Published in final edited form as:

Photochem Photobiol. 2008 ; 84(4): 880–888. doi:10.1111/j.1751-1097.2008.00318.x.

The Lifetimes of *Pharaonis* Phoborhodopsin Signaling States Depend on the Rates of Proton Transfers—Effects of Hydrostatic Pressure and Stopped Flow Experiments[†]

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Abstract

Pharaonis phoborhodopsin (ppR), a negative phototaxis receptor of *Natronomonas pharaonis*, undergoes photocycle similar to the light-driven proton pump bacteriorhodopsin (BR), but the turnover rate is much slower due to much longer lifetimes of the M and O intermediates. The M decay was shown to become as fast as it is in BR in the L40T/F86D mutant. We examined the effects of hydrostatic pressure on the decay of these intermediates. For BR, pressure decelerated M decay but slightly affected O decay. In contrast, with ppR and with its L40T/F86D mutant, pressure slightly affected M decay but accelerated O decay. Clearly, the pressure-dependent factors for M and O decay are different in BR and ppR. In order to examine the deprotonation of Asp75 in unphotolyzed ppR we performed stopped flow experiments. The pH jump-induced deprotonation of Asp75 occurred with 60 ms, which is at least 20 times slower than deprotonation of the equivalent Asp85 in BR and about 10- fold faster than the O decay of ppR. These data suggest that proton transfer is slowed not only in the cytoplasmic channel but also in the extracellular channel of ppR and that the light-induced structural changes in the O intermediate of ppR additionally decrease this rate.

[†]This invited paper is part of the Symposium-in-Print: Photoreceptors and Signal Transduction.

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SUPPLEMENTAL MATERIALS

The following supplemental material is available for this article:

Appendix 1. The influence of the pressure-dependent pH change on the calculated activation volume.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/full/10.1111/j.1751-1097.2008.00318.x>.

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INTRODUCTION

Haloarchaeon *Natronomonas pharaonis* shows a photophobic response mediated by a phototaxis receptor, *pharaonis* phoborhodopsin (*ppR*, also called *pharaonis* sensory rhodopsin II) (1–4). This receptor protein has close similarity to bacteriorhodopsin (BR), a well-studied proton-pump in the purple membrane from *Halobacterium salinarum* (5). Both BR and *ppR* consist of seven transmembrane helices and a chromophore, all-*trans* retinal, bound through a protonated Schiff base to a specific lysine residue in helix G. A photon absorption by the protein-bound retinal triggers its isomerization from all-*trans* to 13-*cis* configuration. Both proteins then undergo a similar photocycle, described by the intermediates called K, L, M, N and O in the order of their appearances. An exception is the N state of *ppR*. The global fitting analysis of the *ppR* photocycle suggested the presence of the N state (absorption maximum at 485 nm) which is apparently in equilibrium with the more pronounced M and O states (6). N is still not a very well-characterized intermediate. In analogy with BR it is expected to be a 13-*cis* intermediate with reprotonated Schiff base. However, FTIR study of *ppR* did not detect the characteristic protein structure corresponding to N of BR (7). The last state of the *ppR* photocycle (O intermediate, *ppR*_O) has all-*trans* retinal as in the case of BR (8).

The formation of M of BR accompanies the primary proton transfer from the protonated Schiff base to its counterion Asp85 as well as subsequent proton release at the extracellular (EC) surface from the proton-releasing group (PRG), which consists of Glu194, Glu204 and water molecules. In the M → N → O transition, the deprotonated Schiff base takes up proton from the cytoplasmic (CP) surface *via* Asp96 located at the middle of the CP channel. Finally, proton transfer occurs from Asp85 to the deprotonated PRG during O → BR transition. As a result of these steps, one proton is translocated from the CP side to the EC side of the membrane.

The sensory protein, *ppR*, also pumps protons outwardly (9,10), but the pattern of proton release and uptake reactions depend on whether the protein is examined in the membrane-reconstituted or the detergent-solubilized states (11,12). In the membrane-reconstituted state, the formation of M of *ppR* (*ppR*_M) is coupled with the proton transfer from the protonated Schiff base to its counterion, Asp75, and is accompanied by the proton release at the EC side from the PRG (11). In *ppR*, the residue corresponding to Glu204 of PRG of BR is conserved as Asp193^{*ppR*} while that to Glu194 in BR is replaced by Pro183^{*ppR*}. In the course of *ppR*_M to *ppR*_O transition, the deprotonated Schiff base takes up a proton from the CP side (12). Because of the lack of a residue corresponding to Asp96 of BR the proton transfer from the surface and reprotonation of the Schiff base is very slow (2 orders of magnitude slower than in BR) (6,13,14). Finally, Asp75 transfers its proton to the deprotonated PRG during *ppR*_O → *ppR* transition. In the detergent-solubilized state of *ppR*, the proton release at the EC side does not occur through PRG—the proton is released from Asp75 to the EC side during the *ppR*_O → *ppR* transition (11).

Despite such similarities in proton transfer reactions, the photocycling rates are quite different in BR and *ppR*. In BR, the M and O intermediates decay in several milliseconds (13), whereas the intermediates of *ppR* decay in several hundred milliseconds to several

seconds (6,14). The fast photocycle of BR is critical for efficient proton pumping at high light intensities, whereas the slow decay of the intermediates in *ppR* helps to activate the cognate transducer at the *ppR_M* and *ppR_O* states by signal transduction (2–4). Thus, understanding the origin of the rate differences of M and O decay should be important for understanding the functional differentiation of these proteins in molecular terms.

The outward tilting of helix F in the late M intermediate of BR has been detected (15–17). The accompanying hydration of the CP channel in BR is widely considered to be critical for the fast proton transfer from Asp96 to the deprotonated Schiff base in M → N transition (5) and reprotonation of Asp96 from the bulk (18). A similar helix motion has also been reported in *ppR* (19–22). However, it is not known whether or not transient hydration occurs also in *ppR*. The slow decay of *ppR_M* is attributed to the slow rate of reprotonation of the Schiff base through the hydrophobic CP channel due to the lack of a proton donating group corresponding to the Thr46^{BR} and Asp96^{BR} pair. The corresponding positions in the *ppR* structure are Leu40^{*ppR*} and Phe86^{*ppR*}. In support of this hypothesis, the substitution of Leu40^{*ppR*} with Thr and Phe86^{*ppR*} with Asp (L40T/F86D) greatly accelerates the decay of *ppR_M* to a rate comparable to that observed in BR (23,24). The decay of *ppR_M* is clearly limited by the rate of reprotonation of the Schiff base. Does the hydrated CP channel contribute to this accelerated *ppR_M* decay similar to BR? The hydration of the CP channel thus seems to be important to understand the conformation change and/or the proton transfer mechanism of *ppR*.

In both BR and *ppR*, the decay of the O intermediate is coupled to proton transfer from protonated Asp85^{BR}/Asp75^{*ppR*} through the EC channel. However, the decay of *ppR_O* is 2 orders of magnitude slower than that of BR (6,13,14). The slow decay of *ppR_O* is partially attributed to a greater hydrophobicity of the EC channel in *ppR* compared to that in BR (25). A previous study showed that replacing hydrophobic residues in the EC channel of *ppR* by the more polar counterparts found in BR (P182S/P183E/V194T) results in a 20-fold increase in the rate of decay of *ppR_O* compared to that of the wild type (26). However, this accelerated *ppR_O* decay is still slower than that observed with BR. Another mutation of Thr204^{*ppR*} (corresponding to Ala215^{BR}) located near the Schiff base also resulted in an acceleration of the decay of *ppR_O*. Especially, the quadruple mutants P182S/P183E/V194T/T204S or T204C have decay rates of *ppR_O* comparable to that of O in BR (24,26). These data imply that, besides the hydrophobicity of the EC channel, there is another mechanism further slowing down the decay of *ppR_O*.

In the present study, we focused on the differences in the decay of M and O intermediates in BR and *ppR*. The dependence of the photocycle kinetics on hydrostatic pressure was examined for each of these proteins as well as for the *ppR*-transducer complex and the L40T/F86D mutant of *ppR*. Hydrostatic pressure is expected to influence the reaction kinetics associated with the large changes in activation volumes, which usually reflect the changes in hydration and packing states of the proteins (27). Furthermore, a time-resolved pH titration was performed for *ppR* to compare the rate of *ppR_O* decay to the rate of deprotonation of Asp75 in the unphotolyzed state, comparing the latter also with analogous data for the rate of deprotonation of Asp85 in BR (28,29). We observed: (1) the effects of pressure on the kinetics of M and O decay and the activation volumes associated with these

transitions are quite different between BR and *ppR*; and (2) the rate of deprotonation of *Asp75^{ppR}* in the dark is much slower than the rate of deprotonation of *Asp85^{BR}*, but it is faster than the decay of *ppR_O* in the photocycle.

MATERIALS AND METHODS

Preparation of samples

Purple membrane was isolated from *H. salinarum* strain S9 using the standard method (30). Histidine-tagged recombinant *ppR*, L40T/F86D mutant and *pHtrII₁₋₁₅₉* were prepared from *Escherichia coli* membranes as previously reported (23,31,32). The N-terminal 159 amino acid residues of the *pharaonis* halobacterial transducer of *ppR* is denoted by *pHtrII₁₋₁₅₉*.

Pressure cell

Flash photolysis under various hydrostatic pressures was measured using a pressure cell constructed by UNISOKU (Osaka, Japan). The cylindrical sample cuvette (5 mm optical path) sealed with PARAFILM (American National Can, Chicago, IL) was set inside the cell filled with water. Using a hand-operated hydraulic pump, the pressure on the sample was changed between 1 and 900 bar using water as a pressurizing medium. The pressure applied was determined by a manometer. Four windows (3 mm diameter) in the pressure cell were used for the monitoring light and also for the actinic laser flash into the cell. These two beams intersected perpendicularly in the sample cuvette. The temperature of the pressure cell was controlled by a circulating water bath. All measurements were performed at 25°C.

Flash photolysis spectroscopy

The laser flash photolysis setup was essentially the same as previously reported (33). At each selected measuring wavelength, 20–100 transient absorption changes in the samples, induced by the laser pulse, were acquired and averaged to improve the S/N ratio.

The samples contained 50 mM sodium phosphate buffer, pH 6.5. For the solubilized *ppR*, *ppR-pHtrII₁₋₁₅₉* complex and L40T/F86D mutant, 0.1% *n*-dodecyl- β -D-maltoside was added. The protein concentrations determined from the optical densities were 13 μ M for BR ($\epsilon_{570} = 63\,000\text{ M}^{-1}\text{ cm}^{-1}$), and 10–15 μ M for *ppR* and its L40T/F86D mutants ($\epsilon_{500} = 40\,000\text{ M}^{-1}\text{ cm}^{-1}$). For the preparation of the *ppR-pHtrII₁₋₁₅₉* complex, *ppR* and *pHtrII₁₋₁₅₉* were purified independently and then mixed with a molar ratio of 1:10 to insure complex formation of all *ppR* with *pHtrII₁₋₁₅₉*. At this molar ratio, the amount of free *ppR* becomes negligible (32).

Data analysis was performed using Origin (Microcal Software). From the pressure dependence of the rate constant, we calculated the activation volume, $V^\ddagger$, which is a volume change in the transition state (27). Here, we used the linear dependence of the logarithm of the rate constant on pressure in the following equation:

$$\ln k = \text{constant} - \frac{P\Delta V^\ddagger}{RT}, \quad (1)$$

where k is the rate constant, P is the hydrostatic pressure, R is the gas constant and T is the absolute temperature.

Time-resolved pH titration of ppR

Time-resolved titrations were performed with a stopped flow apparatus (Kinetic Systems, UK) at 25°C. In the fastest mode, 400 spectra were automatically collected during 1 s after mixing of sample at pH 2 with 100 mM buffer solution, MOPS, pH 7. In this mode the first spectrum is taken 1.3 ms after mixing and the time interval between subsequent spectra is about 2.5 ms.

RESULTS

Pressure dependencies in the BR photocycle

Figure 1a shows the flash-induced absorbance changes in BR at various hydrostatic pressures. The selected wavelengths were 410, 570 and 680 nm. Prominent changes appeared at all three wavelengths. At 410 and 570 nm, the absorbance changes slowed with an increase in pressure. At 680 nm, the signal amplitude became small as the pressure increased and the decay time increased. At these wavelengths, absorption changes resulting from M, ground-state BR and O are mainly observed. Thus, the increase in pressure retarded the M to N decay and the reformation of ground-state BR, and suppressed the accumulation of O. The data for M are in good agreement with earlier results of Váró and Lanyi (27) performed at a higher pH where the O intermediate did not accumulate.

Pressure dependencies in the ppR photocycle

In the ppR photocycle, the effects of pressure were different from those observed in BR (Fig. 1b). In the case of ppR, the selected wavelengths were 390, 500 and 560 nm. As in the case of BR, these wavelengths monitor the concentration changes of ppR_M, ground-state ppR and ppR_O respectively. At 390 nm, only a slight effect of pressure was observed. At 500 nm, the increase in pressure caused acceleration of the recovery of ground-state ppR. Thus, with ppR the effect of pressure is the opposite of that observed with BR. At 560 nm, increased pressure caused a decrease in the amplitude of absorption changes, as with BR (Fig. 1a, 680 nm), but the decay time is significantly decreased (apparent half-times, 1.5 → 0.7 s), indicating faster turnover of the photocycle.

Similar dependencies of hydrostatic pressure on ppR kinetics were also observed in ppR reconstituted with L- α -phosphatidylcholine (data not shown). Moreover, as shown in Fig. 1c, the effects of pressure on the photoreactions of the ppR-pHtrII complex were not significantly different from that on the kinetics of the ppR photocycle.

The effects of pressure were also examined for the photocycle of the L40T/F86D mutant ppR (Fig. 1d). For this mutant, the absorbance changes at 390 nm are shown in the 0–30 ms time range, and those at 500 and 560 nm are in the 0–5 s time range. Although the ppR_M decay rate was fast (comparable with that of M of BR), relatively small effects of pressure were observed (390 nm) and, contrary to the M of BR, pressure accelerated the decay of

ppR_M . The recovery of the 500 and 560 nm signals, associating mainly with $ppR_O \rightarrow ppR$ transition, is also accelerated by pressure as it is in wild type ppR .

For this mutant, besides substantially accelerated decay rate of ppR_M , a decelerated decay rate of ppR_O has been reported previously (23)—the reason for the modification of O decay is not yet clear. These results were confirmed in the present study. In addition, in the current work, somewhat complex changes were also observed in both M and O decay. For M decay (390 nm), the fast, major phase occurs with a time constant of *ca* 5 ms (1 bar), which is comparable to previous observations (23,24). Besides this fast decay, however, an additional very slow component is present. The recovery of signals at 500 and 560 nm, associating with O decay, is also biphasic. Different sample conditions may be responsible for the differences in the data obtained. In previous studies ppR was solubilized with octylglucoside (23) or reconstituted with purple membrane lipids (24). The complex absorbance changes imply the appearances of other intermediates, but their origins are not yet known. In analogy with BR, biphasic decay of the M intermediate might be caused by a back reaction from the following intermediate(s): $M \rightleftharpoons N \rightleftharpoons O$.

Activation volumes associating with M and O decays

To evaluate the effects of pressure on the M and O decay of BR and ppR , we analyzed the absorbance changes by the following procedures. For BR, we adopted the simple scheme of $M \rightarrow N \rightarrow O \rightarrow BR$ and analyzed the absorption changes at 410 and 680 nm, independently. For M decay, the data at 410 nm were fitted by a single exponential. The rate constant obtained by this procedure mainly reflects the rate constant of the $M \rightarrow N$ transition. For O decay, the data at 680 nm were fitted by the sum of two exponentials. This results in apparent rate constants for the formation of O and the subsequent O decay. One rate constant is for the process M to O through N ($M \rightarrow N \rightarrow O$), and another rate constant (the faster one) reflects mainly the rate constant of the $O \rightarrow BR$ transition. For ppR and ppR – $pHtrII$ complex, the fitting analysis was performed according to the method of Miyazaki *et al.* (14), where we adopted the scheme $ppR_M \rightarrow ppR_O \rightarrow ppR$ and simultaneously fitted the data at three wavelengths (390, 500 and 560 nm).

The same method could not be applied to the L40T/F86D mutant. Because this mutant exhibited a somewhat complex photocycle, we tentatively analyzed the data by separating the time regions. For M decay, the 390 nm data up to 30 ms were analyzed by a single exponential plus a constant to determine the rate of the fast, major decay process. For O decay, we tentatively adopted the scheme, $O_1 \rightarrow O_2 \rightarrow \text{ground-state}$. The 500 and 560 nm data from 0.1 to 5 s were then analyzed simultaneously by a similar procedure for wild type ppR and its complex with $pHtrII$. Calculated best-fit curves are shown in Fig. 1 as smooth lines, and the logarithmic values of the rate constants obtained are plotted against pressure in Fig. 2. For BR, the rate constant of M decay ($M \rightarrow N$) and the slow rate constant for the absorbance change at 680 nm ($k_{s,680}$) are very sensitive to pressure when compared with the faster rate constant ($k_{f,680}$). For ppR and its complex with the transducer, the ppR_O decay ($O \rightarrow ppR$) is more sensitive to pressure. For the L40T/F86D mutant, the three rate constants show similar pressure dependences.

As shown earlier, for BR, the $O \rightarrow BR$ transition occurs with a faster rate constant than the $M \rightarrow N \rightarrow O$ transition (34). This relationship of the rate constants was reported in previous studies (13,34–36). The results obtained in this study on a similar pressure dependence of the M decay ($M \rightarrow N$) and the slower rate constant ($k_{s,680}$) are in agreement with this assignment— $k_{s,680}$ reflects the rate constant for the $M \rightarrow N \rightarrow O$ transition and the faster rate constant, $k_{f,680}$, is for the $O \rightarrow BR$ transition, respectively. Thus elevated pressure decreases the accumulation of the O intermediate (Fig. 1a, 680 nm) mostly because of slowing the $M \rightarrow N \rightarrow O$ transition.

From the pressure dependence of the rate constants, the activation volumes for the transition states were determined and are summarized in Table 1. In the present study, we used sodium phosphate buffer, whose pH is known to increase about 0.4 U at 1 kbar (37). The decay of ppR_M is sensitive to the pH change (14)—the rate constant for ppR_M decay becomes about half by the increase in pH from 6.5 to 7. However, this pressure-dependent pH change has only a small influence on the calculated activation volume (about -2.6 mL mol^{-1} for the $ppR_M \rightarrow ppR_O$ transition; see Supplemental Materials). The results in Table 1 show a striking contrast between BR and ppR . For BR, all transitions have positive activation volumes. The $M \rightarrow N$ transition has a large activation volume (47.3 mL mol^{-1}). Because the M2 formation of BR is completed within 1 ms (13), this activation volume mainly reflects the change during the $M2 \rightarrow N$ transition. For this transition, Váró and Lanyi also reported a large activation volume of about 50 mL mol^{-1} (27). The $M \rightarrow N \rightarrow O$ transition also has a large activation volume (37.1 mL mol^{-1}), which is a little smaller than that of the $M \rightarrow N$ transition. This implies that the $N \rightarrow O$ transition occurs with a relatively small activation volume. For ppR , on the other hand, the activation volumes of both transitions are negative. In particular, the $ppR_O \rightarrow ppR$ transition has a large negative activation volume ($-48.4 \text{ mL mol}^{-1}$). These results imply that the conformational changes in BR and ppR occur in very different ways. The complex formation with the transducer does not cause substantial changes in the activation volumes. The magnitudes of the activation volumes become slightly larger for the $ppR_M \rightarrow ppR_O$ transition, and smaller for the $ppR_O \rightarrow ppR$ transition. The negative activation volumes were also obtained for the three rate constants of the L40T/F86D mutant.

Time-resolved pH titration of Asp75 in ppR

The Schiff base, as well as its counterion, Asp75 in ppR (corresponding to Asp85^{BR}), is protonated in ppR_O . During the $ppR_O \rightarrow ppR$ transition, Asp75 returns to the initial deprotonated state. In unphotolyzed ppR , Asp75 protonates at low pH. This acid ppR has a redshifted absorption spectrum, similar to that of ppR_O . In the dark state, the kinetics of deprotonation of Asp75 can be observed by a pH jump (stopped flow). It has been shown that in BR, deprotonation of Asp85 occurs very rapidly upon a shift of the pH between 2 and 7, *ca* 3 ms or faster (28,29). This is close to the time constant of O to BR transition at neutral pH of about 1–3 ms observed in the present study (1.3 ms at 1 bar) as well as in previous studies (13,18). These data show a correlation between the rates of deprotonation of Asp85 in the initial state and in the O intermediate. The fast deprotonation of Asp85 apparently involves a network of hydrogen bonds in the EC domain of BR (38) and its PRG (18). Substitution of one of the key groups constituting the PRG in BR, Glu204, by a nonionizable

residue in the E204Q mutant slowed the deprotonation of Asp85 to 18 ms (29). The decay of the O intermediate was slowed to an even greater extent in this mutant, to 100 ms (34). These results again indicate a correlation between the rates of deprotonation of Asp85 in the initial state and in O in this mutant, though the two rates do not coincide. The O decay was about six-fold slower.

In the EC channel of *ppR*, the hydrogen-bonded network observed in BR is probably disrupted by replacement of the residues in the EC channel with hydrophobic groups. This implies that the deprotonation of Asp75 should be slowed and might contribute to the overall slow photocycle of *ppR*. To check this, we performed a stopped flow experiment with *ppR*, where the time-dependent changes in the absorption spectrum were measured after a pH jump from pH 2 to 7. Figure 3a shows a family of spectra obtained during the first 100 ms after the pH shift. These spectra show a blueshift of the absorption maximum and reformation of *ppR*. The difference spectra in Fig. 3b indicate that the maximum change occurs at 545 nm and that there is an isosbestic point around 515 nm. The kinetics of the absorption changes at 545 nm (Fig. 3c) shows that the redshifted (acid) form of *ppR* disappears with a time constant of about 60 ms. Because deprotonation of Asp85 in BR occurs within 3 ms (28,29), deprotonation of Asp75 in *ppR* is at least 20-fold slower than in BR. The $ppR_O \rightarrow ppR$ transition occurs with a time constant of 560 ms (Fig. 1b, 1 bar), which is consistent with a previous report (14). This transition in the *ppR* photocycle is about 10-fold slower than the deprotonation of Asp75 in the unphotolyzed state.

DISCUSSION

Decay of M intermediate

For BR, the outward tilting of helix F in the late M state enables water molecules to hydrate the CP channel around Asp96 (to stabilize the deprotonated state of Asp96), and to create a transient hydrogen-bonded network between Asp96 and the Schiff base at the early N intermediate (16,39,40). This hydration decreases the pKa of Asp96 and facilitates the fast internal proton transfer between Asp96 and the Schiff base during the M $\rightarrow$ N transition (5). The large activation volume in this transition (47.3 mL mol⁻¹) should correspond with the transient hydration of the CP region (25).

Similar to BR, the outward tilting of helix F in *ppR* is thought to occur within a few milliseconds, corresponding to the M1 $\rightarrow$ M2 transition (19,21). Our data in Fig. 1b–d mainly show the photocycle after the M2 state. Contrary to the results of BR, we observe relatively small and negative activation volumes in the $ppR_M \rightarrow ppR_O$ transition of *ppR* alone (–4.1 mL mol⁻¹), *ppR* associating with the transducer (–9.8 mL mol⁻¹) and the L40T/F86D mutant (–10.3 mL mol⁻¹). These results suggest that the additional hydration of the CP channel does not occur in the $ppR_M \rightarrow ppR_O$ transitions in these three situations. This consideration is based on the data obtained only after formation of the M2 state. However, hydration also seems not to occur during the formation of the M2 state, because such hydration should decrease the rate and yield of ppR_M (M2 state). However, the yield of ppR_M does not change significantly at high pressure (Fig. 1b–d, 390 nm).

In the L40T/F86D mutant of *ppR*, the *ppR*_M decay is greatly accelerated to a level comparable to that in BR. This suggests that the implanted Asp86 functions as the proton donor for the deprotonated Schiff base, analogous to BR. However, the pressure-dependent deceleration of M decay in BR is not observed, even in this mutant. Similar to wild type *ppR*, the fast M decay of this mutant appears to occur without the additional hydration of the CP channel.

The proper pK_a values of the Asp86 and the Schiff base at this state are critically important for the proton transfer between them. In unphotolyzed BR, Asp96 has an extremely high pK_a above 11 (41). In the M and N states, the hydration of the CP channel lowers the pK_a of Asp96 to about 7 (34,42–44), which is lower than the pK_a of the Schiff base, about 8 in these states (45). From the pH dependence of the *ppR*_M decay of the quadruple mutant *ppR* (L40T/F86D/P183E/T204A), which has the additional mutations (P183E/T204A) on the EC side of the Schiff base, the pK_a of Asp86 was deduced to be about 9.5 in the unphotolyzed state (24). The corresponding pK_a of the L40T/F86D mutant has not yet been determined, but its pK_a should be close to 9.5, similar to that of the quadruple mutant. This pK_a value is at least 1.5 U lower than the corresponding pK_a of Asp96 in BR (above 11). The lower pK_a of Asp86 in this mutant might originate from increased hydration of the CP channel even in the unphotolyzed state. This initial hydration could contribute to the fast proton transfer between Asp86 and the Schiff base in the mutant, which can be accomplished by simple displacements of the water molecules initially present in the unphotolyzed state. However, we observed in the current work a small but distinct acceleration of *ppR*_M decay under high pressure. This acceleration indicates some tightening (decrease in volume) in the transition state. Further studies are needed on the photocycle of this mutant and on the hydration effects on the pK_a of Asp86.

A tentative conclusion that we draw from the comparison of the remarkably different effects of pressure on the M decay in BR and *ppR* is that the high activation volume in BR is probably caused by transient changes in hydration, but that no such changes occur in either wild type *ppR* or its L40T/F86D mutant. M decay in *ppR* occurs without a transient increase in volume.

Decay of O intermediate

During the O → BR transition, we observe a small activation volume (9.8 mL mol⁻¹). This suggests that a large conformational change does not occur in this transition, which is coupled with the proton transfer from Asp85 to the PRG. In contrast to this is the decay of the O intermediate in *ppR*, which becomes faster at elevated pressures. The most notable observation is the large negative activation volume in the *ppR*_O → *ppR* transition (−48.4 mL mol⁻¹). Similar to BR, this transition in *ppR* is also coupled with the deprotonation of the counterion, Asp75. For *ppR*, however, the deprotonation of Asp75 should require a large conformational change accompanying the dehydration and/or more dense packing of the protein. These data clearly show the distinct difference between BR and *ppR* in the deprotonation of the counterion (Asp85 in BR and Asp75 in *ppR*) in the last step(s) of the photocycle.

The protein volume contraction during the $ppR_O \rightarrow ppR$ transition was reported by the transient grating method (46). During the same transition, electron paramagnetic resonance spectroscopy and the studies using fluorescent resonance energy transfer detected the recovery of the outwardly tilted helix F of ppR (19,21,47). Thus, this recovery of helix F might be related to the transient decrease in the protein volume observed here. At present, however, we cannot conclude that the helix motion directly causes the decrease in the protein volume because we did not detect the increase in the protein volume for ppR attributable to the outward tilting of helix F. Instead of the change in the CP region, the transient decrease in the protein volume might occur in the EC channel along with the proton transfer reaction in this channel.

Effects of transducer binding on the pressure dependence of ppR

The binding of the transducer does not significantly affect the essential light-induced conformational changes in ppR or the response to an increase in pressure. However, we did observe a modification in the magnitude of the activation volumes. While the change in the $ppR_M \rightarrow ppR_O$ transition might be negligible (-4.1 to -9.8 mL mol⁻¹), a small but distinct reduction (about 30%, -48.4 to -34.0 mL mol⁻¹) appears in the $ppR_O \rightarrow ppR$ transition. Besides the conformational change in ppR , the change in the adjacent transducer might also contribute to the measured activation volumes. Moreover, light-induced changes in the interface between ppR and the transducer might induce changes in the hydration of the contact points. Thus, the change in the activation volume cannot be assigned simply to the conformational change in ppR . However, the most straightforward consideration should be that the adjacent transducer slightly restricts the light-induced conformational changes in ppR and reduces the conformational difference between ppR_O and its initial state.

Deprotonation of the counterion in the unphotolyzed ppR

The stopped flow experiments reveal a remarkable difference in the rate constant of deprotonation of Asp75 in ppR and Asp85 in BR. In BR (in a suspension of purple membranes), deprotonation occurs with a time constant faster than 3 ms (28,29). In ppR it takes 60 ms. This 20-fold decrease in the rate of deprotonation might be explained by a more hydrophobic composition of the EC domain in ppR . In BR, Glu204, Thr205, Glu194 and Ser193 form a hydrogen bonding system, which provides a pathway for proton release from Asp85 to the EC surface. In ppR , Glu204^{BR} is conservatively substituted with Asp193^{ppR}; however, other residues (Thr205^{BR}, Glu194^{BR} and Ser193^{BR}) are substituted with hydrophobic amino acids, Val194^{ppR}, Pro183^{ppR} and Pro182^{ppR}, respectively, and so the effective hydrogen bonding system in BR should not be present in ppR . A previous mutation study of ppR revealed a 20-fold acceleration of the $ppR_O \rightarrow ppR$ transition in the triple mutant of P182S/P183E/V194T (26).

Previous estimates (13,34,35) indicate that in wild type BR, the time constant of deprotonation of Asp85 in the $O \rightarrow BR$ transition is 3 ± 2 ms, which is close to our determined value of 1.3 ms. These time constants are approximately as fast as diffusion of a proton in the dark initiated by a pH jump from 2 to 7. In ppR , the ppR_O decay (560 ms) is 2 orders of magnitude slower than in BR, and about 10-fold slower than the rate of deprotonation of Asp75^{ppR} in the unphotolyzed state. These data indicate that during the

photocycle, substantial structural changes occur and ppR_O maintains a distorted conformation, which creates a barrier for deprotonation of Asp75. The distorted conformation in ppR_O may be consistent with the results of the high-pressure experiments. The $ppR_O \rightarrow ppR$ transition requires a large-scale conformational change accompanied by a transient decrease in the protein volume, whereas the $O \rightarrow BR$ transition does not require this. The conformational change in the $ppR_O \rightarrow ppR$ transition may be essential for the subsequent deprotonation of Asp75.

A distorted conformation in ppR_O , as well as the hydrophobic nature of the EC channel, most likely serves the purpose of slowing deprotonation of Asp75 in the $ppR_O \rightarrow ppR$ transition. These changes might be important to prolong the active signaling state for efficient signal transduction, because the O intermediate is also a signaling state (48).

CONCLUSION

In this article, we have shown striking differences in the effect of increasing hydrostatic pressure on the decay of M and O in BR and ppR . In BR, these reactions become slower at higher pressure whereas in ppR they become faster. In BR, they are accompanied by positive activation volumes, and in ppR the activation volumes are negative. Assuming that the two major contributions to activation volume are hydration and protein repacking, we conclude the following. For BR, the reprotonation of the Schiff base during the M decay is facilitated by the hydration of the CP channel. For ppR as well as its L40T/F86D mutant, positive contributions resulting from hydration were not detected, indicating the absence of the transient hydration. These observations apparently reflect differences in local environments in the CP and EC channels. The L40T/F86D mutant exhibits a fast reprotonation of the Schiff base, with a rate comparable with that in BR, but this still exhibits a negative activation volume.

The O decay of BR appears to be accompanied by a rather small conformational change. On the other hand, a large negative activation volume, suggesting a large conformational change, occurs during the decay of ppR_O . Unlike BR, the distorted conformation of ppR_O apparently prolongs the lifetime of ppR_O itself, one of the signaling states. pH-jump experiments show a 20-fold decrease in the rate of Asp75 deprotonation in the ground-state compared to Asp85 in BR, in agreement with the lack of a hydrogen-bonded network connecting the counterion of ppR with the bulk. Thus, a drastic decrease in the rate of proton transport reactions is observed not only in the CP, but also in the EC, channel, and these are utilized in ppR to extend the lifetime of the signaling states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Janos K. Lanyi for helpful suggestions and improving the manuscript. This work was supported in part by NIH grant HL16101 (to R.G.).

References

1. Kamo N, Shimono K, Iwamoto M, Sudo Y. Photochemistry and photoinduced proton-transfer by *pharaonis* phoborhodopsin. *Biochemistry (Mosc)*. 2001; 66:1277–1282. [PubMed: 11743872]
2. Klare JP, Gordeliy VI, Labahn J, Büldt G, Steinhoff HJ, Engelhard M. The archaeal sensory rhodopsin II/transducer complex: A model for transmembrane signal transfer. *FEBS Lett*. 2004; 564:219–224. [PubMed: 15111099]
3. Moukhametzianov R, Klare JP, Efremov R, Baeken C, Göppner A, Labahn J, Engelhard M, Büldt G, Gordeliy VI. Development of the signal in sensory rhodopsin and its transfer to the cognate transducer. *Nature*. 2006; 440:115–119. [PubMed: 16452929]
4. Spudich JL, Luecke H. Sensory rhodopsin II: Functional insights from structure. *Curr Opin Struct Biol*. 2002; 12:540–546. [PubMed: 12163079]
5. Lanyi JK. Bacteriorhodopsin. *Annu Rev Physiol*. 2004; 66:665–688. [PubMed: 14977418]
6. Chizhov I, Schmies G, Seidel R, Sydor JR, Lüttenberg B, Engelhard M. The photophobic receptor from *Natronobacterium pharaonis*: Temperature and pH dependencies of the photocycle of sensory rhodopsin II. *Biophys J*. 1998; 75:999–1009. [PubMed: 9675200]
7. Furutani Y, Iwamoto M, Shimono K, Kamo N, Kandori H. FTIR spectroscopy of the M photointermediate in *pharaonis* phoborhodopsin. *Biophys J*. 2002; 83:3482–3489. [PubMed: 12496114]
8. Imamoto Y, Shichida Y, Hirayama J, Tomioka H, Kamo N, Yoshizawa T. Chromophore configuration of *pharaonis* phoborhodopsin and its isomerization on photon absorption. *Biochemistry*. 1992; 31:2523–2528. [PubMed: 1547236]
9. Schmies G, Lüttenberg B, Chizhov I, Engelhard M, Becker A, Bamberg E. Sensory rhodopsin II from the haloalkaliphilic *Natronobacterium pharaonis*: Light-activated proton transfer reactions. *Biophys J*. 2000; 78:967–976. [PubMed: 10653809]
10. Sudo Y, Iwamoto M, Shimono K, Sumi M, Kamo N. Photo-induced proton transport of *pharaonis* phoborhodopsin (sensory rhodopsin II) is ceased by association with the transducer. *Biophys J*. 2001; 80:916–922. [PubMed: 11159458]
11. Iwamoto M, Hasegawa C, Sudo Y, Shimono K, Arais T, Kamo N. Proton release and uptake of *pharaonis* phoborhodopsin (sensory rhodopsin II) reconstituted into phospholipids. *Biochemistry*. 2004; 43:3195–3203. [PubMed: 15023069]
12. Iwamoto M, Shimono K, Sumi M, Koyama K, Kamo N. Light-induced proton uptake and release of *pharaonis* phoborhodopsin detected by a photoelectrochemical cell. *J Phys Chem B*. 1999; 103:10311–10315.
13. Chizhov I, Chernavskii DS, Engelhard M, Mueller KH, Zubov BV, Hess B. Spectrally silent transitions in the bacteriorhodopsin photocycle. *Biophys J*. 1996; 71:2329–2345. [PubMed: 8913574]
14. Miyazaki M, Hirayama J, Hayakawa M, Kamo N. Flash photolysis study on *pharaonis* phoborhodopsin from a haloalkaliphilic bacterium (*Natronobacterium pharaonis*). *Biochim Biophys Acta*. 1992; 1140:22–29.
15. Radzwill N, Gerwert K, Steinhoff HJ. Time-resolved detection of transient movement of helices F and G in doubly spin-labeled bacteriorhodopsin. *Biophys J*. 2001; 80:2856–2866. [PubMed: 11371459]
16. Sass HJ, Büldt G, Gessenich R, Hehn D, Neff D, Schlesinger R, Berendzen J, Ormos P. Structural alterations for proton translocation in the M state of wild-type bacteriorhodopsin. *Nature*. 2000; 406:649–653. [PubMed: 10949308]
17. Subramaniam S, Henderson R. Molecular mechanism of vectorial proton translocation by bacteriorhodopsin. *Nature*. 2000; 406:653–657. [PubMed: 10949309]
18. Balashov SP. Protonation reactions and their coupling in bacteriorhodopsin. *Biochim Biophys Acta*. 2000; 1460:75–94. [PubMed: 10984592]
19. Bordignon B, Klare JP, Holterhues J, Martell S, Krasnaberski A, Engelhard M, Steinhoff HJ. Analysis of light-induced conformational changes of *Natronomonas pharaonis* sensory rhodopsin II by time-resolved electron paramagnetic resonance spectroscopy. *Photochem Photobiol*. 2007; 83:263–272. [PubMed: 16961434]

20. Sato Y, Hata M, Neya S, Hoshino T. Computational analysis of the transient movement of helices in sensory rhodopsin II. *Protein Sci.* 2005; 14:183–192. [PubMed: 15576566]
21. Wegener AA, Chizhov I, Engelhard M, Steinhoff HJ. Time-resolved detection of transient movement of helix F in spin-labelled *pharaonis* sensory rhodopsin II. *J Mol Biol.* 2000; 301:881–891. [PubMed: 10966793]
22. Yoshida H, Sudo Y, Shimono K, Iwamoto M, Kamo N. Transient movement of helix F revealed by photo-induced inactivation by reaction of a bulky SH-reagent to cysteine-introduced *pharaonis* phoborhodopsin (sensory rhodopsin II). *Photochem Photobiol Sci.* 2004; 3:537–542. [PubMed: 15170482]
23. Iwamoto M, Shimono K, Sumi M, Kamo N. Positioning proton-donating residues to the Schiff-base accelerates the M-decay of *pharaonis* phoborhodopsin expressed in *Escherichia coli*. *Biophys Chem.* 1999; 79:187–192. [PubMed: 10443011]
24. Klare JP, Schmies G, Chizhov I, Shimono K, Kamo N, Engelhard M. Probing the proton channel and the retinal binding site of *Natronobacterium pharaonis* sensory rhodopsin II. *Biophys J.* 2002; 82:2156–2164. [PubMed: 11916871]
25. Seidel R, Scharf B, Gautel M, Kleine K, Oesterhelt D, Engelhard M. The primary structure of sensory rhodopsin II: A member of an additional retinal protein subgroup is coexpressed with its transducer, the halobacterial transducer of rhodopsin II. *Proc Natl Acad Sci USA.* 1995; 92:3036–3040. [PubMed: 7708770]
26. Iwamoto M, Sudo Y, Shimono K, Arais T, Kamo N. Correlation of the O-intermediate rate with the pK_a of Asp-75 in the dark, the counterion of the Schiff base of *pharaonis* phoborhodopsin (sensory rhodopsin II). *Biophys J.* 2005; 88:1215–1223. [PubMed: 15533927]
27. Váró G, Lanyi JK. Effects of hydrostatic pressure on the kinetics reveal a volume increase during the bacteriorhodopsin photocycle. *Biochemistry.* 1995; 34:12161–12169. [PubMed: 7547956]
28. Druckmann S, Ottolenghi M, Rouso I, Friedman N, Sheves M. Time-resolved titrations of the Schiff base and of the Asp⁸⁵ residue in artificial bacteriorhodopsins. *Biochemistry.* 1995; 34:12066–12074. [PubMed: 7547945]
29. Richter HT, Needleman R, Kandori H, Maeda A, Lanyi JK. Relationship of retinal configuration and internal proton transfer at the end of the bacteriorhodopsin photocycle. *Biochemistry.* 1996; 35:15461–15466. [PubMed: 8952499]
30. Becher BM, Cassim JY. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep Biochem.* 1975; 5:161–178. [PubMed: 1144313]
31. Shimono K, Iwamoto M, Sumi M, Kamo N. Functional expression of *pharaonis* phoborhodopsin in *Escherichia coli*. *FEBS Lett.* 1997; 420:54–56. [PubMed: 9450549]
32. Sudo Y, Iwamoto M, Shimono K, Kamo N. *Pharaonis* phoborhodopsin binds to its cognate truncated transducer even in the presence of a detergent with a 1:1 stoichiometry. *Photochem Photobiol.* 2001; 74:489–494. [PubMed: 11594066]
33. Kikukawa T, Arais T, Shimozawa T, Mukasa K, Kamo N. Restricted motion of photoexcited bacteriorhodopsin in purple membrane containing ethanol. *Biophys J.* 1997; 73:357–366. [PubMed: 9199800]
34. Balashov SP, Lu M, Imasheva ES, Govindjee R, Ebrey TG, Othersen B 3rd, Chen Y, Crouch RK, Menick DR. The proton release group of bacteriorhodopsin controls the rate of the final step of its photocycle at low pH. *Biochemistry.* 1999; 38:2026–2039. [PubMed: 10026285]
35. Ames JB, Mathies RA. The role of back-reactions and proton uptake during the N → O transition in bacteriorhodopsin's photocycle: A kinetic resonance Raman study. *Biochemistry.* 1990; 29:7181–7190. [PubMed: 2169875]
36. Gillbro T. Flash kinetic study of the last steps in the photoinduced reaction cycle of bacteriorhodopsin. *Biochim Biophys Acta.* 1978; 504:175–186. [PubMed: 708721]
37. Neuman RC Jr, Kauzmann W, Zipp A. Pressure dependence of weak acid ionization in aqueous buffers. *J Phys Chem.* 1973; 77:2687–2691.
38. Luecke H, Schobert B, Richter HT, Cartailler JP, Lanyi JK. Structure of bacteriorhodopsin at 1.55 Å resolution. *J Mol Biol.* 1999; 291:899–911. [PubMed: 10452895]
39. Maeda A, Gennis RB, Balashov SP, Ebrey TG. Relocation of water molecules between the Schiff base and the Thr46-Asp96 region during light-driven unidirectional proton transport by

- bacteriorhodopsin: An FTIR study of the N intermediate. *Biochemistry*. 2005; 44:5960–5968. [PubMed: 15835885]
40. Schobert B, Brown LS, Lanyi JK. Crystallographic structures of the M and N intermediates of bacteriorhodopsin: Assembly of a hydrogen-bonded chain of water molecules between Asp-96 and the retinal Schiff base. *J Mol Biol*. 2003; 330:553–570. [PubMed: 12842471]
41. Száraz S, Oesterhelt D, Ormos P. pH-induced structural changes in bacteriorhodopsin studied by Fourier transform infrared spectroscopy. *Biophys J*. 1994; 67:1706–1712. [PubMed: 7819502]
42. Cao Y, Váró G, Klinger AL, Czajkowsky DM, Braiman MS, Needleman R, Lanyi JK. Proton transfer from Asp-96 to the bacteriorhodopsin Schiff base is caused by a decrease of the pK_a of Asp-96 which follows a protein backbone conformational change. *Biochemistry*. 1993; 32:1981–1990. [PubMed: 8448157]
43. Dioumaev AK, Brown LS, Needleman R, Lanyi JK. Coupling of the reisomerization of the retinal, proton uptake, and reprotonation of Asp-96 in the N photointermediate of bacteriorhodopsin. *Biochemistry*. 2001; 40:11308–11317. [PubMed: 11560478]
44. Zscherp C, Schlesinger R, Tittor J, Oesterhelt D, Heberle J. In situ determination of transient pK_a changes of internal amino acids of bacteriorhodopsin by using time-resolved attenuated total reflection Fourier-transform infrared spectroscopy. *Proc Natl Acad Sci USA*. 1999; 96:5498–5503. [PubMed: 10318912]
45. Brown LS, Lanyi JK. Determination of the transiently lowered pK_a of the retinal Schiff base during the photocycle of bacteriorhodopsin. *Proc Natl Acad Sci USA*. 1996; 93:1731–1734. [PubMed: 8643698]
46. Inoue K, Sasaki J, Morisaki M, Tokunaga F, Terazima M. Time-resolved detection of sensory rhodopsin II-transducer interaction. *Biophys J*. 2004; 87:2587–2597. [PubMed: 15454453]
47. Taniguchi Y, Ikehara T, Kamo N, Yamasaki H, Toyoshima Y. Dynamics of light-induced conformational changes of the phoborhodopsin/transducer complex formed in the *n*-dodecyl β -D-maltoside micelle. *Biochemistry*. 2007; 46:5349–5357. [PubMed: 17432830]
48. Yan B, Takahashi T, Johnson R, Spudich JL. Identification of signaling states of a sensory receptor by modulation of lifetimes of stimulus-induced conformations: The case of sensory rhodopsin II. *Biochemistry*. 1991; 30:10686–10692. [PubMed: 1931988]

Figure 1.

Flash-induced absorbance changes at various hydrostatic pressures for BR (a), *ppR* (b), *ppR*-*pHtrII*₁₋₁₅₉ complex (c) and L40T/F86D mutant *ppR* (d). The tested wavelengths are denoted in the panels. Pressures applied on samples were 1, 180, 360, 540, 720, 900 bar for BR (a) and *ppR* (b), 1, 225, 450, 675, 900 bar for *ppR*-*pHtrII* complex (c). For L40T/F86D mutant *ppR* (d), only two or three data are plotted here to show the pressure dependences clearly. These pressures are 1, 450 and 900 bar for 390 nm, and 1 and 900 bar for 500 and 560 nm, respectively. The bestfit curves are shown by the smooth lines (see text for details).

Figure 2.

Pressure dependence of rate constants of BR, ppR, ppR-*pHtrII*₁₋₁₅₉ complex and L40T/F86D mutant ppR. $k_{f,680}$ and $k_{s,680}$ in BR denote the apparent rate constants for the concentration changes of the O intermediate (determined from a fit of the absorption changes observed at 680 nm). For the L40T/F86D mutant, the data in early and later time regions were analyzed independently. For the data in later time regions, we tentatively adopted the scheme, O1 → O2 → ground-state (see text for details).

Figure 3.

(a) Absorption spectra of *ppR* taken after changing the pH from 2 to 7 during the first 100 ms. Curves 1 through 12 are measured at 1.3, 3.8, 6.4, 9.0, 11.5, 16.6, 24.3, 29.4, 37.1, 50, 75.5 and 101 ms after the pH jump. (b) Difference absorption spectra obtained by subtracting spectrum 1 on panel (a) from the subsequent spectra. (c) Kinetics of absorption changes at 545 nm.

Table 1

Activation volumes for the photocycle reactions after formation of the M intermediate* (mL mol⁻¹).

Reaction	BR	ppR	ppR-pHtrII ₁₋₁₅₉	L40T/F86D
M → O	47.3 ± 1.8 [†] 37.1 ± 2.2 [‡]	-4.1 ± 1.4	-9.8 ± 0.8	-10.3 ± 1.0
O → ground-state	9.8 ± 2.5	-48.4 ± 2.6	-34.0 ± 4.3	-20.7 ± 6.1 [§] -19.2 ± 1.8 ^{//}

* Activation volumes and their standard errors were calculated from slopes in Fig. 2.

^{†,‡} Activation volumes for M → N and M → N → O transitions, respectively.

^{§,||} Activation volumes for O₁ → O₂ and O₂ → ground-state transitions, respectively.