asp-212 in bR_{570}, and the protonation of Asp-85 during M formation, these studies do not detect protonation of Asp-212 in the M intermediate [see He et al. (1993) for a more detailed discussion]. Recent work also indicates that Asp-96 is protonated in both bR_{570} and the O^{Y185F} species (Bousché et al., 1992). For this reason, a shift toward the O-like species in Y185F relative to wild-type bR could occur if the effective pK_a's of Asp-85 and/or Asp-212 were elevated for the all-trans configuration of the chromophore. In this case, we would expect that only at relatively high pH or higher ionic strength would a pure bR_{570}^{Y185F} species be observed, in agreement with our measurements. At a lower or neutral pH, the increased

[4] Because of this slow decay, the postulated equilibrium between bR_{570}^{Y185F} and O^{Y185F} should be considered a quasi-equilibrium.

protonation of these residues would result in shifting the equilibrium toward O^{Y185F}.

Disruption or alteration of a polarizable hydrogen bond, which normally exists between Asp-212 and Tyr-185 in light-adapted bR (Rothschild et al., 1990, 1992; Henderson et al., 1990; Duñach et al., 1990a; He et al., 1992) but not in the 13-cis component of dark-adapted bR (Roepe et al., 1988), could provide a mechanism to increase the effective pK_a of Asp-85 and Asp-212 for only the all-trans component of bR. As pointed out previously (Rothschild et al., 1990; Bousché et al., 1992), it is possible that the hydroxyl group of Tyr-185 is replaced by a water molecule in Y185F which is capable of acting as a part of the proton wire, thus explaining why a protonation signal of Asp-212 is still observed in the Y185F mutant (Bousché et al., 1992; He et al., 1993). Such a substitution could lead to an increase in the protonation state of Asp-212. While it is difficult to predict how this substitution would affect the pK_a of Asp-85 because of the complexity of the active site and the interaction of these groups with the positively charged Schiff base and Arg-82, one mechanism which might act to raise the pK_a of Asp-85 is related to cooperative interactions between these groups. For example, the overall free energy difference between bR_{570} and the O intermediate could be lowered if the Tyr-185→Phe mutation altered the stability of the active site by disrupting nearby interactions including those involving Tyr-57, Trp-86, and the protonated Schiff base which are in a position to interact directly with Asp-212 (Henderson et al., 1990). From this point of view, the effective pK_a of both Asp-85 and Asp-212 may be directly related to the overall conformational energy for the transition between bR_{570} and the O species and not just to local changes occurring near Asp-212. We also note that although replacement of Asp-212 with a neutral residue does not lead to a similar phenotype as Y185F (Mogi et al., 1988; Subramaniam et al., 1990; Duñach et al., 1990b; Marti et al., 1991; Needleman et al., 1991), this is most likely a consequence of the removal of a net negative charge (i.e., Asp-212) located near the Schiff base which does not occur in replacement of Tyr-185 by Phe.

Relation between the O Species and the Acid-Induced Blue Membrane. Since the Y185F mutation increases the pK_a for the acid-induced blue transition from 2.7 to 3.7, the O-like species which forms at higher pH cannot be attributed to this low-pH acid-induced blue form of Y185F. However, the acid-induced blue membrane consists of a mixture of two forms containing all-trans and 13-cis isomers of retinal (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Fahmy & Siebert, 1990). Although it is unlikely that the the O intermediate is equivalent to the acid-induced blue membrane (Smith & Mathies, 1985) as previously suggested (Moore et al., 1978; Edgerton et al., 1978; Lam et al., 1982), the possibility remains that the all-trans component of the acid-induced blue membrane (BM^T) is similar to both the O intermediate and the O-like species we observe here. In fact, FTIR difference spectra for pH titration of wild-type bR between the purple and acid-induced blue form (Marrero & Rothschild, 1987) and more recent studies on light-adapted blue membrane (Fahmy & Siebert, 1990) reveal similarities in the vibrational spectra between the all-trans species formed at low pH and the O-like species we observe at higher pH in Y185F (He et al., 1993; Rath et al., 1993). In contrast, acid-induced blue formation observed in Y185F in the dark may involve predominantly a bR_{555}^{Y185F}→BM^C (blue membrane with 13-cis structure of retinal) transition. This possibility is supported by the finding that dark-adapted Y185F contains predominantly the 13-*cis*-bR_{555}^{Y185F} species (Rath et al., 1993), unlike wild-type bR which contains a mixture of both *all-trans*- and 13-*cis*-retinal [see Rath et al. (1993) for a further discussion on this point]. Thus, an interesting possibility is that formation of an O-like species at higher pH in Y185F may be due to a split in the pK_a's for the formation of the all-trans and 13-cis components of the acid-induced blue membrane, with the pK_a for the all-trans component elevated in Y185F relative to wild-type bR.

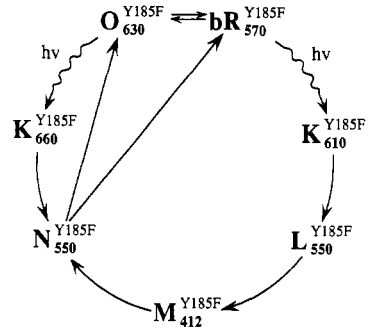

FIGURE 7: Photocycles of Y185F. In this model, bR_{570}^{Y185F} and O_{630}^{Y185F} are shown in equilibrium. The bR_{570}^{Y185F} photocycle involves all of the normal intermediates and includes a branching reaction of N which allows decay either directly back to bR_{570}^{Y185F} or through the O intermediate. The O_{630}^{Y185F} photocycle involves a red-shifted primary photointermediate, K_{660}, and a long-lived N intermediate which can directly decay back to O_{630}^{Y185F} or bR_{570}^{Y185F}.

Photocycle of the O^{Y185F} Species in Y185F. In the context of the above discussion, the two photocycles of Y185F as observed here should arise from a bR_{570}^{Y185F} species very similar to that of native light-adapted bR and an O species which exists in equilibrium with bR_{570}^{Y185F}. Since the O chromophore has an *all-trans*-retinal configuration (Smith et al., 1983), its photocycle is expected to involve photointermediates with a 13-*cis*-retinal structure. In agreement with this, low-temperature FTIR spectroscopy reveals that there exists a long-lived intermediate in the O^{Y185F} photocycle with a structure similar to the N intermediate (He et al., 1993). Thus, as shown in Figure 7, the photocycle of the O^{Y185F} species includes formation of a primary photoproduct (K_{660}^{Y185F}) which has an absorption maximum near 660 nm and is red-shifted relative to the primary K photoproduct of the bR_{570} photocycle. This red shift may be a consequence of the destabilizing interaction of the protonated Schiff base with neutralized Asp-85 and possibly Asp-212 in the active site. This red-shifted product then appears to decay to an N-like intermediate. The possibility exists that this N-like intermediate formed from O^{Y185F} also exists in equilibrium with the M intermediate as has been previously postulated for the bR_{570} photocycle (Otto et al., 1989; Holz et al., 1989), although this is difficult to determine directly from our data without using photoselection techniques because of the presence of the two photocycles. The return to the O^{Y185F} species could occur either by a direct N→O transition or through a branching pathway to bR_{570}^{Y185F}. Since bR_{570}^{Y185F} and the O^{Y185F} species exist in equilibrium, the final concentration of these species after the photocycles are completed should remain the same as prior to the flash. However, it should be noted that if the equilibrium between bR_{570}^{Y185F} and O^{Y185F} species is sufficiently fast, the two photocycles might not be independent (see below).

One consequence of the existence of the two photocycles for Y185F is that a partial cancellation of the O bands near 630 nm due to each separate photocycle should occur, thus providing an explanation for why a positive band associated

with the O intermediate is not observed in the visible absorption difference spectra of Y185F at a pH below 9. The exact degree of cancellation would depend on the initial concentrations of O^{Y185F} and bR_{570}^{Y185F}, the exciting frequency of the flash, the kinetics of both photocycles, and the degree of O^{Y185F} production by bR_{570}^{Y185F}. Interestingly, an N→bR_{570}^{Y185F} decay branch in both photocycles as shown in Figure 7 would tend to enhance this cancellation. First, the branch would delay the repopulation of O^{Y185F} depleted by photoexcitation, allowing it to cancel the O produced by the bR_{570}^{Y185F} photocycle.[5] Second, the amount of O produced from the bR_{570}^{Y185F} photocycle would be reduced by such a branch, reducing the level of O produced and causing more cancellation. It should be recognized that the postulated equilibrium between bR_{570}^{Y185F} and O^{Y185F} in the late photocycle is expected to alter the relative concentrations of these two species, allowing the bR_{570}^{Y185F} created by the branch to re-form the O^{Y185F} species.[5] A consequence of this model is that at high pH and high salt concentration, where the O^{Y185F} is not detected in equilibrium with bR_{570}^{Y185F}, a positive O band should be detected in the Y185F photocycle as observed here. A similar explanation also applies to the time-resolved FTIR difference spectra (Bousché et al., 1992), where positive bands associated with the O^{Y185F} intermediate are observed in hydrated films of Y185F which contain high salt concentrations. Finally, the slow decay of the O^{Y185F} intermediate, which we observe, might be a consequence of a higher energy barrier for the O→bR_{570} transition associated with the removal of Tyr-185, which is believed to form a hydrogen bond with Asp-212 during this last step of the photocycle [see Bousché et al. (1992) for discussion on this point].

CONCLUSIONS

Our study of Y185F produced in *H. halobium* and the work reported in the accompanying papers (Rath et al., 1993; He et al., 1993) have allowed us to further characterize the defects produced by this mutation and understand its effect on a molecular level. Our results demonstrate that a red-shifted species exists in light-adapted Y185F which is distinct from the acid-induced blue membrane, in agreement with an earlier study on eY185F. By postulating the existence of an equilibrium between bR_{570} and the O intermediate, the last step in the bR photocycle, we can account for many of the altered properties of Y185F determined here and in earlier studies (Ahl et al., 1988, 1989; Braiman et al., 1988b; Jang et al., 1990; Duñach et al., 1990a,b).

On a molecular level, a shift toward the O intermediate as a stable species in light-adapted Y185F might be due to disruption of a hydrogen bond between Tyr-185 and Asp-212. This would require, however, that the removal of this interaction affect not only the effective pK_a of Asp-212 but also that of Asp-85 which is found to be protonated in the O^{Y185F} species and ionized in bR_{570}^{Y185F} (Rath et al., 1993). An interesting possibility is that a coupling exists between the effective pK_a's of these residues and the overall conformational energy for the two states.

One consequence of our work on Y185F is that it provides a conceptual framework to examine the behaviour of other bR mutants. From this viewpoint, many properties of these mutants, especially those which involve active-site residues, can be explained in terms of a shift in equilibrium to other forms of bR which are normally not present under physiological conditions in wild-type bR. For example, the mutants R82A and R82Q also exhibit formation of a red-shifted species upon light adaptation (Duñach et al., 1990b). Since Arg-82 is in a position to interact with Asp-212 and Asp-85 (Rothschild et al., 1990), its substitution may also decrease the stability of the active site. Thus, characterization of the light–dark adaptation, photocycle kinetics, and vibrational spectra of these mutants can further test our understanding of the forces which stabilize the structure of native bR_{570}.

[5] The ratio of the two negative bands at 570 and 630 nm appears to reverse in the late stages of the Y185F photocycles, indicating that such a branch occurs followed by O and bR_{570}^{Y185F} reequilibration.

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