BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 4457–4465 4457
DOI: 10.1021/bi901746b

Binding of Anions to Proteorhodopsin Affects the Asp97 pK_a[†]

Yamit Sharaabi,[‡] Vlad Brumfeld,[§] and Mordechai Sheves*,[‡]

[‡]*Department of Organic Chemistry and* [§]*Department of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel*

Received October 10, 2009; Revised Manuscript Received April 18, 2010

ABSTRACT: Proteorhodopsin (PR), a retinal protein of marine proteobacteria, is a light-driven proton pump. Light excitation of PR initiates a photocycle that triggers the translocation of a proton from the cytoplasmic to the extracellular side. Asp97 is located near the retinal-protonated Schiff base and serves as the proton acceptor during the photocycle. The pK_a of Asp97 is unusually high (~7.0), especially in comparison with that of its bR equivalent residue Asp85 (~2.6). We have studied possible anions binding to PR (produced from gene vector eBAC31A08 and expressed in *Escherichia coli*) and their effect on its absorption maxima, Asp97 pK_a, and the photocycle. We found that chloride, sulfate, trifluoroacetate, trichloroacetate, and tribromoacetate anions bind to PR and regulate Asp97 pK_a. Asp97 has a pK_a of ~7.8 in water, but the value decreases to ~7.0 in the presence of sulfate and chloride anions. Halogeno-acetate anions elevated Asp97 pK_a and compete with chloride anions. The most significant effect was detected with tribromoacetate anions that increase the Asp97 pK_a to a value of >9.5. The possibility that PR has at least two binding sites for these anions is discussed. In addition, we have demonstrated that these anions bind to PR also at high pH (above Asp97 pK_a) because they affect the rate of growth and thermal decay of the M intermediate in the photocycle of PR.

Retinal proteins are widely distributed in nature, and one can find them in higher organisms (visual pigments) as well as in lower organisms (light-activated ion pumps, phototaxis sensors, etc.). In a manner independent of their specific function and despite a marked difference in the amino acid sequences of these two groups, all of these proteins share common structural motifs. For example, they are membrane proteins composed of seven transmembrane α-helices that enclose a binding pocket for a retinal chromophore covalently bound to an ε-amino group of a conserved lysine in the seventh helix via a protonated Schiff base (PSB)[1] linkage (for recent reviews, see refs (*1*−*4*)). Light-induced transfer of the protonated Schiff base proton within the binding pocket is crucial for the function of both transport and sensory rhodopsins (*5*). In visual pigments, the retinal chromophore adopts an 11-*cis* configuration, and photoisomerization converts it to all-*trans* (*1*). In the pigments of lower organisms, it is in the form of the all-*trans* isomer that photoisomerizes to 13-*cis* (*2*). Retinal proteins provide environmental perturbation that tunes the absorption maximum of the retinal chromophore over a very wide spectral range from 360 to 635 nm. The term opsin shift (OS) (*6*) was introduced to refer to the energy difference (in cm^{-1}) between the absorption maximum of retinal PSB in methanol and that in the protein environment. All of these proteins exhibit a light-induced cascade of spectroscopically identifiable intermediates that reflect changes in the structure of both polyene and opsin. These spectroscopic changes are coupled to the physiological response such as visual transduction [visual rhodopsin (Rh)], proton and chloride pumping [bacteriorhodopsin (bR) and halorhodopsin, respectively], and phototaxis-signal transduction [sensory rhodopsin (SR)] (*5*).

In the past few years, a new type of retinal protein named proteorhodopsin derived from the bacterial domain has been discovered through genomic analyses of naturally occurring marine bacterioplankton (*7*). Proteorhodopsin (PR) was encoded in the genome of an uncultivated marine bacterium of the SAR86 group of γ-proteobacteria and was expressed in *Escherichia coli*. Following retinal binding, the protein forms an active light-driven proton pump. The γ-proteobacteria harboring the proteorhodopsins are widely distributed in the Pacific Ocean, the Red Sea, and other similar environments and are known to use CO_2 as their sole carbon source. This suggests the possibility of a previously unrecognized phototrophic pathway that may influence the flux of carbon and energy in the ocean's photic zone worldwide. Following its initial discovery in Monterey Bay (California) (*7*), several variants of proteorhodopsin have been identified throughout the world (*7*, *8*). The amino acid sequence of PR shows a high degree of similarity to the sequences of the archaeal rhodopsins (*7*, *9*). Proteorhodopsin-expressing variants can be classified into two major groups depending on their absorption maxima as the green-absorbing (GPR; $\lambda_{max} = 525$ nm) and blue-absorbing (BPR; $\lambda_{max} = 490$ nm) forms. The absorption maxima of the pigments are tuned to the depth at which the specific bacteria live. Those living in the upper layers of the ocean absorb at longer wavelengths, whereas those that exist in deeper layers absorb at shorter wavelengths, in keeping with the available light able to penetrate the water (*9*−*11*). Light absorption by proteorhodopsin triggers a series of conformational changes associated with the chromophore and the protein environment

[†]This work was supported by grants from the Kimmelman Center for Biomolecular Structure and Assembly and the United States-Israel Binational Science Foundation (BSF). M.S. holds the Katzir-Makineni Professorial Chair in Chemistry.

*To whom correspondence should be addressed: Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel. Phone: +972 8 9344320. Fax: +972 8 9343026. E-mail: mudi.sheves@weizmann.ac.il.

[1]Abbreviations: bR, bacteriorhodopsin; DM, dodecyl maltoside; PR, proteorhodopsin; PSB, protonated Schiff base; TBA^-, tribromoacetate; TCA^-, trichloroacetate; TFA^-, trifluoroacetate.

Published on Web 04/20/2010

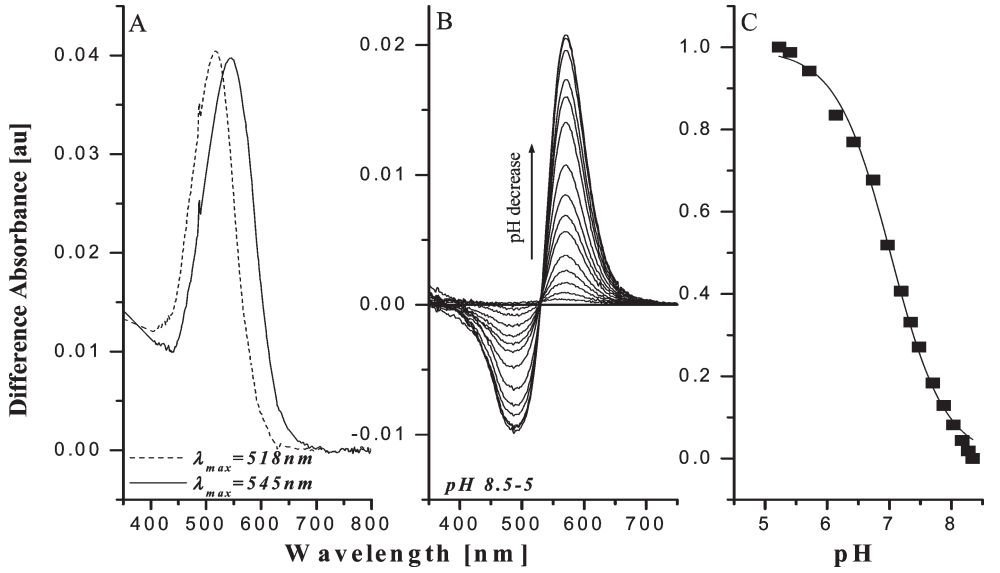

FIGURE 1: pH dependence of the PR sample incubated with 300 mM NaCl. (A) Basic (---) and acidic (—) forms. (B) Difference spectra in the pH range of 8.5−5.0. All spectra were subtracted from pH 8.5. (C) Titration curve at 570 nm, where the solid line represents the best fit (pK_a = 7.0).

and results in the translocation of a proton from the cytoplasmic side to the extracellular surface (*7*, *9*, *12*). The electrochemical gradient generated by this process is used to drive ATP synthesis for fueling metabolic processes within the cell (*13*). The photocycle of GPR is similar to that of the bR photocycle (*10*, *12*, *14*−*19*) with a turnover time that is slightly slower than that of bR but considerably faster than those of sensory photoreceptors such as sensory rhodopsins I and II (SR I and SR II, respectively). This property is characteristic of transport proteins (*7*, *10*, *20*). PR shares great similarity with bR with regard to its amino acid sequence and most of the key residues in the binding pocket. In particular, those involved in proton transport are conserved. The most studied proteorhodopsin is that expressed from the genetic material ebac31A08 that was also used in this study. The absorption maximum of the chromophore in PR is reversibly pH-dependent and shifts from 518 nm at pH 10 (basic form) to 546 nm at pH 4 (acidic form). Such an absorption shift has also been observed in bR and has been attributed to protonation of Asp85 which has a pK_a of ~2.6. In PR, the residue that corresponds to Asp85 (bR) is Asp97, which is known to have a uniquely higher pK_a ranging from 7.1 to 7.68 (depending on conditions) (*10*, *12*, *21*). The binding of anions to retinal proteins has been the subject of many studies. Anions affect the photocycle of halorhodopsin (*22*) and have high binding affinity for bacteriorhodopsin (bR) (*23*). In addition, it is well-known that cations bind to bR and significantly affect its function mainly by affecting the pK_a of the major accepting proton residue (Asp85) following light absorption. The question of whether anions and cations bind similarly to PR and affect its Asp97 residue pK_a arises.

In this study, we have examined possible binding of anions to PR and their effect on its properties. In particular, we studied the effect of anions (organic and inorganic) on the protein residue Asp97 pK_a and the PR photocycle. Our results revealed that anions bind to pR. Specifically, chloride, sulfate, trifluoroacetate, trichloroacetate, and tribromoacetate anions significantly affect Asp97 pK_a. Tribromoacetate anions dramatically elevate Asp97 pK_a (> 9.5), thereby affecting the PSB pK_a as well. Studies of the PR photocycle indicated that anions affect the kinetics of photocycle intermediates.

MATERIALS AND METHODS

Sample Preparation. PR was expressed in *E. coli* and isolated using a previously published method (*9*). Anion exchange of the DM-solubilized protein was achieved with Amicon Ultra centrifugal filter devices; more specifically, the sample was treated against the appropriate salt. The final sample was incubated with the appropriate concentration of the desired salt (NaX), 50 mM Tris buffer, and 0.06% DM. Buffer was not added in samples lacking ions or characterized by low ion concentrations.

Absorption Measurements. Absorption spectra were recorded with an Agilent 4583 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA).

Titration Experiments. Titrations were conducted in the dark using base or acid to achieve the desired pH (typically ~2 μL of 0.0001−1 M NaOH/HX, as appropriate), and then the absorption maxima were monitored. The differences in absorbance at a λ_{max} of 570 nm were plotted versus the pH, and the pK_a values were determined using the following equation:

$$F(x) = 1/[1 + 10^{n(pK_a - x)}]$$

where n is the number of protons participating in the transition, x is the measured pH, and pK_a is the midpoint of the observed transition.

Pulsed Laser Photolysis. Pulsed laser photolysis was conducted using laser pulses from a Nd:YAG laser (532 nm, 9 ns). Light-induced absorbance changes were recorded using a continuous 100 W xenon lamp, a photomultiplier, and a LeCroy oscilloscope. Raw data were fed into a computer through a LabView program. Unless otherwise mentioned, 20 pulses were averaged and analyzed. We minimized photolytic effects due to the monitoring beam by placing an interference filter centered at the exciting wavelength (532 nm, 20 nm bandwidth) between the lamp and the sample and by using a mechanical shutter synchronized with the laser pulse. The concentration of the protein ranged from 1.32×10^{-5} to 2.2×10^{-5} M (or 0.6−1 OD).

RESULTS AND DISCUSSION

Anions Binding to PR and Their Effect on Asp97 pK_a. We studied the effect of several anions on the pK_a values of Asp97. It

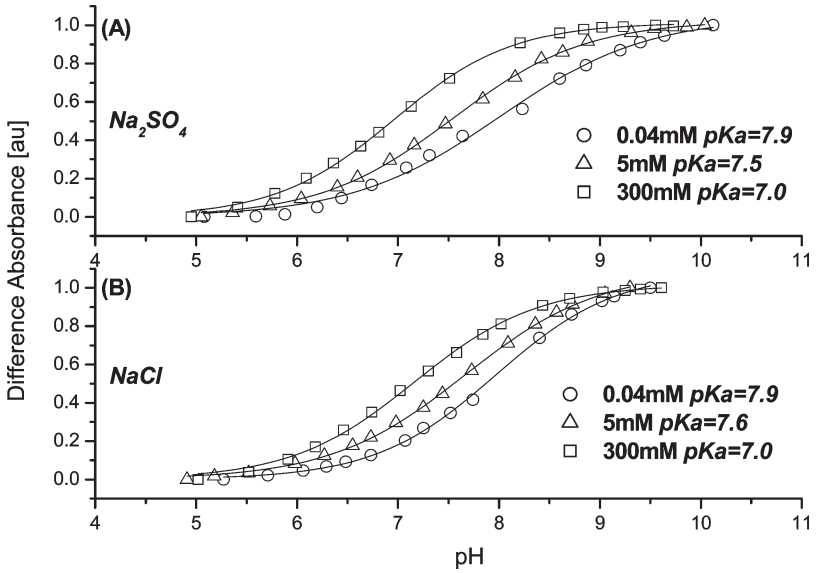

FIGURE 2: pH dependence of PR samples incubated with variable concentrations of Na_2SO_4 (A) and NaCl (B). Each symbol represents a different concentration of the respective anion (0.04−300 mM). The solid lines represent the best fits.

Table 1: pK_a Values of PR Samples Incubated with Variable NaCl and Na_2SO_4 Concentrations (0.04−300 mM)[a]

[Na_2SO_4]/[NaCl] (mM)	pK_a(Na_2SO_4)	pK_a(NaCl)	[Na_2SO_4]/[NaCl] (mM)	pK_a(Na_2SO_4)	pK_a(NaCl)
0	7.9	7.9	30	7.3	7.4
0.04	7.9	7.9	50	7.1	7.3
0.5	7.8	7.9	80−90	7.0	7.2
5	7.5	7.6	150	7.0	7.0
10	7.5	7.6	200−300	7.0	7.0

[a]The pK_a values were extracted from titration curves monitored at 570 nm of difference spectra.

is clear that the absorption maximum of PR is pH-dependent shifting from a λ_{max} of 519 nm (basic form) to a λ_{max} of 546 nm (acidic form) in the presence of chloride anions (> 150 mM) (Figure 1). The transition has a pK_a of 7.0 and is attributed to the titration of Asp97 (the prime PSB counterion), which was suggested previously (*12*). The pK_a is salt concentration-dependent at NaCl concentrations ranging from 0.04 to 300 mM (Figure 2 and Table 1), and its value ranges from 7.9 to 7.0, decreasing as the NaCl concentration increases. Saturation has been achieved at ~150 mM NaCl with a binding constant of ~30 mM. A similar phenomenon has been observed with Na_2SO_4, and as the salt concentration increases, the Asp97 pK_a decreases (at Na_2SO_4 concentrations ranging from 0.04 to 300 mM) (Table 1). It is evident that titration with Na_2SO_4 reached saturation at a lower salt concentration. It may be due to the higher affinity of sulfate relative to chloride anions. However, this result can also be explained by higher sodium cation concentrations in the Na_2SO_4 reducing the pK_a.

Other salts that were studied consisted of monovalent and trivalent anions. These salts, which include bromide, formate, acetate, chloroacetate, and phosphate at high concentrations (300 mM), exhibited pK_a values similar to those detected in PR samples incubated with 150−300 mM NaCl or Na_2SO_4. The absorption maxima and Asp97 pK_a were similar for all those salts over a pH range of 5−10 (Table 2). We noted that all salts, including NaCl and Na_2SO_4, exhibited a similar pK_a value (7.9) at a low salt concentration (0.04 mM). This value is identical to the value detected in water. It was established that cations affect the pK_a of the protein residues of bR by affecting the surface

Table 2: pK_a Values of PR Samples Incubated with Variable Anions (300 mM)[a]

salt (300 mM anion)	pK_a
chloride	7.0
bromide	6.8
sulfate	7.0
phosphate	6.8
$NaOOCCH_2Cl$ monochloroacetate	7.0
$NaOOCCH_3$ acetate	6.9
NaOOCH formate	6.9

[a]The pK_a values were extracted from titration curves monitored at 570 nm of difference spectra.

potential of the protein and/or its membrane (*24*−*26*). Free (Guy−Chapman) or bound metal cations on the protein−membrane surface compete with protons and thus determine the local proton concentration around the protein. Increasing the cation concentration increases the pH at the protein surface, thereby affecting the pK_a values of protein residues. Several studies attributed the effect of the cations to specific binding sites (inside the protein) or close to the surface (*27*−*31*). The question of whether the effect we have observed in PR is solely attributed to the effect of the cations or can be attributed to the binding of the anions arises.

To shed further light on the possibility of anions binding, we studied the effect of halogeno-acetate salts on Asp97 pK_a. Interestingly, we found that a unique effect was observed on Asp97 pK_a when acetate anions were substituted for halogens such as trichloroacetate (TCA^-), trifluoroacetate (TFA^-), and

Table 3: pK_a Values of PR Samples Incubated with Variable Concentrations of (A) TFA^-, (B) TCA^-, and (C) TBA^- with and without 300 mM NaCl[a]

(A) [TFA^-] (mM)	pK_a (without NaCl)	pK_a (300 mM NaCl)
0.07	7.9	7.0
1	7.9	7.0
10	7.6	7.2
100	7.3	7.15
300	7.8	7.4
	pK_a (without Na_2SO_4)	pK_a (300 mM Na_2SO_4)
100	7.3	7.0
300	7.8	7.2
(B) [TCA^-] (mM)	pK_a (without NaCl)	pK_a (300 mM NaCl)
0.15	7.9	7.0
10	7.8	7.3
30	7.8	7.5
70	7.9	7.8
100	8.0	7.8
300	8.3	8.0
500	8.4	8.2
(C) [TBA^-] (mM)	pK_a (without NaCl)	pK_a (300 mM NaCl)
0.5	7.9	7.1
1	7.8	7.1
5	7.8	7.3
7	7.9	7.9
10[b]	~9.3	7.9
100	~10.3	~9.5

[a]The pK_a values were extracted from titration curves monitored at 570 nm of difference spectra. [b]For >10 mM TBA^-, the pK_a values are estimated and cannot be exactly calculated because of an additional process taking place simultaneously with Asp97 titration.

tribromoacetate (TBA^-) over a pH range of 4.5−10.0. Although a significant effect on the pK_a was detected, the absorption maxima of the acidic and basic forms were not affected by these anions, and the absorption maxima were similar to those monitored in the presence of chloride, sulfate, and the other anions previously studied.

Sodium trifluoroacetate (NaTFA) affects Asp97 pK_a differently at high and low concentrations. At relatively low concentrations (≤100 mM), the pK_a value gradually decreases to a value of 7.3. At >100 mM NaTFA, the pK_a value gradually increases, reaching a value of 7.8 at 300 mM NaTFA (Table 3A). This result clearly indicates that TFA^- anions affect Asp97 pK_a. To elucidate possible Cl^- binding effects and its possible effect on Asp97 pK_a, we conducted titration experiments with PR samples incubated with different concentrations of NaTFA at a constant NaCl concentration of 300 mM. The results reveal competition between these two anions (Table 3A). Comparison of Asp 97 pK_a in the presence of TFA^- with and without Cl^- indicated different values for the pK_a. For example, 300 mM NaTFA yields a pK_a of 7.8 which is 0.8 pK_a unit higher than that observed for 300 mM NaCl [without NaTFA (Table 1)]. However, in the presence of 300 mM NaCl, 300 mM NaTFA exhibited an Asp97 pK_a value of 7.4. Therefore, the TFA^- anions elevated the pK_a by only 0.4 pK_a unit [relative to 300 mM NaCl (7.0)]. Since the NaCl effect reached full saturation at a concentration of 300 mM, we cannot attribute the weaker effect of the TFA^- anions to screening of sodium cations, and we can conclude that Cl^- anions compete with TFA^- and reduce its effect on the pK_a. Therefore, Cl^- anions bind to PR as well and thereby reduce Asp97 pK_a. To determine whether sulfate anions bind to PR as well, we investigated the effect of TFA^- on the pK_a of Asp97 in the presence of 300 mM Na_2SO_4 instead of 300 mM NaCl. The pK_a was 7.0 in the presence of 100 mM TFA^- and 300 mM Na_2SO_4 and 7.2 in 300 mM TFA^- and 300 mM Na_2SO_4 (Table 3A). Therefore, the presence of 300 mM Na_2SO_4 decreases the pK_a from 7.8 (Table 3) to 7.2 in the case of 300 mM TFA^-. This effect cannot be attributed to the screening of sodium cations, since saturation of the salt effect (if it exists) is reached below 300 mM salt (Table 1). These results led us to conclude that sulfate anions also bind to PR and reduce Asp97 pK_a. The effect of the sulfate anions seems stronger than that of chloride anions, and sulfate anions may compete more efficiently with the TFA^- anions. The different effect of NaTFA at high and low concentrations may point to the existence of two binding sites in which binding to the first one somewhat decreases the pK_a while occupying the second one increases it. The possibility that the pK_a decrease at a low concentration of NaTFA is caused by sodium cation binding cannot be completely excluded. However, the pK_a value of Asp97 in the presence 10 mM NaTFA and 300 mM NaCl [7.2 (Table 3A)] is higher compared with that detected for samples containing only 300 mM NaCl [7.0 (Table 1)]. Therefore, it is likely that TFA^- anions already bind at a concentration of 10 mM and affect the pK_a. Moreover, the latter result supports the possibility that at 10 mM TFA^- the anion occupies a different binding site versus that occupied at high concentrations.

Trichloroacetate (TCA^-) binds to PR as well, affecting Asp97 pK_a, and it exhibited an interesting behavior (Table 3B). With up to 70 mM NaTCA, Asp97 pK_a reached a value of ~7.9, but above this concentration, the pK_a value increased and reached a value of 8.4 at 300−500 mM NaTCA. To elucidate whether chloride anions compete with TCA^- anions and affect Asp97 pK_a, we have measured the Asp97 pK_a of PR samples incubated with different concentrations of TCA^- in the presence of 300 mM NaCl (Table 3B). The results indicate competition between these two anions, which affected Asp97 pK_a. For example, the pK_a is 8.3 in the presence of 300 mM NaTCA (without NaCl) but is 8.0 in a sample incubated with 300 mM NaTCA and 300 mM NaCl together. As in the case of TFA^-, the difference cannot be attributed to the screening effect of sodium cations since the effect of NaCl reached saturation at 300 mM NaCl (Table 1). Therefore, the reducing effect on the pK_a value should be attributed to the chloride anions that bind to the protein and reduce the pK_a. The competition with Cl^- strongly implies that TCA^- and Cl^- occupy the same binding site. Similarly, it is clear that at 500 mM TCA^- and together with the presence of 300 mM Cl^-, Asp97 pK_a increases and reaches its highest value [8.2 (Table 3)]. This value is lower than that detected for 500 mM TCA^- in the absence of Cl^- which further supports the binding of chloride anions. The results presented in Table 3 indicate that TCA^- binds to PR at a low concentration [10 mM (Table 3B)]. Even at this concentration, the TCA^- anions increase the pK_a in the presence of 300 mM NaCl from 7.0 [without NaTCA (Table 1)] to 7.3. This result strongly supports the suggestion that that TCA^- anions bind to the protein at a low concentration, inducing a pK_a value of 7.8 [without chloride anions (Table 3)]. We cannot exclude the possibility that sodium cations have some effect on the pK_a as well. This possibility should be the subject of future studies.

Tribromoacetate (TBA^-) exhibits a trend similar to that with TCA^- anions, but the effect on the Asp97 pK_a was much stronger. With up to 7 mM NaTBA, the Asp97 pK_a value is not affected (Table 3C and Figure 3A). However, above 7 mM

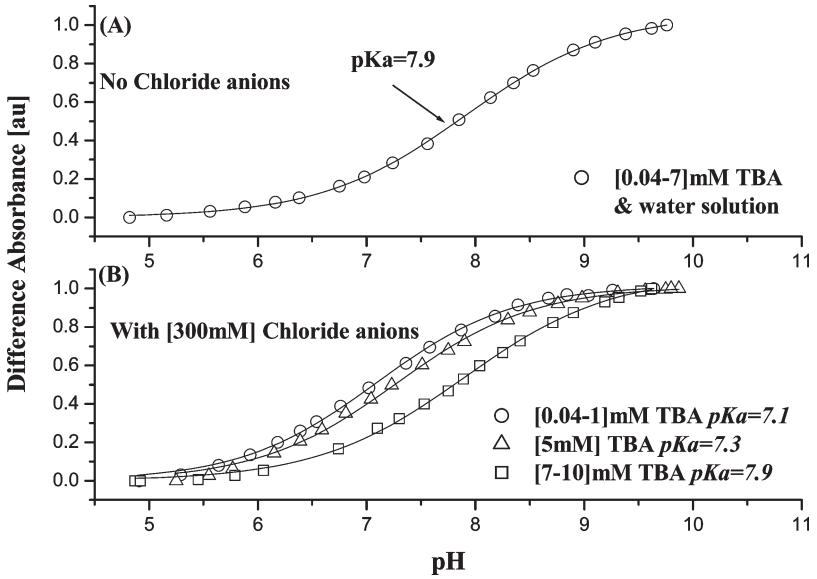

FIGURE 3: pH dependence of PR samples incubated with variable TBA^- concentrations in the absence of Cl^- (A) and in the presence of 300 mM Cl^- anions (B): $pK_a = 7.1$ (○), $pK_a = 7.3$ (△), and $pK_a = 7.9$ (□). Each symbol represents a different TBA^- concentration from 0.04 to 10 mM, and the solid lines represent best fits.

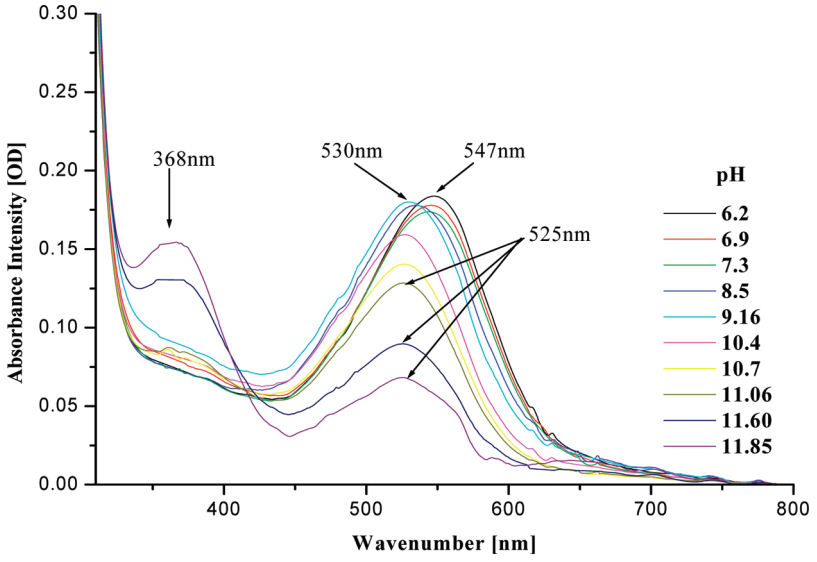

FIGURE 4: Absorption spectra of a PR sample incubated with 70 mM TBA^- at different pH values.

NaTBA, the Asp97 pK_a value increases dramatically, exceeding a value of > 10. We noted that above 10 mM NaTBA, the titration of Asp97 is not fully achieved. The blue-shifted transition reaches a λ_{max} value of 525−527 nm instead of a λ_{max} of 518−520 nm, which was observed in the titrations with other anions as well as with TBA^- anions up to 7 mM described above. Possibly, Asp97 titration stops at a λ_{max} of ~525 nm because of the titration of another protein residue that affects and increases Asp97 pK_a following its deprotonation. We noted that a similar phenomenon was observed during the titration of the protonated Schiff base in bR artificial pigments derived from fluorinated retinals that have a pK_a value of ~9 (*32*).

Moreover, we have observed a continuous decrease in the intensity of the basic form (λ_{max} = 525−527 nm), along with a simultaneously growing band at a λ_{max} of 368 nm. This band's appearance is probably due to a titration of the protonated Schiff base (PSB) (Figure 4 shows a titration example of PR incubated with 70 mM NaTBA). Asp97 serves as a counterion to the protonated Schiff base, and therefore, its protonation decreases the pK_a value of the PSB. Neutralization of PSB counterion induces a PSB pK_a decrease which was observed by us in the D97N PR mutant (data not published), and in the bacteriorhodopsin D85N mutant (*33*). Since in the presence of TBA^- anions the Asp97 pK_a is significantly increased, the titration of the PSB takes place before full deprotonation of Asp97 is achieved. Therefore, it is difficult to extract an exact value for Asp97 pK_a, and hence, a λ_{max} of 530 nm was chosen as the median of Asp97 titration. This assumption is also based on the fact that the basic form of PR has a similar absorption maximum in the presence of TBA^- and all other studied anions. Consequently, we have derived an estimation for the Asp97 pK_a value for a TBA^- concentration of > 7 mM (Table 3C). In addition, we have conducted titration experiments of the PR pigment incubated with different NaTBA concentrations in the presence of 300 mM NaCl (Table 3C). Importantly, we found that addition of NaCl to PR samples incubated with different TBA^- concentrations

decreases the Asp97 pK_a value relative to the values detected without NaCl. With up to 7 mM NaTBA, the pK_a is not appreciably affected but TBA^- anions bind to the protein, as manifested by the increased pK_a in the presence of 300 mM NaCl [7.9 (Table 3C, right column)] relative to 300 mM NaCl alone [pK_a = 7.0 (Table 1)] because of competition between TBA^- and chloride anions. Therefore, we can conclude that TBA^- anions bind to the protein at a concentration of 7 mM and induces a pK_a value for Asp97 of 7.9. As indicated above, it is possible that sodium cations affect the pK_a as well by affecting the surface potential. A significant increase in the pK_a is detected above 10 mM NaTBA, which indicates a difference in behavior. Therefore, we suggest that TBA^- anions occupy two binding sites in which, upon binding to the first one, the effect on the pK_a is modest relative to the effect of water (pK_a $\sim$ 7.8), whereas occupying the second one significantly increases the pK_a. Further support for competition between TBA^- and chloride is obtained from the experiment at 7 mM NaTBA with and without 300 mM NaCl. The pK_a in both cases is 7.9 (Table 3), in spite of the fact that the concentration of sodium cations and chloride anions significantly increased. Since the chloride anions decreased the pK_a below 7 mM NaTBA, we propose that chloride anions do compete with TBA^- anions on the first binding site but at 7 mM the TBA^- anions prevail and chloride anions do not decrease the pK_a. Chloride anions (300 mM) do compete on the second binding site (for example, at 100 mM NaTBA) and decrease the pK_a (Table 3).

The pK_a of Asp97 can be regulated by several mechanisms. During the bR photocycle, the pK_a of Asp85 (the equivalent of Asp97 in PR) is considerably increased. This alteration can be attributed to the Asp85 hydrophobic environment prevailing following light absorption and retinal chromophore isomerization. It has been proposed that the low pK_a of Asp85 and high pK_a of the PSB prior to light absorption are due to a very effective hydrogen bonding of these ions with bridging bound water (*34*, *35*) . Following light absorption and retinal isomerization, the relative geometry of these residues is altered, and the hydrogen bonding network is disturbed, inducing a considerable elevation of the Asp85 pK_a, as well as proton transfer from the PSB to Asp85. The pK_a of Asp85 is considerably affected as well by electrostatic interactions with positive charges such as the PSB and Arg82 (*36*), but the hydrogen bonding network may also play a significant role. A remote effect on protein residues' pK_a values in bR is demonstrated in its photocycle. Protonation of Asp85 induces the pK_a alteration of the proton release complex located in the extracellular part of the protein close to the protein surface. Asp85 protonation induces the movement of Arg82 closer to the protein's surface and hydrogen bonding rearrangement and thereby alters the pK_a of the proton release complex which releases a proton.

Our studies demonstrate that anions such as chloride, sulfate, and halogeno-acetate bind to the protein and affect the pK_a of Asp97. Since the absorption maxima of both the acidic and basic forms of the protein are not affected by the anions binding, it is reasonable to assume that the anions do not bind near Asp97. In this regard, it was recently demonstrated (*37*) that mutation of Ala178 to Arg in the E-F cytoplasmic loop of PR induced a pK_a increase of Asp97 of 1 pK_a unit. This point mutation is more than 20 Å from Asp97, and therefore, the effect must be via alteration of the protein conformation and/or modification of the hydrogen bonding network. Further recent studies revealed that the change in the pK_a is specific to PR and to its Ala178 residue, and that it

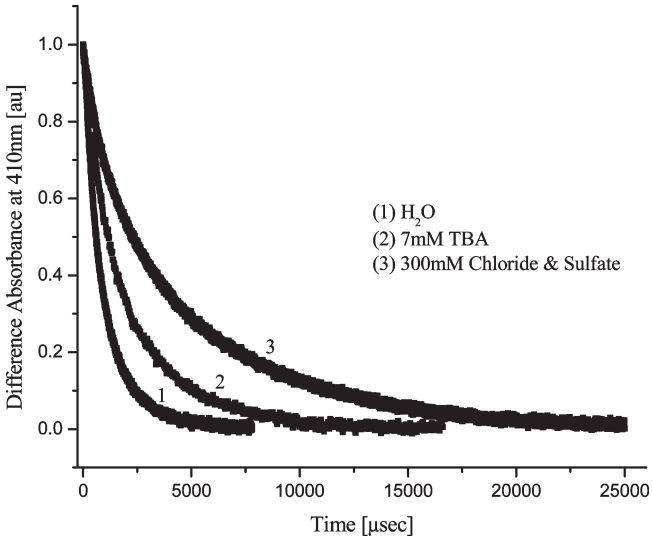

FIGURE 5: Laser pulses induced absorbance changes in the PR sample at 410 nm following the thermal decay of the M-like intermediate (410 nm) in the presence of water (1), 7 mM TBA^- (2), and 300 mM Cl^- and SO_4^{2-} (3). Measurements were taken at pH ~9.4.

Table 4: Lifetimes Detected for the Induction Phase and Thermal Decay of the M-like Intermediate Observed at 410 nm[a]

	formation of M (410 nm)		decay of M (410 nm)	
anion	fraction 1 (μs)	fraction 2 (μs)	fraction 1 (ms)	fraction 2 (ms)
chloride	10	53	0.77	4.1
sulfate	9.6	55	0.77	3.9
7 mM TBA^-	–	42	0.69	2.2
H_2O	–	30	0.5	1.4

[a]This was detected for pR in the presence of 300 mM $Cl^-$and SO_4^{2-}, 7 mM TBA^- anions, and a water solution contains no anions. Measurements were taken at pH ~9.4.

does not occur in bR (*38*). The structure of PR has not yet been determined, and it is impossible at this stage to propose a defined location for anion binding. The cytoplasmic side is highly hydrophobic in microbial rhodopsins, and therefore, one would expect anions to bind at the more hydrophilic extracellular side. However, possible binding at the cytoplasmic side cannot be excluded especially since mutation of Ala178 in the cytoplasmic site affects the Asp97 pK_a.

Halogeno-acetic acids interact with ion pairs and form strong hydrogen bonds with negative charges and weak hydrogen bonds with positive charges because of the electron withdrawal capability of the halogen atoms. Model studies in solutions of retinal protonated Schiff base demonstrated that halogeno-acetic acids can induce a red shift in the absorption maximum of the retinal-protonated Schiff base (*39*). It is conceivable that the halogeno-acetate anions will bind in PR to ion pair and induce protein alteration, which consequently will increase the Asp97 pK_a. The anion will bind to a positively charged residue (lysine or arginine) but, in addition, can bind to and stabilize a carboxyl moiety that interacts as a carboxylate with the positively charged residue prior to the halogeno-acetate binding. The strongest effect was detected for TBA^- which might be attributed to its largest size inducing the most significant protein alteration. The chloride or sulfate anions will bind only to positive charges and therefore may induce

different protein alterations relative to halogeno-acetate anions in spite of the fact that the anions bind to the same binding sites as they compete with each other. In addition, because of the binding to ion pairs and possibly also since they can form additional hydrogen bonding with protein residues, the affinity of acetate anions substituted for halogens is stronger than that of inorganic anions.

Pulsed Laser Photolysis at High pH Values (*~9.4*). At high pH values, the PR protein is known to function as a proton pump and transfers a proton from the cytoplasmic to the extracellular side of the membrane (*12*, *40*). This proton translocation process is associated with a light-induced photocycle.

Table 5: Intensity Fractions Detected for the Induction Phase and Thermal Decay of the M-like Intermediate Observed at 410 nm[a]

	formation of M (410 nm)		decay of M (410 nm)	
anion	fraction 1	fraction 2	fraction 1	fraction 2
chloride	0.1	0.9	0.3	0.7
sulfate	0.08	0.92	0.2	0.8
7 mM TBA^-	–	1	0.51	0.49
H_2O	–	1	0.77	0.23

[a]This was detected for pR in the presence of 300 mM Cl^- and SO_4^{2-}, 7 mM TBA^-, and a water solution contains no anions. Measurements were taken at pH ~9.4.

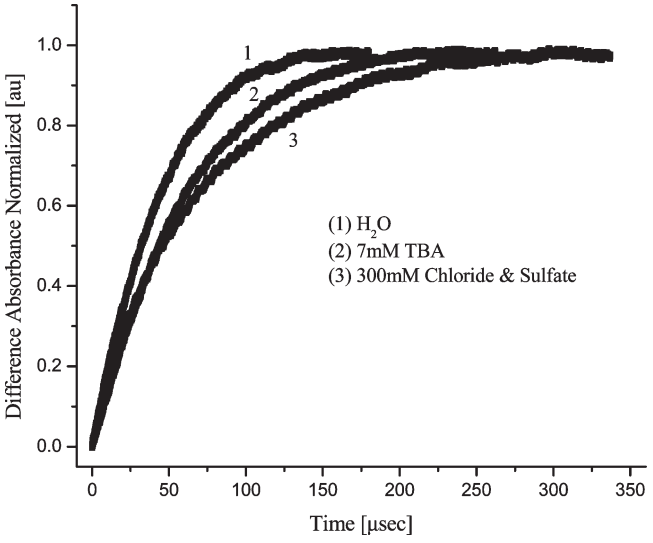

FIGURE 6: Formation of M-like intermediate detected at 410 nm in the presence of water (1), 7 mM TBA^- (2), and 300 mM Cl^- and SO_4^{2-} (3). Measurements taken at pH ~9.4.

The photocycle of PR shares a high degree of similarity with that of bacteriorhodopsin (bR) and exhibits M, N, and O intermediates (*7*, *9*, *16*–*19*, *41*). The effect of SO_4^{2-}, Cl^-, and TBA^- anions on the PR photocycle at high pH values (9.4, above the Asp97 pK_a) was studied using pulsed laser photolysis experiments.

The M intermediate decay exhibits a biphasic behavior for both the Cl^- and SO_4^{2-} anions (Figure 5), characterized by lifetime values of ~0.8 ms (τ_1) and ~4 ms (τ_2), respectively (Table 4), in which the fraction of the first phase was 0.2–0.3 (Table 5). However, PR samples incubated with deionized water and with 7 mM NaTBA had different parameters. TBA^- anions exhibited a shorter lifetime value of ~0.7 ms (τ_1) for the first phase and approximately half the lifetime for the second phase ($\tau_2 \sim 2$ ms) compared with the Cl^- and SO_4^{2-} anions (Table 4). The fraction of each phase changed to ~0.5 (Table 5). The largest differences were observed for PR samples incubated with a deionized water solution. The first phase lifetime was somewhat shorter ($\tau_1 \sim 0.5$ ms), whereas the second phase was almost 3 times shorter relative to those of Cl^- and SO_4^{-2} [$\tau_2 \sim 1.4$ ms (Table 4)]. However, the fraction of the first phase was increased dramatically [~0.8 (Table 5)]. Apparently, the anions affect the thermal decay of the M intermediate and decrease the decay rate relative to that of a sample that lacks anions. Therefore, we can conclude that at this high pH (9.4), Cl^-, SO_4^{2-}, and TBA^- occupy a binding site that affects the M intermediate decay rate and fractions of its decay phases, especially for its second phase decay.

The M species grows via a monophasic process in the presence of 7 mM TBA^- ($\tau \sim 40$ μs) and deionized water ($\tau \sim 30$ μs), whereas Cl^- and SO_4^{2-} anions induce a biphasic kinetics (Figure 6) with lifetime values of ~10 μs (τ_1; ~0.1 fraction) and ~53 μs (τ_2) (Tables 4 and 5). The fact that 7 mM TBA^- and deionzied water exhibited monophasic kinetics for the growth of the M intermediate in contrast with Cl^- and SO_4^{2-} anions lends further support to the notion that the latter anions bind to PR at pH ~9.4. Moreover, this phase is somewhat faster with TBA^- ($\tau_2 \sim 40$ μs) than with deionized water ($\tau_2 \sim 30$ μs) (Table 4). Therefore, it is conceivable that TBA^- anions do bind as well. We noted that the effect on formation of the M intermediate cannot be attributed to the effect of sodium cations via alteration of surface potential and the pH effect, since the rise kinetics of M was not affected significantly with the change in pH to 7.5 in the presence of 300 mM NaCl and was similar to that shown in trace 3 of Figure 6. The photocycle studies described above provide further evidence that TBA^- anions bind to PR at a low concentration of 7 mM and at a high pH of ~9.4.

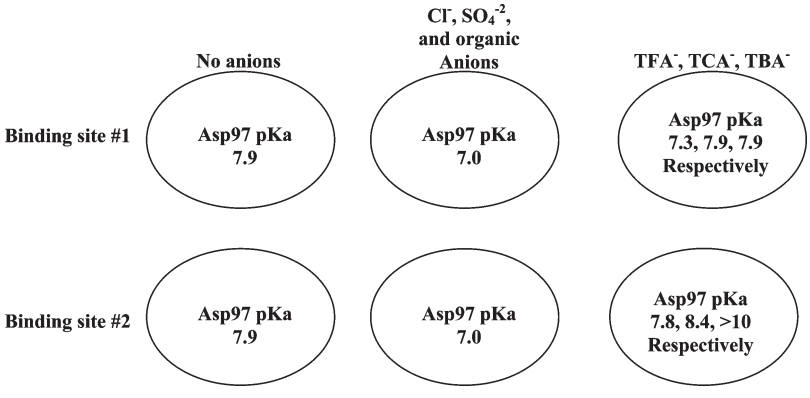

FIGURE 7: Schematic presentation of the effect of binding of anions on Asp97 pK_a.

We have found in the experiments described above that with up to 7 mM TBA^-, the anions bind to PR but do not alter the Asp97 pK_a relative to water. It is conceivable that the same binding site affects both the Asp97 pK_a and the M intermediate kinetics.

Pulsed laser photolysis experiments have also been conducted at pH 5 (below Asp97 pK_a) with the anions described above. At this pH, the M intermediate has not been observed previously (*12*, *19*) probably since its formation involves Asp97 serving as a proton acceptor. At pH 5, Asp97 is probably protonated and cannot accept a proton (*19*). The question of whether the studied anions will be able to replace Asp97 as a proton acceptor, and consequently will induce M formation, arises. However, we did not observe formation of the M intermediate in the presence of the anions; moreover, significant differences in the photocycle were not detected at the detected wavelengths (510, 570, and 620 nm). As described above, support for Cl^-, SO_4^{2-}, and TBA^- binding to PR at pH 9.4 (above Asp97 pK_a) was obtained by studying the photocycle. Clarifying whether anions also bind at pH values as low as 5 will need to await further studies in the future.

CONCLUSIONS

The pK_a of the primary protonated Schiff base counterion of PR is relatively high and close to the pH of its natural environment. Therefore, to impose the deprotonated state of Asp97 and to allow the biological activity of the pigment, the pK_a of Asp97 should be maintained below the pH of the environment. We found that the pK_a is sensitive to and is affected by the binding of anions to the protein. We propose that the protein has two binding sites for anions, and occupation of these binding sites affects the pK_a differently (Figure 7). It appears that the effect depends on the nature of the anion.

Whereas chloride and sulfate anions reduce the pK_a following binding to the protein, acetate anions substituted for halogens (especially trichloroacetate and tribromoacetate) behave differently. At low concentrations, they bind to the protein but do not alter the pK_a considerably (relative to water). At high concentrations, halogeno-acetate anions significantly elevate the pK_a of Asp97. This observation raises the possibility that these anions occupy two binding sites. Tribromoacetate (TBA^-) has the strongest effect and increases the pK_a to a very high value (> 10) since Asp97 adopts a very hydrophobic environment. Such a high pK_a was detected for Asp85 in a bR M photochemically induced intermediate (*42*). It is possible that cations also affect the pK_a of Asp97 either by modifying the protein surface potential or by occupying specific binding sites, which was suggested previously for bR (*24*−*31*). This possibility should be the subject of future studies. It was demonstrated that anions bind to halorhodopsin and to bR (at low pH values) and its mutants. However, anions that bind near the PSB affect pK_a and absorption. This study indicates that the anions probably do not bind in PR near the PSB but still exhibit an effect on Asp97 pK_a. This effect may be unique to PR, and future studies should reveal the specific location of anion binding.

Our results underscore the important role that chloride or sulfate anions play in reducing the pK_a of Asp97 to a value ($\sim$7) that may allow pR to function in its native environment.

REFERENCES

1. Haupts, U., Tittor, J., and Oesterhelt, D. (1999) Closing in on bacteriorhodopsin: Progress in understanding the molecule. *Annu. Rev. Biophys. Biomol. Struct. 28*, 367–399.
2. Klare, J. P., Gordeliy, V. I., Labahn, J., Buldt, G., Steinhoff, H. J., and Engelhard, M. (2004) The archaeal sensory rhodopsin II/transducer complex: A model for transmembrane signal transfer. *FEBS Lett. 564*, 219–224.
3. Lanyi, J. K. (2000) Molecular mechanism of ion transport in bacteriorhodopsin: Insights from crystallographic, spectroscopic, kinetic, and mutational studies. *J. Phys. Chem. B 104*, 11441–11448.
4. Sasaki, J., and Spudich, J. L. (2000) Proton transport by sensory rhodopsins and its modulation by transducer-binding. *Biochim. Biophys. Acta 1460*, 230–239.
5. Spudich, J. L., Yang, C. S., Jung, K. H., and Spudich, E. N. (2000) Retinylidene proteins: Structures and functions from archaea to humans. *Annu. Rev. Cell Dev. Biol. 16*, 365–392.
6. Nakanishi, K., Baloghnair, V., Arnaboldi, M., Tsujimoto, K., and Honig, B. (1980) An External Point-Charge Model for Bacteriorhodopsin To Account for Its Purple Color. *J. Am. Chem. Soc. 102*, 7945–7947.
7. Beja, O., Spudich, E. N., Spudich, J. L., Leclerc, M., and DeLong, E. F. (2001) Proteorhodopsin phototrophy in the ocean. *Nature 411*, 786–789.
8. Sharma, A. K., Spudich, J. L., and Doolittle, W. F. (2006) Microbial rhodopsins: Functional versatility and genetic mobility. *Trends Microbiol. 14*, 463–469.
9. Beja, O., Aravind, L., Koonin, E. V., Suzuki, M. T., Hadd, A., Nguyen, L. P., Jovanovich, S., Gates, C. M., Feldman, R. A., Spudich, J. L., Spudich, E. N., and DeLong, E. F. (2000) Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. *Science 289*, 1902–1906.
10. Wang, W. W., Sineshchekov, O. A., Spudich, E. N., and Spudich, J. L. (2003) Spectroscopic and photochemical characterization of a deep ocean proteorhodopsin. *J. Biol. Chem. 278*, 33985–33991.
11. Man, D. L., Wang, W. W., Sabehi, G., Aravind, L., Post, A. F., Massana, R., Spudich, E. N., Spudich, J. L., and Beja, O. (2003) Diversification and spectral tuning in marine proteorhodopsins. *EMBO J. 22*, 1725–1731.
12. Friedrich, T., Geibel, S., Kalmbach, R., Chizhov, I., Ataka, K., Heberle, J., Engelhard, M., and Bamberg, E. (2002) Proteorhodopsin is a light-driven proton pump with variable vectoriality. *J. Mol. Biol. 321*, 821–838.
13. Gomez-Consarnau, L., Gonzalez, J. M., Coll-Llado, M., Gourdon, P., Pascher, T., Neutze, R., Pedros-Alio, C., and Pinhassi, J. (2007) Light stimulates growth of proteorhodopsin-containing marine Flavobacteria. *Nature 445*, 210–213.
14. Bergo, V., Amsden, J. J., Spudich, E. N., Spudich, J. L., and Rothschild, K. J. (2004) Structural changes in the photoactive site of proteorhodopsin during the primary photoreaction. *Biochemistry 43*, 9075–9083.
15. Amsden, J. J., Kralj, J. M., Bergo, V., Spudich, E. N., Spudich, J. L., and Rothschild, K. J. (2007) Structural changes in the photoactive site of blue-absorbing proteorhodopsin in the primary photoreaction. *Biophys. J.* 148A.
16. Krebs, R. A., Devita, A. M., Partha, R., Alexiev, U., Dunmire, D., and Braiman, M. S. (2002) Purification and spectroscopic studies of proteorhodopsin. *Biophys. J. 82*, 224A.
17. Dioumaev, A. K., Brown, L. S., Shih, J., Spudich, E. N., Spudich, J. L., and Lanyi, J. K. (2002) Proton transfers in the photochemical reaction cycle of proteorhodopsin. *Biochemistry 41*, 5348–5358.
18. Varo, G., Brown, L. S., Lakatos, M., and Lanyi, J. K. (2002) Proton transfers in the photochemical reaction cycle of proteorhodopsin. *Biochemistry 41*, 5348–5358.
19. Lakatos, M., Lanyi, J. K., Szakacs, J., and Varo, G. (2003) The photochemical reaction cycle of proteorhodopsin at low pH. *Biophys. J. 84*, 3252–3256.
20. Lutz, I., Sieg, A., Wegener, A. A., Engelhard, M., Boche, I., Otsuka, M., Oesterhelt, D., Wachtveit, J., and Zinth, W. (2001) Primary reactions of sensory rhodopsins. *Proc. Natl. Acad. Sci. U.S.A. 98*, 962–967.
21. Imasheva, E. S., Balashov, S. P., Wang, J. M., Dioumaev, A. K., and Lanyi, J. K. (2004) Selectivity of retinal photoisomerization in proteorhodopsin is controlled by aspartic acid 227. *Biochemistry 43*, 1648–1655.
22. Mevorat-Kaplan, K., Brumfeld, V., Engelhard, M., and Sheves, M. (2005) Effect of anions on the photocycle of halorhodopsin. Substitution of chloride with formate anion. *Biochemistry 44*, 14231–14237.
23. Kelemen, L., Galajda, P., Szaraz, S., and Ormos, P. (1999) Chloride ion binding to bacteriorhodopsin at low pH: An infrared spectroscopic study. *Biophys. J. 76*, 1951–1958.
24. Szundi, I., and Stoeckenius, W. (1987) Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A. 84*, 3681–3684.

25. Szundi, I., and Stoeckenius, W. (1988) Purple-to-blue transition of bacteriorhodopsin in a neutral lipid environment. *Biophys. J. 54*, 227–232.
26. Szundi, I., and Stoeckenius, W. (1989) Surface pH controls purple-to-blue transition of bacteriorhodopsin. *Biophys. J. 56*, 369–383.
27. Ariki, M., and Lanyi, J. K. (1986) Characterization of metal ion-binding sites in bacteriorhodopsin. *J. Biol. Chem. 261*, 8167–8174.
28. Zhang, Y. N., Sweetman, L. L., Awad, E. S., and El-Sayed, M. A. (1992) Nature of the individual Ca^{2+} binding sites in Ca^{2+}-regenerated bacteriorhodopsin. *Biophys. J. 61*, 1201–1206.
29. Yoo, S. K., Awad, E. S., and El-Sayed, M. A. (1995) Comparison between the binding of Ca^{2+} and Mg^{2+} to the two high-affinity sites of bacteriorhodopsin. *J. Phys. Chem. 99*, 11600–11604.
30. Fu, X., Bressler, S., Ottolenghi, M., Eliash, T., Friedman, N., and Sheves, M. (1997) Titration kinetics of Asp-85 in bacteriorhodopsin: Exclusion of the retinal pocket as the color-controlling cation binding site. *FEBS Lett. 416*, 167–170.
31. Eliash, T., Weiner, L., Ottolenghi, M., and Sheves, M. (2001) Specific binding sites for cations in bacteriorhodopsin. *Biophys. J. 81*, 1155–1162.
32. Zadok, U., Asato, A. E., and Sheves, M. (2005) Titration of the bacteriorhodopsin Schiff base involves titration of an additional protein residue. *Biochemistry 44*, 8479–8485.
33. Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., and Heyn, M. P. (1990) Substitution of amino acids Asp-85, Asp-212, and Arg-82 in bacteriorhodopsin affects the proton release phase of the pump and the pK of the Schiff base. *Proc. Natl. Acad. Sci. U. S. A. 87*, 1018–1022.
34. Rousso, I., Friedman, N., Sheves, M., and Ottolenghi, M. (1995) pKa of the protonated Schiff base and aspartic-85 in the bacteriorhodopsin binding-site is controlled by a specific geometry between the two residues. *Biochemistry 34*, 12059–12065.
35. Gat, Y., and Sheves, M. (1993) A mechanism for controlling the pKa of the retinal protonated Schiff base in retinal proteins: A study with model compounds. *J. Am. Chem. Soc. 115*, 3772–3773.
36. Balashov, S., Govindjee, R., Kono, M., Imasheva, E., Lukashev, E., Ebrey, T., Crouch, R., and Menick, D. (1993) Effect of the arginine 82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release and photochemical cycle. *Biochemistry 32*, 10331–10343.
37. Yoshitsugu, M., Shibata, M., Ikeda, D., Furutani, Y., and Kandori, H. (2008) Color change of proteorhodopsin by a single amino acid replacement at a distant cytoplasmic loop. *Angew. Chem., Int. Ed. 47*, 3923–3926.
38. Yoshitsugu, M., Yamada, J., and Kandori, H. (2009) Color-Changing Mutation in the E-F Loop of Proteorhodopsin. *Biochemistry 48*, 4324–4330.
39. Baasov, T., and Sheves, M. (1985) Model compounds for the study of spectroscopic properties of visual pigments and bacteriorhodopsin. *J. Am. Chem. Soc. 107*, 7524–7533.
40. Krebs, R. A., Alexiev, U., Partha, R., Devita, A. M., and Braiman, M. S. (2002) Detection of fast light-activated H^+ release and M intermediate formation from proteorhodopsin. *BMC Physiol. 2*, 5.
41. Varo, G., Brown, L. S., Lakatos, M., and Lanyi, J. K. (2003) Characterization of the photochemical reaction cycle of proteorhodopsin. *Biophys. J. 84*, 1202–1207.
42. Braiman, M. S., Dioumaev, A. K., and Lewis, J. R. (1996) A large photolysis-induced pK_a increase of the chromophore counterion in bacteriorhodopsin: Implications for ion transport mechanisms of retinal proteins. *Biophys. J. 70*, 939–947.