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Weakened coupling of conserved arginine to the proteorhodopsin chromophore and its counterion implies structural differences from bacteriorhodopsin

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Abstract

In wild-type proteorhodopsin (pR), titration of the chromophore's counterion Asp^{97} occurs with a pK_a of 8.2±0.1. R94C mutation reduces this slightly to 7.0±0.2, irrespective of treatment with ethylguanidinium. This contrasts with the homologous archaeal protein bacteriorhodopsin (bR), where R82C mutation was previously shown to elevate the pK_a of Asp^{85} by ~5 units, while reconstitution with ethylguanidinium restores it nearly to the wild-type value of 2.5. We conclude there is much weaker electrostatic coupling between Arg^{94} and Asp^{97} in the unphotolyzed state of pR, in comparison to Arg^{82} and Asp^{85} in bR. Therefore, while fast light-driven H^+ release may depend on these two residues in pR as in bR, no tightly conserved pre-photolysis configuration of them is required.

Keywords: Sensory rhodopsin; Light-driven proton pumping; Red to purple transition; Blue membrane; Fast H^+ release; Photocycle

1. Introduction

Proteorhodopsin (pR), a retinal protein that functions as a light-driven proton pump, is the first archaeal rhodopsin homolog identified in the domain *Bacteria*. Recently, pR was discovered in the genomes of several species of uncultivated marine γ-proteobacteria present in the Eastern Pacific Ocean, Central North Pacific Ocean and Southern Ocean, Antarctica [1–3]. Subsequently, genes of pR variants have been identified in samples from the Mediterranean and Red Seas [4,5], the Sargasso Sea [6] and the Japanese Sea [7].

On comparison to its better-known archaeal homolog bacteriorhodopsin (bR), most of the active site residues of known importance to the bR mechanism are conserved in pR [1]. Homologues of the active-site residues Arg^{82}, Asp^{85} (the primary proton acceptor), Asp^{212} and Lys^{216} (the retinal Schiff base binding site) in bR are conserved as Arg^{94}, Asp^{97}, Asp^{227} and Lys^{231} in pR. However, in pR, there are no carboxylic acid residues directly homologous to Glu^{194} or Glu^{204} of bR, which are thought to be involved in the proton release pathway at the extracellular surface.

It seems likely that pR functions throughout the Earth's oceans as a light-driven H^+ pump, by a mechanism similar to that of bR. As in bR, the retinal chromophore of pR is covalently bound to the apoprotein via a protonated Schiff's base at Lys^{231}. The configuration of the retinal chromophore in unphotolyzed pR is predominantly all-*trans*, and changes to 13-*cis* upon illumination with light [8,9]. Several models of the complete pR photocycle have been proposed, based on FTIR and UV–visible spectroscopy [9–11]; they resemble established photocycle models for bR.

Abbreviations: pR, proteorhodopsin; bR, bacteriorhodopsin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; OG, β-octyl-D-glucoside; TCM, triple cysteine mutant of pR [C(107, 156, 175)S]; QM, quadruple mutant of pR [C(107, 156, 175)S, R94C]; NpSRII, *Natronobacterium pharaonis* sensory rhodopsin II

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It is well known that in bR, the chromophore undergoes a transition in appearance from purple (570 nm absorbance maximum) to blue (605 nm absorbance maximum) when the pH is lowered. The mechanism of formation of blue membrane in bR is due to the protonation of the retinylidene Schiff base group's principal counterion, Asp^{85}, which is conserved in pR as Asp^{97}. Analogous pH-dependent spectral shifts in natural variants of pR have been reported to have pK_a values in the range 7.1–8.5 [12].

The role of Arg^{82} in the purple-to-blue transition of bR was revealed by using a combination of site-directed mutagenesis and chemical modification [13–15]. Mutation of Arg^{82} in bR causes the protein to change from purple- to blue-colored at neutral pH. This effect is almost wholly due to the upshifted pK_a of the purple-to-blue transition, to near 7.4 in R82C bR [13]. Similar pK_a upshifts had previously been observed in R82Q and R82A mutants [15].

Based on such experiments, we expected the R94C mutation in pR to cause an increase in the pK_a of the Schiff base counterion Asp^{97}, as in R82C bR [13], but were surprised instead to see a small decrease. The upshifted pK_a of the chromophore counter-ion in wild-type pR, as compared with wild-type bR, and the lack of any further upshift when Arg^{94} is mutated, suggest a weakened electrostatic interaction between Arg^{94} and Asp^{97} in pR. Our results, as well as those recently reported by others on the D97N and D97E mutants of pR [16], are consistent with an altered position of Arg^{94} in pR as compared to bR, making it doubtful that fast H^+ release in this family of proteins is dependent on a particular initial position of this residue.

2. Materials and methods

2.1. Protein expression

Wild-type pR was expressed in *E. coli* as described [17]. The UT5600 strain without the plasmid was recovered by growing the pR-expressing strain under non-selective conditions, and then testing plasmid exclusion by determining ampicillin resistance of single colonies. This strain was retransformed with mutated plasmids, prepared from the wild-type expression plasmid by using the Stratagene QuikChange® site-directed mutagenesis kit.

2.2. Protein purification

Crude extracted pR in β-octyl-D-glucoside (OG), prepared as described previously [17], was then further purified by using either of three alternative procedures, which (for wild-type pR) gave equivalent results. (a) The His-tagged pR in 3% OG, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonicacid)+10 mM imidazole, pH 7.1, was directly applied to a column containing ProBond™ Ni resin (Invitrogen, Carlsbad, CA), using 4 mL hydrated resin for ~4 mg impure pR. The column was washed with 15 mL of the loading buffer, then eluted with the same buffer containing 200 mM imidazole (pH 7.1). This single step yielded pR with A_{280}/A_{540} of 4.0 or lower. (b) Partial purification on a Phenylsepharose™ column with a gradient of 0.5–2.0% OG in 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.1, yielded material with A_{280}/A_{540}=5–10. Further purification on a hydroxylapatite column with a 0–600 mM phosphate gradient yielded material with A_{280}/A_{540} of 2.0 or better [17]. (c) Citrate purification procedure: wild-type pR was subjected to a novel purification by selective precipitation [18]. The *E. coli* membrane extract containing pR in 3.0% OG in buffer A, was diluted 6-fold (to 0.5% OG), by the addition of 5 volumes of 100 mM sodium citrate, pH 5.5. It was centrifuged immediately at 5000 rpm for 10 min at 4 °C. The faint-red supernatant, containing the pR, was separated from the white pellet, containing impurities. The supernatant was then further diluted to 0.3% OG, by adding 2/3 volume of the same 100 mM sodium citrate (pH 5.5) solution used previously. After thorough mixing, the suspension was immediately centrifuged at 3000 rpm for 5 min at 4 °C. Typically, on fresh wild-type pR, the pellet at this point consisted once again of colorless (white) proteinaceous material, and was discarded. The supernatant was removed, and then stored at 4 °C for 12–18 h, at which time a new purple precipitate was clearly visible. The suspension was centrifuged at 6000 rpm for 30 min at 4 °C. The purple pellet was re-solubilized in 3.0% OG in 5 mM Tris–Cl (pH 9.5). The citrate-induced selective precipitation was repeated twice to obtain ~50% pure pR ($A_{280}/A_{560} \cong 5$). This could be further purified on the Ni-NTA column described in (a) to obtain >95% purity of the pR.

Purified pR in OG was concentrated, and then washed several times with 5 mM HEPES pH 7.1, by using 20 mL conical ultrafiltration tubes with 5000 MW cutoff.

2.3. Absorption spectroscopy

Concentrated protein solution (typically 1 mg/mL in 3% OG, 5 mM HEPES, pH 7.1) was diluted with 7 parts buffer (acetate, HEPES, glycylglycine, glycine; final concentration: 100 mM each containing 3% OG). Different aliquots of the buffer had previously been adjusted to pH values between 4.5 and 10.5. Absorption spectra were recorded at 2-nm resolution and room temperature by using a UV-265 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A sample volume of 0.8 ml in a masked quartz cuvette was used for all measurements.

3. Results

3.1. UV–visible absorption spectra of pR mutants

As reported [17], purified wild-type pR in OG at pH 7.0 has an absorption maximum at 545 nm (Fig. 1), shifted

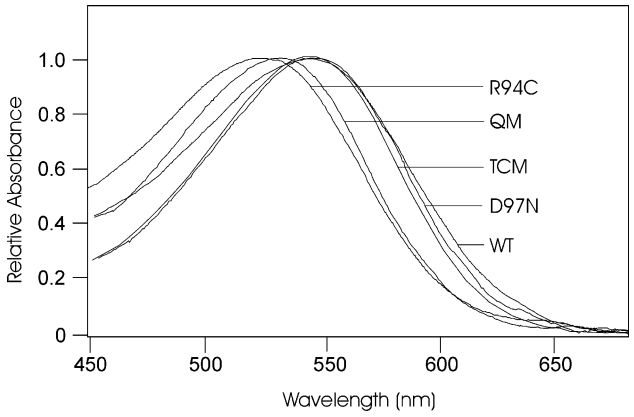

Fig. 1. Absorption spectra of wild-type pR and mutants. λ_{max} values were as follows: wild type, 547 nm; TCM, 545 nm; R94C, 525 nm; D97N, 547 nm; and QM, 532 nm. Measurements were made on pR in 3.0% OG, pH 7.1, and purified using a Phenylsepharose™ column followed further by a hydroxylapatite column.

somewhat when compared to the absorption peak of 520 nm measured previously on unpurified membranes using photobleaching difference spectroscopy [1]. The triple cysteine mutant (TCM), in which all three native cysteines were replaced by serines, and D97N pR both have an absorption spectrum indistinguishable from the wild type at pH 7.0. However, significant blue shifts of ~10–15 nm are seen when Arg^{94} is mutated to cysteine, either singly (R94C) or in the quadruple mutant (QM) combination, in which the three native cysteines are mutated to serines.

3.2. pH dependence of the absorption spectrum of wild-type and mutant pR samples

Fig. 2 shows a pH titration of wild-type pR in OG. The visible absorption maximum shifts from 521 to 544 nm when the pH is lowered from 10 to 6.5. The 811-cm^{-1} red shift over this pH range is somewhat smaller than the 1015-cm^{-1} red shift seen in the purple-to-blue transition of bR, which also occurs at a substantially lower pH range. In pR, the pH-induced transition is phenomenologically better described as "red-to-purple". The pR titration was fully reversible over the pH range pH 6.5–10.0. At higher and lower pH values, a portion of the protein denatured irreversibly.

Our pK_a of 8.2±0.1, obtained by fitting A_{510} to the Henderson–Hasselbalch equation (Fig. 2, inset), differs from the values of 7.1 [12,16] and 7.68 [9] previously reported. Similar pK_a values were obtained for wild-type pR purified using any of the three methods as described in the protein purification section. Our titrations were done using different detergent conditions and in the absence of any salt, contrary to the presence of 100–150 mM NaCl used in earlier experiments. However, we also performed spectroscopic titrations (data not shown) of wild-type samples with 0.5 M NaCl and found <0.1-unit shift in the pK_a value resulting from the addition of salt. We did obtain a pK_a value of ~7.25 (data not shown) when titrations were performed using samples of wild-type pR solubilized in OG that had been stored at 4 °C for ~2 months.

In the R94C mutant, the absorption maxima shifted from 515 nm at pH 8.5 to 521 nm at pH 6.5, with a pK_a for this transition of 7.0±0.2 (Fig. 3 inset). Unlike the wild type, the R94C mutant showed a significant decrease in maximum absorbance as the pH was lowered, and therefore no isosbestic point was observed within the range of the visible absorption band. Such an absorbance decrease is unexpected; it was not seen for the purple-to-blue transition in the R82Q mutant of bR [19]. The R94C protein was also stable over a somewhat narrower pH range than the wild type. The onset of pH-dependent protein unfolding could lead to the presence of more than two species over the titration range, which might account for the apparently shifting isosbestic point near 440–480 nm.

When 10 mM ethylguanidinium chloride was added to the R94C mutant in an attempt to chemically reconstitute the guanidino group of Arg^{94}, no changes in the spectra were observed (data not shown). This is in contrast to the result seen with the R82C mutant of bR in which the H^+-

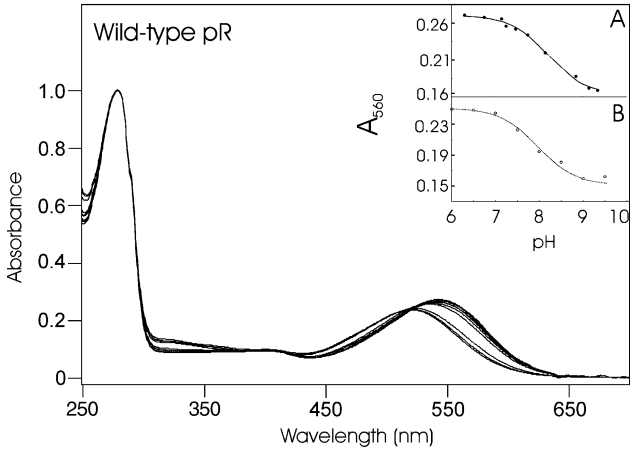

Fig. 2. Absorption spectra of wild-type pR that had been purified using the citrate precipitation method followed by a ProBond™ Ni column. All samples were in 3.0% OG, and the protein concentration was adjusted so that all had the same A_{280}=1.0. Spectra were obtained at (in order of decreasing wavelength of the visible absorption maximum) pH 6.3, 6.75, 7.15, 7.25, 7.47, 7.75, 8.15, 8.85, 9.15 and 9.35. Inset, titration curves obtained from A_{560}, shown for two different pR sample preparations. (A) Measurements (solid circles) were taken from the spectra shown in the main figure, i.e., using pR purified by citrate precipitation followed by Ni column. (B) Measurements (open circles) were averages from two different samples of pR that had been purified without citrate, i.e., using the ProBond™ Ni column alone. These samples were less pure, so the absorbances were rescaled to give A_{530}=0.24 (corresponding to the isosbestic point). For both insets, the measured absorbance values at 560 nm were fitted using a nonlinear least-squares routine to the Henderson–Hasselbalch equation, yielding a best-fit pK_a of 8.2 with n=0.50 (A, solid line) or 8.0 with n=0.25 (B, dotted line). Even when the fits were limited to single-group titration (n=1), the pK_a obtained was 8.2 for A and 7.9 for B (fits not shown). When other wavelengths (500, 575, and 580 nm) were examined, fits with pK_a values in the range 7.9–8.3 were obtained (not shown).

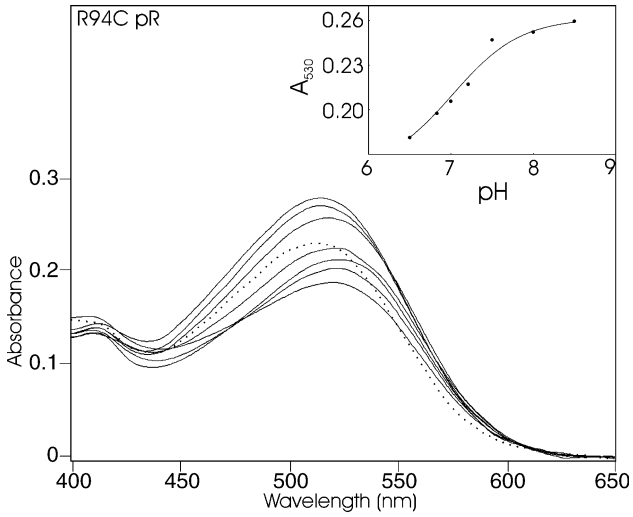

Fig. 3. Absorption spectra of R94C pR taken at (from lower to upper solid curves) pH 6.5, 6.8, 7.0, 7.2, 8.0 and 8.5. A corresponding spectrum obtained at pH 9.0 (dotted curve) represented partially denatured protein. Measurements were made on pR in 3.0% OG that had been purified using a ProBond™ Ni column. Inset, titration curves obtained from the absorbance at 530 nm (solid circles). These absorbance values were fitted using a least-squares routine to the Henderson–Hasselbalch equation, yielding a best-fit pK_a of 7.0, n=1.05 (solid line). A similar titration curve (not shown) with pK_a of 7.3 was obtained by using data at 560 nm.

pumping functionality is restored and the pK_a is dropped by several units in the presence of guanidinium salts [13].

As reported previously by others [16], the D97N mutant of pR does not show any red-to-purple transition (Fig. 4). The absorbance maxima remains around ~540–546 nm over the pH range 10.5 to 5.5. This is the basis for concluding that the red-to-purple transition in pR is analogous to the purple-to-blue transition in bR, i.e., is also caused by protonation of Asp^{97} (the homolog of Asp^{85} in bR), even though they occur at significantly different pH values. Denaturation of the D97N protein occurs below pH 5.0. To confirm that the pH-dependent spectral changes in R94C are also caused by the changes in the protonation state of Asp^{97}, a titration of the double mutant (R94C, D97N) pR (Fig. 5)

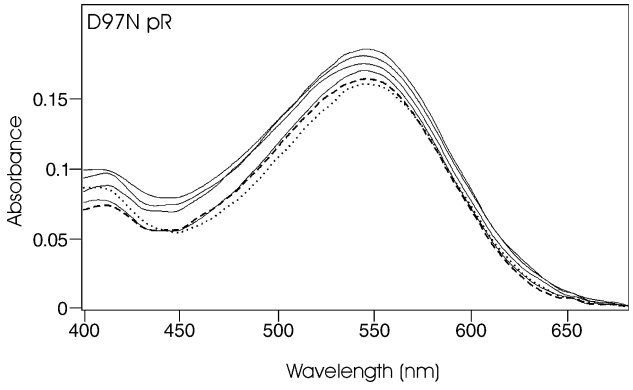

Fig. 4. Absorption spectra of D97N pR taken at (from lower to upper solid curves) pH 6.5, 7.0, 7.5, 8.0; and at pH 9.0 (dashed curve) and 9.5 (dotted curve). Measurements were made on pR in 3.0% OG that had been purified using a Phenylsepharose™ column.

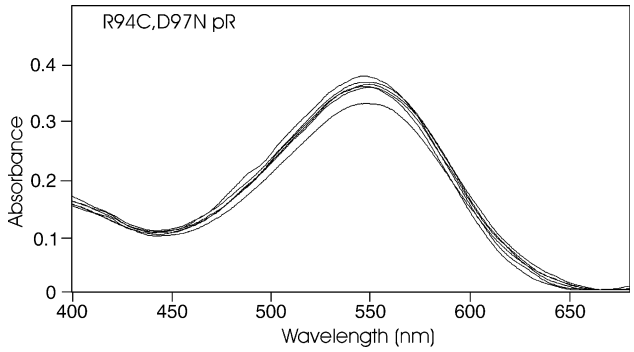

Fig. 5. Absorption spectra of R94C,D97N pR taken at (from lower to upper solid curves) pH 6.0, 6.5, 7.0, 7.5, 8.0; and at pH 8.5. Measurements were made on pR in 3.0% OG that had been purified using a using a ProBond™ Ni column.

was performed over the pH range 5 to 9.5. The absorbance maxima remained around 545 nm, i.e., the titration behavior was eliminated as in the D97N mutant.

4. Discussion

With bR, the so-called blue membrane may be prepared by acidification of [20–22] or removal of cations from [23,24] purple membrane. Subramaniam et al. [15] identified Asp^{85} as the group becoming protonated. The pK_a of Asp^{85} in bR is also considerably affected by the mutation of Arg^{82}, increasing from 2.5 in the wild type to 7.2–7.4 in R82A, R82Q, and R82C mutants [13,19]. A slightly lower pK_a (~6.9) was observed for R82A in monomeric state in lipid-detergent micelles [14,15].

In pR in OG, the corresponding pH-dependent transition is markedly different. Phenomenologically, it is rather a red-to-purple transition, with a smaller shift of 811-cm^{-1} compared to the 1015-cm^{-1} shift seen in bR. It has already been noted by others that the pK_a of this transition in wild-type pR is higher than that of the purple-to-blue transition in bR [9,16]. The size of the pK_a elevation, ~5.5 pH units, is remarkable considering the overall conservation of amino acid residues in the active sites of the two proteins, but even more so considering the apparent similarity in function: both pR, like bR, appears to function as a light-driven outward H^+ pump under physiological conditions.

The significant additional result we report here is the absence of any further upshift in the pK_a of the primary proton release group Asp^{97} when Arg^{94} is mutated to the neutral cysteine (Figs. 2 and 3). Instead, there appears to be a small but significant downshift. This is in contrast to what is seen in bR, where there is an upshift of ~5.0 units in this pK_a upon going from wild type to R82C [13]. Furthermore, addition of guanidinium compounds to the R94C mutant of pR did not result in any change to its absorption maxima, in contrast to their effect on the R82C mutant of bR. All these results suggest that arginine in pR

has less of an electrostatic interaction with Asp^{97} than that which exists between Arg^{82} and Asp^{85} in bR.

4.1. The pK_a value of Asp^{97} in wild-type pR

Other workers have reported pK_a values for the wild-type pR of 7.1 [12,16] and 7.68 [9]. There are several factors that might account for our observing a value somewhat higher than these (8.2±0.1). The protein purification procedures differed, especially in the usage of salt and detergents/lipids. However, in our preparations, the addition of salt shifts the pK_a value for wild-type pR only minimally (<0.1 pH unit; data not shown). A similar result was recently reported by others using pR samples reconstituted into lipid vesicles [9]. This is in contrast to the effects of salt seen in bR, in which the surface pH is believed to control the purple-to-blue transition [25,26]. For example, in the R82A mutant of bR, the pK_a is decreased to 6.4 from 6.9 [15] when the titration is performed in 2 M KCl versus 0.15 M KCl. This has been explained as arising from a lowering of the surface pH of the acid-rich purple membrane under low salt concentrations [25].

Acidic residues corresponding to Glu^{194} and Glu^{204} of bR, which are closest to Asp^{85} of all the surface charges, in its 3-D structure, are conspicuously absent in pR (see next subsection for details). Furthermore, OG and residual *E. coli* lipids surrounding pR are less negatively charged, when compared to the negatively charged lipids around bR in native purple membranes. For all these reasons, it is likely that the surface pH in pR is much less affected (less acidified) under low-salt conditions than in bR. This could explain why the apparent pK_a is almost unaffected by salt concentration.

One factor that might account for a different pK_a in our samples from that reported earlier is that we used 3.0% OG in our sample preparation, whereas earlier workers used 0.2% *n*-dodecyl-β-D-maltoside [16], or else thoroughly removed detergent and replaced it by polar lipids from halobacterial purple membranes [9]. To reduce light scattering, other workers also measured their pK_a values on pR encased in polyacrylamide gels [9,16]. An additional concern is the storage conditions of pR samples. Krebs et al. [17] reported that a post-translational modification in *E. coli*-expressed pR is removed (hydrolyzed) upon storage at 4 °C, pH 7.1, over an extended period of time. We observed a 0.8-unit down-shift in the pK_a value of the red-to-purple transition in pR samples stored for 6–8 weeks after initial preparation (data not shown). Interestingly, SDS-PAGE analysis shows a decrease in heterogeneity of pR samples over the same time frame [17]. Sample heterogeneity in freshly prepared pR samples, with different pK_a values for Asp-97 in the subpopulations, is the simplest explanation for values of n<1 in our Henderson–Hasselbalch fits (see Results).

It is unlikely that a change in the state of cysteine(s) could account for the differences between our results and those reported earlier. Cysteine-less (TCM) pR has an absorption maximum almost identical to that of wild-type pR in OG. This is not surprising given the cysteine–retinal distances. The three native cysteines of pR are located in helix C (Cys^{107}), helix E (Cys^{156}) and third cytoplasmic interhelical loop (Cys^{175}). In 3-D structures of bR [27], the corresponding residues are all >4 Å from the retinylidene moiety.

4.2. Electrostatic coupling of R94 to the chromophore counterion: similarities and differences with bR and other homologues

We observe a small (450–750 cm^{-1}) blue shift of the absorbance maximum, relative to the wild type, at pH 7.0 in both of the pR samples that contained the R94C mutation (the single mutant, as well as the QM that also included all three cysteine-to-serine mutations). The homologs of Arg^{94} (Arg^{82} in bR, Arg^{108} in halorhodopsin (hR), Arg^{72} in sensory rhodopsin II) have long been recognized as playing key roles in chromophore color regulation and ion transport [28–30]. However, this blue shift is in the opposite direction from the large red shift caused by the R82C mutation in bR at the same pH.

The altered effects of Arg^{94} on the pK_a of Asp^{97} in pR raise questions about how similar a role it plays to that of Arg^{82} in bR. It is thought that pR functions as a light-driven proton pump, like bR. However, the pK_a of the primary aspartate counterion (pK_a=8.2, shown in Fig. 2) is more similar to that of the primary counterion in sensory rhodopsin II, a halobacterial phototaxis receptor. The pK_a of Asp^{75} in reconstituted *Natronobacterium pharaonis* sensory rhodopsin II (NpSRII) is 5.6 and its absorption maximum is near 500 nm [31].

Crystal structures of NpSRII [30,32] have revealed that guanidinium group of Arg^{72} is oriented towards the extracellular surface of the protein. Furthermore, there is only a single nearby negative charge arising from Asp^{193} (the homolog of Glu^{194} in bR); that is, no charged homolog of Glu^{204} is present. As a result, the closest approach of the Arg^{72} guanidino nitrogens to the Asp^{75} carboxylate oxygens is 2 Å further away in NpSRII than for the corresponding residues in bR. The shifted position of Arg^{72} was concluded to be responsible for most of the spectral blue shift in NpSRII [33]. This shifted position is likely the result of replacement of Phe^{208} in bR by Ile^{197} in NpSRII, which opens up space to allow the repositioning of Arg^{72} [30]. Substitution of amino acids Pro^{183} and Asp^{192} in NpSRII for Glu^{194} and Glu^{204} in bR could also partly account for the repositioning of Arg^{72} in NpSRII.

These explanations suggest the following possibilities, which are not mutually exclusive:

(1) The direct electrostatic coupling between Asp^{97} and Arg^{94} is substantially reduced in pR relative to bR, similar to what is thought to occur in NpSRII. Arg^{94} in pR may be located close to the position of the corresponding residue in NpSRII. However, Phe^{208} in bR is replaced in pR by Tyr^{223}, which unlike Ile^{197}

of NpSRII does not allow as much space to be opened up for Arg^{94}.

(2) There is altered electrostatic coupling of Arg^{94} to acidic residues at the external surface of the protein. In bR, the extracellular H^+ release group has been proposed to be a complex involving Arg^{82} [34,35], Glu^{194} [36], Glu^{204} [37–41] and water molecules [35,42,43]. As mentioned in the preceding subsection, a significant primary sequence difference between pR and bR is the absence of amino acids corresponding to Glu^{194} and Glu^{204} in bR (see [8] for a sequence alignment). Both these residues are replaced by leucines in pR. This could be directly related to the different pK_a of Asp^{97} in pR, either through loss of direct electrostatic interactions with the other carboxylates (no homologs in pR of bR residues Glu^{194} or Glu^{204}), or indirectly by helping to cause the repositioning of pR residue Arg^{94} (relative to its bR homolog Arg^{82}). Previous studies on the E194Q and E204Q mutants in bR have indicated that an acid-induced purple-to-blue transition is still observed [44], casting some doubt on the importance of electrostatic interactions of these residues in explaining the observed differences between pR and bR. It is still possible that the differences in the acid titration behavior of bR and pR are closely related to hydrogen bonding by glutamic acid residues 194 and 204 in bR, which might be involved in maintaining the position of water molecules in the extracellular channel, and (less directly) the position of Arg^{82}. In the bR mutants E194Q and E204Q, this H-bonding might be maintained by the glutamines, whereas the corresponding leucines in pR would not be expected to do so.

A high-resolution crystal structure is the best way to distinguish if either or both of the two preceding explanations is correct. Regardless of the outcome, we predict based on our data that pR has a substantially different initial configuration of the conserved residues that take part in the fast H^+ release in bR. Nevertheless, Krebs et al. [17] detected fast (~10 μs) light-activated H^+ release and formation of a M-like intermediate in pR samples reconstituted into mixed micelles containing short-chain phospholipid. It was also previously observed that in the R82Q mutant of bR, guanidinium can restore the wild-type chromophore absorption maximum without restoring fast H^+ release [45]. All the preceding observations indicate that the process of fast H^+ release is not directly dependent on the initial configuration of the highly conserved arginine residue and its surrounding environment.

Therefore, we conclude that the mechanism of fast H^+ release is unlikely to be deduced from the crystal structure of the unphotolyzed state of bR and/or pR. Instead, we speculate that there is a conserved mechanism of fast H^+ release in pR and bR that will be deduced only from commonalities in the structures around $Arg^{82/94}$ in the M (and perhaps L) photoproducts of these two proteins.

Acknowledgements

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