# Effect of Anions on the Photocycle of Halorhodopsin. Substitution of Chloride with Formate Anion†

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*Received July 11, 2005; Revised Manuscript Received August 24, 2005*

ABSTRACT: Halorhodopsin from *Natronomonas pharaonis* is a light-driven chloride pump which transports a chloride anion across the plasma membrane following light absorption by a retinal chromophore which initiates a photocycle. It was shown that the chloride anion bound in the vicinity of retinal PSB can be replaced by several inorganic anions, including azide which converts the chloride pump into a proton pump and induces formation of an M-like intermediate detected in the bR photocycle but not in native halorhodopsin. Here we have studied the possibility of replacing the chloride anion with organic anions and have followed the photocycle under several conditions. It is revealed that the chloride can be replaced with a formate anion but not with larger organic anions such as acetate. Flash photolysis experiments detected in the formate pigment an M-like intermediate characterized by a lifetime much longer than that of the O intermediate. The lifetime of the M-like intermediate depends on the pH, and its decay is significantly accelerated at low pH. The decay rate exhibited a titration-like curve, suggesting that the protonation of a protein residue controls the rate of M decay. Similar behavior was detected in *N. pharaonis* pigments in which the chloride anion was replaced with $NO_2^-$ and $OCN^-$ anions. It is suggested that the formation of the M-like intermediate indicates branching pathways from the L intermediate or basic heterogeneity in the original pigment.

Retinal proteins serve as photoreceptors and are found in lower and higher organisms. They play a key role in signal transduction by signaling the presence and characteristics of ambient light and triggering important concomitant functions. These systems are associated with a wide range of light-induced biological functions such as vision (rhodopsin, Rho), proton pump-driven photosynthesis (bacteriorhodopsin, bR),[1] chloride pump (halorhodopsin, HR), and phototaxis (sensory rhodopsin, SRI and SRII). Halorhodopsin (HR) was discovered in the archaea *Halobacterium salinarum* (*1*). The pigment serves as an inward-directed chloride pump (*2*), and its function is based on a retinal chromophore (all-trans) covalently bound to a lysine residue to form a PSB linkage.

Various analogues of HR were found, but the most studied pigments are from *H. salinarum* (sHR) and *Natronomonas pharaonis* (pHR) (*3*). These pigments are involved in transport of chloride into the cell (*4*). An important difference between sHR and pHR is the ratio between trans and cis retinal isomers. In the dark-adapted form, sHR comprises 45% all-trans isomer, while the percentage increases to 75% following the light adaptation process. In pHR, however, the all-trans percentage (85%) does not change in the dark and light forms (*5*, *6*). Importantly, in contrast to sHR, the 13-cis pigment does not have a detectable photocycle (*5*) which simplifies the studies of the pHR all-trans retinal photocycle.

The amino acid sequence of HR was determined (*7*) and is considerably similar to that of bR. Consistent with the lack of proton pumping activity of HR, sequence alterations were found in crucial residues. HR is lacking the equivalent of Asp85 which serves as the SB proton acceptor in the bR photocycle. This residue is replaced in both pHR and sHR with Thr which is part of the chloride binding site. In bR, Asp96 is the proton donor to the SB in the second half of the photocycle and is substituted with Ala in HR.

The chloride is bound in the vicinity of the SB as detected by resonance Raman spectroscopy (*8*, *9*). Recently, the high-resolution structure of sHR indicated that the chloride anion is part of the SB counterion and replaces Asp85 of bR (*10*). Light absorption by the retinal chromophore initiates a photocycle in which the chloride anion is transferred between several binding sites, resulting in a net translocation of one chloride from the extracellular membrane to the cytoplasmic side (*11*, *12*). The photocycle of sHR and pHR was studied by several methods. The intermediates of the photocycle and their absorption spectra were determined by time-resolved absorption spectroscopy (*5*, *13*, *14*). In addition, the photocycle was studied by infrared spectroscopy (*15*−*17*) as well as by resonance Raman spectroscopy (*18*−*20*). The details of the photocycle are still under debate, but it is generally accepted that the photocycle comprises (besides the ultrafast

† This work was financially supported by the Fund for Basic Research (administered by The Israel Academy of Sciences and Humanities), by the Human Frontier Science Program and the Kimmelman Center for Biomolecules Structure (to M.S.), and by the Deutsche Forschungsgemeinschaft (to M.E.). M.S. holds the Katzir-Makineni professorial chair in chemistry.

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[1] Abbreviations: bR, bacteriorhodopsin; DM, *N*-dodecyl β-D-maltoside; HR, halorhodopsin; pHR, *N. pharaonis* halorhodopsin; PSB, protonated Schiff base; SB, Schiff base; sHR, *H. salinarum* halorhodopsin; SR, sensory rhodopsin.

10.1021/bi051323g CCC: $30.25 
Published on Web 10/04/2005

primary events) K, L, N, O, HR′, and HR intermediates. The O intermediate is accumulated in the pHR photocycle but not in sHR (*21*). In pHR, the chloride anion is released between N and O intermediates and the uptake process takes place either between O and HR′ intermediates (*11*, *22*, *23*) or in a transition between two spectrally silent O intermediates (*12*).

The chloride anion can be replaced with several anions, including bromide, iodide, nitrate, fluoride, sulfate, and azide (*24*), but the PSB does not undergo a deprotonation process during the photocycle except in the case of azide (*24*, *25*). In the presence of azide, the chloride pump is converted into a proton pump in a manner similar to that of bR (*26*). The M-like intermediate of HR formed in the presence of azide was studied both under steady-state conditions and via flash photolysis experiments (*24*, *25*, *27*). The rate of M-like formation and decay was in the range of the photocycle kinetic, and the M intermediate was suggested to exist in equilibrium with photocycle intermediates (*25*).

In this report, we have further studied the possibility of replacing the chloride anion in its strong binding site with several anions. We have revealed that it is possible to replace the chloride with an organic anion (formate) which induces, following flash photolysis, an M-like intermediate. The decay of the M intermediate was much slower than that of the O intermediate, clearly indicating two separate photocycles. It is concluded that the M-like intermediate is formed in a branching process from the L intermediate or due to heterogeneity in the initial pigment.

## MATERIALS AND METHODS

*Sample Preparation.* pHR was expressed in *Escherichia coli* and isolated according to a previously published method (*28*).

Anion exchange of the DM-solubilized protein was achieved by a 12 h dialysis of the sample against the appropriate salt. The final medium composition was 25 mM buffer, 0.1 M NaX (where X is $OCN^-$, $SCN^-$, $HCOO^-$, $NO_2^-$, or $N_3^-$), and 0.05% DM.

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

*Titration Experiments.* Titrations were performed in the dark by addition of small amounts of base (typically, 2 $\mu$L of 0.01−0.1 M NaOH, as appropriate) to the pigment without the buffer, followed by absorption measurements. The absorption at the $\lambda_{max}$ of the PSB was plotted against the pH, and the p$K_a$ value was 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 pH, and p$K_a$ is the midpoint of the observed transition.

*Pulsed Laser Photolysis.* Pulsed laser photolysis was carried out using laser pulses from a Nd:YAG laser (532 nm, 9 ns). Light-induced absorbance changes were recorded using a continuous 75 W xenon lamp, a photomultiplier, and a TDS Tektronix digitizer. Unless otherwise mentioned, 150 pulses were averaged and analyzed. Photolytic effects due to the monitoring beam were minimized by placing an interference filter between the lamp and the sample and by using a mechanical shutter synchronized with the laser pulse. The concentration of the protein ranged between $7 \times 10^{-6}$ and $1.4 \times 10^{-5}$ M.

Table 1: Absorption Maxima and p$K_a$ Values of SB Titration of pHR Incubated with Various Salts

| salt[a] | $\lambda_{max}$ at pH 7 (nm) | p$K_a$[b] |
|---|---|---|
| NaCl | 578 | 10 |
| NaSCN | 571 | 9.8 |
| $NaNO_2$ | 575 | 9.8 |
| $NaN_3$ | 568 | 9.3 |
| NaOCN | 573 | 8.8 |
| NaHCOO | 568 | 8.5 |

[a] At 0.1 M. [b] The error for p$K_a$ values is ±0.1.

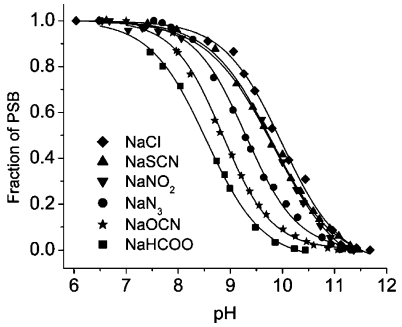


FIGURE 1: Effect of the various anions (0.1 M) on the p$K_a$ of the retinal SB. Absorbance changes monitored at the $\lambda_{max}$ of each pigment.

## RESULTS

Previous studies have shown that chloride ion can be substituted with various anions in pHR without significantly affecting the pigment absorption maximum (*24*). The chloride anion was substituted with $Br^-$, $I^-$, $F^-$, $BrO_3^-$, $N_3^-$, and $NO_3^-$. We have tried to introduce into the pHR binding site an organic anion and to enlarge the series of anions with anions characterized by different p$K_a$ values. The chloride anion was replaced with $HCOO^-$, $OCN^-$, $SCN^-$, $NO_2^-$, and $N_3^-$ using a dialysis process to yield stable pigments. Interestingly, exchange with $CH_3COO^-$, $CF_3COO^-$, and $Cl_2CHCOO^-$ failed to yield stable pigments. In addition, phthalate anion led to formation of ~30% modified pigment (565 nm), but the pigment was unstable and difficult to handle. Table 1 summarizes the absorption maxima of various pigments and indicates that the absorption maxima were dependent on the nature of the anion. For the $HCOO^-$ anion, the absorption maximum was also measured at different pH values. Following the pH decrease, the absorption maximum shifted from 571 nm at pH 8 to 560 nm at pH 4, in keeping with similar studied anions (*24*). The anions also affected the p$K_a$ of the SB as shown in Table 1. The p$K_a$ shifted from 10 with chloride ion to 8.5 with $HCOO^-$ (Figure 1). The p$K_a$ of $HCOO^-$ is similar to the p$K_a$ measured previously for the blue form of pHR (p$K_a$ = 8.5) (*24*).

*Deprotonation of the PSB following Flash Photolysis.* Previous studies have established that replacement of chloride ion with azide ion allows for PSB deprotonation following flash photolysis, resulting in formation of the M-like photointermediate similar to that detected in bR. We have studied the photocycles of the various pigments in which the chloride ion was replaced with different anions and examined factors

Table 2: A Correlation between the p$K_a$ Values of the Various Anions and the Formation of the M-like Intermediate

| salt | p$K_a$ of the corresponding acid[a] | appearance of the M-like intermediate at pH 7 |
|---|---|---|
| NaCl | less than −1 | no |
| $NaNO_3$ | −1.44 | no |
| NaSCN | 0.9 | no |
| $NaNO_2$ | 3.22 | yes |
| $NaN_3$ | 4.63 | yes |
| NaOCN | 3.45 | yes |
| NaHCOO | 3.73 | yes |

[a] Values are in water.

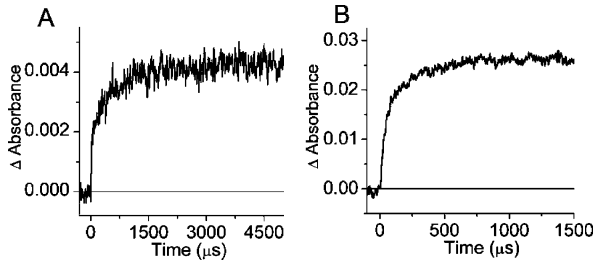


FIGURE 2: Laser-induced absorbance changes of pHR pigments monitored at 420 nm (M-like intermediate). (A) Pigment incubated with 0.1 M NaHCOO at pH 7. (B) Pigment incubated with 0.1 M NaOCN at pH 7.4.

Table 3: Decay Rates of the M-like Intermediate of pHR Incubated with Various Salts Monitored at 420 nm at Different pH Values

| | $k$ for decay at 420 nm ($ms^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| salt | pH 8 | pH 7 | pH 6 | pH 5 | pH 4 | p$K_a$[c] |
| $NaNO_2$ | 0.004 | 0.03 | 0.316 | 1.22 | 1.2 | 5.8 |
| $NaN_3$ | 0.306[a] | 0.235 | 0.93 | 1.01[b] | | 6.6 |
| NaHCOO | 0.002 | 0.004 | 0.051 | 0.42 | 0.54 | 5.3 |
| NaOCN | | 0.03 | | | | |

[a] At pH 7.88. [b] At pH 5.4. [c] The error for p$K_a$ values is ±0.1.

controlling the formation and decay of the M-like intermediate. As shown in Table 2, only anions for which their corresponding acid has a p$K_a$ above 3.22 yielded the M-like intermediate (measured at 420 nm). For $HCOO^-$ ($k_1 = 0.037$ $\mu s^{-1}$; $k_2 = 0.0019$ $\mu s^{-1}$) and $OCN^-$ ($k_1 = 0.034$ $\mu s^{-1}$; $k_2 = 0.0071$ $\mu s^{-1}$), a biexponential formation of the 420 nm absorption was detected (Figure 2). For $NO_2^-$, the biexponential kinetics was less pronounced and at several pH values was not detected at all. In the pHR($N_3^-$) pigment, the formation of the M-like intermediate was monoexponential.

The decay rate of the absorption at 420 nm was strongly dependent on the pH of the sample (Table 3 and Figure 3), and was significantly accelerated at low pH. A similar pH dependence was observed previously for pHR($N_3^-$) (*26*). In pHR($HCOO^-$), pHR($NO_2^-$), and pHR($N_3^-$), the decay rate exhibited a titration-like curve. As shown in Figure 4, the p$K_a$ values for the different pigments are 5.3 for pHR($HCOO^-$), 5.8 for pHR($NO_2^-$), and 6.6 for pHR($N_3^-$).

*Photocycle of pHR Incubated with Specific Anions*. The photocycles of several pHR pigments incubated with different salts to substitute the chloride anion were studied by monitoring absorption changes at four different wavelengths. The traces of the corresponding absorbance changes of pHR($HCOO^-$) and pHR($NO_2^-$) at two pH values are presented as representative examples in Figures 5 and 6. As described above, laser excitation of pHR pigments in which the $Cl^-$ was replaced with $HCOO^-$, $NO_2^-$, $OCN^-$, and $N_3^-$

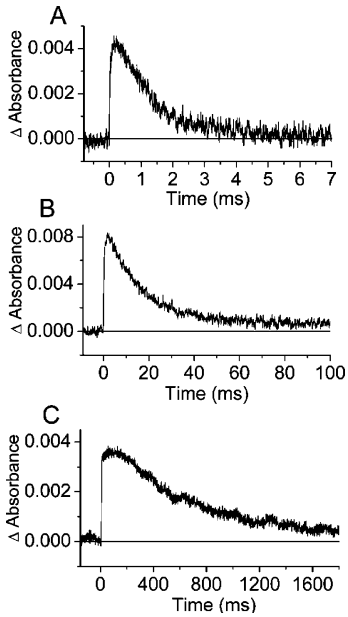


FIGURE 3: Laser-induced absorbance changes of pHR incubated with 0.1 M NaHCOO at 420 nm (M-like intermediate) as a function of pH: (A) pH 4, (B) 6, and (C) 8.

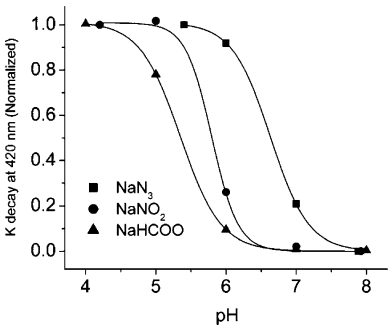


FIGURE 4: Rate of decay of the M-like intermediate (at 420 nm) as a function of pH in pHR incubated with NaHCOO, $NaNO_2$, and $NaN_3$.

yielded an M-like intermediate detected at 420 nm, which exhibited a pH dependence of the decay rate. Strikingly, in both $HCOO^-$- and $NO_2^-$-bound complexes, the lifetime of the M-like intermediate (at pH 7) was much longer than that of the O intermediate (Figures 5A and 6A). In pHR($HCOO^-$), the M-like intermediate lifetime ($k = 0.004$ $ms^{-1}$) was ~200 times longer than that of the O intermediate ($k = 0.0009$ $\mu s^{-1}$), while in pHR($NO_2^-$), it was ~70 times longer ($k = 0.03$ $ms^{-1}$ vs 0.002 $\mu s^{-1}$). When the pH was lowered, the M-like intermediate decay rate was accelerated, approaching that of the O intermediate, such that in pHR($HCOO^-$) at pH 5 the M-like intermediate lifetime was only 2 times longer than that of the O intermediate (Figure 5B). The experiments described above were carried out in the presence of 0.1 M NaHCOO; however, increasing the concentration to 0.5 M NaHCOO did not affect significantly the photocycle, and the M lifetime was much longer than that of the O intermediate (data not shown). In pHR($NO_2^-$) at pH 4, the M-like intermediate decayed before the O intermediate (Figure 6B), while in pHR($N_3^-$), the M-like intermediate and O had similar lifetimes. We note that excitation of pHR($N_3^-$) above pH 6 did not lead to O intermediate accumulation which prevented comparison of M-like and O intermediate lifetimes (data not shown). The L intermediate completed

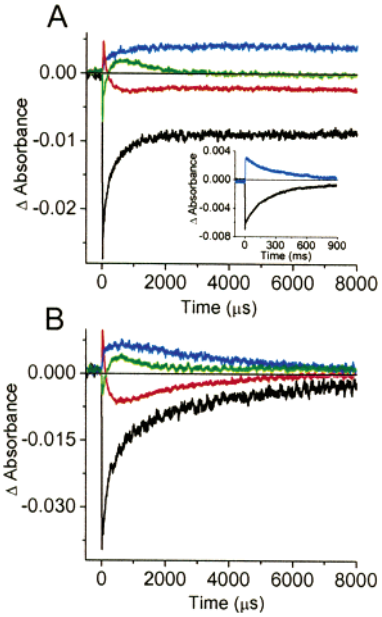


FIGURE 5: Laser-induced absorbance changes of pHR incubated with 0.1 M HCOONa, monitored at 570 (black), 420 (blue), 500 (red), and 650 nm (green), at pH 7 (A) and 5 (B).

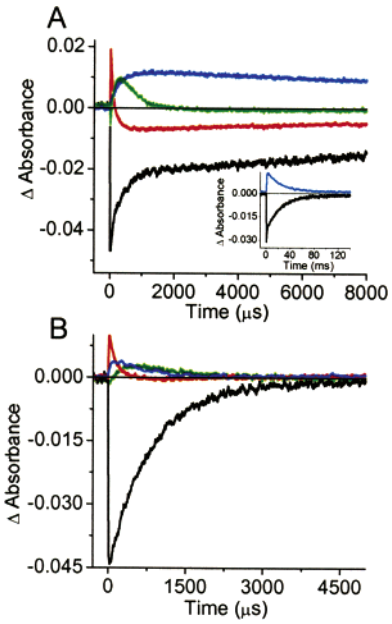


FIGURE 6: Laser-induced absorbance changes of pHR incubated with 0.1 M $NaNO_2$, monitored at 570 (black), 420 (blue), 500 (red), and 650 nm (green) at pH 7 (A) and 4.2 (B).

its decay in ~100 $\mu$s at all measured pH values. PHR($SCN^-$) does not exhibit M-like formation (Figure 7A), probably since the corresponding acid of $SCN^-$ has a p$K_a$ of 0.9 (Table 2). In contrast, the M-like intermediate was detected for pHR($OCN^-$) (Figure 7B) in which the corresponding acid of its anion ($OCN^-$) has a p$K_a$ of 3.45.

Since pHR($N_3^-$) exhibited a similar decay rate for M and O intermediates, we have looked for a possible separation of their decay rates. We have revealed that pHR($Cl^-$) experienced retinal hydrolysis following warming to 338 K and pigment re-formation upon cooling to 298 K (K. Mevorat-Kaplan and M. Sheves, unpublished results). However, the re-formed pigment exhibited a modified photocycle in which the decay of the O intermediate is significantly

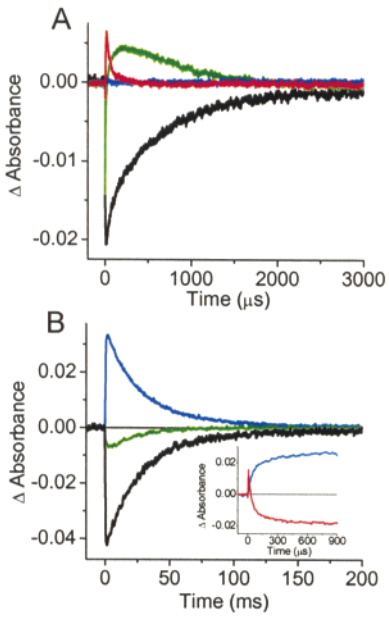


FIGURE 7: (A) Laser-induced absorbance changes of pHR incubated with 0.1 M NaSCN at pH 7, monitored at 570 (black), 420 (blue), 500 (red), and 650 nm (green). (B) Laser-induced absorbance changes of pHR($OCN^-$) in 0.1 M NaOCN at pH 7.4. Absorption was monitored at 570 (black), 420 (blue), 500 (red), and 650 nm (green).

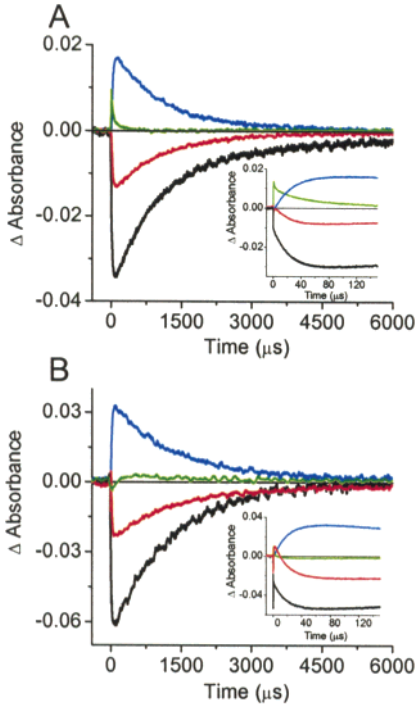


FIGURE 8: Laser-induced absorbance changes of pHR incubated with 0.3 M $NaN_3$. (A) Regenerated pigment following hydrolysis at 65 °C and incubation at 25 °C. Measurements were carried out at pH 6.4, and the absorbance was monitored at 570 (black), 500 (red), 420 (blue), and 650 nm (green). (B) pHR($N_3^-$) in 0.3 M $NaN_3$ before hydrolysis at pH 6.4.

accelerated. Therefore, we have warmed pHR($N_3^-$) to 338 K to begin the hydrolysis process and pigment re-formation at 298 K. Figure 8 compares the photocycle of pHR($N_3^-$) before and after the process. Interestingly, a clear effect on the decay rate of the O intermediate is observed in which O decays faster than M. This result further supports the above-

described observations in several pHR pigments in which M decays slower than the O intermediate.

## DISCUSSION

It was previously shown that the chloride anion of the pHR complex can be replaced with $Br^-$, $NO_3^-$, $BrO_3^-$, and $N_3^-$ anions (*24*). The azide anion significantly affected the photocycle and induced the formation of an M-like intermediate. Similar behavior was observed in HR from *H. salinarum*. In this study, we have checked the ability of additional anions to bind and to modify the photocycle. The formate anion is the first observed organic anion that can replace the chloride, but we have failed to introduce larger anions probably due to steric constraints. The binding site cannot accommodate even acetic acid anion characterized by an additional methyl group relative to formate. The absorption maxima are affected by the nature of the anion probably due to different electrostatic interactions between the positively charged SB linkage and the counterion. It is well-known that this interaction is a major factor affecting the absorption maxima of retinal proteins in which weaker electrostatic interaction red shifts the absorption maximum (*29*−*31*). The p$K_a$ of the PSB is affected as well by the nature of the anion. In bR, the p$K_a$ of the SB is unusually high (ca. 13) (*32*, *33*), whereas the p$K_a$ of pHR SB is 9.6 in 150 mM NaCl (*24*). In the binding site of pHR, a chloride anion is substituted with an Asp85 anion present in the vicinity of bR SB. This difference may lead to the relatively large alteration in the SB p$K_a$. However, this study demonstrates that incorporating a carboxylate counterion instead of the chloride does not reinstate the high SB p$K_a$ found in bR and even induces a lower p$K_a$ relative to that of the chloride anion. It was suggested (*34*) that the specific geometry between the positively charged SB and its counterion controls the hydrogen bonding to these two groups, thereby regulating the p$K_a$ of the SB. This mechanism can account for the relatively low SB p$K_a$ in pHR (relative to bR) and explains the observation that the nature of the anion will modify the PSB p$K_a$ in pHR as observed for the various anions used in this study (Table 1).

Following light absorption, bR PSB experiences a deprotonation process to form an M intermediate which initiates the proton pumping activity. In contrast, the M intermediate does not form in the native pHR photocycle but was observed following substitution of the chloride with azide anion (*25*). The hydrogen bonding network in the retinal binding site of bR is considerably disturbed following light absorption such that the p$K_a$ of the PSB is significantly reduced and that of Asp85 is elevated (*34*). These changes induce transfer of a proton from the PSB to Asp85. In pigments in which such alterations do not occur, the M intermediate is not produced, but its formation can be stimulated by intrinsically reducing the PSB p$K_a$. For example, in the Y57N mutant (*35*) or in the 13-cis photocycle (pH 7) (*36*), the M intermediate was formed only by substitution of the retinal chromophore with a 14-F retinal, having the PSB p$K_a$ reduced by ca. 2 p$K_a$ units. We suggest that in pHR the p$K_a$ of $Cl^-$ anion is too low to serve as a proton acceptor even though the p$K_a$ of the PSB is significantly reduced. Therefore, substitution of the $Cl^-$ anion with one with a relatively high intrinsic p$K_a$ provides a potential proton acceptor and induces a proton transfer and formation of an M-like intermediate. These studies indicate that an anion with a p$K_a$ of $\geq 3.22$ (Table 2) is capable of serving as a proton acceptor. Therefore, an M intermediate was observed in several anions, including the formate anion.

Strikingly, it is observed that in several cases the M-like intermediate lifetime is much longer than that of the O intermediate. This observation can best be explained by the presence of two L intermediates in which one decays to the O-like intermediate which consequently decays to the original pigment, whereas the second L decays to an M-like intermediate. The M and O intermediates decay to the original pigment in parallel and with different rates which suggests photocycle heterogeneity. The presence of two L intermediates existing in equilibrium was suggested also for pHR($Cl^-$) (*23*). We note that the O intermediate possibly being a member of a 13-cis photocycle is very unlikely since as mentioned this retinal isomer does not display a photocycle (*5*). In addition, the 13-cis fraction in the pigment mixture as revealed by HPLC analysis (data not shown) is much smaller [ca. 20% in pHR($HCOO^-$)] than that of the photocycling pigment fraction related to the O intermediate as can be derived from the re-formed pigment fraction following O decay. The longer M lifetime can also be explained by protein heterogeneity in which the M intermediate is formed in a formate-bound pigment and the O intermediate is due to a formate-unbound pigment or to a $Cl^-$-bound "normal" pigment. This possibility is unlikely since increasing the formate concentration from 0.1 to 0.5 M did not significantly affect the photocycle.

A fundamental point in understanding the functional mechanism of bR relates to the explanation of the complex kinetics of the photocycle, which is still controversial even after decades of intensive investigations (*37*). In fact, in some works a basic heterogeneity of the bR system has been invoked (*38*, *39*). Recently, studies of retinal binding to the apomembrane pointed also to a heterogeneity in the binding process (*40*). Despite these investigations, the currently prevailing hypothesis is that bR is structurally and mechanistically homogeneous and that the complexity of the photocycle kinetics is only due to its intrinsic properties (such as branching pathways, cooperativity, and pH-induced heterogeneity). Our study indicating two distinct decay pathways, especially in pHR($HCOO^-$) and pHR($NO_2^-$), can be explained either by the basic heterogeneity of pHR or by branching pathways possibly following formation of L. We note that in pHR($HCOO^-$) and pHR($OCN^-$) a biexponential formation of M was clearly observed. As in bR, it can be explained by the heterogeneity of the M-like intermediate and an equilibrium between at least two M intermediates. The presence of two O intermediates were suggested for pHR($Cl^-$) (*13*). Possible mechanisms are described in the following scheme:

*a*)
$$pHR(HCOO^-) \xrightarrow{h\nu} L^1_{520} \Leftrightarrow L^2_{520} \Leftrightarrow O_{600} \Leftrightarrow\Leftrightarrow pHR(HCOO^-)$$
$$\Updownarrow$$
$$M_1 \Leftrightarrow M_2 \Leftrightarrow\Leftrightarrow pHR(HCOO^-)$$

*b*)
$$pHR^1(HCOO^-) \xrightarrow{h\nu} \longrightarrow L^1_{520} \Leftrightarrow O^1_{600} \Leftrightarrow\Leftrightarrow pHR^1(HCOO^-)$$

$$pHR^2(HCOO^-) \xrightarrow{h\nu} \longrightarrow L^2_{520} \Leftrightarrow M^2_1 \Leftrightarrow M^2_2 \Leftrightarrow\Leftrightarrow pHR^2(HCOO^-)$$

At low pH, the decay rate of the M-like intermediate is accelerated while the decay of O is not significantly affected. Therefore, it is plausible that the kinetic separation between the two pathways is diminished and the photocycle can be viewed as a uniform pathway. This interpretation might also hold for pHR($N_3^-$). It is possible that azide anion accelerates the rate of M decay, as previously observed for bR, preventing kinetic separation of the decay of M and O intermediates. This view is strongly supported by the experiment with the reconstituted pigment which clearly indicates a separation between the rates of decay of O and M intermediates also in pHR($N_3^-$). In this case, the M lifetime is much longer than that of O. The question of whether the branching process or heterogeneity exists in pHR($Cl^-$) in which the M intermediate is not formed exists. Clearly, the photocycle of pHR($Cl^-$) can be analyzed as one photocycle comprised of several equilibrium processes. However, the possibility of branching/heterogeneity processes should be the subject of future studies.

The acceleration of M-like decay at low pH is probably associated with a titration of a protein residue. The p$K_a$ of this residue is affected by the anion nature possibly through hydrogen bonding network modification. The decay of the M-like intermediate to the original pigment is associated with several events, including SB protonation, retinal double bond isomerization, and possibly relocation of the anion to its original location. The M-like decay in the pigments studied here is monoexponential, and therefore, it is plausible that SB protonation is the rate-determining step and probably occurs prior to the thermal double bond isomerization, since the barrier for thermal isomerization is significantly lower in the PSB (*41*). It was suggested that the anion is relocated to the cytoplasmic side following retinal isomerization and probably accepts the proton from the PSB to form the M-like intermediate. At present, it is not possible to identify the group that donates a proton to the SB during M decay, and it may be the protonated anion itself or a protein residue. However, it is plausible that the SB has to change its p$K_a$, and this process should probably be associated with hydrogen bonding network modification accelerated by the protonation of a protein residue.

## ACKNOWLEDGMENT

We thank Annika Goeppner for excellent technical help.

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BI051323G