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Vibrational Spectroscopy of Bacteriorhodopsin Mutants: I. Tyrosine-185 Protonates and Deprotonates During the Photocycle

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ABSTRACT **The techniques of FTIR difference spectroscopy and site-directed mutagenesis have been combined to investigate the role of individual tyrosine side chains in the proton-pumping mechanism of bacteriorhodopsin (bR). For each of the 11 possible bR mutants containing a single Tyr→Phe substitution, difference spectra have been obtained for the bR→K and bR→M photoreactions. Only the Tyr-185→Phe mutation results in the disappearance of a set of bands that were previously shown to be due to the protonation of a tyrosinate during the bR→K photoreaction [Rothschild et al.: *Proceedings of the National Academy of Sciences of the United States of America* 83:347, (1986)]. The Tyr-185→Phe mutation also eliminates a set of bands in the bR→M difference spectrum associated with deprotonation of a Tyr; most of these bands (e.g., positive 1272-cm^{-1} peak) are completely unaffected by the other ten Tyr→Phe mutations. Thus, tyrosinate-185 gains a proton during the bR→K reaction and loses it again when M is formed. Our FTIR spectra also provide evidence that Tyr-185 interacts with the protonated Schiff base linkage of the retinal chromophore, since the negative $C{=}NH^+$ stretch band shifts from 1640 cm^{-1} in the wild type to 1636 cm^{-1} in the Tyr-185→Phe mutant. A model that is consistent with these results is that Tyr-185 is normally ionized and serves as a counter-ion to the protonated Schiff base. The primary photoisomerization of the chromophore translocates the Schiff base away from Tyr-185, which raises the pK_a of the latter group and results in its protonation.**

Key words: proton transport, energy transduction, purple membrane, proton wire, Schiff base counter-ion

INTRODUCTION

Membrane proteins are not well understood, in part because high-resolution structures have not been available for identifying their active-site residues. However, a variety of site directed mutagenesis methods now permit the alteration of any individual amino acid residue.[12,13,26,38] Mutant proteins can be studied by a number of physical techniques; infrared difference spectroscopy is particularly well suited for detecting changes in protonation states of amino acid side chains.[7] Thus, combining site-directed mutagenesis with infrared difference spectroscopy should permit the identification of specific protein residues undergoing protonations and deprotonations. Such reversible ionizations are likely to be important steps in the molecular mechanisms of a wide variety of membrane proteins, particularly those that transport protons.

BR, the light-driven proton pump of *Halobacteria halobium*, is an attractive candidate for experiments combining infrared spectroscopy and site-directed mutagenesis. While electron density maps of this integral membrane protein [1,16] have not yet permitted the localization of individual residues, the primary structure of the protein is established[22] and the gene has been cloned[9] and expressed in *Escherechia coli*.[21] Furthermore, a great deal is known about the structure and mechanism of bR based on a variety of physical and biochemical techniques (for examples, see references 32,39). Detailed information about structural changes in the retinylidene Schiff base chromophore during the proton-pumping photocycle has come from vibrational spectroscopy, especially from resonance Raman experiments (for reviews, see references 45,46). These studies have shown that the primary photoreaction of bR (λ_{max} = 570 nm) to K (λ_{max} = 630 nm) is a *trans→cis* isomerization about the chromophore's $C_{13} = C_{14}$ bond.[6] The chromophore then undergoes a well-defined sequence of structural

Abbreviations used: a.u., absorbance units; bO, bacterio-opsin; bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, dimyristoyl phosphatidylcholine; FTIR, Fourier transform infrared; SDS, sodium dodecyl sulfate; sh, shoulder.

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changes, including transient deprotonation of the Schiff base linkage at the M (412 nm) state, followed by reprotonation and reisomerization.

A plausible proton-translocating mechanism for bR probably requires proton transfers between amino acid side chains.[29,47] Several models involving specific residues have been proposed; in a number of these,[27] a role for tyrosines is suggested. In support of such models, recent FTIR studies of bR containing isotopically labeled tyrosines has shown that one or more of the 11 Tyr residues in bR undergoes protonation changes during the primary (bR→K) photoreaction[8,40] as well as during subsequent steps in the photocycle.[8,35]

For the current work, the most important results from the isotope-labeling studies are as follows. Vibrational assignments were made for a number of lines in bR→K and bR→M difference spectra. A key assignment was for an isotope-sensitive vibration observed near 1275 cm^{-1} in difference spectra of bR and some of its photoproducts. This line was assigned to the $C-O^-$ stretching vibration of tyrosinate, based on comparisons with tyrosinate model compounds. The bR→K difference spectrum exhibits negative tyrosinate peaks (including the 1277-cm^{-1} $C-O^-$ stretch as well as a characteristic tyrosinate band at 833 cm^{-1}) and positive peaks assignable to protonated (un-ionized) tyrosine. Thus, a tyrosine residue starts out ionized in the bR state and picks up a proton during the primary photoreaction to K. The bR→M spectrum exhibits the same negative tyrosinate peaks as the bR→K spectrum (e.g., 1277 cm^{-1}), as well as new positive tyrosinate peaks (e.g., 1272 cm^{-1}). Therefore, a tyrosinate group is formed some time during the decay of K to M—more specifically during the L→M step.[35]

The isotopic substitutions labeled all 11 tyrosines in bR uniformly and therefore did not reveal which specific residues gave rise to the tyrosine and tyrosinate peaks in the difference spectra or even whether each observed tyrosine peak in the difference spectrum was a contribution from one or from several different residues. With site-directed mutagenesis, however, it is possible to modify each of the 11 tyrosines individually to determine whether it contributes to the observed peaks. FTIR difference spectra of the resulting mutant proteins show that all of the tyrosines but one can be changed to phenylalanine without eliminating the tyrosinate peaks. The FTIR spectra of site-directed mutants thus complement the isotope-labeling experiments and permit identification of the particular tyrosine undergoing protonation changes during the bR photocycle.

MATERIALS AND METHODS

Preparation of bR Mutant Samples

The construction of genes coding for the 11 Tyr→Phe mutants and the preparation of mutant bacterio-opsin (bO) apoproteins using a heterologous (*E. coli*) expression system have been described previously.[13,28] To make bR samples suitable for FTIR difference spectroscopy, the purified bO was regenerated with retinal and reconstituted in vesicles in a 1:1 weight ratio with polar lipids from *H. halobium*[25] using the procedure devised by Popot et al.[33] We sometimes employed 13-*cis*-retinal because it resulted in faster regenerations and, subsequent to light-adaptation, gave spectral results that were identical with those obtained from samples regenerated with *all-trans*-retinal.

This regeneration/reconstitution procedure was selected over a number of others, including several that gave both a higher fractional regeneration and a lower lipid:protein ratio, because it resulted in FTIR difference spectra that were most similar to those obtained previously by using native bR (purple membrane) from *H. halobium*.[35,40] Samples made by the procedure of Popot et al.[33] are also the only ones in which a two-dimensional crystalline lattice could be formed, as determined by X-ray scattering[33] (S. Flitsch, H.G. Khorana, T. Kahn, and D. Engelman, unpublished results).

The 11 mutant apoproteins were also successfully regenerated and reconstituted using this procedure, which generally produced samples with visible absorption maxima shifted slightly to the red, relative to the same mutants regenerated in DMPC/CHAPS micelles.[2,28] (Complete visible absorption spectra of these samples will be published elsewhere.) The fraction of mutant apoprotein that bound a chromophore was generally in the range 40–60%; the unregenerated protein did not contribute any signal to the FTIR difference spectra because it did not undergo photoreactions under visible illumination.

FTIR Difference Spectroscopy

The regenerated bR vesicles were pelleted (5 min, 5,000*g*), and then a portion of the pellet was spread on a silver chloride window to give a protein surface concentration of 100–200 $\mu g\text{-}cm^{-2}$. The samples were air-dried and rehydrated, and FTIR difference spectra of the bR→K and bR→M transitions were measured in a Nicolet Analytical Instruments (Madison, WI) 60SX spectrometer as described previously.[35,42] In all cases the spectral resolution was 4 cm^{-1}.

RESULTS

Tyr→Phe Mutations at Residues 26, 43, 64, 79, 131, 133, 147, and 150 Do Not Affect the bR→K or bR→M Difference Spectrum

Figures 1 and 2 compare the bR→K and bR→M difference spectra, respectively, of wild-type bR (top) to the corresponding spectra of the mutants (below, as labeled). Of the 11 Tyr→Phe mutants, only Y57F, Y83F, and Y185F* give spectra that differ signifi-

*The bR mutants with Tyr→Phe substitutions are indicated by a residue number between the letters "Y" and "F", the latter being the standard 1-letter abbreviations for tyrosine and phenylalanine. Thus "Y26F" designates the mutant in which the tyrosine at residue 26 has been replaced by phenylalanine.

cantly from the wild type. These three mutants are discussed individually below. The other eight Tyr→Phe mutants give spectra that are the same as the wild type, down to very small details.† The minor differences that are observed can generally be ascribed to sample-to-sample variations in water content. This affected the bR→K spectra of the Y131F and Y150F samples most markedly in the region below 900 cm^{-1}, where strong background water absorption resulted in somewhat poorer signal/noise ratios. The need to subtract a large background absorption band also explains minor variations in the appearance of the 1650-cm^{-1} (water and amide I) and 1545-cm^{-1} (amide II) regions of the 8 "normal" mutants (especially noticeable in the bR→M differences in Fig. 2).

The most important observation for these eight mutants is that they all exhibit a positive shoulder at 827 cm^{-1} and negative peaks at 833 and 1277 cm^{-1} in the bR→K spectrum (Fig. 1), as well as negative peaks at 833 and 1277 cm^{-1} and positive peaks at 1272 and 1507 cm^{-1} in the bR→M spectrum (Fig. 2). These are peaks that have been assigned to tyrosine based on isotope labels incorporated in bR.[35,40] Using different isotope labels, the 1277 cm^{-1} negative peak was assigned to tyrosine independently by a second group.[8] Based on the spectra in Figures 1 and 2, none of these peaks can be attributed to vibrations of tyrosines 26, 43, 64, 79, 131, 133, 147, or 150.

The Mutant Tyr-185→Phe

Both the bR→K and bR→M difference spectra are affected by this mutation, and all of the aforementioned lines that have been assigned to tyrosine or tyrosinate are specifically eliminated in both (Figs. 1, 2). In the bR→K spectrum, the negative band at 1277 cm^{-1} (the tyrosinate $C-O^-$ stretch[8,40]) is completely absent, leaving only a broader positive band on which it was superimposed. A broad positive shoulder at 1240–1245 cm^{-1} (unmarked in Fig. 1), which corresponds in frequency to the C–OH stretch of tyrosine model compounds,[8,40] also appears to be eliminated in the Y185F mutant. Likewise, the negative 833- and 827-cm^{-1} bands (assigned to a component of a Fermi resonance characteristic of tyrosinate and tyrosine, respectively[40], are eliminated. However, small negative peaks at 842 and 854 cm^{-1} (unlabeled in Fig. 1), which were previously attributed to tyrosine(s),[40] are still present with this mutation. Additionally, the shape of the large positive peak near 1515 cm^{-1} is altered. This peak, which is predominantly due to C=C stretches of the chromophore in K, is known to include some contribution caused by an intense tyrosine vibration.[8,40] Other peaks, some of which are assignable to chromophore vibrations, are also affected. For example, the negative 1641-cm^{-1} and positive 1608-cm^{-1} Schiff base vibrations,[42] as well as several of the hydrogen out-of-plane vibrations (e.g., positive 942-cm^{-1} peak unlabeled), of wild-type bR are shifted or eliminated. Despite these changes, it is clear from the overall appearance of the spectrum and especially from the intense fingerprint vibrations at 1100–1300 cm^{-1} that the same all-trans→13-cis isomerization is occurring during the bR→K reaction in the Y185F mutant as in the wild type.

Likewise, in the bR→M spectrum of this mutant (Fig. 2), the tyrosine/tyrosinate peaks at 1507, 1277, 1272, and 833 cm^{-1} are eliminated. The perturbations on the rest of the spectrum are somewhat larger than for the bR→K spectrum. For example, the negative (bR) Schiff base vibration is shifted to 1635 cm^{-1}. Furthermore, the negative (bR) and positive (M) ethylenic peaks (near 1530 and 1570 cm^{-1}, respectively) are broadened, with several components clearly distinguishable. This suggests that several different photoproduct structures are present in the photosteady state at 250°K, indicating that new photochemical and/or thermal decay pathways have opened up for this mutant. Nevertheless, the normal appearance of the fingerprint region (1100–1300 cm^{-1}) indicates that the chromophore is still undergoing isomerization and deprotonation, and the major features in the 1750-cm^{-1} region indicate that protonation changes of several Asp groups[10,11,43] still occur in the Y185F mutant.

Although the tyrosine/tyrosinate peaks are small, their presence in the wide-type sample and their absence in the Y185F mutant are both highly reproducible. Samples of the Y185F mutant (and of a wild-type control) were originally prepared from the *bop* gene, which had been cloned from *Halobacteria halobium*.[13,26] Subsequent samples were prepared by mutating the synthetic *bop* gene.[28,30] Although the resulting gene constructions coded for proteins with minor differences at the N- and C-termini, the FTIR difference spectra of the two types of Y185F mutant samples were completely identical; in particular, both showed the characteristic changes discussed above.‡

†With the Y26F mutation, several of the positive bands in the bR→K spectrum (Fig. 1) are split, e.g., 1,510 and 958 cm^{-1}. This may indicate some heterogeneity of the primary photoproduct; however, the presence of the "normal" frequencies as the major components of these bands suggests that whatever side reaction pathway(s) may have opened, the bulk of the protein is photoreacting normally. In any case, none of the bR→K difference peaks associated with tyrosine are affected by this mutation.

‡All of the spectra shown in Figures 1 and 2 were obtained from samples coded by constructions based on the synthetic *bop* gene; the spectra of the Y185F and wild-type samples coded by the prior construction are not shown.

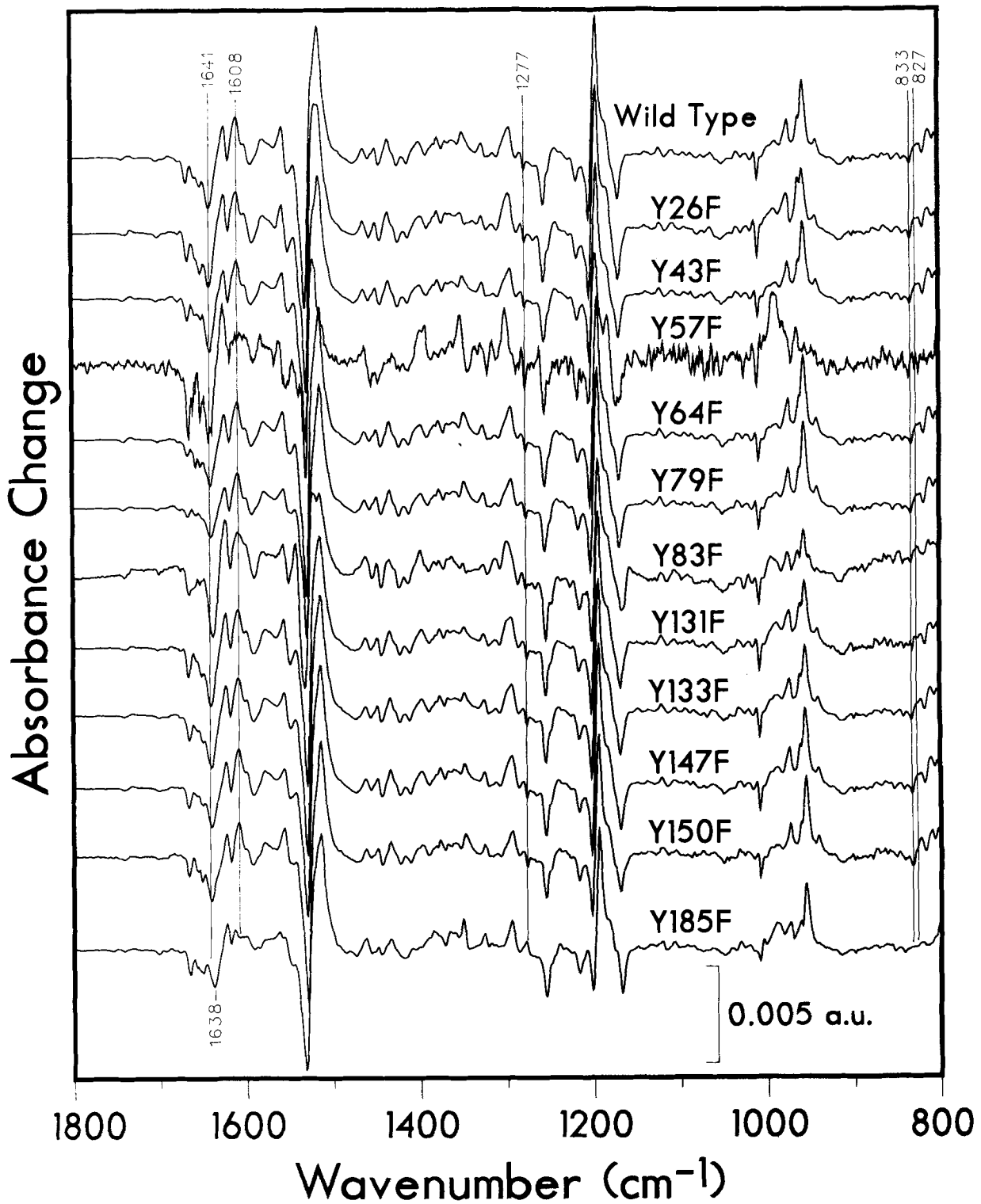

Fig. 1. bR→K absorbance difference spectra of wild-type-bR and Tyr→Phe mutants, as indicated. Spectra were measured at 78°K using samples which had been light-adapted at 280–290°K prior to cooling in the dark. Spectra obtained during alternating 15-min periods of green (500 nm) and red (650 nm) illumination were ratioed and converted to an absorbance scale; each spectrum in this figure represents an average from 20–60 such cycles of illumination. This procedure was modified for the Y57F and Y83F spectra (see text for details). The eleven mutant difference spectra have been expanded along the y-axis to fill approximately the same scale as the wild-type spectrum. This expansion corrects for variations among the mutants in sample thickness, fractional regeneration, and percentage of sample photocycling. The expansion factor was no greater than 2 for any of the samples except Y57F (see discussion of Y57F in "Results" for details). The indicated peaks at 1277, 833, and 827 cm^{-1} are due to tyrosine vibrations[40]; those at 1641 (1638 for Y185F) and 1608 cm^{-1} are due to the C = N stretch of the Schiff base group of the chromophore.

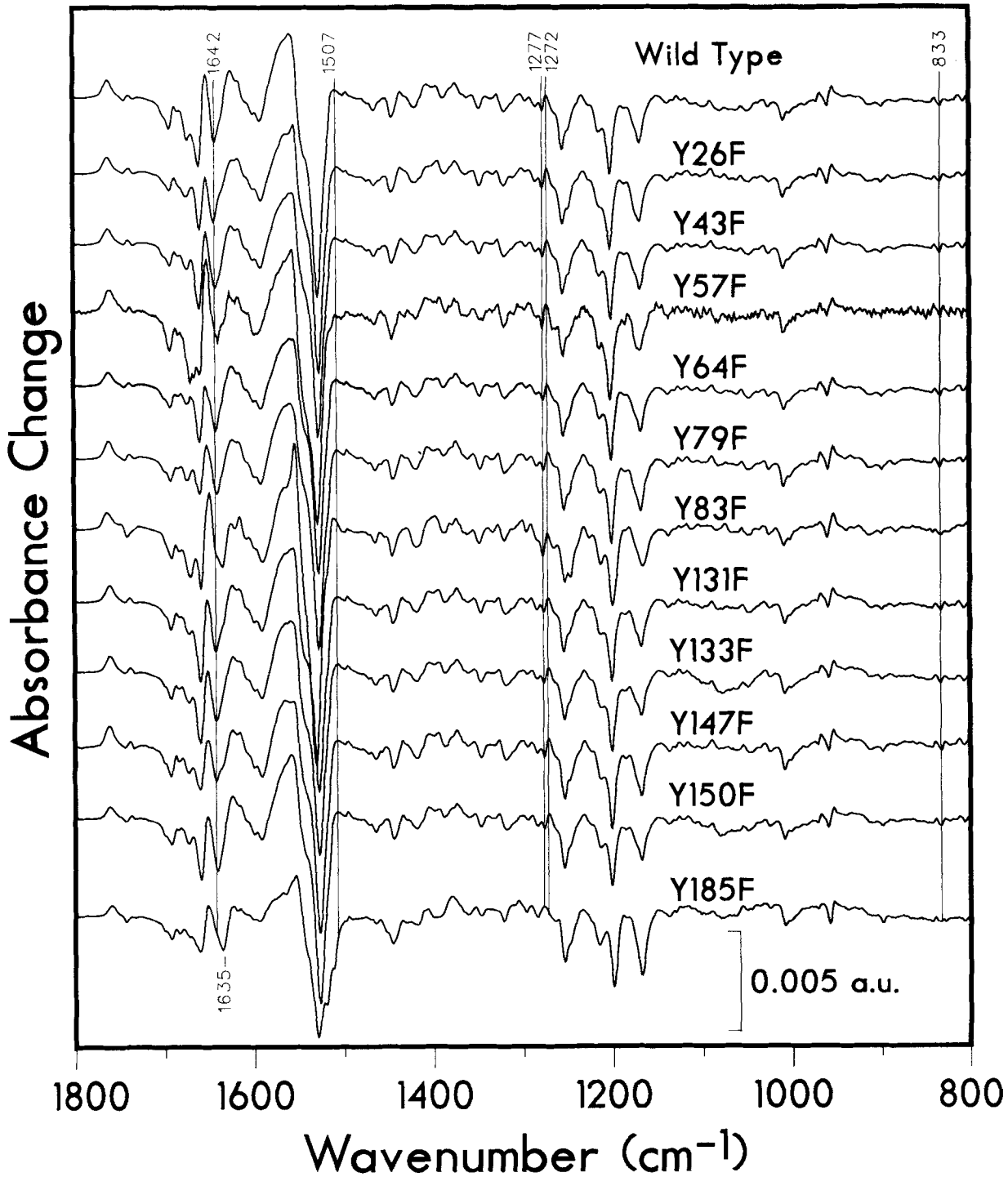

Fig. 2. bR→M difference spectra of wild-type bR and Tyr→Phe mutants, as indicated. The bR→M measurements were made at 250°K with alternating 15-min periods of yellow ($\lambda > 500$ nm) illumination and darkness.[35] Spectra were averaged and scaled as in Figure 1. The indicated peaks at 1507, 1277, 1272, and 833 cm^{-1} are due to tyrosine vibrations[35]; those at 1,642 and 1,635 cm^{-1} are due to the C = N stretch of the Schiff base group of the chromophore.

The reproducibility of the spectra obtained from protein samples coded by genes constructed in two different ways confirms that these spectral changes are due to the Y185F mutation.

The Mutant Tyr-83→Phe

The Y83F mutation caused changes in both chromophore and protein bands in the spectra. For the bR→K reaction, the first difference spectrum measured after cooling to 78°K (green illumination minus dark) was quite similar to the wild-type difference spectrum. However, it was clear that red illumination did not fully photoreverse this mutant, since subsequent difference spectra became more and more abnormal. The original difference spectrum could be reproduced only by warming the sample to room temperature and recooling. Unfortunately, spectral regions where tyrosine peaks are located were among the most affected by prolonged illumination at 78°K. Thus, to obtain the Y83F spectrum of Figure 1, it was necessary to average data from only the first few cycles of illumination obtained following three separate cooldowns. It is clear that this spectrum is quite perturbed, based on the splitting of the positive (K) ethylenic peak near 1515 cm^{-1} and the change in the appearance of the hydrogen out-of-plane vibrations near 950 cm^{-1}. Nevertheless, despite their intensity decrease the tyrosine/tyrosinate peaks at 827, 833, and 1277 cm^{-1} are still identifiable.

The bR→M spectrum of this mutant is also highly perturbed, as evidenced by the small frequency upshift of the negative (bR) ethylenic stretch near 1530 cm^{-1} and the unusual shape of the positive (M) ethylenic stretch band at slightly higher frequency. Furthermore, the negative C=NH+ stretch band near 1640 cm^{-1} is shifted down to 1636 cm^{-1}; this was the largest shift observed for this band other than with the Y185F mutant. Again, however, the tyrosine-associated peaks at 1272 and 1277 cm^{-1} are both clearly present, and the peaks at 833 and 1507 cm^{-1} are identifiable although somewhat altered in appearance. As with bR→K difference spectrum of this mutant, it appears that the bR→M spectrum is somewhat altered, but the characteristic tyrosine/tyrosinate vibrations are still present.

The Mutant Tyr-57→Phe

To obtain the spectra of this mutant shown in Figures 1 and 2, it was necessary to use procedures different from those used for the other samples. This is because the "bR→K" spectrum of Y57F measured by the usual procedure (data not shown) resembled most closely the difference spectrum obtained from the 13-cis component of dark-adapted bR (bR_{548}) and its 78°K photoproduct.[36] Superimposed on the features associated with this bR_{548} photoreaction were smaller peaks characteristic of the normal bR→K spectrum. Likewise, when the "bR→M" spectrum was obtained using the usual procedure, the initial difference spectra were abnormal in appearance. However, in this case the subsequent difference spectra gradually became more like the normal bR→M spectrum (although all the features were substantially smaller than normal). Therefore, in averaging to produce the bR→M spectrum labeled "Y57F" in Figure 2, data from the first 15 illumination cycles were omitted. The spectrum shown included data from the following 50 cycles, over the course of which the difference spectrum did not change significantly.

A hypothesis that could explain these results is that the Y57F mutant has a greatly increased thermal rate of conversion from all-trans to 13-cis, so that "dark adaptation" in the last few seconds before freezing leaves the sample predominantly in the 13-cis form. Only by cooling to 250°K is the rate of dark adaptation slowed sufficiently to trap the all-trans (light adapted) state. The long period of illumination required to bring about a fully light-adapted state at this temperature could reflect the slowing of thermal processes within the "13-cis" photocycle that are required for light adaptation to occur.

Based on this hypothesis, we predicted that a more normal bR→K spectrum could be obtained from this mutant if it were subjected to "light-adaptation" at 250°K before cooling to 78°K. This prediction was borne out and enabled us to obtain the bR→K spectrum of the Y57F mutant shown in Figure 1. For this spectrum, the sample was first cooled to 250°K and was exposed to the normal illumination pattern used to obtain bR→M difference spectrum (see Fig. 2 legend). When difference spectra obtained from sequential light-dark cycles became constant in appearance (15 cycles), the sample was kept in the dark at 250°K for an additional 30 min and was then cooled in the dark to 78°K. At this temperature the normal pattern of alternating red and green illumination was used to obtain the bR→K difference spectrum (Fig. 1, labeled Y57F). This spectrum shows features that are generally characteristic of the photolysis of "all-trans" (i.e., light-adapted) bR. Most importantly, the spectrum shows the negative peak at 1277 cm^{-1}, which is characteristic of a tyrosinate anion being protonated during the bR→K transition.[40] The relatively large size of this feature is consistent with the assumption that we are observing a "high-temperature" bR→K difference spectrum as previously observed at 135°K,[41] possibly since the protein was frozen in a partially relaxed conformation when it was cooled after light adaptation at 250°K. A number of other spectral features (e.g., the shift of the large hydrogen-out-of-plane mode from 957 to 984 cm^{-1}) are also similar to features in the "high-temperature" bR→K spectrum,[41] and therefore support this assumption.

The magnitude of the Y57F difference peaks were smaller than those of the other mutants: the bR→K spectrum of Y57F in Figure 1 was expanded ~50× relative to the wild type (which had a similar total

protein concentration). For the bR→M spectrum in Figure 2, the relative expansion factor was 5×. The diminished size of the peaks in these spectra can probably be explained by a combination of factors: for this mutant only a small fraction (~20%) of protein was successfully regenerated with retinal[28]; there was furthermore some permanent photobleaching that occurred each time the sample was light adapted; and (evidently) the quantum yields for some of the bR photoreactions are altered by this mutation. For the bR→K spectrum, the resulting poor signal/noise ratio makes it impossible to see whether the 833- and 827-cm^{-1} lines are present. Therefore, our conclusion that the tyrosinate protonation still occurs during the bR→K reaction with this mutant depends on our observation of a single peak (1277 cm^{-1}) in a somewhat perturbed spectrum. However, the bR→M result is much stronger, since the entire spectrum—including peaks at 1507, 1277, 1270, and 833 cm^{-1} that have previously been assigned to tyrosine or tyrosinate on the basis of isotope labeling—is essentially unaffected by the Y57F mutation.

DISCUSSION

Our results enable us to identify Tyr-185 as a residue undergoing protonation changes during the bR photocycle. Substitution of Tyr-185 by Phe results in the disappearance of a set of lines in bR→K and bR→M difference spectra that are known to be due to tyrosine and tyrosinate. The earlier assignment of these peaks to tyrosines rather than other groups in the protein was unambiguous, because it was based on several different tyrosine isotope labels incorporated isomorphously into bR from *H. halobium* and into model compounds.[8,35,40] We conclude that the *FTIR difference bands assigned to tyrosinate* (e.g., 1277 cm^{-1}) arise from a single residue, based on the complete set of control experiments that we have performed. For example, the 1277-cm^{-1} negative band disappears when Tyr-185 is mutated, whereas it is clearly present in the other ten tyrosine mutants.** The null results obtained for these ten mutants, along with the earlier isotope labeling experiments that showed that the 1277-cm^{-1} peak must be due to some tyrosine, allow us to assign this peak to Tyr-185. The use of exhaustive controls thus allows us to assign tyrosinate bands definitively to Tyr-185, even if secondary effects in the Y185F mutant caused additional changes in bands that were not due to tyrosine.

What specifically do these spectra tell us about the structure of tyrosine groups during the bR, K, and M states of the photocycle?

**In one other mutant, Y83F, there is a decrease in the intensity in this 1277 cm^{-1} band in the bR→K spectrum. However, we consider it unlikely that a tyrosinate-83 contributes intensity to this band, for reasons discussed below (see "The Role of Other Tyrosines").

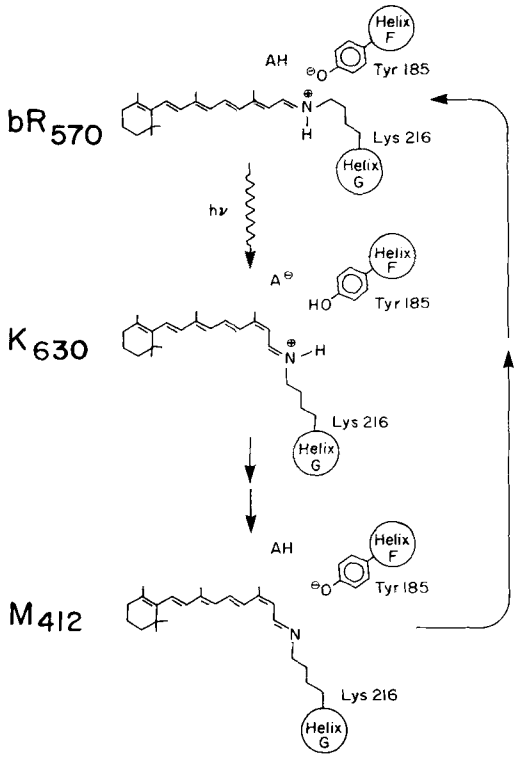

Fig. 3. Model for steps in bR mechanism involving Tyr-185. In the bR_{570} state (**top**), the *all-trans*-retinal configuration positions the positively charged Schiff base close to Tyr-185, stabilizing this residue in its anionic state. The primary photoisomerization of the chromophore pulls the Schiff base group away from the tyrosinate, leading to an increase in its pK_a and thus to its protonation by an (unidentified) proton donor group, AH. In the resulting K_{630} structure (**middle**), energy is stored as a result of increased charge separation. Tyr-185 becomes deprotonated again by the time that M_{412} (**bottom**) is formed. It is unclear how the deprotonation of the Schiff base group at this step is coupled to Tyr-185 *deprotonation*.

The Role of Tyr-185 in the Photocycle

Figure 3 depicts a model for a part of the active site in the bR, K, and M states. The chromophore structures are based principally on previous resonance Raman experiments.[6,45] Based on the FTIR work presented here and elsewhere[8,35,40] we have incorporated protonation changes of Tyr-185 into this active site model. Our most important conclusions are discussed below.

Tyr-185 is deprotonated in bR, protonated in K, and deprotonated in M

Based on previous work with isotope labels, and especially on a lack of sensitivity of some vibrations to solvent deuterium exchange, it was concluded that a tyrosinate residue is present in bR. This tyrosinate becomes protonated during the bR→K reaction, giving rise to negative peaks at frequencies characteristic of ionized tyrosine (e.g., 833 and 1277 cm^{-1}) and to positive peaks at frequencies characteristic of unionized tyrosine (e.g., 827 cm^{-1}).[40] A number of addi-

tional bands characteristic of these protonation changes could be discerned only in spectra of samples containing isotopically labeled tyrosines.[8,35,40] We have not yet incorporated these isotopically labeled tyrosines into the bR mutants; however, it is already clear from the current data that the Tyr group that protonates during the bR→K reaction is Tyr-185, as indicated in our model (Fig. 3).

It was previously concluded that the negative bands at 833 and 1277 cm^{-1} in the bR→M spectrum (Fig. 2) are due to the same Tyr residue that gives rise to bands at these frequencies in the bR→K spectrum.[8,35] Our results confirm this conclusion. It was also suggested that this residue remains protonated in the M state, and that a *different* Tyr becomes deprotonated and gives rise to the positive C–O^- stretch band at 1272 cm^{-1}.[35] However, the clear loss of the 1272-cm^{-1} peak in the Y185F mutant and the lack of even a small frequency shift of this peak in any of the other mutants argue strongly that the 1272-cm^{-1} tyrosinate band should also be assigned to Tyr-185. Thus, Tyr-185 protonates during the bR→K transition and then loses its proton to form an anion once again in the M state (Fig. 3, bottom). Presumably, the positive (1272 cm^{-1}) and negative (1277 cm^{-1}) tyrosinate-185 bands in the bR→M spectrum do not cancel each other because a change in the chemical environment around this group shifts the vibrational frequency.

Tyr-185 interacts with the Schiff base group of the chromophore and may be its primary counter-ion in the bR_{570} state

The Y185F substitution results in a perturbation of the C=NH stretching mode in both the bR and K states. In the case of bR, this mode shifts down 3–5 cm^{-1} from its normal frequency of 1,641 cm^{-1} (cf. negative peaks in Figs. 1, 2). The direction of this shift is consistent with removal of a negative counterion near the Schiff base, since this should promote delocalization of the positive charge through the chromophore's conjugated π-orbital system and a decrease in C=N bond strength.[42] Likewise the C=N stretch mode of K (positive peak at 1608 cm^{-1}, as assigned in reference 42) shifts in the Y185F mutant (cf. Fig. 1). However, from the data obtained so far we cannot say in which direction this peak shifts.

The effects of Tyr-185 substitution on the Schiff base stretching mode suggest that these two groups interact closely. Thus, our data support the previously proposed hypothesis that the tyrosinate group in light-adapted bR is stabilized by electrostatic interaction with the positively charged Schiff base.[39,40] This hypothesis is in turn consistent with a simple mechanism that was proposed for coupling the primary photoisomerization to the protonation of the tyrosine group[39,40]: the chromophore geometry change moves the positively charged Schiff base group away from the tyrosinate (see Fig. 3), destabilizing the anion relative to its conjugate acid.

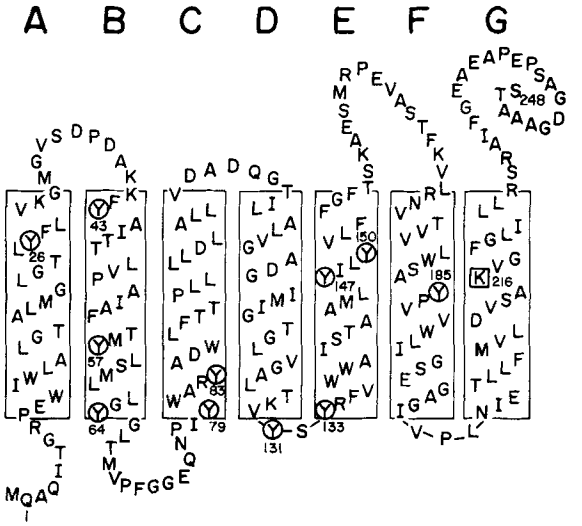

Fig. 4. Model for transmembrane folding pattern of bR, essentially as presented previously.[20,28] The standard one-letter abbreviations for the amino acids are employed. The 11 tyrosine (Y) residues are circled, and the retinal Schiff base attachment site (Lys-216) is boxed.

Our proposal that Tyr-185 is located near the Schiff base group is consistent with a model for the transmembrane folding pattern of bacteriorhodopsin (Fig. 4) that positions Tyr-185 near the center of helix F, i.e., immediately adjacent to the chromophore binding site (Lys-216) on the neighboring helix G.[20] A three-dimensional atomic model of helices F and G (K.J. Rothschild and M.S. Braiman, unpublished) shows that retinal can fit into a binding site with Tyr-185 positioned near the Schiff base and with Trp-182, Trp-189, and Pro-186 forming a pocket around the polyene chain. Furthermore, the Y185F mutant exhibits a red-shifted absorption relative to the wild-type species[2,13,28]; this too is an expected consequence of removing a negative counterion from the vicinity of the Schiff base.[19] Finally, the rate of dark adaptation, which involves protein-catalyzed isomerizations around the C_{13}=C_{14} and Schiff base double bonds,[15,45] is greatly slowed by the Y185F mutation (M.S. Braiman, T. Mogi, and H.G. Khorana, unpublished data), suggesting that Tyr-185 is an important nearby residue.

In summary, the present study and others provide experimental evidence that Tyr-185 is located in the active site of bR, close to the Schiff base linkage between retinal and Lys-216. The effective pK_a of the tyrosine could thus be strongly dependent on the Coulombic interaction with the protonated Schiff base. It was recently shown by FTIR[8,36] that a tyrosine protonation occurs during dark adaptation, which involves just a slight movement of the Schiff base group

associated with the dual "bicycle-pedal" isomerization.[15,45] The frequencies of the tyrosinate bands that are depeleted during dark adaptation are nearly identical with those that are depleted during the bR→K photoreaction, suggesting that the same residue (Tyr-185) undergoes protonation during either step. Thus, it appears that the protonated Schiff base stabilizes the ionized form of Tyr-185 only when the bR chromophore is all-trans. The idea that Tyr-185 and the Schiff base group interact more closely when the chromophore is in the all-trans configuration is further supported by the observation that the Y185F mutation causes a >20-fold decrease in the rate of chromophore regeneration with *all-trans*-retinal, while the regeneration rate with *13-cis*-retinal is completely unaffected.[13]

The Role of Other Tyrosines

Besides Y185F, the other Tyr-185→Phe mutations that led to significant changes in the FTIR difference spectra were Y57F and Y83F. As discussed in the "Results" section, the photochemical behavior of these two mutants at low temperatures (as well as their light-dark adaptation at room temperature) was considerably altered from that of the wild-type protein. This led to the formation of photoproducts other than those observed with the wild-type bR. For example, the negative peak near 1738 cm^{-1} in the "bR→M" difference spectrum of Y83F (cf. Fig. 2) is a feature that is characteristic of the bR→L difference spectrum, which is normally observed only at temperatures somewhat below 250°K.[35]

Thus, the spectral changes exhibited by these two mutants are mostly secondary effects that result from production of entirely different photoproducts, as opposed to primary spectral effects, which are directly attributable to vibrations of the altered residue. Significantly, none of the bR→M difference bands previously associated with a tyrosinate group (1272, 1277, 833 cm^{-1}) is completely eliminated by the Y57F or Y83F mutations. The observation of an intensity decrease in the negative 1277-cm^{-1} band in the Y83F mutant (Fig. 1) raises the possibility that Tyr-83 contributes to this peak in addition to Tyr-185. However, this interpretation would require that bR contain not just one but several ionized tyrosine groups, an unlikely possibility considering that tyrosinates have as yet been observed in only a handful of proteins at neutral pH. Furthermore, the reduction in the 1277 cm^{-1} band is seen only in the bR→K difference spectrum, whereas this band is actually found to increase in intensity in the bR→M difference spectrum of the Y83F mutant (Fig. 2). Overall, the simplest explanation for our data is that the tyrosinate vibrations arise solely from Tyr-185 and that changes in their intensity in the Y57F and Y83F mutants reflect secondary effects.

While the Y57F and Y83F mutations do affect several bR→K and bR→M bands characteristic of unionized tyrosine (for example, at 839, 1240–1250, and 1517 cm^{-1}), these peaks in the wild-type bR spectrum could arise from changes in the environment of Tyr-57 and/or Tyr-83 rather than from a change in their protonation state. Indeed, previous FTIR results with bR containing deuterated tyrosine have suggested that a tyrosine residue changes its environment, without losing or gaining a proton, during the bR→K and bR→M reactions.[8,24] Based on our results, it seems most likely that this is Tyr-57 or Tyr-83. Incorporation of a deuterated tyrosine label into the mutants would help to identify the residue more definitively.

Previously, ultraviolet (UV) visible absorbance spectra and proton-pumping measurements of bR that had been treated with tetranitromethane indicated that Tyr-26 and Tyr-64 undergo protonation changes during the photocycle.[23,44] Recent FTIR and UV difference spectra of photointermediates of similarly modified bR argued against a role for Tyr-26 in the formation of the K and M intermediates but still supported the conclusion that Tyr-64 was involved in the bR photocycle.[37] According to a different group of investigators, however, FTIR spectra of chemically modified bR show that Tyr-64 is remote from the active site and affects the photocycle through steric interaction only.[24] Our results with the mutants confirm the conclusion[37] that Tyr-26 does not contribute to peaks in the bR→K or bR→M FTIR difference spectra. However, in contradiction to a proposal based on chemical modification results,[37] the mutant data show that Tyr-64 also does not contribute peaks to the bR→K or bR→M difference spectra. In particular, all of the FTIR difference peaks assigned to tyrosine appear normal when Tyr-64 is changed to Phe. As reported earlier,[28] the Y64F and Y26F mutants also exhibit normal visible absorption spectra in the light- and dark-adapted states as well as normal chromophore regeneration and proton-pumping rates. Site-directed mutagenesis has the advantage that it is more specific than chemical modification, which can alter other tyrosines as well as the retinal chromophore.[18] Therefore, the results with the mutants provide strong evidence against a major role for Tyr-64 or Tyr-26 in the photocycle.

Despite their disagreement with some chemical modification results, our conclusions from the site-directed mutants are quite consistent with a recent experiment performed under somewhat different conditions.[31] It was reported that when bR is first partially denatured by treatment with SDS, it can be nitrated by tetranitromethane selectively at Tyr-83 (or Tyr-79) and Tyr-185. Furthermore, this modification affects the kinetics of Schiff base deprotonation, as might be expected based on our model (Fig. 3).

Our conclusion that the only tyrosines with important roles in the photocycle are Tyr-185 and, to a lesser extent, Tyr-57 and Tyr-83 is also reasonable from an evolutionary point of view. These are the

only tyrosine residues conserved between the sequences of bacteriorhodopsin[22] and halorhodopsin.[4]

Results from kinetic UV[5,17] and low-temperature UV and FTIR difference spectroscopy[35,37] have been used to conclude that there is a net tyrosine deprotonation during the bR→M transition. We cannot completely exclude the possibility that in addition to the protonation changes at Tyr-185, a second tyrosine residue is deprotonated in M. Tyr-57 or Tyr-83 would be the most likely candidates based on the spectral perturbations observed when they are mutated. However, the present results are not easily explained by such a model. None of the mutants other than Y185F eliminates or reduces a positive peak in the 1270 cm^{-1} region (cf. Fig. 2), as would be expected if a second tyrosinate group deprotonated during the bR→M photoreaction. If there is no net tyrosine deprotonation during the bR→M reaction, then the 295-nm "tyrosine deprotonation" signal observed in kinetic[5,14,17] and low-temperature[37] UV/visible differences spectra of the bR→M reaction must be accounted for in some other way. While changes in the environment of a (protonated) tyrosine group have been ruled out based on the shape of the observed UV difference spectrum, the possibility remains that the UV absorbance increases between bR and M could be caused not by formation of a new tyrosinate, but rather by a change in the environment of a tyrosinate group that already exists in bR, i.e., Tyr-185. In agreement with this model, it was found that the Y185F mutation eliminates most, if not all, of the "tyrosine deprotonation" signal in UV difference spectra.[2]

Is Tyrosine Protonation/Deprotonation Needed To Pump Protons?

We have determined that the side chain of Tyr-185 alternates between protonated and deprotonated states during the proton-pumping photocycle of bR. As with the cyclic deprotonation/reprotonation of the Schiff base during the same photocycle, an unresolved question is whether these events actually reflect transfers of a proton on its way through the membrane. It is possible that the observed tyrosine protonation changes aid in the regulation of structural changes of the chromophore near the Schiff base, but do not directly contribute to the net transport of a proton across the membrane. The observation that all 11 Tyr→Phe mutants pump protons—although the rate is halved for Y185F—is consistent with this view.[28]

However, there are alternative explanations for the retention of proton-pumping activity by the Y185F mutant that would still allow for the direct participation of Tyr-185 in proton transport. For example Tyr-185 may be a component of just one of several proton relays normally functioning in bR. Several groups have, in fact, measured a stoichiometry of ≥ 2 protons pumped per bR photocycle (e.g., reference 34). Thus, removing Tyr-185 could block one of the proton pathways, while leaving the other(s) intact.

Alternatively, there may exist substitute proton transport mechanisms in the Y185F mutant which do not contribute greatly to the normal functioning of wild-type bR. For example, it seems likely that some other anion (one that is normally unionized in wild-type bR) must take the place of tyrosinate-185 in the Y185F mutant; otherwise there would be a destabilizing electrical charge imbalance in the binding pocket of this very hydrophobic protein. This type of compensation for a destabilizing mutation would be somewhat analogous to that observed in crystal structures of T4 lysozyme mutants.[3] The hypothesized substitute anion might be sufficiently close in pK_a to tyrosine to substitute as well in some of the proton transfer reactions that normally involve Tyr-185. However, the only other residues present in bR which are likely to substitute for a tyrosinate anion are Asp and Glu, and the FTIR difference spectrum of the bR→K transition of the Y185F mutant (Fig. 1) is inconsistent with the idea that a carboxylate group is being protonated in place of the tyrosinate. There is still the intriguing possibility that a small solute anion (possibly even hydroxide) could fit into the space left by the elimination of the Tyr-185 hydroxyl group and could substitute for tyrosinate in the proton-pumping mechanism.

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