His-75 in Proteorhodopsin, a Novel Component in Light-driven Proton Translocation by Primary Pumps

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Proteorhodopsins (PRs), photoactive retinylidene membrane proteins ubiquitous in marine eubacteria, exhibit light-driven proton transport activity similar to that of the well studied bacteriorhodopsin from halophilic archaea. However, unlike bacteriorhodopsin, PRs have a single highly conserved histidine located near the photoactive site of the protein. Time-resolved Fourier transform IR difference spectroscopy combined with visible absorption spectroscopy, isotope labeling, and electrical measurements of light-induced charge movements reveal participation of His-75 in the proton translocation mechanism of PR. Substitution of His-75 with Ala or Glu perturbed the structure of the photoactive site and resulted in significantly shifted visible absorption spectra. In contrast, His-75 substitution with a positively charged Arg did not shift the visible absorption spectrum of PR. The mutation to Arg also blocks the light-induced proton transfer from the Schiff base to its counterion Asp-97 during the photocycle and the acid-induced protonation of Asp-97 in the dark state of the protein. Isotope labeling of histidine revealed that His-75 undergoes deprotonation during the photocycle in the proton-pumping (high pH) form of PR, a reaction further supported by results from H75E. Finally, all His-75 mutations greatly affect charge movements within the PR and shift its pH dependence to acidic values. A model of the proteorhodopsin proton transport process is proposed as follows: (i) in the dark state His-75 is positively charged (protonated) over a wide pH range and interacts directly with the Schiff base counterion Asp-97; and (ii) photoisomerization-induced transfer of the Schiff base proton to the Asp-97 counterion disrupts its interaction with His-75 and triggers a histidine deprotonation.

A variety of unicellular microorganisms contain primary proton pumps that convert solar energy into a transmembrane electrochemical proton gradient, which is subsequently used by membrane ATP synthases to generate chemical energy. Well known examples of such pumps are the halooarchael rhodopsins, photoactive, seven-helix membrane proteins, which include the well studied proton pump bacteriorhodopsin (BR) from Halobacterium salinarum and BR homologs in other haloarchaea. Recently, a much larger new family of light-driven proton pumps, the proteorhodopsins (PRs), was identified in marine proteobacteria throughout the oceans (1–3). Despite the diverse properties of PRs, including different visible absorption maxima and photocycle rates (4–6), they all share with BR several key conserved residues as well as an all-trans-retinylidene chromophore in their unphotolyzed state, which is covalently bound to transmembrane helix G via a protonated Schiff base linkage.

Many of the molecular events that occur in PRs following light activation are similar to those of BR, including an initial ultrafast all-trans→13-cis-retinal isomerization, which triggers a sequence of protein conformational changes, including several intramolecular proton transfer reactions. The two key carboxylate groups involved in proton pumping in helix C of BR are conserved in PRs, and in the first found and most commonly studied PR, the Monterey Bay variant eBAC31A08, also known as green-absorbing proteorhodopsin (GPR), the helix C residues Asp-97 and Glu-108 undergo protonation changes during the photocycle similar to those of the homologous carboxylate residues in BR. Initial FTIR studies on GPR identified the role of Asp-97 as the Schiff base counterion and proton acceptor during Schiff base deprotonation and concomitant M formation and Glu-108 as the proton donor that reprotonates the Schiff base during N formation (7, 8). Studies of other variants indicate these roles of the two carboxylic acid residues are general in the proteorhodopsin family.

One major difference between BR and the PRs is the presence of a highly conserved histidine residue at position 75, near the middle of transmembrane helix B in the latter pigments. The His-75 homolog is not present in BR nor thus far found in other microbial rhodopsins (9). The proximity of His-75 to the pro-
tein active site and specifically to the Schiff base counterion. Asp-97 inferred from the x-ray crystal structure of BR suggests its involvement in spectral tuning of the visible absorption (10) and potentially PR photochemical reactions. Because the pK_a of histidine in solution is close to neutral pH (11), its imidazole group often plays a major role in intramolecular proton transfers in enzymes, including NADPH oxidase (12), alcohol dehydrogenase (13), carbonic anhydrase II (14), and serine proteases (15).

In this study we have used a combination of time-resolved FTIR difference spectroscopy, visible absorption spectroscopy, isotope labeling, kinetic charge displacement measurements, and site-directed mutagenesis to study the role of His-75 in GPR. We report evidence that protonated His-75 interacts directly with Asp-97 in the unphotolyzed protein and during the photocycle undergoes a deprotonation in response to the protonation of Asp-97.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The Monterey Bay eBAC31A08 variant of PR (also designated as green-absorbing PR (GPR)) cloned in a pBAD-TOPO® (Invitrogen) vector was used to construct the mutants H75A, H75E, and H75R using Stratagene Quik-Change® (Stratagene, La Jolla, CA) site-directed mutagenesis kit. All mutations were verified by DNA sequencing.

Protein Expression and Purification—Wild-type and mutant PR were heterologously expressed in Escherichia coli strain UT5600 using the pBAD-TOPO® plasmid as described elsewhere (1). Cultures grown overnight were diluted to 1:50 and grown to 0.4 absorbance units at 600 nm at 37 °C. At this stage, the cells were induced with 0.2% (+)-L-arabinose for 3 h at 37 °C. For flash photolysis and proton pumping experiments, 5 μM (final concentration) all-trans-retinal was added with the (+)-L-arabinose. Procedures for purification of His-tagged PR and its reconstitution in E. coli polar lipids were similar to those used previously (16). After the induction period, the cells expressing PR were centrifuged at 1000 × g, resuspended in 5 mM MgCl_2 and 150 mM Tris-HCl, pH 7.0, and disrupted by sonication. The membranes containing pigment were collected by centrifugation (39,000 × g, 30 min) and solubilized in a wash buffer (50 mM KP_v, 300 mM NaCl, 5 mM imidazole, and 1.5% octyl glucoside (OG), pH 7.0) for at least 1 h at 4 °C. Unsolubilized membranes were removed by centrifugation at 28,000 × g for 30 min. The supernatant was incubated with a His-binding resin on a shaker for 4 h at 4 °C to remove a histidine label. The bound resin was washed with 3 × volumes of wash buffer followed by elution buffer (50 mM KP_v, 300 mM NaCl, 250 mM imidazole, and 1.0% OG, pH 7.0). Purified His-tagged PR was reconstituted in the E. coli polar lipids (Avanti, Alabaster, AL) at 1:10 protein-to-lipid (w/w) ratio. Lipids initially dissolved in chloroform were dried under argon and resuspended in the dialysis buffer (50 mM potassium phosphate, 300 mM NaCl, pH 7.0) to which OG was added to the final concentration of 1%. The lipid solution was incubated with the OG-solubilized protein for 1 h on ice and dialyzed against the dialysis buffer with three buffer changes every 24 h. The reconstituted protein was centrifuged for 15 min and resuspended in the sample buffer (50 mM CHES, 150 mM NaCl, pH 9.5).

Protein Isotope Labeling—Wild-type and H75E mutant PR was heterologously expressed in the histidine auxotroph E. coli strain JM1100 using the pBAD-TOPO® plasmid. The JM1100 strain was obtained from the E. coli Genetic Stock Center at Yale University. The histidine-labeling protocol (17) was modified as described previously (18) to minimize the consumption of labeled media. Briefly, E. coli cultures were grown in 0.5-liter volume using LB medium to the cell density of at least 0.4 absorbance units at 600 nm, collected by centrifugation at 1000 × g, washed with an equal volume of 1× M9 medium (Sigma), and transferred to an equal volume of minimal growth medium (18) supplemented with 10 μM U-13C6-L-histidine (Cambridge Isotope Labs, Andover, MA) or alternatively nonlabeled L-histidine (Sigma) and incubated for 1 h to remove unlabeled metabolites. The cells were subsequently induced with 0.2% (+)-L-arabinose to which 5 μM (final concentration) all-trans-retinal was added and incubated for additional 5 h. In a control experiment, cell cultures transferred to the minimal growth medium lacking histidine did not exhibit any detectable protein expression (data not shown). The label incorporation was verified by comparing the absolute absorption FTIR spectra of unlabeled and labeled protein samples (data not shown). To demonstrate the absence of isotope label scrambling, purified PR samples were digested with trypsin and sequenced using an ABI 4800 Plus matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). The mass and peptide fragmentation pattern obtained from the internal fragment GVWIETGDSPTVFR (amino acids 81–94), which does not contain a histidine residue, was identical between the labeled and nonlabeled protein samples (supplemental Fig. 1). The data indicate that the 13C isotope label was not transferred to other amino acids.

FTIR Difference Spectroscopy—Protein films were prepared by depositing 5−10 ml of the proteoliposome suspension in the sample buffer, pH 9.5, onto a polished 2-mm thick, 25-mm diameter CaF_2 window (Wilmad, Buena, NJ) and drying the sample under a gentle stream of argon. Films were rehydrated via the vapor phase and then sealed in a temperature-controlled IR cell (model TFC, Harrick Scientific Corp., Ossining, NY) using a second CaF_2 window. Rapid scan time-resolved FTIR spectra were recorded with a Bruker IFS 66 v/s FTIR spectrometer (Bruker Optics, Germany) at 5 °C as described previously (19) at 4 cm⁻¹ spectral resolution and 240-kHz scanner velocity corresponding to the data acquisition window of 18 ms. Between 10 and 20 individual spectra were averaged to produce the final spectrum. The low temperature FTIR measurements were performed using a Bio-Rad FTS-60A FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) (20).

Charge Movements—Laser flash induced charge movements in GPR and its mutant forms were recorded in suspensions of E. coli cells expressing the rhodopsins as described (21).
RESULTS

Visible Absorption of His-75 Mutants at Acidic and Alkaline pH—PR mutants were prepared in which His-75 was replaced with a neutral (Ala), acidic (Glu), or basic (Arg) residue. The formation of a pigment was observed for all substitutions, although the protein yield was lower in the case of H75E and H75R. The H75R mutant also exhibited more rapid bleaching, possibly because of destabilization of the retinal-binding pocket.

Visible spectra of the wild-type and mutant PRs were recorded using octyl glucoside-solubilized purified protein (Fig. 1). In agreement with earlier reports (5), the spectrum of wild-type GPR has a visible absorption maximum near 522 nm at pH 9, which is red-shifted to 545 nm at pH 5. This shift is attributable to the spectral transition due to protonation of the Schiff base counterion Asp-97 at acidic pH (7, 8). A similar transition also occurs in H75A and H75E GPR. However, the H75A absorption maximum is blue-shifted from the wild-type pigment by 11 nm at pH 9 and red-shifted by 11 nm at pH 5. As a result, the mutant undergoes a considerably larger shift between the acidic and alkaline forms than the wild type. As described below, the light-induced transfer of the Schiff base proton to Asp-97 is also blocked during M formation when the Schiff base proton is normally transferred to Asp-97.

Interaction between the Counterion Asp-97 and His-75—To explore the effect of His-75 on the protein changes, which occur upon light activation, FTIR difference spectra of the wild-type and mutant GPR were measured in the millisecond time window using rapid-scan spectroscopy (19) (Fig. 2A). The wild-type spectra are very similar to those reported previously (7, 8). Negative bands reflecting vibrations in the unphotolyzed PR state appear in the configurationally sensitive C-C stretch region characteristic of the all-trans-retinal chromophore at 1198 (793), 1235 (797), and 1252 (797) cm⁻¹, whereas positive bands at 1170 and 1187 cm⁻¹ arise from the formation of a mixture of late photocycle intermediates similar to the M, N, and O intermediates in BR (8). The negative ethylenic band at 1537 cm⁻¹
agrees with the expected position based on an empirical inverse correlation of vibrational frequency and visible absorption wavelength (see Ref. 16 and references therein). Strong bands appear in the amide I region at 1689 (+), 1660 (+), and 1674 (−) cm\(^{-1}\) reflecting mainly protein backbone movements (7). In the carboxylic acid region, a positive band at 1757 cm\(^{-1}\) in the wild-type PR arises from protonation of the counterion Asp-97, which accepts a proton from the Schiff base during the formation of the M intermediate (8). The pair of bands at 1727 (−)/1742 (+) cm\(^{-1}\) reflects a change in hydrogen bond strength of Glu-108. Similarly to Asp-96 in BR, this group functions as a proton donor to the Schiff base (8) and is expected to undergo a transient deprotonation during the decay of the M intermediate as in BR (22).

**H75E and H75A**—These mutations do not cause large perturbations in the FTIR difference spectra. However, evidence is found for changes in the protein environments of both the Schiff base proton acceptor Asp-97 and the proton donor Glu-108 as well as altered kinetics of the late photocycle. The positive peak at 1757 cm\(^{-1}\) in the wild-type GPR difference spectra is upshifted by ~3 cm\(^{-1}\) in H75A (Fig. 2B, middle trace) and to a smaller degree in the H75E mutant (Fig. 2B, bottom trace) indicating a more hydrophobic hydrogen-bonding environment of the protonated counterion in the light-activated pigment (23). Note that this frequency upshift does not appear to result from changes in the relative concentrations of the late photointermediates (see below) because the 1757 cm\(^{-1}\) peak undergoes a downshift during the late photocycle in wild-type PR (8). The negative 1727 cm\(^{-1}\) peak associated with Glu-108 is still present in both the mutant spectra, but the 1742 cm\(^{-1}\) positive peak absent. The absence of a positive peak indicates that Glu-108 does not reprotonate until reformation of the unphotolyzed PR state in the His-75 mutants. Finally, a new negative peak appears near 1715 cm\(^{-1}\) in H75E but does not in H75A spectra. Its frequency is characteristic of the C=O stretching vibration of a glutamic acid side chain located in a hydrophilic environment (23). Thus, this band most likely reflects deprotonation of the Glu residue introduced at position 75, in response to protonation of Asp-97. As discussed below, a similar deprotonation of His-75 is indicated on the basis of isotope labeling His-75 in the wild-type pigment.

In addition to the carboxylate peaks, the H75A and H75E mutations cause changes in the retinal fingerprint and ethylenic stretching regions. In particular, the increase of intensity of the 1187 cm\(^{-1}\) peak assigned to photointermediate(s) containing the 13-cis chromophore (8) is observed in the mutants (Fig. 2C) indicating altered kinetics of the retinal reisomerization to the all-trans form. Also, the split of the 1537 cm\(^{-1}\) negative band (Fig. 2A) resulting in two minima at 1530 and 1541 cm\(^{-1}\) can be explained by appearance of a positive band with a frequency close to 1537 cm\(^{-1}\), most likely due to accumulation of an intermediate with the chromophore C=C stretching vibration close to that of the unphotolyzed state.

Changes in the photocycle kinetics evident from the decay rates of FTIR difference bands show that both H75A and H75E mutants exhibit significantly faster return to the protein initial state (Fig. 3A). The analysis of absorption changes in the visible range (16) also reveals an accelerated photocycle (Fig. 3B), although the rate of dark state recovery, which is monitored at 500 nm, appears to be more similar between H75A and wild-type GPR. The differences from the FTIR data may be attributable to the different sample properties (hydrated proteoliposomes versus E. coli cell suspensions). Also the final intermediate in the PR photocycle may have absorption very close to that of the unphotolyzed state, and therefore its decay may not produce a pronounced absorption change at this wavelength (8). In any case, both measurements show acceleration of the photocycle by the His-75 mutations.

**H75R**—This mutation had the most drastic effect on the FTIR difference spectra relative to wild-type spectra (Fig. 4). In the first 50 ms (*top trace*), a strong 1525 cm\(^{-1}\) (+) ethylenic band appears along with a retinal fingerprint peak near 1183 cm\(^{-1}\) (+). Both peaks are characteristic of the early K-like intermediate (16) and are also present in the low temperature static H75R spectra recorded at 215 K, where the transition to subsequent intermediates is blocked (Fig. 4, 2nd trace). How-
Proton Transfer in His-75 Mutants—Proton transport is greatly affected in all His-75 mutants (Fig. 5). Although both wild-type GPR and H75A exhibit outward charge movements indicative of the Schiff base proton transfer to Asp-97 and rep-protonation of the Schiff base, the overall proton translocation is inhibited by at least 50% in the mutant. H75E shows a complex kinetics involving both outward and inward charge movements, whereas mutation of His-75 to Arg completely eliminates light-induced proton transfer.

The lack of proton transfers in the latter mutant correlates with the absence of the 1757-cm\(^{-1}\) band in the FTIR spectra and further supports the finding that H75R substitution blocks proton transfer from the Schiff base to Asp-97. The blocking of proton transfer is also confirmed by visible flash-induced absorption changes. Laser flash photolysis shows no absorption change at 400 nm (Fig. 6), a wavelength indicative of the Schiff base-deprotonated intermediate M. Rather, depletion of the unphotolyzed pigment band monitored at 500 nm results in transient appearance of a red-shifted species not preceded by M formation.

The pH dependences of absorption changes and charge movements in H75A and H75E are acid-shifted as compared with wild type by \(\sim 0.5\) and \(\sim 0.7\) units, correspondingly (data not shown). The shift in pK\(_a\) of Asp-97 in H75R mutant is evidently greater because color changes are not observed down to pH 5. Further acidification led to precipitation of the protein. Effects of mutations on the pK\(_a\) of Asp-97 support the conclusion discussed below of direct interaction of His-75 and Asp-97.

Protonation Changes of His-75 during the Photocycle—To identify difference bands that arise from structural changes of His-75 in the FTIR spectra, the wild-type protein was uniformly labeled with \([^{13}\text{C}_6]\)histidine (see “Experimental Procedures”), and spectra were compared with unlabeled PR. Except for the recombinant C-terminal His\(_{16}\) tag, which is not perturbed by photoactivation (see below), His-75 is the only histidine residue

![FIGURE 4. Comparison of the early and late photocycle FTIR difference spectra of H75R PR. Time-resolved spectra were recorded during the first 50 ms and 150–200 ms of the photocycle. Spectra are compared with those measured by static FTIR spectroscopy at 215 K, where the transition from early to late photocycle is blocked, and at 248 K, where accumulation of the late photocycle intermediates is observed.](image-url)

![FIGURE 5. Charge movements in the wild-type (WT) PR and H75A, H75E, and H75R mutant proteins in response to a 6-ns flash from a Nd-YAG laser (\(\lambda = 532\) nm). y axis, integral over time of the macroscopic photocurrent in suspensions of \(E.\ coli\) cells expressing the pigment, flashed along the direction between electrodes (for details see Ref. 21). The spectra represent an average of 100 signals with 5-s intervals measured at pH 7.6 and 23 °C. Upward shifts correspond to outwardly directed charge movements.](image-url)
in the GPR sequence; therefore, any spectral changes observed can be unambiguously assigned to His-75 vibrations. The labeled and unlabeled spectra closely resemble each other as expected because isotope labeling should not alter the structure or conformational changes that GPR undergoes during the photocycle (Fig. 7, top and middle traces). However, reproducible changes were detected in several spectral regions that are characteristic of histidine side-chain vibrations (27–29). The negative signal near 1610 cm\(^{-1}\) is reduced in the \([^{13}\text{C}_6]\)histidine PR spectrum, consistent with existence of a small negative band at this frequency, which is altered because of histidine labeling. The spectra of fully protonated HisH\(_2^+\) exhibit a strong C4–C5 mode near 1631 cm\(^{-1}\) that shifts to lower frequency upon increased hydrogen bonding (28, 29). In contrast, this mode is considerably weaker and appears below 1600 cm\(^{-1}\) in the spectra of neutral HisH. Because \(^{13}\text{C}\) labeling downshifts the C4–C5 band by as much as 38 cm\(^{-1}\) (28), the likely assignment of the 1610 cm\(^{-1}\) (−) peak is to fully protonated His-75 in the PR initial state. A similar assignment of the 1617 cm\(^{-1}\) peak to HisH\(_2^+\) in the FTIR difference spectra of photosystem II was suggested on the basis of \(^{13}\text{C}\) His labeling by Hienerwadel et al. (28). Our results do not rule out the possibility that histidine difference bands also appear at higher frequency. Although no reproducible spectral changes because of the isotope labeling were found between 1630 and 1650 cm\(^{-1}\) (supplemental Fig. 2), weaker bands in this region may be obscured by larger noise resulting from the strong background water absorption.

Substitution with \([^{13}\text{C}_6]\)histidine also results in disappearance of a small band at 1108 cm\(^{-1}\) (+) superimposed on top of a larger positive peak, which is not affected by the labeling. The strong C-N stretching mode of the imidazole ring near 1100 cm\(^{-1}\) is not significantly affected by the environment, such as ligand binding, and therefore serves as a highly specific IR marker of the histidine protonation state (30). This band is found at 1102–1108 cm\(^{-1}\) in the spectra of HisH (N3-protonated nitrogen), and at 1088–1094 cm\(^{-1}\) in HisH\(_2^+\) (29, 30). On this basis, we assign the observed spectral feature to the C-N stretching vibration of neutral His-75 in the PR light-activated state. Isotope labeling also affects the peak near 1268 cm\(^{-1}\), which has different intensity in the spectra of protonated and neutral histidine (29).

The isotope-induced spectral changes at 1108 (+) and 1610 (−) cm\(^{-1}\) indicate that His-75 undergoes a structural change during the late photocycle consistent with its deprotonation. Confirming that these spectral differences are attributable to His-75, and not to the His\(_a\)-terminal tag, the spectra of isotope-labeled and unlabeled H75E mutant of PR, which has a photocycle similar to the wild-type, do not exhibit the differences at 1108, 1268, and 1610 cm\(^{-1}\) (Fig. 7, bottom traces). The latter result is not surprising because the solvent-exposed residues in the His\(_a\) tag are expected to remain unprotonated because of the high pH (9.5) of the sample buffer.

**DISCUSSION**

The above results reveal a significant role of His-75, a highly conserved residue in the proteorhodopsin family, on spectral tuning and photochemical reactions of GPR. In the predicted folding structure (Fig. 8), this residue is located near the middle of transmembrane helix B. An x-ray crystal structure of a PR is not available, but substituting a histidine residue for the homologous residue Met-56 into the crystal structure of bacteriorhodopsin (31) predicts that His-75 is in a position to directly interact with the Schiff base counterion Asp-97 (Fig. 8, inset), which was previously shown.
using the energy minimization method (10). This proximity suggests that the protonation state of His-75 would have a significant effect on various properties of PR, including its color (e.g., visible absorption spectrum) and photocycle. Furthermore, because histidine has a near-neutral $pK_a$, a protonated form may form a salt bridge with the negative counterion. Our results indicate that His-75 does interact directly with the Schiff base counterion Asp-97 and exists in a protonated state even at pH as high as 9. The evidence includes the information below.

**His-75 When Substituted with a Positively Charged Arg Does Not Shift the Visible Absorption**—The absence of a shift is especially significant in view of the related result that the substitutions H75A and H75E both substantially affect the $\lambda_{max}$ of PR in the low pH (5) and high pH (9) forms. Given that the substitution of Arg for His is not structurally conservative unless His carries a positive charge like Arg, this result indicates that His-75 exists in unphotolyzed GPR in a protonated form.

**H75R Blocks the Acid-induced Protonation of the Schiff Base Counterion**—This result would be explained if Arg-75 interacted directly with the Schiff base counterion Asp-97, thereby preventing it from undergoing a protonation, which normally occurs at low pH. This would occur if the Asp-97 salt bridge with Arg-75 is more stable than that with His-75, effectively preventing Asp-97 protonation at pH 5. An earlier study has shown that the elevated $pK_a$ of Asp-97 compared with the homologous residue in BR most likely results from a weaker interaction between the carboxylate group and Arg-94 because of a larger distance between these residues (32). Thus, it is possible that the replacement of nearby His-75 with an arginine restores a strong Asp-97-arginine counterion interaction, which would significantly lower the $pK_a$ of Asp-97. The $pK_a$ of Asp-97 is shifted to more acidic values in each of the three His-75 substitutions made, further supporting a salt-bridge interaction between His-75 and Asp-97 that raises the Asp-97 $pK_a$.

**His-75 Mutations Have Strong Effects on the Schiff Base Counterion in the Light-activated Protein**—In addition to the effect described above, Asp-97 does not appear to undergo a protonation reaction during the H75R photocycle. Instead, the decay of the early K-like intermediate is significantly prolonged, and no photointermediate possessing deprotonated Schiff base is detected. The H75A and H75E substitutions seem to be less perturbing and do not block the counterion protonation. However, the environment of Asp-97 is more hydrophobic in these mutants.

**Histidine Isotope Labeling and the Substitution with Glu-75 Indicate That His-75 Undergoes a Deprotonation during the Photocycle**—These findings favor a model whereby the protonation of the counterion Asp-97 disrupts its interaction with His-75, thereby causing His-75 to deprotonate. Such a mechanism may be a part of the overall proton ejection pathway. In fact, the initial study performed under similar pH as this study showed existence of a fast proton release in PR coincident with the formation of M, leading to the suggestion that the PR mechanism may involve different groups than those in BR (33). However, in another study performed at lower pH, proton release to the extracellular medium was not observed in GPR until after M formation (8), suggesting that under certain conditions there may exist a delay in His-75 deprotonation, or alternatively there...
may be additional residues acting as a relay in the proton ejection pathway. One possibility is Glu-142, which has recently been found to exist in a protonated state in GPR but in an ionized state in blue-absorbing proteorhodopsin (34).

It is notable that the molecular changes because of His-75 mutations extend outside the Schiff base region and affect Glu-108 on the protein cytoplasmic side. Similar to BR, a proton transfer pathway exists in the late photocycle intermediates of GPR that connects Glu-108 to the Schiff base (8). This structure is likely to be altered in the mutants, which causes Glu-108 reprotonation to be delayed until the end of the photocycle. On the other hand, the mutations do not change the environment of Glu-108 in the initial state of the protein as evident from the identical frequency of the negative band assigned to this residue (1727 cm\(^{-1}\)) in the wild-type and mutant PRs. The presence of Glu-108 predominantly in the unprotonated form is in agreement with the observed larger accumulation of the 13-cis photointermediates in the mutants. This correlation is analogous to BR where the 13-cis→all-trans chromophore isomerization does not occur until re-protonation of the homologous group Asp-96 (22).

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Note Added in Proof—While this article was under review, Luecke et al. (Luecke, H., Schobert, B., Stagno, J., Imasheva, E. S., Wang, J. M., Balashov, S. P., and Lanyi, J. K. (2008) Proc. Natl. Acad. Sci. USA 105, 16561–16565) reported the crystal structure of xanthorhodopsin, a homologous microbial rhodopsin that contains a His residue at the position corresponding to His-75 in the proteorhodopsin studied here. The His residue in xanthorhodopsin is closely associated with the Schiff base counterion aspartate with the ND1 of the His residue hydrogen-bonded to the OD1 of the Asp residue. The atomic structure is in full agreement with the conclusions of this study, suggesting that proton exchange reactions involving histidine are a general feature of light-driven proton transport by eubacterial pumps.

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