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from *Natronomonas pharaonis*

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2013

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Abbreviations

BR	bacteriorhodopsin
CCCP	carbonyl cyanide <i>m</i> -chlorophenylhydrazone.
CP	cytoplasmic
DDM	dodecyl- β -D-maltoside
EC	extracellular
<i>E.coli</i>	<i>Escherichia coli</i>
<i>f</i>	fraction of N of N/O equilibrium
FTIR	Fourier transform infrared
HR	halorhodopsin
HsHR	HR from <i>Halobacterium salinarum</i>
$K_{d, N \leftrightarrow O}$	dissociation constant of Cl ⁻ at the N/O-equilibrium
K_d'	dissociation constant of Cl ⁻ at the assumed Cl ⁻ binding site at O
k_i	decay kinetic constant of P _i (i =1~4)
NpHR	HR from <i>Natronomonas pharaonis</i>
OD	optical density
SG	skewed Gaussian
SR	Sesory rhodopsin
PDB	Protein data bank
P _i	i-th photochemically defined intermediate (i =1~4)
PSB	Protonated Schiff base
$\Delta V_{N \rightarrow O}$	partial molar volume change between N and O with reference to N (see Eq. 6)
ΔV_i^*	activation volumes associated with the decay of P _i intermediates

ABSTRACT

Halorhodopsin (HR) is an inwardly-directed light-driven halogen ion pump, and NpHR is a HR from *Natronomonas pharaonis*. We made various mutants of Thr218 that is located one-half turn up from retinal, chromophore to the cytoplasm (CP) channel, and analyzed the photocycle using a sequential irreversible model. Four photochemically-defined-intermediates (P_i , $i = 1 - 4$) were adequate to describe the photocycle. The third component, P_3 was a quasi-equilibrium complex between N and O-intermediates, where $N \leftrightarrow O + Cl^-$ equilibrium attained. The $K_{d, N \leftrightarrow O}$ values of this equilibrium for various mutants were determined, and the value of Thr (wild-type) was the largest. From the pressure dependence of $K_{d, N \leftrightarrow O}$, the partial molar volume differences between N and O, $\Delta V_{N \rightarrow O}$ were estimated. Comparison between $K_{d, N \leftrightarrow O}$ and $\Delta V_{N \rightarrow O}$ led to the conclusion that the water entry possibly by the F-helix opening at O may occur, which may increase $K_{d, N \leftrightarrow O}$. For some mutants, however, large $\Delta V_{N \rightarrow O}$ were found while $K_{d, N \leftrightarrow O}$'s were small. This suggests that the special coordination of water molecule with the OH group of Thr is necessary. The k_2 ($L_2 \rightarrow N$ transition) presents possibly the rate constant of the transition in which Cl^- may be transferred from the extracellular (EC) to CP channel across the Schiff base. The wild-type showed the largest. Thus, Thr of wild-type is tuned to accomplish the fast transfer of Cl^- from EC to the CP space. The rate constant of k_3 is that of Cl^- transfer from EC space to the binding site near the Schiff base, and thus previous reports described the increase in k_3 with an increase in Cl^- concentration. This rate in the present experiment, however, seemed to show convex Cl^- dependency: In high Cl^- concentration region: the rate decreased with an increase in Cl^- concentration. This was explained by a putative binding site at an exterior part of EC channel, from where Cl^- may move to the binding site near Schiff base. The rate constant k_3 was the product of O-concentration (decrease with Cl^- concentration) and the degree of saturation of this putative site (increase with Cl^- concentration). The slowest rate was k_4 in which NpHR' (whose structure may be very similar to NpHR) transforms to the original NpHR for the wild-type. This implies that the rates of photocycle (turnover rates) are determined by this process, and then it is anticipated that the modification of T218 which modulates the equilibrium of $N \leftrightarrow O$ and the rates of k_2 and k_3 may have little effect on the pumping activity. However, the mutation changed the activity greatly. This implies that the interior negative membrane potential shifts $N \leftrightarrow O$ equilibrium toward N, and then k_3 becomes the slowest process. The large $K_{d, N \leftrightarrow O}$ is necessary not to decrease the activity in the presence of membrane potential, and in this sense, Thr at position 218 should be the best suitable residue.

Introduction

In the exchanges of such substances across the cell membrane, some are spontaneous and others are involuntary. The free energy for enabling involuntary exchanges is supplied by the primary active transporters, which generate from the external energy, such as chemical energy and light energy. Then, the secondary active transporters take substances in and out using the energy made by the primary active transporters. The key in both primary and secondary active transporters is the nature of the internal step that separates the release of a substrate at one surface from the uptake at the other, which is also the central question in the study of active transporter. In order to investigate this question, the light-driven ion pump is assumed to be more optimal model than chemical energy driven ion pump because light driven ion pump can turn its own activity on and off immediately by illumination and each step of ion transport can be traced easily by absorbance change.

Archaeal rhodopsin as the model of active transporter

Archaeal rhodopsin is a retinal membrane protein archaea have. The history of archaeal rhodopsin dates back to the discovery of bacteriorhodopsin (BR) in Haloarchaea *Halobacterium salinarum* in 1971 (1). BR transport one proton from cytoplasmic (CP) side to extracellular (EC) side through the membrane utilizing light, which generates proton gradient fuel for ATP production by ATP synthases. After the discovery of BR, three homologous pigments with different functions were found in the same archaeon; a light-driven inward chloride pump halorhodopsin (HR), a positive phototaxis (attractant) sensor sensory rhodopsin I (SR I), and a negative phototaxis (repellent) sensor sensory rhodopsin II (SR II, also called phoborhodopsin) (Fig 1). Currently, a large number of variants of BR, HR, SR I, SR II have been reported in related halophilic archaea, such as *Natronomonas pharaonis*. and *Haloarcula vallismortis*. These in archaea genetically termed “archaeal rhodopsin”. Four archaeal rhodopsins, BR, HR, SR I and SR II in *H.salinarum*, are very homologous and have in common in the presence of a retinal chromophore near the center of helical bundle, despite

these functions differ. (Fig 1) (2). The comparison of crystal structure of BR (3 4), HR in *H. salinarum* (5), HR (6) and SR II in *Natronomonas. pharaonis* (7,8) demonstrated that the structures of these archaeal rhodopsins are almost the same (9) BR can convert into the anion inward pump like HR by one residue substitution (D85S or D85T) (10-15) and the acidification (16,17). SR II shows proton pump pumping activity like BR in the absence of the transducer (18,19). HR can also convert into the proton outward pump like BR in the presence of azide (20,21). Thus, archaeal rhodopsin is the suitable model for the discussion of the structural similarity and the functional difference.

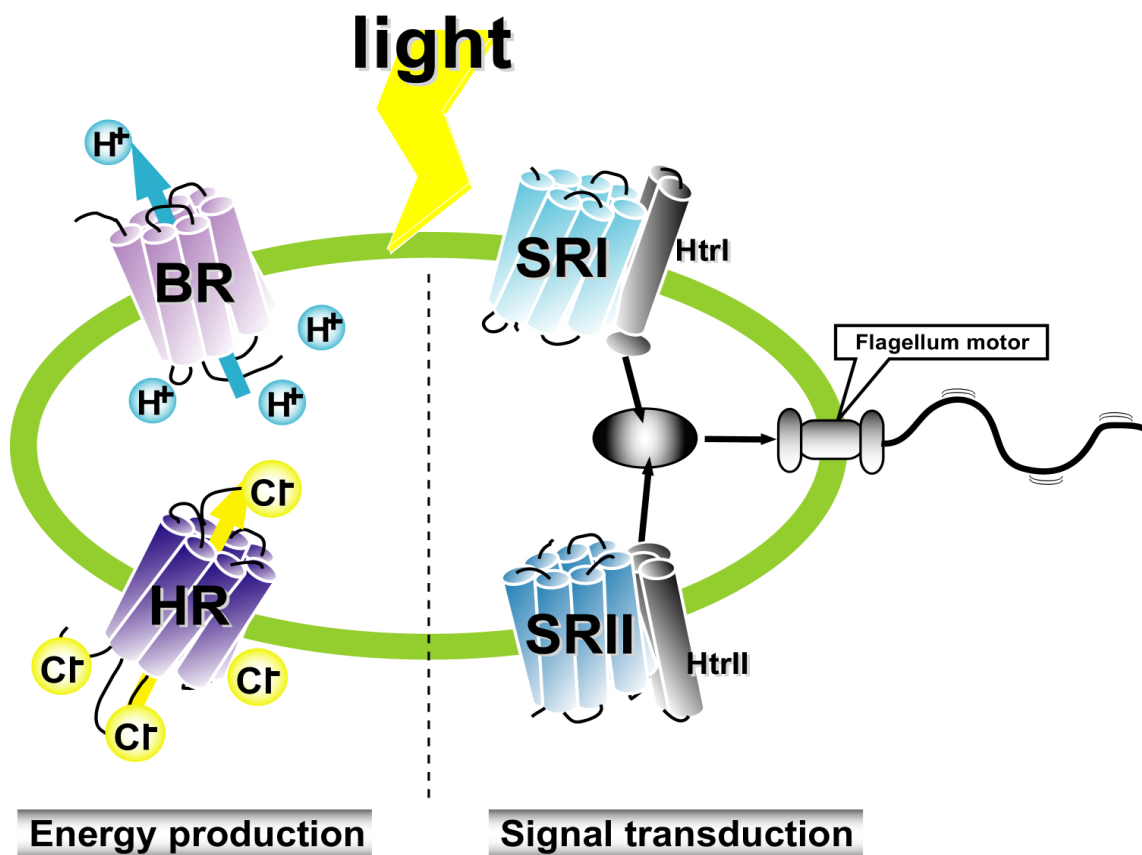


Figure 1. The function of archaeal rhodopsins

Ion pump → BR: bacteriorhodopsin, HR: halorhodopsin

Light sensor → SR I :sensory rhodopsin I , SR II : sensory rhodopsin II

Bacteriorhodopsin (The best characterized ion pump)

As a basis for understanding halorhodopsin (HR), we start with a brief description of the structure model and function of bacteriorhodopsin (BR) which is the best characterized ion pump. Bacteriorhodopsin is a small retinal-protein and function as a light-driven proton pump in the cytoplasmic membrane of halophilic archaea. *Halobacterium salinarum* (*H.salinarum*), a kind of halophilic archaea thrive in high-temperature saturated salt brines that are exposed to bright sunlight. To extract energy as well as information provided by the light and its spectral composition, haloarchaea possess a set of four related retinal proteins in their cytoplasmic membrane: bacteriorhodopsin (BR), halorhodopsin (HR), and sensory rhodopsin I and II (SR I and SR II).

BR converts the energy of “green” light (500-650 nm) into an electrochemical proton (H^+) gradient, which is used for ATP production by ATP synthases by transporting H^+ from cytoplasmic (CP) side to extracellular (EC) side.

BR folds into a seven-transmembrane helix topology with short interconnecting loops, which forms a pathway of ion. The helices (named A to G) are arranged in an arc-like structural model and tightly surround a retinal molecule that forms a Schiff base (SB) with a conserved lysine on helix G (K216 in BR). The cross section of BR with residues important for proton transfer is shown in Fig 2. Retinal separates a cytoplasmic (CP) from an extracellular (EC) half channel that is lined by amino acids crucial for efficient proton by BR (especially Asp96 in the cytoplasmic and Asp85 in the extracellular half channel). The geometry of the retinal, the protonation state of the SB, and its precise electrostatic interaction with surrounding charges (Asp85, Asp212, and R82 in BR) and dipoles tune the absorption maximum to fit its biological function.

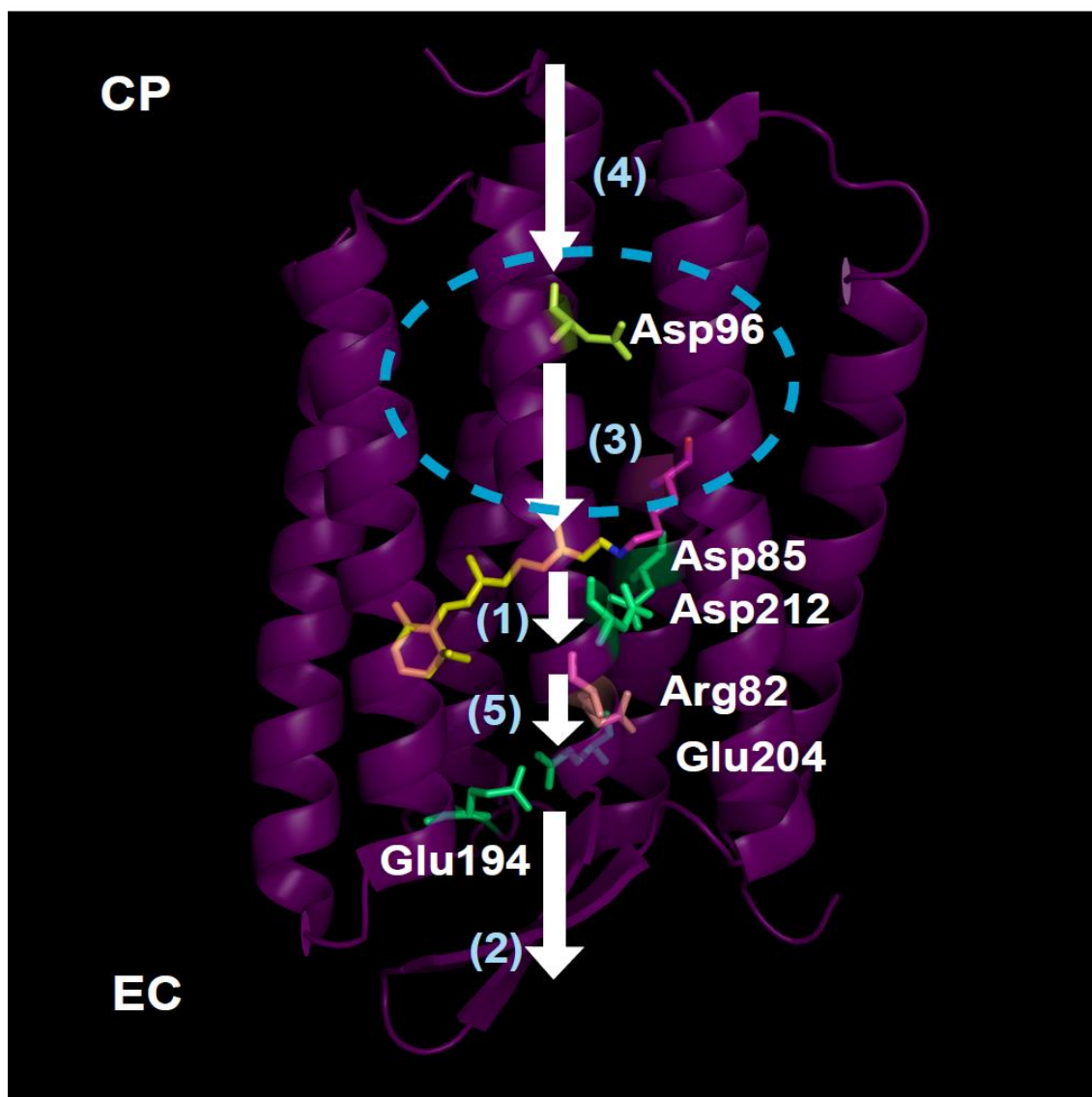


Figure 2. Cross section of the structure of BR (pdb code: 1c3w)

Selected residues important for proton transfer steps are shown in stick representation. The possible path of proton is indicated by arrows. The number of the parenthesis stands for the order of H^+ transfer.

- (1) PSB (Protonated Schiff Base)→D85 (Asp85)
- (2) E194 (Glu194) or E204 (Glu204)→Extracellular (EC) space
- (3) D96 (Asp96)→SB(Schiff Base)
- (4) Cytoplasmic (CP) space→D96 (Asp96)
- (5) E194 (Glu194) or E204 (Glu204)→D85(Asp85)

In the dark, BR exists in the dark-adapted form (DA-BR) absorbing at 568 nm containing two distinct different isomer of the retinal chromophore, which is a mixture of 13-*cis* retinal pigment (BR^{13-*cis*}) and all-*trans* retinal pigment (BR^{all-*trans*}) with a ratio of 1:1 (22, 23, 24). Irradiation of DA-BR with light yields light-adapted form (LA-BR) containing 100% all-*trans* retinal (BR^{all-*trans*}).

Absorption of photon by (BR^{all-*trans*}) leads the all-*trans* to 13-*cis* isomerization of the retinal chromophore, which causes BR to perform the series of structural change called “photocycle” which is a multistep thermal interconversion pathway of several intermediates in an alphabetical order, leading back to the initial state. BR’s photocycle is described as: BR $\xrightarrow{(h\nu)}$ $\rightarrow K \leftrightarrow L \leftrightarrow M_1 \rightarrow M_2 \leftrightarrow N \leftrightarrow O \rightarrow BR$ (Fig 3). Initiation of BR’s photocycle sets off a series of H⁺ transfers inside the protein and at the two membrane surfaces that result in the net translocation of a H⁺. The lettered states are the nomenclature of photointermediate in the photocycle. Each photointermediate has different configurational state of the retinal, the protonation state of various groups, or the conformation of the protein. The study of these intermediates has provided much information about the proton transfer steps that make up the trajectory of the transported H⁺. Previously, the relation of H⁺ translocation steps and the photointermediates in the photocycle of BR was generally agreed (25).

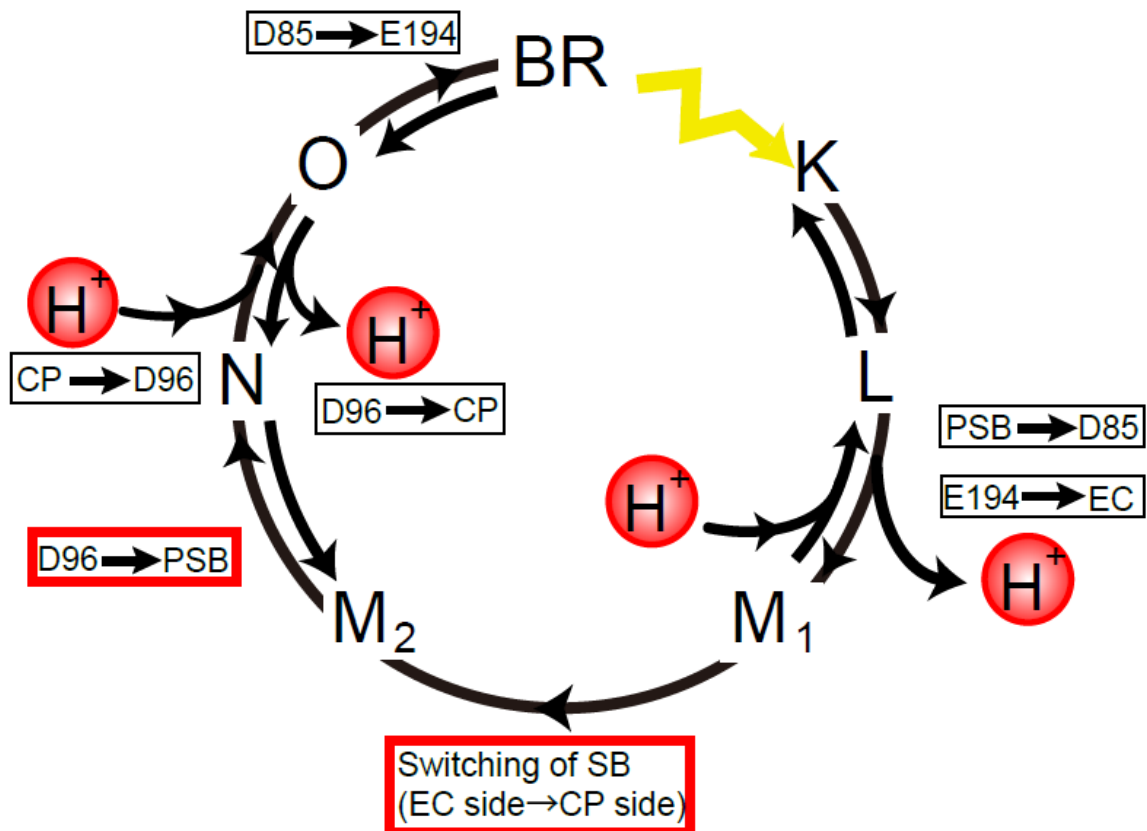


Figure 3. The photocycle scheme of bacteriorhodopsin (BR)

Square stands for the proton translocation step. Red square means the step in which BR causes a large structural change.

PSB, protonated Schiff base; D, Aspartic acid residue (Asp); EC, extracellular; CP, cytoplasmic

The key in active transport, i.e. the internal step that separates the release of a proton at one surface from the uptake at the other in BR corresponds to the change in the connectivity of the retinal Schiff base in the $M_1 \rightarrow M_2$ step (Fig 3). In the light-adapted ground state, it is to Asp85 with access to the extracellular surface and function as proton acceptor from the protonated Schiff base (PSB). At the N-intermediate in the photocycle it is to Asp96 with access to the cytoplasmic surface. According to the model (25), The isomerization of retinal from all-*trans* to 13-*cis* accompanies a change in the geometry of the Schiff base, Asp85, and bound water (Brown et al., 1994;), and distortions of the retinal chain (Maeda et al., 1994; Yamazaki et al., 1995) during the K $\rightarrow$ L reaction. This cause the transfer of a proton from the retinylidene group to the anionic Asp85 followed by proton transfer from PRG (proton releasing group, such as Glu204 and Glu194) to the interior of cell in the L $\rightarrow$ M₁ step ((1), (2) in Fig 2 and Fig 3). Next, in the M₁ $\rightarrow$ M₂ step, the access of the Schiff base will change from EC to CP side. Then, in the M₂ $\rightarrow$ N step, one H⁺ is transferred from the initially protonated Asp96 to the Schiff base. A ((3) in Fig 2).

In this “reprotonation switch”, a large-scale conformational change of BR was expected to occur, because the loss of the Schiff base-counterion interaction upon deprotonation of the Schiff base and the rearrangement of the access of the Schiff base proton from EC to CP side. The structural change in the “reprotonation switch” was detected by diffraction (29-31) estimated to be the tilt of CP end of helix F away from the center of the protein. This structural change occurs during the lifetime of the M state, and although its rise has not been possible to follow, it appears to decay with the N state (32).

Then, why does the CP end of helix-F open? This answer lies in the structure of archaeal rhodopsin including BR. As mentioned before, they consist of α -helices forming the channel, which is separated by the covalently bound retinal chromophore located in the middle of channel. There are few internal water molecules and hydrophilic residues in their channel connecting to the CP surface (CP half channel), unlike that connecting to the EC surface (EC half channel) is hydrophilic (Fig 4). Thus, in the CP half channel, there is a large energetic barrier against ion transport.

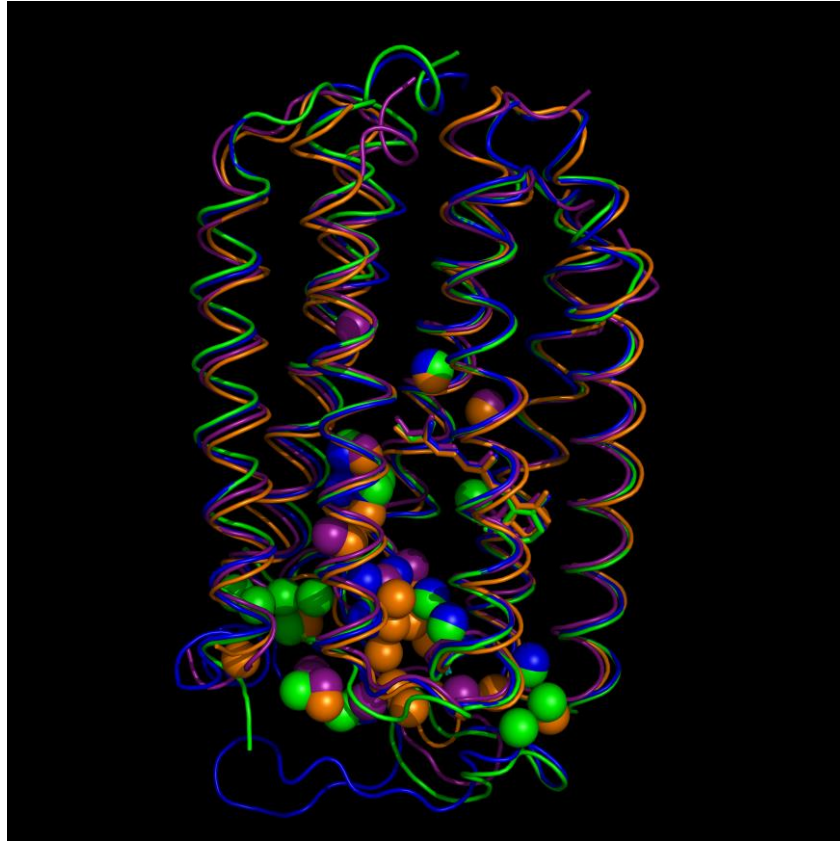


Figure 4. The Structural comparison between BR, SR I , HsHR, and NpHR
 Purple, bacteriorhodopsin (BR); Orange, sensory rhodopsin I (SR I);
 Yellow-green, halorhodopsin from *Halobacterium salinarum* (HsHR); blue,
 HR from *Natronomonas pharaonis*. Sphere stands for the bound water in
 protein. There are many water molecules in the extracellular half channel
 (upper half channel), however, few in the cytoplasmic channel (lower half
 channel). Therefore, some large structural change may be necessary for
 irreversible ion transfer.

In order for BR to uptake one H^+ through the CP half channel, It was assumed to be necessary to increase hydration in the CP half channel by the conformational change of BR during the M-decay (Fig 6). This postulate was proved mainly from good correspondence between the conclusions drawn from the inhibitory effects of decreased water activity (i) and hydrostatic pressures (ii) on the reprotonation of the Schiff base.

- (i) The specifically inhibitory effect of the $M \leftrightarrow N$ equilibrium by membrane suspensions containing BR upon adding osmotically active solutes (32).
- (ii) A significant volume increase between M_1 and M_2 of D96N mutant of BR (approximately 32 mL/mol) and a large positive (approximately 50 mL/mol) activation volume for H^+ exchange between D96 and the Schiff base of the wild type protein, which are calculated from the observed steep pressure dependence (Fig 5) (33).

In general, the volume change of protein is decomposed into two groups, “hydration” and “cavity”. The contribution of hydration is the volume change caused by the interaction of protein and water molecules. The contribution of cavity is the solvent-inaccessible core due to imperfectly packing of protein. In the case of the M decay of BR, the volume change is considered to be almost completely contribution of hydration, because the magnitude of this volume increase (32 mL/mol) is about as what would be expected from the change of surface area as calculated from the increase of the lattice constant in the M state by diffraction. The ΔV mL/mol of BR during M decay was estimated to be equivalent to the binding of about 20 water molecules because the bound water involve the transition from the liquid state to the ice state (33). This concluded that BR tilts of CP end of helix F away during M decay, widening of the CP half channel, allowing water molecules to enter and interact with Asp96, thereby decreasing its pK and rendering it a proton donor. After that, Reversal of its tilt promotes H^+ uptake from the CP surface to D96, which was consistent with the diffraction changes that implicate helix F in the structural change (31). The volume change during H^+ uptake from the CP surface to D96 were estimated to be negative (the $M_2 \rightarrow BR$ reaction of the D96N mutant) or small (the $N \rightarrow BR$ reaction of wild-type) (See Fig 5).

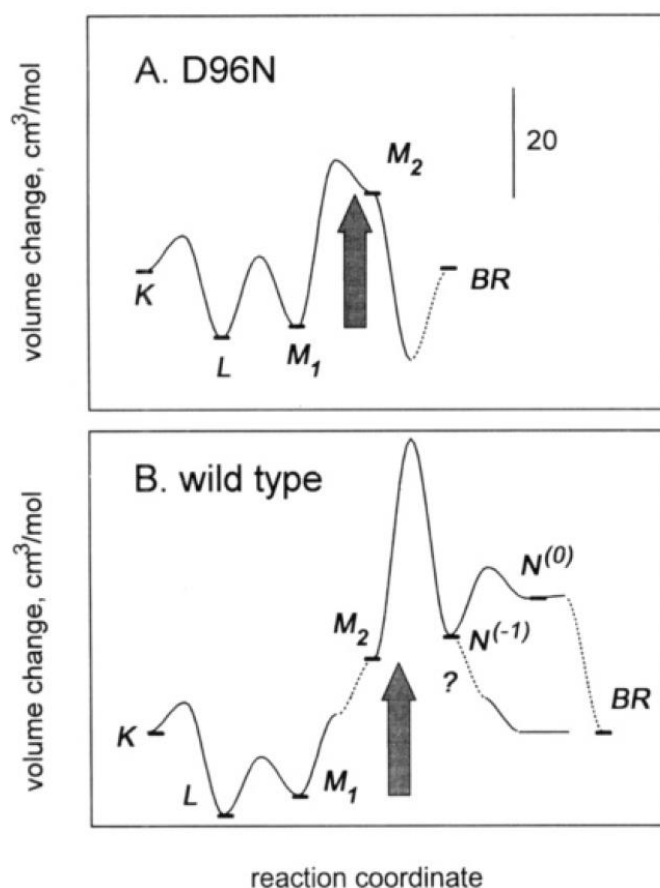


Figure 5. Graphic representation of the calculated activation volumes and volume changes in the photocycles of D96N bacteriorhodopsin (A) and wild-type bacteriorhodopsin (B). (Cited from Varo&Lanyi 1995 (33))

The vertical calibration bar applies to both A and B. The solid lines show the changes calculated directly from the pressure dependencies. The dotted lines indicate changes for which direct measurements are not available. The shaded arrows indicate the largest volume change in the photocycle, which corresponds to the protein conformation change. The volume of K is assumed not to be much different from the unphotolyzed state. In the wild type, The volume of relative to the volume of M₁ as in D96N was drawn, since the N(O) reaction in the wild type is limited by proton concentration, the activation volume at this step might not originate from a conformation change occurring in the protein. For this reason they have drawn in, with a question mark, a second alternative for the recovery of the volume increase at this reaction.

(33)

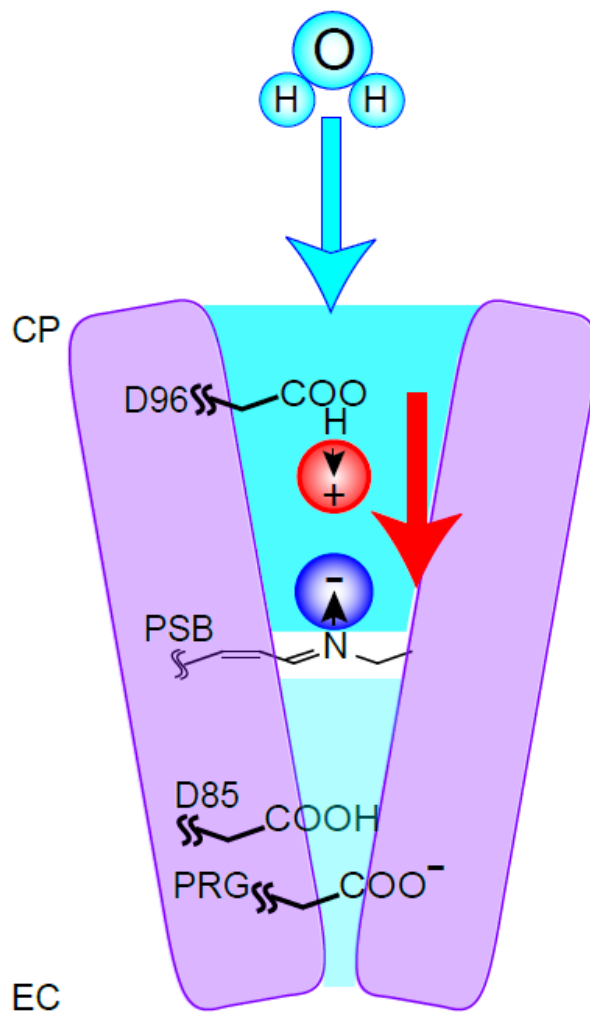


Figure 6. The model of proton transfer from Asp96 (D96) to Schiff base (SB) through the hydrophobic CP channel

First, BR widens the CP channel by tilting of the CP end of F-helix. Next, water inflow and bind to the CP channel. This hydration causes proton transfer from Asp96 (D96) to Schiff base (SB) through the hydrophobic CP channel.

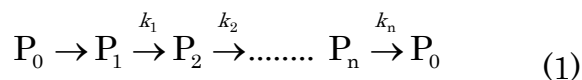
Halorhodopsin (The ion pump similar but different from BR)

HR transports Cl^- from extracellular (EC) side to cytoplasmic (CP) side through the membrane utilizing light energy alike BR (34-37), which creates a negative interior membrane potential. Halorhodopsin (HR) belongs to archaeal rhodopsin group just same as BR. HR has many similarities to BR. For example, Both of them use retinal as a chromophore, which bind to specific lysine residue of the last and seventh helix via a protonated Schiff base (38).: Absorption of light by retinal results in its isomerization from all-trans to 13-cis and initiates a photocycle in which the photo-excited state returns to the original pigment through various photo-intermediates with names such as K, L, M, N and O. Each spectrum and of photo-intermediate is similar between BR and HR. During a photocycle, HR releases one Cl^- which bind to the site near the Schiff base to the cytoplasmic (CP) space and uptakes another Cl^- from the extracellular (EC) space.

Since its discovery in the archaeon *Halobacterium salinarum*, various types of HRs have been reported. The most extensively studied are HR from *H. salinarum* (HsHR) and *Natronomonas pharaonis* (NpHR). NpHR has advantages over HsHR. First, no light-dark adaptation of NpHR has been found (39, 40), and HsHR presents this adaptation (41-43). Retinal isomers of HsHR in the dark are approximately 1:1 all-*trans*: 13-*cis*. Illumination increases the all-*trans* content, which decreases to the dark-adapted value during incubation in the dark. In contrast, for NpHR, all-*trans* retinal is a major component independent of the light- or dark-adapted states. Second, the 13-*cis* pigment in NpHR does not have a detectable photocycle in contrast to HsHR (40, 42). Third, the functional expression of NpHR in *Escherichia coli* cells has been achieved (44, 45); these cells have a much shorter doubling time than halobacterial cells. Functional expression of HsHR in *E. coli* cells has not been successful, although it was reported that active HsHR was obtained by solubilization of cells in the presence of excess retinal (46). For all these reasons, we employed NpHR in the present study.

The photocycle of NpHR has been investigated previously. In contrast to that of BR, the deprotonated Schiff base does not appear because the

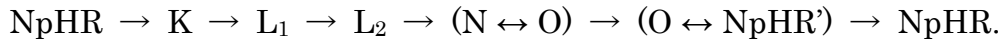
proton-acceptor residue BR Asp85 is replaced with a non-proton-accepting threonine (Thr, T) for both HRs (38). The photocycle scheme and pumping mechanism are still in debate. Ludmann et al. (47) stated that the cycle is $\text{NpHR} \rightarrow \text{K} \rightarrow \text{L}_1 \leftrightarrow \text{L}_2 \leftrightarrow \text{N} \leftrightarrow \text{O} \leftrightarrow \text{NpHR}' \rightarrow \text{NpHR}$. The absorption maxima (λ_{max}) are 580 for unphotolyzed NpHR, 585 for K, 530 for L (L_1 and L_2), 580 for N and 615 for O (40). NpHR' has λ_{max} of almost the same as that of NpHR (40), suggesting that its conformation may be only slightly different from the original NpHR. Essen proposed the conformational restoration of some residues around Arg(R)123 or a change in the orientation of Arg123 itself during the NpHR' to NpHR transition (48). Chizhov and Engelhard proposed a photocycle scheme (49) having some differences from that of Ludmann et al.: The representative differences are 1) the inclusion of two O, which were denoted by O^1_{600} and O^2_{600} , and 2) the appearance of N after O (O^2_{600}). We analyzed the flash-induced absorbance changes of NpHR using an irreversible sequential model (50) that was originally introduced by Chizhov et al. (51). This model is described as follows:



where P_0 stands for the unphotolyzed pigment, P_i ($i = 1 \sim n$) stand for the photo-chemically defined intermediates, which are kinetically distinguishable in the analysis according to this irreversible transition model and each k_i stands for the rate constant of the transition of P_i to P_{i+1} respectively.

The P_i intermediates may contain a few physically defined intermediates such as L, N and O when the reverse reactions exist between them. The “physically defined” means that these intermediates are spectrally distinguishable due to their physical differences. The assignment and name of the intermediates are usually based on BR nomenclature. Here, n is the smallest number of photo-chemically defined intermediates required to adequately simulate the data. We found that n of 4 is sufficient (50), and we determined the spectra of P_i ($i = 1 \sim 4$). Note that, using our apparatus, the K intermediate is not clearly observable, so exactly 5 photo-chemically defined intermediates are necessary. From the spectra of the estimated P_i we

assigned that P_1 , P_2 , P_3 and P_4 are composed of L_1 , L_2 , a quasi-equilibrium complex of N and O, and a quasi-equilibrium complex of a red-shifted species (potentially O, see the scheme of Ludmann et al. (47)) and $NpHR'$, respectively. Based on comparison with the scheme of Ludmann et al. (47), we describe our scheme as follows (Fig 7):



The parentheses indicate the quasi-equilibrium complexes. The equilibrium of $(O \leftrightarrow NpHR')$ shifted greatly to $NpHR'$ because only small amounts of O were observed. Other reports have also proposed the existence of two L (42, 49, 52, and 53). Our scheme indicates that in the scheme of Ludmann et al. (47), the rates of $L_2 \rightarrow L_1$ and $N \rightarrow L_2$ are negligible. Ludmann et al. (47) calculated the time-dependent concentrations of the respective photo-intermediates and revealed a similar time course of N and O content to that proposed in our scheme.

Váró et al. found that the rate constants of both $O \rightarrow NpHR'$ and $O \rightarrow N$ were dependent on the external Cl^- concentration, suggesting that O decay accompanies Cl^- uptake from the outside and that O is a Cl^- -free form (54), which is possible based on its absorption maximum (Fig 7). Supporting evidence was obtained from transient grating measurements (55) and from the absorbance changes of bacterioruberin, an intrinsic membrane-potential indicator of the halobacterial cell membrane (in preparation). Lanyi and Vodyanoy (56) and Ames et al. (57) assumed that O in HsHR was in a Cl^- -free form. Fourier transform infrared (FTIR) spectroscopy also concluded that O in $NpHR$ is Cl^- free form (58). In our analysis adopting the N-O equilibrium, the following equilibrium equation holds:



where the dissociation constant of Cl^- ($K_{\text{d, N} \rightarrow \text{O}}$) is described as

$$K_{\text{d, N} \leftrightarrow \text{O}} = \frac{[\text{O}][\text{Cl}^-]}{[\text{N}]} \quad (3)$$

After the Cl^- release during the O formation, the subsequent Cl^- uptake is considered to occur during the O to NpHR' transition. This notion is consistent with the Cl^- dependent acceleration of O to NpHR' transition rate described above (54) and the closely resembled absorption spectra between NpHR' and the initial dark state (49, 50, 54). On the contrary to this notion, the experiments for NpHR-induced electrogenicities did not detect the Cl^- movement assigned to the Cl^- uptake during this transition (47, 73). Thus, the details for the Cl^- uptake are still under debate.

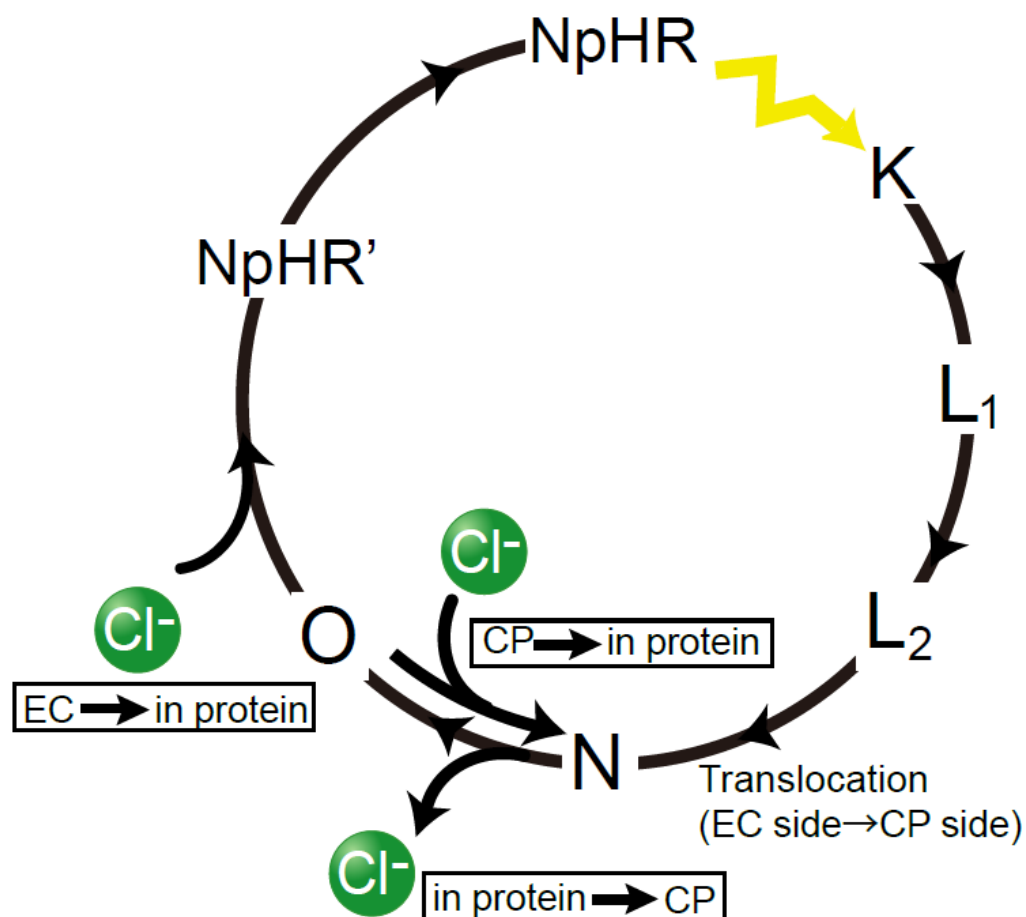


Figure 7. The photocycle scheme of NpHR proposed by our group

The switching of the accessibility of Schiff base (SB) from extracellular (EC) to cytoplasmic (CP) direction occurs during the reaction of $L_2 \rightarrow N$. Next, in the $N \rightarrow O$ step, NpHR release Cl^- into the interior of cell, followed by Cl^- uptake during $O \rightarrow \text{NpHR}'$.

Several functionally important amino acid residues have been reported based on mutagenesis experiments (59-65). Of the residues in the EC channel, NpHR Arg123, Thr126 and Ser130, which correspond to HsHR Arg108, Thr111 and Ser115, respectively, were found to be important (Fig 8). These residues constitute the Cl⁻-binding site in the dark, and their mutation inhibited Cl⁻ binding and/or distorted the photocycle. The putative important residues in the Cl⁻-interacting (binding) site in the CP channel are Lys215 and Thr218, corresponding to HsHR Arg200 and Thr203, respectively (60, 63, 66). These residues are hydrophilic or relatively hydrophilic compared to the large population of hydrophobic residues in the CP channel (Fig 8). Thr218 is located about only one helix turn away from the Schiff base. Previously, we mutated this residue to Val and found a significant change in the photocycle: O intermediate becomes hardly measurable (63). The importance of this residue in HsHR was also reported by Rüdiger and Oesterhelt (60). For HsHR, the replacement to Val slowed down the turnover rate of the photocycle and thereby reducing the Cl⁻ transport activity. Due to the close position to the Schiff base, Kolbe et al. proposed that the Cl⁻ probably forms hydrogen bond with OH group of the Thr after the passage of Cl⁻ over the Schiff base (66).

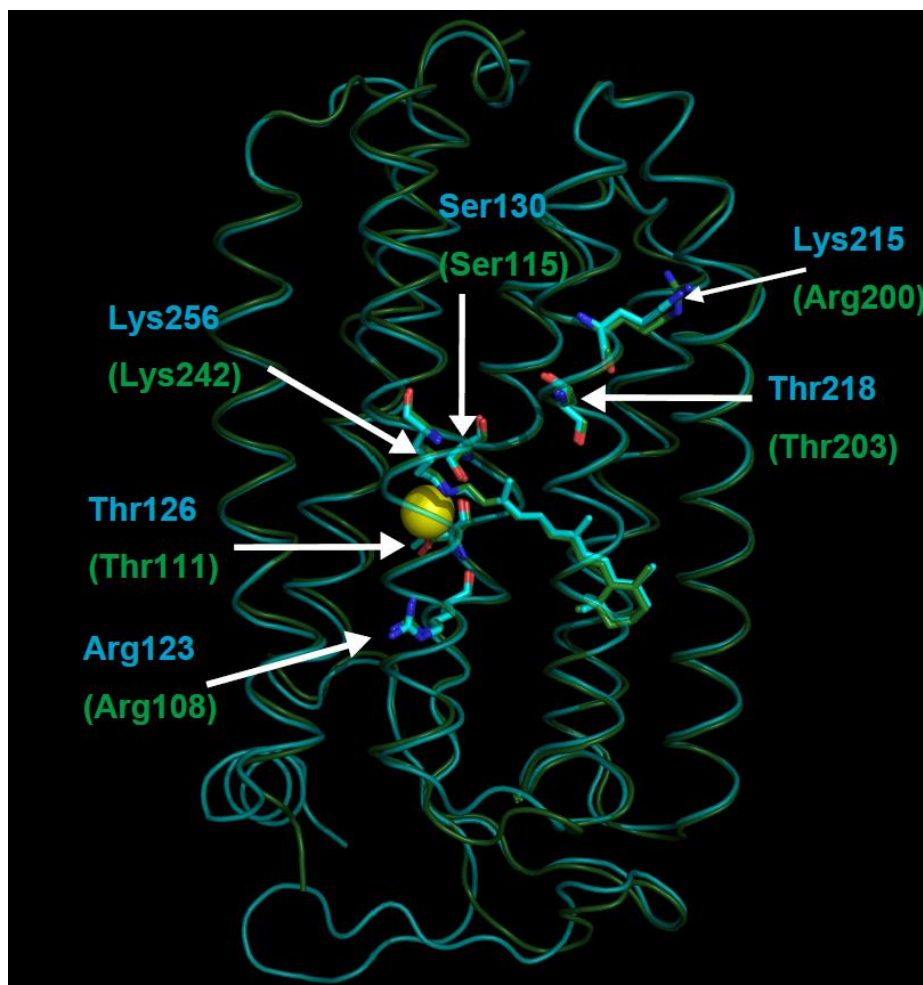


Figure 8. The comparison of structure and important residues of NpHR and HsHR (pdb code; 3a7k (NpHR), 1e12 (HsHR))

Important residues are preserved in both NpHR and HsHR.

In the present study, we mutated Thr218 to a variety of amino acid residues and measured changes in the Cl⁻-dependent N/O intermediate ratio (determination of $K_{d, N \rightarrow O}$ in Eq. 3). The hydropathy index of amino acid mutated is shown in Table 1. Needless to say, a large $K_{d, N \rightarrow O}$ indicates a low affinity for Cl⁻. The partial molar volume changes between N and O ($\Delta V_{N \rightarrow O}$) were estimated from the pressure dependence of $K_{d, N \rightarrow O}$. The volume change might be due to water entry at O as observed for the M-to-N transition of BR (67). The values of both $K_{d, N \rightarrow O}$ and $\Delta V_{N \rightarrow O}$ were the largest for the wild type and T218S mutant, and the relationship between $K_{d, N \rightarrow O}$ and $\Delta V_{N \rightarrow O}$ proposed the formation of a special coordination of the entered water molecule with the OH group of Thr or Ser. The rate constants k_2 and k_3 are those of the formation of the N-O complex and its decay, respectively. These values were also largest for the wild type and the serine mutant. Thus, Thr (wild type) is best suited for the transfer of Cl⁻. The effect of the mutation on the NpHR activity is also discussed.

Table 1. The hydropathy index of amino acid mutated at the 218th position
^a; the wild-type, ^b; from ref. (78)

AA residues at the 218 th position	Hydropathy index ^b
Gln(Q)	-0.85
Glu(E)	-0.74
Ser(S)	-0.18
Thr(T) ^a	-0.05
Cys(C)	0.29
Ala(A)	0.62
Met(M)	0.64
Trp(W)	0.81
Leu(L)	1.06
Val(V)	1.08
Ile(I)	1.40

Materials and Methods

Construction of Expression Plasmids of Mutants with a Histidine Tag.

Plasmids for the expression of various Thr218 mutants were constructed with a Quikchange Site-directed Mutagenesis kit (Stratagene, Agilent Tech. Tokyo, Japan) and a modified pET-21c (+) vector (Merck Japan, Tokyo, Japan) to create a histidine-tagged region. The mutations introduced were confirmed by a standard method using an automated DNA sequencer (model 3100, Applied Biosystems, Foster City, CA). The expression of the recombinant NpHR in *E. coli* cells (strain BL21 (DE)) and the solubilization procedure were described previously (45). Fractions of the proteins separated with a Ni-NTA agarose (Qiagen, Hilden, Germany) column were collected by elution with buffer E (50 mM Tris-HCl (pH 7.0), 400 mM NaCl, 150 mM imidazole, 0.1% n-dodecyl- β -D-maltopyranoside (DDM) (Dojindo Laboratory, Kumamoto, Japan). The samples were then placed in appropriate buffer solutions by passage over Sephadex-G25 in a PD-10 column (GE Healthcare Japan, Tokyo, Japan).

Flash Photolysis Spectroscopy under Normal Pressure.

The computer-controlled flash-photolysis apparatus used was the same as that reported previously (63). The transient absorption changes (λ from 410 to 710 nm, every 10 nm) induced by a light pulse ($\lambda = 532$ nm, 7 ns, ~ 5 mJ) of a Q-switched Nd: YAG laser (Surelite I-10; Continuum, Santa Clara, CA) were acquired every 0.5 μ s between -44 and 218 ms. At each selected wavelength, 30 laser pulses were used to improve the S/N ratio. Data points on a logarithmic time scale were picked from the observed data for the following analysis. Temperature was kept constant by circulating thermostated water at 25 °C. The NpHR concentration was 10 ~ 15 μ M, which was determined using an extinction coefficient of 54,000 at 580 nm. Experiments were performed with varying concentrations of Cl⁻, whereas the ionic strength was maintained equivalent to 4 M NaCl through the use of appropriate concentrations of Na₂SO₄ for both the normal and the following high-pressure experiments. The pH of the sample was 6.0 and buffered using 10 mM MES.

Flash Photolysis Spectroscopy under High Pressure

The pressure cell was constructed by UNISOKU (Osaka, Japan) (Fig 9). 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 1480 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 Nd: YAG laser into the cell. These two beams intersected perpendicularly in the sample cuvette (Fig 9). The temperature of the pressure cell was controlled by a circulating water bath (68). All measurements were performed at 25°C.

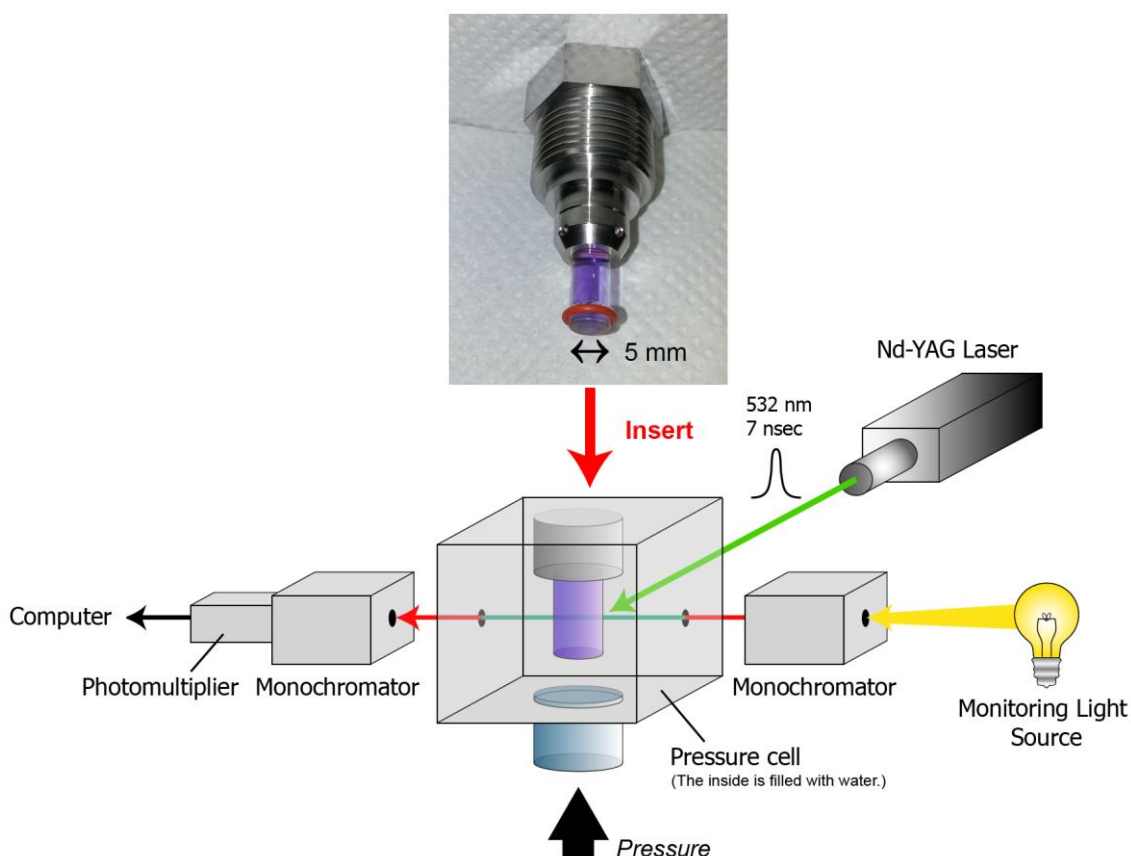
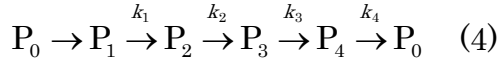


Figure 9.

The high pressure Flash-Photolysis Apparatus (lower) and the pressure cell containing the sample (upper)

Analysis of Flash Photolysis Data

As described in the introduction, we employed an irreversible sequential model and found that $n = 4$ was sufficient to simulate the data because the reduction in the standard deviation of the weighted residuals were saturated at $n = 4$ (50). This means that the NpHR photocycle can be described by the following sequential scheme:



As described above, K intermediate is not detected in our apparatus due to the slow response. As judging from the determined decay rate constants, our P_1 - P_4 may correspond to P_3 - P_6 , respectively, in the previous analysis by Chizhov and Engelhard (16). In this assignment, our P_3 corresponds to P_5 of Chizhov and Engelhard. The spectra of these two intermediates show similar Cl^- dependence as described below. The further analysis was performed as described previously (50, 51, 63). Briefly, by using the determined k_i values and the sequential irreversible model, the time-dependent concentration changes of P_i intermediates ($P_i(t)$) could be calculated by the following equation (51).

$$P_i(t) = \sum_{j=1}^i a_{i,j} \exp(-k_j t), \quad a_{i,j} = \frac{1}{\prod_{m=1}^{i-1} k_m \prod_{\substack{m=1 \\ m \neq j}}^i (k_m - k_j)} \quad (5)$$

Thus, the time-dependent absorbance changes at all wavelengths ($\Delta A(\lambda, t)$; $\lambda = 410 - 710$ nm) were fitted simultaneously by the following multi-exponential function (51):

$$\Delta A(\lambda, t) = \sum_{i=1}^n B_i(\lambda) \cdot \exp(-k_i \cdot t), \quad B_i(\lambda) = Fc \times \Delta \varepsilon_i(\lambda) \times \sum_{j=1}^i a_{i,j} \quad (6)$$

Fc is defined as the fraction of sample molecule initiating the photocycle. Using the fitting results, the rate constant of the decay of the P_i state (k_i) and the absorption differences between P_i and P_0 ($\Delta \varepsilon_i$) were calculated. Independently, the measured spectrum of the P_0 state was fitted with the following 4 factors: (1) skewed Gaussian (SG) function (18) of the main absorption band approximately 580 nm, (2) the background scattering ($A +$

B/λ^4 ; λ in nm), (3) SG function of the β -band approximately 400 nm and (4) SG function of the band from the aromatic residues approximately 280 nm. The SG function is defined by four parameters, $\lambda_{\max}$ (in nm), $A_{\max}$ (amplitude at $\lambda_{\max}$), ρ (skewness of the absorption band) and $\Delta\nu$ (half-band width in cm^{-1}). The sum of the SG functions of (1) and (3) expresses the retinal spectrum at P_0 state. Typical parameter values were $\lambda_{\max} = 575$ nm, $\rho = 1.62$ and $\Delta\nu = 3152$ cm^{-1} for the SG function of (1) and $\lambda_{\max} = 392$ nm, $\rho = 1.70$ and $\Delta\nu = 8210$ cm^{-1} for the SG function of (3), respectively. The relative $A_{\max}$ value of the SG function (3) and (1) was approximately 0.11. By adding this retinal spectrum to the absorption difference ($\Delta\epsilon_i$), we obtained the absolute spectra of the P_i state. Further details of this method have been described previously (50, 63).

Decomposition of the Spectra of the P_3 State

As described previously (50) and below (results section), P_3 was a quasi-equilibrium mixture of N and O whose relative fractions depended on the Cl^- concentration (see Eqs. 2 and 3). In addition to the respective main absorption bands, the P_3 intermediate spectra included the respective β -bands. Assuming both β -bands to be identical with that of the P_0 state, the P_3 spectrum is expressed as:

$$\text{Spectrum of } P_3 = f \times \text{Abs}(N, \lambda) + (1 - f) \times \text{Abs}(O, \lambda) + \text{Abs}(\beta, \lambda) \quad (7)$$

where f , $\text{Abs}(N, \lambda)$, $\text{Abs}(O, \lambda)$ and $\text{Abs}(\beta, \lambda)$ stand for the fraction of N in the P_3 state, the main band of N, main band of O and the β -band, respectively. By employing the SG functions to express the three absorption bands, we decomposed the P_3 spectra to estimate f . In this calculation, we assumed f to be the only parameter varying with the Cl^- concentration. Typical parameter values of SG functions were $\lambda_{\max} = 518$ nm, $A_{\max} = 0.65$, $\rho = 2.07$ and $\Delta\nu = 4348$ cm^{-1} for $\text{Abs}(N, \lambda)$, and $\lambda_{\max} = 607$ nm, $A_{\max} = 0.87$, $\rho = 1.74$ and $\Delta\nu = 3509$ cm^{-1} for $\text{Abs}(O, \lambda)$, respectively. The $A_{\max}$ s described above are relative to the $A_{\max}$ for the main absorption band of the P_0 state.

Determination of $K_{d,N\leftrightarrow O}$ of the Wild Type and Mutants

Values of f and $1-f$ (fractions of N and O at P_3 state, respectively) were plotted against Cl^- concentrations. In these plots, the two curves crossed each other at $f = 0.5$ (see Fig. 12). The concentration of Cl^- giving $f = 0.5$ is the $K_{d, N\leftrightarrow O}$. For some mutants, another method below was adopted because their $K_{d, N\leftrightarrow O}$ values were so low for plotting of f against Cl^- , in which we transformed the Eq. 8 defining the relationship between the N fraction (f) and $K_{d, N\leftrightarrow O}$ and made the Eq. 9. According to Eq. 9, we obtained the slope of -1.0 when $\log [(1-f)/f]$ is plotted against $\log [Cl^-]$.

$$f = \frac{[Cl^-]}{K_d + [Cl^-]} \quad (8)$$

$$\log \left[\frac{1-f}{f} \right] = \log K_{d,N\leftrightarrow O} - \log [Cl^-] \quad (9)$$

As shown in Fig. 13, however, the slopes of the plots for some mutants (E, A, W) were not -1.0. Thus, we derived Eq. 10 by introducing the Hill coefficient (n_H) to Eq. 9.

$$\log \left[\frac{1-f}{f} \right] = n_H \cdot \log K_{d,N\leftrightarrow O} - n_H \cdot \log [Cl^-] \quad (10)$$

According to Eq. 10, we can estimate $K_{d, N\leftrightarrow O}$ and n_H . The $K_{d, N\leftrightarrow O}$ values estimated by Eq. 10 were almost same as those estimated from the Cl^- concentration dependence of f , where the concentration at $f = 0.5$ gave the $K_{d, N\leftrightarrow O}$ (Panels C and D of Fig. 12). Interestingly, the value of n_H has a negative linear relation with $\Delta V_{N\rightarrow O}$ except for the Val and Ser mutants (Panel C of Fig. 13). The reason for this linearity is not clear, and further investigation will be needed.

NpHR Activity Measurements by Photo-Induced Proton Transfer

The activities of various mutants were estimated using *E. coli* suspensions. Cl^- transport by NpHR hyperpolarizes the cell membrane and causes passive proton uptake. Thus, the activity is evaluated as the pH increase of the suspension. It is worth noting that we used fresh cells without carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). The rationale for this is elaborated in the discussion. For these experiments, all procedures were performed as quickly as possible. The *E. coli* cells were harvested at $3600 \times g$ for 5 min at 4 °C and washed twice with cold unbuffered basal solution containing 150 mM NaCl, 50 mM MgSO_4 , 40 mM KCl and 0.5 mM CaCl_2 . Concentrated cell suspensions were obtained. For activity measurements, the original suspensions were diluted with the basal solution. The cuvette was thermostated at 25 °C and the sample volume was 1.5 mL. The pH was measured with a glass electrode, and the initial pH values of the samples were about 6.5. Illumination was provided with a 590 ± 8.5 nm LED (LXHL-LL3C, Philips Lumileds Lighting Co., San Jose, CA). Within 3-5 min of illumination, the pH increase became saturated. After each measurement, the pH increase was calibrated to the amount of proton uptake into the cells by adding a known amount of HCl. To determine the relative pigment concentrations in the cuvette, the flash photolysis yields at 580 nm were recorded for the original cell suspensions. Compared with the de-energized cells (see below), the light-induced pH increases were small, and accurate measurements were difficult to obtain due to relatively large baseline fluctuation. Thus, the pH measurements were performed for 5-8 samples diluted at various times from the original cell suspensions. The diluted samples had optical densities at 660 nm (OD_{660}) of 0.1-1.3. The calibrated amounts of proton uptake were plotted against the relative pigment concentrations, which are the products of the flash yields and the dilution ratios. The slopes of the plots indicate the relative Cl^- -pumping activities of the pigments. Independently, the proton uptake experiments were also performed using de-energized cells. For these experiments, washed cells were shaken gently for 2 h at 25 °C in the presence of 10 μM CCCP. After that, the cells were washed again and diluted to an OD_{660} of 0.5 with the basal solution supplemented with 10 μM CCCP. The light-induced pH changes were relatively large. Thus, pH measurements were performed for only the samples at OD_{660} of 0.5. The relative pigment concentrations were

also determined from the flash photolysis yields for original concentrated cell suspensions and the dilution ratios. The proton uptake was proportional to the pigment concentration. The relative pumping activities, which are comparable to those of the intact cells, were determined by dividing the proton uptake by the relative pigment concentrations.

RESULTS

Agreement of the Observed Flash-Induced Transient Absorbance Changes with Simulation Curves using $n = 4$ in Eq. 1 and 4

Previously, we showed that the photocycle of NpHR was adequately described with 4 photo-chemically defined intermediates (50). This was also valid in the present experiments using various mutants and high pressures. Figure 10 shows some examples of the excellent agreement between the observed flash-induced light-dark difference spectra and the simulated curves described by the sum of four exponential functions, which assume the existence of four photo-chemically defined intermediates (P_i , $i=1\sim4$). Using the determined fitting parameters i.e. k_i , the difference spectra between P_i and P_0 ($\Delta\epsilon_i(\lambda)$), the absolute spectra of P_i ($i=1\sim4$) were calculated as described previously (50, 63). The calculated time-dependent concentration changes of P_i intermediates ($P_i(t)$), $\Delta\epsilon_i(\lambda)$ and spectra of P_i of wild-type under 500 mM Cl^- are shown in Figure 11.

In Fig 10, the top row (Panels A, B and C) show the data from the wild type at 62.5 mM Cl^- (A), 500 mM Cl^- (B) and 4000 mM Cl^- (C) under 1 bar. The concentrations of three panels in the second row (D, E and F) are the same as those of the top (A, B and C, respectively), but the pressure is 1480 bar. The panels in the third and fourth row are the data from the T218V mutant. The concentrations of panels G and J are 7.5 mM Cl^- , those of H and K are 25 mM Cl^- , and those of I and L are 500 mM Cl^- . The third row represents the data under 1 bar, and the panels in the fourth row are data under 1480 bar. The differences in the Cl^- concentration of the wild-type protein and the T218V mutant are due to the differences in K_d , $N \leftrightarrow O$ in Eq. 3. These 12 panels indicate that with an increase in Cl^- concentration, the photo-products at the longer wavelength (maybe O-intermediate) decrease, which agrees with the equilibrium equation of Eq. 2. For the wild-type protein (panels A – F), an increase in pressure results in a lower yield of the long-wavelength product, O, whereas the T218V intermediate shows the opposite tendency (panels G – L). This is because the opposite partial molar volume changes between N and O for these two species.

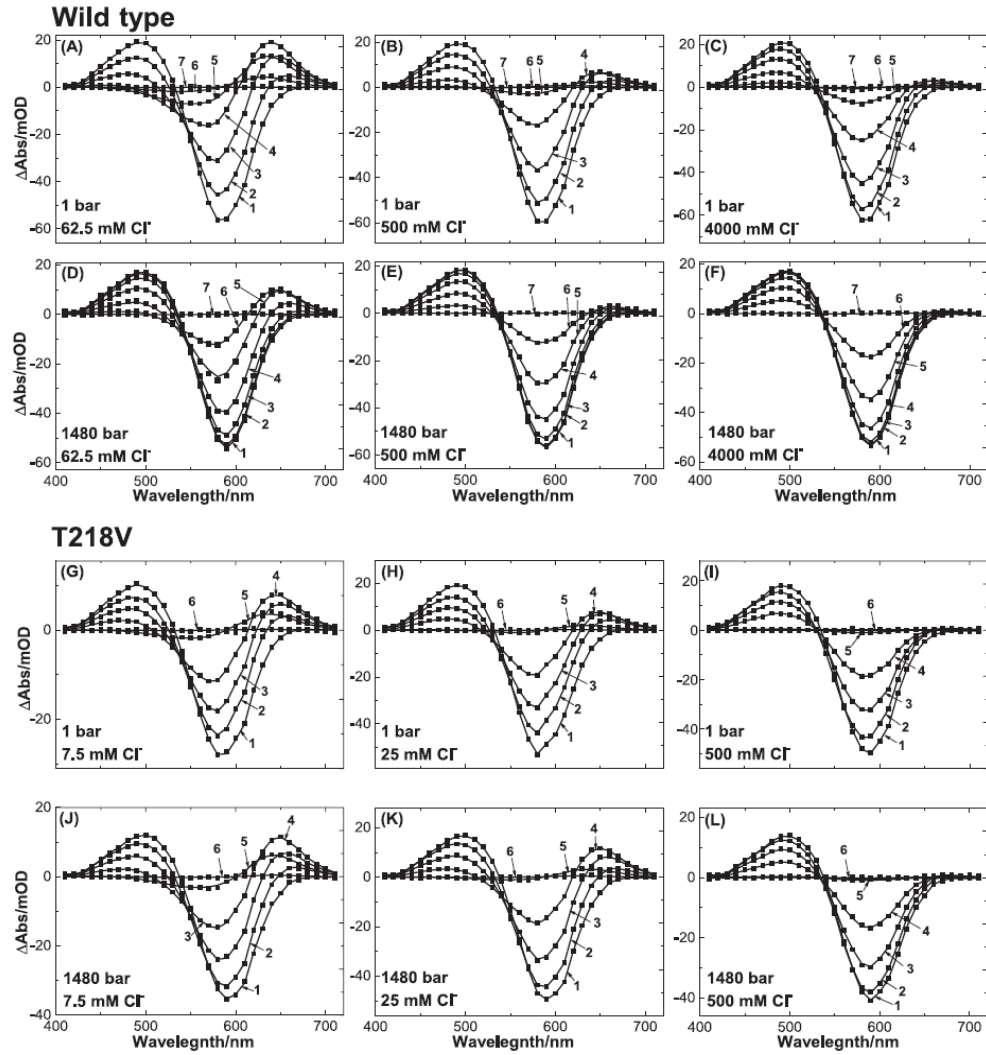


Figure 10. Good agreements of observed (closed mark) and simulated (line) flash-induced light-dark difference spectra. The top row (Panels A, B and C) shows the wild-type data at 62.5 mM Cl⁻ (A), 500 mM Cl⁻ (B) and 4000 mM Cl⁻ (C) under 1 bar. The concentrations of the three panels in the second row (D, E and F) are the same as those of the top (A, B and C, respectively), but the pressure is 1480 bar. The times of the data after the flash are as follows: 1, 0.09; 2, 0.32; 3, 0.63; 4, 1.17; 5, 2.23; 6, 4.23; 7, 29.4 ms. The panels in the third and fourth row are the data of T218V. The Cl⁻ concentrations of panel G and J are 7.5 mM, those of H and K are 25 mM, and those of I and L are 500 mM. The third row represents the data under 1 bar, and the panels in the fourth row are data under 1480 bar. The times of the data after the flash for 218V are as follows: 1, 0.32; 2, 2.23; 3, 4.25; 4, 8.11; 5, 29.4; 6, 106 ms.

Figure 11 shows the data obtained in the analysis for wild-type protein at 1 bar (upper panel) and 1480 bar (lower panel) in the presence of 500 mM Cl⁻. The left panels (A and D) represent the calculated time-dependent concentration changes of P_i intermediates. As shown here, pressure decelerated the formation and decay of all P_i intermediates, although the respective pressure dependencies were different.

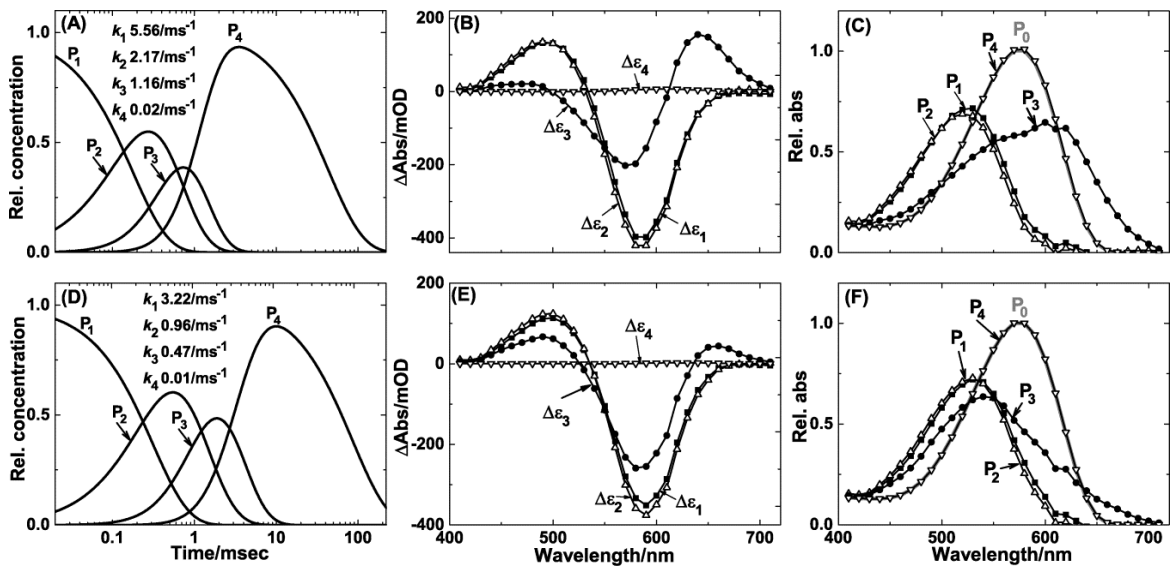


Figure 11. Absolute spectra of P₀ and P_i intermediates and the related data obtained by analysis according to the sequential irreversible model. These are the analysis results for wild-type protein at 1 bar (upper panel) and 1480 bar (lower panel) in the presence of 500 mM Cl⁻. The other sample conditions were the same as in Fig 10. The left panels (A and D) show the time-dependent concentration changes of P_i (i = 1 - 4), whose decay rate constants, k_i , are indicated in the panels. The middle panels (B and E) show the difference spectra between P_i and P₀ ($\Delta\epsilon_i(\lambda)$). The final obtained absolute spectra of P_i are shown in the right panels (C and F) with the spectrum of the P₀ state (thick gray line). The relative amplitudes are plotted against the P₀ state.

Estimation of $K_{d, N \leftrightarrow O}$ values for the Wild-Type Protein and Various Mutants under Normal Pressure

The spectra of P_3 are composed of a quasi-equilibrium mixture of N and O (50), which has typical $\lambda_{\max}$ of 516 and 607 nm, respectively. The open symbols in Panels A and B of Figure 12 are the P_3 spectra for the wild type (A) and T218V (B) at varying concentrations of Cl^- . Here, the absorbances are shown when P_0 is taken as unity. The longer-wavelength product (O) decreases with an increase in Cl^- concentration, whereas the shorter-wavelength products (N) increase. For these P_3 spectra, we performed the fitting analyses according to Eq. 7. The solid lines denote the best-fit curves. This analysis simultaneously determines the spectra of pure N (Abs (N, λ)) and O (Abs (O, λ)) and the fraction of N (f). The fraction of O is given by $1 - f$. The broken lines in Panels A and B denote the determined spectra of pure N and O. The fractions of N and O are plotted in panel C for the wild type and in panel D for T218V. The open circles indicate the O fraction, and closed circles are the N fraction. These two lines cross each other at $f = 0.5$, and the concentrations at these points are the $K_{d, N \leftrightarrow O}$. T218V has a much smaller $K_{d, N \leftrightarrow O}$ than the wild-type protein. The $K_{d, N \leftrightarrow O}$ values of wild type and various mutants were also determined from the linear relationships between $\log[(1-f)/f]$ and $\log[Cl^-]$ (Fig 13). the determined values are almost the same as those described above. Thus, $K_{d, N \leftrightarrow O}$ values of some mutants were determined by the latter procedure. The $K_{d, N \leftrightarrow O}$ values for Gln, Met, Phe, Ile and Leu mutants were not able to be estimated because their $K_{d, N \leftrightarrow O}$ s were very small (less than ~ 5 mM); under these dilute solutions we could not distinguish their photocycle from that of the Cl^- -free form. The estimated values of $K_{d, N \leftrightarrow O}$ are listed in Table 2 with the hydropathy index of the amino acid at the 218th position. The value of $K_{d, N \leftrightarrow O}$ for the wild type (Thr) is the largest.

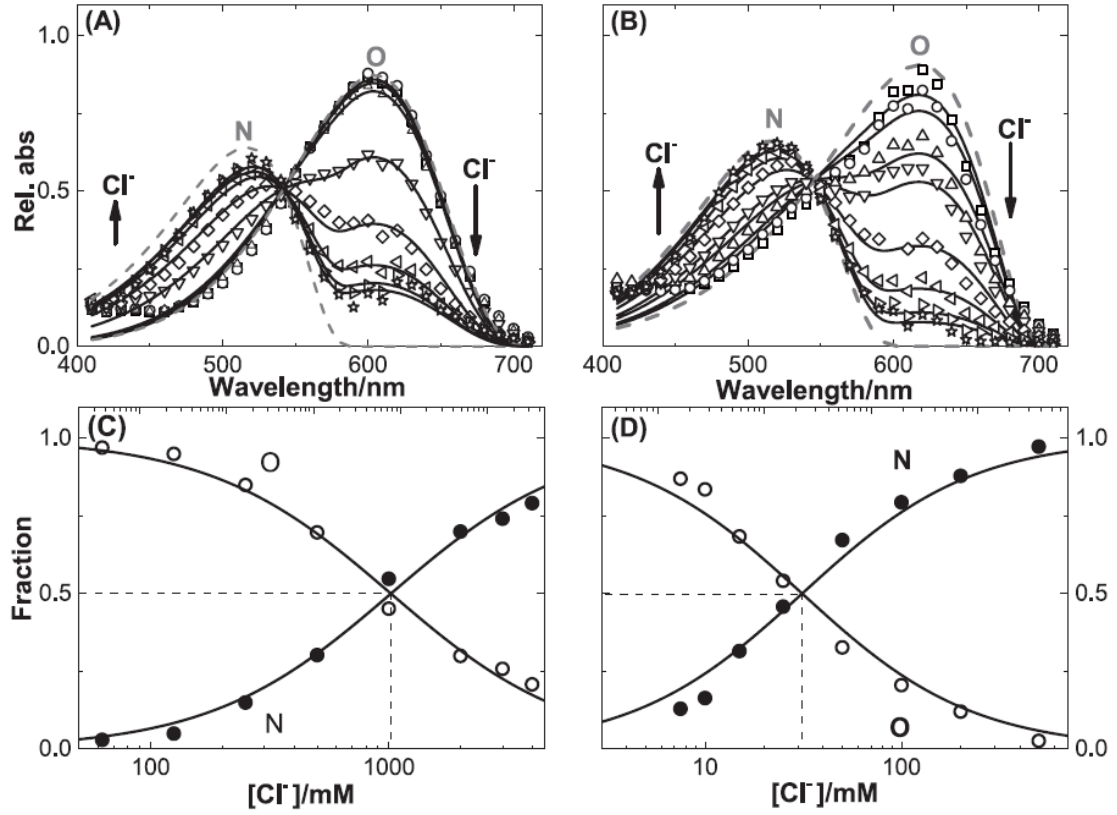


Figure 12. Determination of $K_{d, N \leftrightarrow O}$. Symbols in panels A and B show the P3 spectra of the wild type (A) and T218V (B) at Cl^- concentrations of 62.5 (square), 125 (circle), 250 (triangle), 500 (left triangle), 1000 (right triangle), 2000 (inverted triangle), 3000 (diamond) and 4000 mM (star) for wild type, and 7.5 (square), 10 (circle), 15 (triangle), 25 (left triangle), 50 (right triangle), 100 (inverted triangle), 200 (diamond) and 500 mM (star) for T218V. Solid lines are the calculated best-fit curves from Eq. 5. The determined spectra of N and O are shown by broken lines in panels A and B, and their fractions are plotted in panels C (wild type) and D (T218V) against the Cl^- concentration. Two curves in panels C and D cross each other at $f=0.5$, at the $K_{d, N \leftrightarrow O}$ concentration.

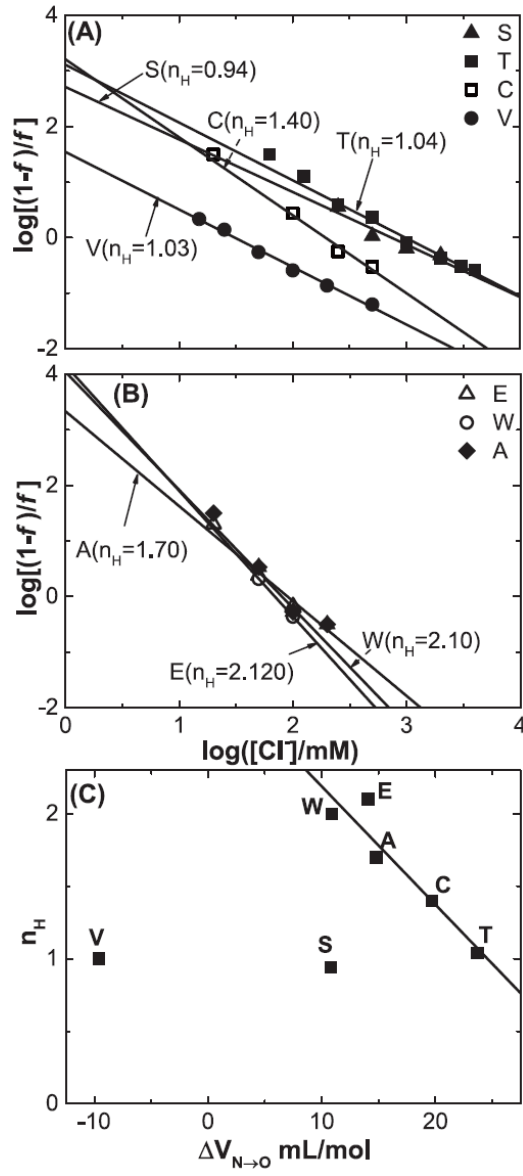


Figure 13. Determination of $K_{d, N \leftrightarrow O}$ by Eq. 8. Panels A and B are the plots of $\log [(1-f)/f]$ against $\log [Cl^-]$. The plots for three mutants (E, W, A) with large n_H (~ 2.0) are shown in Panel B. Panel C is the plot of n_H against $\Delta V_{N \rightarrow O}$.

Pressure dependences of P_i spectra

We obtained the P_i spectra ($i = 1-4$) for wild type and various mutants under various pressures. Figure 14 shows the spectra of wild type and T218V. The spectra of P_1 , P_2 and P_4 did not depend on the pressure. This was also observed for other mutants. However, the P_3 spectra of T218V showed different pressure dependence from wild type and other mutants. P_3 is the quasi-equilibrium mixture of N (short-wavelength product) and O (long-wavelength product). With increasing pressure, the N fraction increased for wild type and other mutants, whereas the O fraction increased for T218V. This pressure dependence reflects the difference in the partial molar volume between N and O. As summarized in Table , for the wild type and other mutants, the volume of O is larger than that of N. For T218V, however, the volume of O is smaller than that of N. The reason is not known at present. For wild type and other mutants, the larger volume of O probably reflects the liquid-to-crystalline transition of the water that binds to the protein interior. For T218V, the water might remain in a state different from the wild type and other mutants.

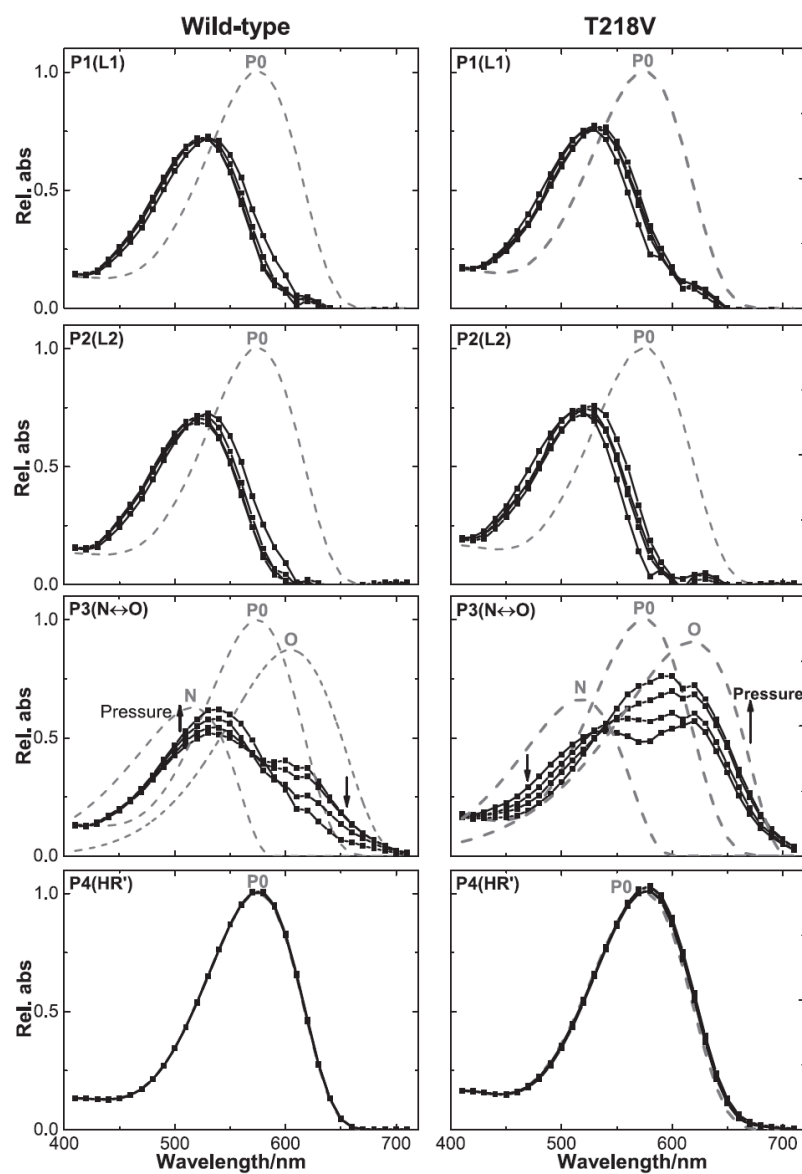


Figure 14. Pressure dependence of P_i spectra of the wild type and T218V. The buffer solution contained 1000 mM Cl^- for wild type and 25 mM Cl^- for T218V. This difference in Cl^- concentration is due to the difference in $K_{d,N\leftrightarrow O}$. The pressure applied to the samples was 1, 370, 750 and 1480 bar. Other sample conditions were the same as described in Fig 1.

Partial Molar Volume Changes between N and O

Because we assume equilibrium between N and O in P_3 , the pressure dependence of $K_{d, N \leftrightarrow O}$ results in partial molar volume changes in accordance with Eq. 11.

$$\left(\frac{\partial \log K_{d, N \leftrightarrow O}}{\partial P} \right)_T = - \frac{\Delta V_{N \rightarrow O}}{2.3RT} \quad (11)$$

where $\Delta V_{N \rightarrow O}$ is the partial molar volume change between N and O with reference to that of N, P is the hydrostatic pressure, R is the gas constant, and T is the absolute temperature. This equation describes the pressure-dependent shift of the N-O equilibrium governed by Le Chatelier's principle: the application of the pressure shifts the equilibrium toward the state having a smaller volume. In Fig 15 $\log K_{d, N \leftrightarrow O}$ is plotted against the pressure. The slope gives $\Delta V_{N \rightarrow O}$, which is listed in Table 2. The contribution of Cl^- volume is not taken into account because the values for all mutants and the wild type should be the same. The $K_{d, N \leftrightarrow O}$ values under high pressure were determined to be the same as those under normal pressure (see Fig 12). only the T218V mutant shows a negative value; the others are positive. In Fig 14, the P_1 spectra of the wild-type protein and T218V under various pressures are shown. The P_3 spectra indicate the reverse pressure dependence of N and O components for the wild type and T218V. P_1 , P_2 and P_4 show small pressure dependence.

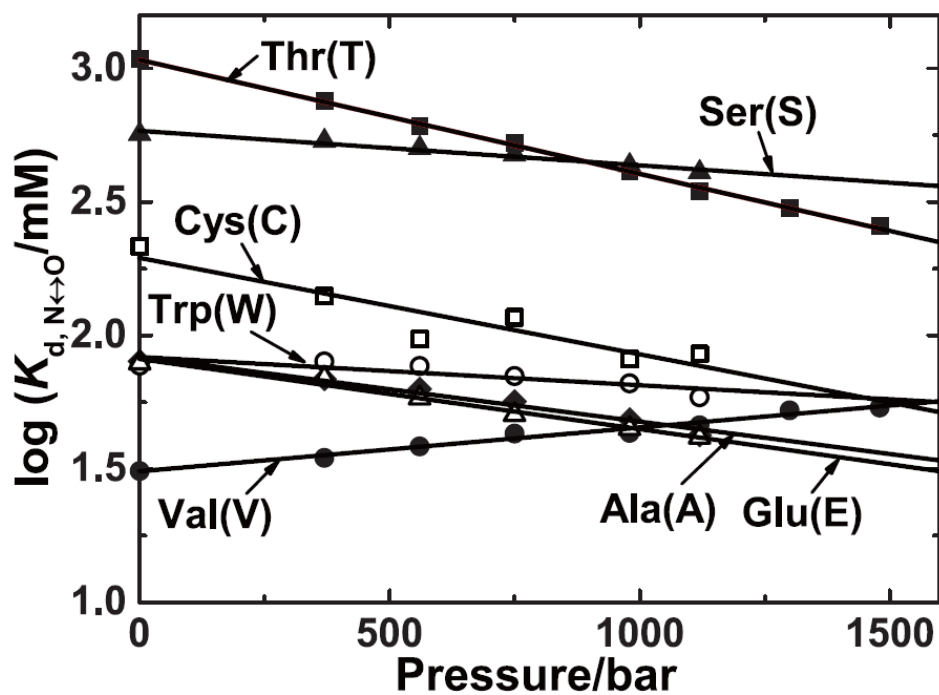


Figure 15. Pressure dependence of $K_{d,N \leftrightarrow O}$. According to Eq. 11, the slopes give $\Delta V_{N \rightarrow O}$, which are listed in Table 2.

Table 2. $K_{d, N \leftrightarrow O}$ and $\Delta V_{N \rightarrow O}$ for various mutants at the 218th position and hydrophathy index of the mutated residue

AA residue at the 218 th position	hydropathy index ^b	$K_{d, N \leftrightarrow O}$ /mM at 1 bar	$\Delta V_{N \rightarrow O}$ /(mL/mol)
Glu (E)	-0.74	80	14.1
Ser (S)	-0.18	526	7.7
Thr (T) ^a	-0.05	1000	23.7
Cys (C)	0.29	215	19.7
Ala (A)	0.62	75	14.8
Trp (W)	0.81	70	10.9
Val (V)	1.08	30	-9.6

^a; the wild type

^b; from ref. (78)

The $K_{d, N \leftrightarrow O}$ of the Gln, Met, Phe, Ile and Leu mutants was not estimated. Their $K_{d, N \leftrightarrow O}$ s were very small (less than ~5 mM), so that we could not distinguish the photocycle from that of the Cl⁻ free cycle.

Rate Constant Dependence on the Chloride Concentration

Figure 16 shows the observed rate constants of the wild-type protein (Thr (T)) and various mutants under normal pressure. Panels are arranged in the order from hydrophilic (left) to hydrophobic (right) AA residues at the 218th position. The rate constant k_1 ($\square$) is that of the transition from $P_1(L_1)$ to $P_2(L_2)$ and is faster than other rate constants for all mutants and the wild-type protein. They seem to be independent of the Cl^- concentrations, implying that this process proceeds without interaction with Cl^- in the medium. Although the molecular event underlying this transition is not clear at present, the following data have been reported. FTIR showed a change in the amide I/II vibration mode in NpHR (67), and low-temperature FTIR showed changes in the protein and water O-H stretching bands in HsHR (53) and the deprotonation of E234 at the EC surface in NpHR (68). k_2 ($\circ$) is the rate constant of the $P_2(L_2)$ -to- $P_3(N \leftrightarrow O)$ transition, and values increase roughly linearly with an increase in external Cl^- concentrations (logarithmic scale), suggesting the necessity of the presence of external Cl^- . The binding of this ion might need to occur for the P_2 -to- P_3 transition to proceed. k_3 ($\square$) is the rate constant of the $P_3(N \leftrightarrow O)$ to $P_4(O \leftrightarrow NpHR')$ transition, in which the external Cl^- enters from the EC channel to bind at the binding site near the Schiff base, because the main component of P_4 is NpHR'. Therefore, these rates should increase with an increase in the external Cl^- concentration. However, the data shows a convex curve as the function of Cl^- concentration. One plausible reason will be discussed below. The appearances of O in both $P_3(N \leftrightarrow O)$ and $P_4(O \leftrightarrow NpHR')$ implies that there exist two O intermediates as proposed by Chizhov and Engelhard (49). They denoted them as $O^{1_{600}}$ and $O^{2_{600}}$, respectively, and proposed a concept that, during $O^{1_{600}}$ to $O^{2_{600}}$ transition, the accessibility changes from the CP to EC channel. The value of k_4 (∇) is the transition rate of the $P_4(O \leftrightarrow NpHR')$ to NpHR (the original pigment) transition. Except for the wild type (Thr), the rate does not depend on the external Cl^- concentration. The molecular nature of this transition is not clear at present, as mentioned above.

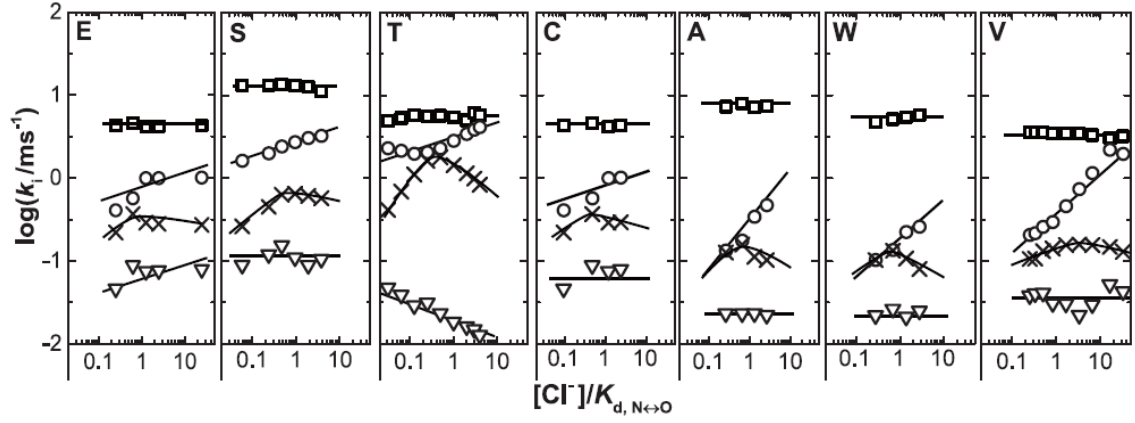


Figure 16. Cl^- concentration dependence of k_i . The Cl^- concentrations are expressed as $[\text{Cl}^-]/K_{d, \text{N} \leftrightarrow \text{O}}$, where the $K_{d, \text{N} \leftrightarrow \text{O}}$ values of the wild type and mutants are listed in Table 2. The rate constant k_1 ($\square$) is that of the transition from $\text{P}_1(\text{L}_1)$ to $\text{P}_2(\text{L}_2)$. k_2 ($\circ$) is the rate constant of the $\text{P}_2(\text{L}_2)$ to $\text{P}_3(\text{N} \leftrightarrow \text{O})$ transition. k_3 ($\times$) is the rate constant of the $\text{P}_3(\text{N} \leftrightarrow \text{O})$ to $\text{P}_4(\text{O} \leftrightarrow \text{NpHR}')$ transition. k_4 (∇) is the transition rate of the $\text{P}_4(\text{O} \leftrightarrow \text{NpHR}')$ to NpHR (the original pigment) transition. The behavior of k_3 is discussed in the discussion section.

Activation volumes associated with the decay of P_i intermediates

From the pressure dependences of the rate constants (k_i), we can estimate the activation volumes associated with the decay of P_i intermediates (ΔV_i^*) from the following relationship (12):

$$\left(\frac{\partial \log k_i}{\partial P} \right)_T = - \frac{\Delta V_i^*}{2.3RT} \quad (12)$$

Estimated values are plotted in Fig 17 against the Cl⁻ concentration divided by $K_{d, N \leftrightarrow O}$ value. The panels are arranged in order from hydrophilic (left) to hydrophobic (right) residues at the 218th position. Most activation volumes are positive (< 30 mL/mol), indicating the volume increase at the transition state. However, a systematic dependency on the Cl⁻ concentration or the hydrophobicity of the 218th residue was not found.

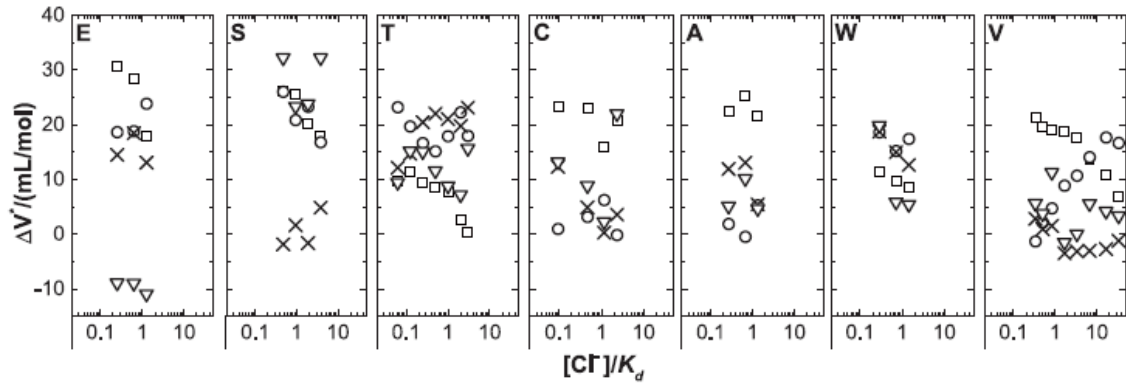


Fig 17. Cl⁻ concentration dependence of ΔV_i^* . The Cl⁻ concentrations are expressed as $[Cl^-]/K_{d, N \leftrightarrow O}$, where $K_{d, N \leftrightarrow O}$ values of the wild-type and mutants are listed in Table 2. The value of ΔV^*_1 (□) is that of the transition from P₁(L₁) to P₂(L₂). The value of ΔV^*_2 (○) is that of P₂(L₂)-to-P₃(N $\leftrightarrow$ O) transition. The value of ΔV^*_3 (×) is that of the P₃(N $\leftrightarrow$ O)-to-P₄(O $\leftrightarrow$ NpHR^{''}) transition. The value of ΔV^*_4 (▽) is that of the P₄ (O $\leftrightarrow$ NpHR^{''}: mainly NpHR^{''})-to-NpHR (the original pigment) transition.

Pumping Activities of the Wild-Type Protein and Various Mutants

The pumping activities were measured by the proton uptake under constant illumination with a glass pH electrode. Intact cells were used. The preparation (harvest and washing) of cells was carried out as quickly as possible in the cold. The photo-induced proton movements were measured in the absence of CCCP, under which condition the cells have interior negative membrane potential. The results are shown in Fig 18. The abscissa is the relative pigment concentration in the cuvette, which was estimated by the 580 nm flash yields of the original concentrated cell suspensions (see Materials and Methods for details). For clarity, the results are shown in four panels. The slopes of the lines in Fig 5, which represent relative pumping activities, are indicated in histogram in Fig 6A, where the plots are arranged in order from hydrophilic (left) to hydrophobic (right) AA residues at the 218th position. The wild type (T) shows the largest activity. The Q, M, L, F and I mutants are not listed in Table 2, but the activities are shown here. Note that the $K_{d, N \leftrightarrow O}$ values of these mutants were very small (less than 5 mM). Neither $K_{d, N \leftrightarrow O}$ nor ΔV_{N-O} were estimated due to the contamination of the Cl⁻-free photocycle. In these experiments, however, all pigments are the Cl⁻-bound form because the medium in this experiment contained approximately 190 mM Cl⁻. In Fig 6C, the pumping activities are plotted against $K_{d, N \leftrightarrow O}$, indicating that the activities increase with an increase in $K_{d, N \leftrightarrow O}$.

We also measured pumping activities using de-energized cells. These cells were prepared by gentle shaking for 2 h in the presence of CCCP. The main difference between intact and de-energized cells is the presence or absence of the membrane potential. Interestingly, the pumping activities by de-energized cells are almost equal to one another (see Fig 19B). The intriguing effect of the negative interior membrane potential will be discussed below. Proton uptake measurements for de-energized cells were performed only for samples at OD₆₆₀ of 0.5. In Fig 19B, the activities are indicated by the proton uptake divided by the pigment concentration (see Materials and Methods for details). These activities correspond to the slopes of Fig 18 and thus are comparable with the plots in Fig 19A. The smaller activities of the intact cells are due to the uphill transport in the intact cells.

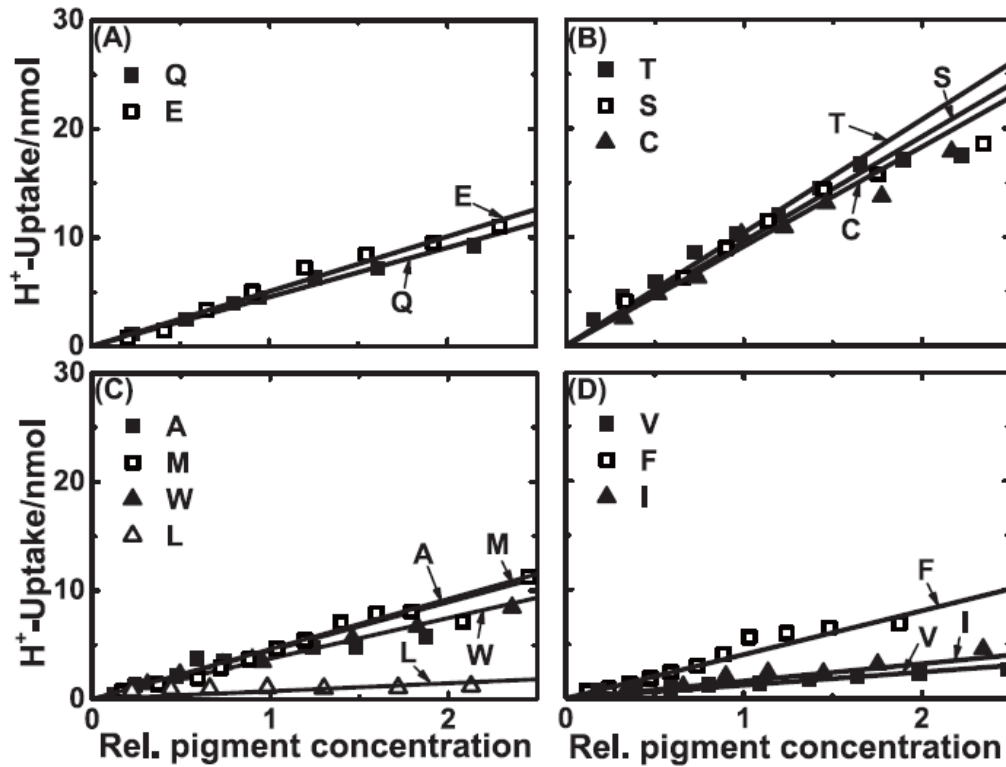


Figure 18. Pumping activities measured by photo-induced proton uptake. Amounts of photo-induced proton uptake are plotted against the relative concentrations of pigments that were estimated from flash-photolysis yields. Intact cells were used, and measurements were made in the absence of the protonophore CCCP. The medium was 150 mM NaCl, 50 mM MgSO₄, 40 mM KCl and 0.5 mM CaCl₂. For clarity, the results are shown in four separate panels. The slopes represent the pumping activity.

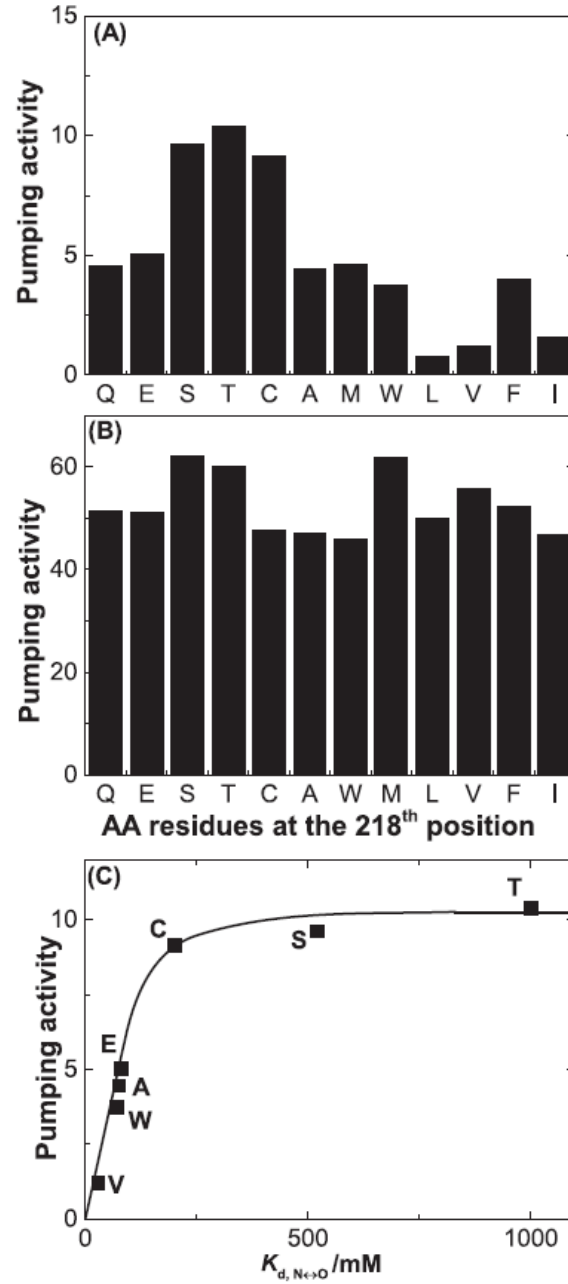


Figure 19. The correlation between $K_{d, N \leftrightarrow O}$ and the pumping activity in the presence or absence of membrane potential. Panel A: the values of the slopes in Fig 18, which represent the pumping activities, are shown as a histogram. Panel B: corresponding histogram for the de-energized cells in the presence of 10 μ M CCCP. It is noteworthy that all mutants show similar magnitudes, in contrast to Panel A. Panel C: plot of the pumping activities of intact cells (shown in panel A) against $K_{d, N \leftrightarrow O}$. The relative pumping activity increases with an increase in $K_{d, N \leftrightarrow O}$.

DISCUSSION

Cl⁻ Position in Each Photo-Intermediate

As described above, O is a Cl⁻ free form in which Cl⁻ is released into the CP space. Because N and O are in quasi-equilibrium, it is natural to consider that at the N state, Cl⁻ is in the CP channel. There is supporting evidence for this assumption. A NpHR mutant engineered to bind with the NpHtrII (halobacterial transducer II from *N. pharaonis*) appreciably decreased the pumping activity by the complex, and no N was found in the photocycle of the complex (50). In sensory rhodopsin I and II (SRI and SRII, also called phoborhodopsin), pigment proteins themselves have photo-induced proton pumping activity, but the complexes with the Htr (halobacterial transducer) lose activity (18, 19). This is interpreted in terms of 'CP channel closure'. Protons are considered to circulate only within the EC channel. Similarly, in the NpHR-NpHtrII complex, illumination may induce the Cl⁻ circulation within the EC channel, resulting in no transport activity and no appearance of the N intermediate. Muneyuki et al. showed that charge (probably negative charge of Cl⁻) movement occurred during the formation and decay of N (69). The N-to-O transition is the process of releasing Cl⁻ from the protein, and the production of electrogenicity at the N decay is then conceivable. Another conclusion of Muneyuki et al. (69) was electrogenicity at the N-state formation. This might be considered the Cl⁻ translocation from EC to CP across the Schiff base from the precursor to N because Cl⁻ is located in the vicinity of retinal in the EC channel in the unphotolyzed state. The X-ray structure of L1 of HsHR revealed that Cl⁻ remained in the EC channel (70). Low-temperature FTIR of HsHR showed that Cl⁻ stayed in the EC channel near the Schiff base at both L₁ and L₂ (53). The three possibilities regarding the timing of the Cl⁻ movement across retinal are the K-to- L₁, L₁-to- L₂ and L₂-to-N transitions. The observations described above suggest strongly that during the transition from L₂-to-N, Cl⁻ moves from EC to CP across retinal. From an electrogenicity experiment using an NpHR-entrapped gel, Ludman et al. located the 'extracellular-to-cytoplasmic switch' at the L₂-to-N step (47), which is consistent with our assumption. The presence of negative charge (Cl⁻) in the vicinity of retinal in the CP channel at N is similar to the case of N of BR in which deprotonated Asp96 is located in the CP channel.

k_2 (Transition of L_2 -to-($N \leftrightarrow O$) Complex) and k_3 (Transition of ($N \leftrightarrow O$) Complex-to-NpHR') are Largest for the Wild Type

Although Fig 16 shows the rate constants of k_i ($i = 1 \sim 4$), only k_2 and k_3 at the $K_{d, N \leftrightarrow O}$ are shown in Fig 20. Note that the ordinate scale is logarithmic in Fig 20. The transition rate constant of L_2 to ($N \leftrightarrow O$) is k_2 , and those of the wild-type (T) and Ser mutant are the largest. As described above, in this process, we assume that Cl^- transfers from EC to CP across the Schiff base. Thr or its Ser homolog may interact with Cl^- to generate prompt N formation, and the OH group of the residue may be important. It is noted that in the dark state of NpHR, Cl^- interacts with the OH group of Ser130 and Thr126 as well as with one water molecule and the protonated Schiff base (6). The importance of Ser130 and Thr126 on Cl^- binding in the dark was revealed experimentally (61, 62). The position of the Cl^- -binding/interacting site in the CP channel at N is not clear at present. It is reasonable, however, to assume that the interaction with the OH group of Thr218 induces the prompt formation of N. The rate constant k_3 represents the process of Cl^- uptake from the EC space, and the value is largest for the wild type. The reason why the wild-type k_3 value is the largest is not understood. Although the event occurs in the EC channel, Thr218 is located in the CP channel. Note that Thr218 is located close to the Schiff base.

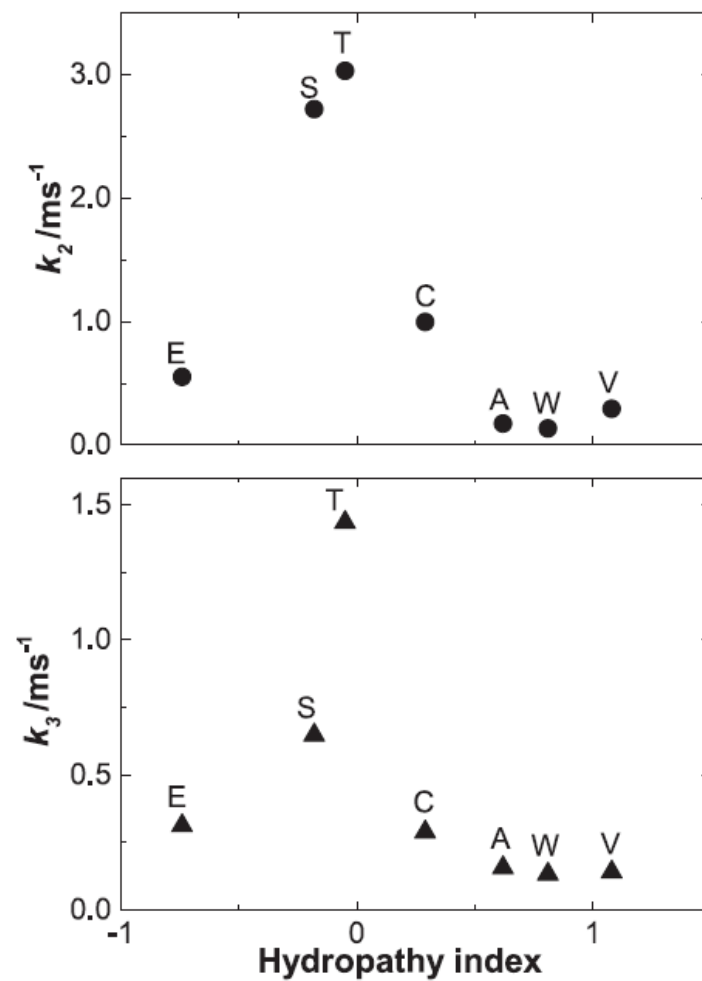


Figure 20. The plots of k_2 and k_3 at the $K_{d, N \leftrightarrow O}$ against the hydropathy index of the residue at the 218th position. Those of the wild type (T) and Ser (S) mutant are the largest.

Special Coordination of Water with the OH Group of Thr218 May be Necessary

As shown in Table 2, the largest value of $K_{d, N \leftrightarrow O}$ is given by Thr (wild type), and although hydrophilicity or hydrophobicity may not be important, the OH group of Thr may be essential. As described above, we measured the partial molar volume change from N to O ($\Delta V_{N \rightarrow O}$) (see Table 2) from the pressure dependence of $K_{d, N \leftrightarrow O}$. Large volume changes of the proteins usually reflect changes in the hydration and packing states of the proteins. Volume increases were also observed in the M-to-N transition of the BR photocycle (33,63). For BR; it has been widely considered that the outward tilting of helix F in the late M state enables water molecules to hydrate the CP channel and to create a transient hydrogen-bonded network (71-74). The liquid water probably binds to the protein interior in a crystalline-like state, so the water entry from the bulk phase could in part contribute to the volume increase. Similar to BR, water may enter NpHR at O (except the V mutant), probably by the opening of helix F. In Fig 21, $K_{d, N \leftrightarrow O}$ values are plotted against $\Delta V_{N \rightarrow O}$. Both $K_{d, N \leftrightarrow O}$ and $\Delta V_{N \rightarrow O}$ are largest for the wild-type protein. Except for T218V, the amounts of water entered do not vary much for the mutants. This tendency is also seen in the inset of Fig 21 where $\Delta V_{N \rightarrow O}$ of various mutants is plotted against the hydropathy index. Thus, we infer that for the Cl^- release (the increase in $K_{d, N \leftrightarrow O}$), water entry is necessary but not sufficient. Special coordination between the entered water molecule and the OH group of Thr may be necessary. The X-ray structure showed the formation of a hydrogen-bonding network between Thr218 (F helix), Ala255 (G helix) and Trp222 (F helix). The proper interaction of the OH group of Thr218 with the entered water disturbs the hydrogen-bonding network, which induces the conformational changes of F and G helices and increases $K_{d, N \leftrightarrow O}$. These three residues are also conserved in BR as Thr178, Ala215 and Trp182, respectively. For BR, the photo-induced switches of the hydrogen-bonding network are considered to cause the subsequent conformational changes in the CP channel (74).

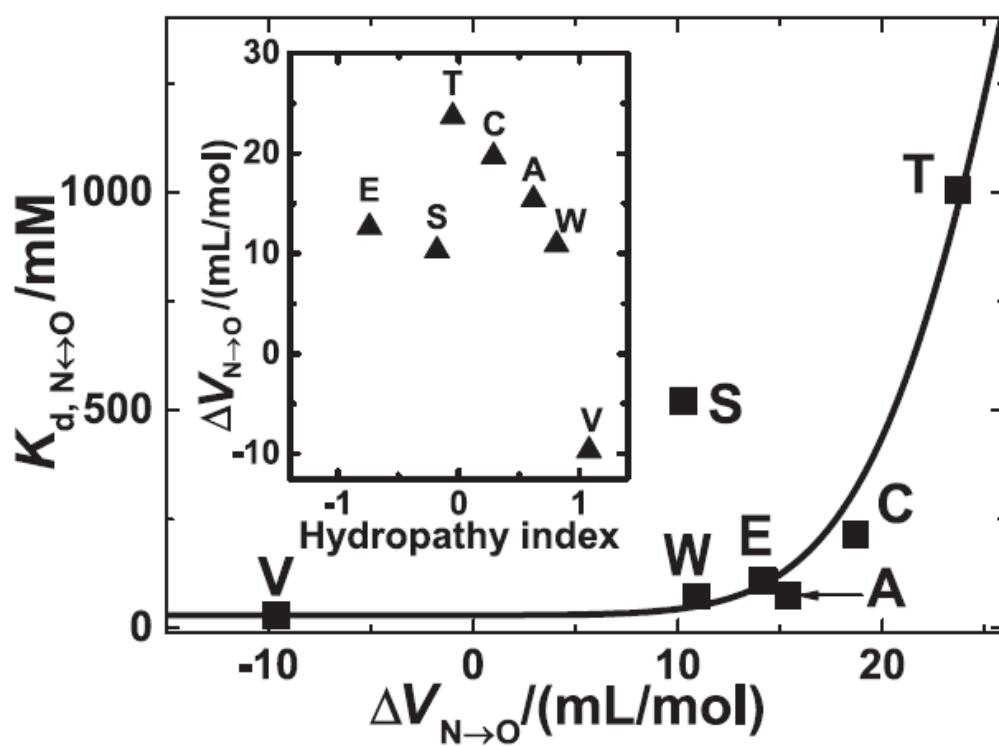


Figure 21. The plot of $K_{d,N \leftrightarrow O}$ values against $\Delta V_{N \rightarrow O}$. In the inset, $\Delta V_{N \rightarrow O}$ is plotted against the hydropathy index of the residue in the 218th position.

Cl⁻ Dependence of k_3 .

The Cl⁻ concentration dependence of this rate constant is peculiar (see Fig 3). The curve is convex. This rate constant is for the O decay. Exactly this is the decay of P₃ (quasi-equilibrium between N and O). However we assume that this decay is that of O in which the ‘external’ Cl⁻ entered into the protein through the EC channel to the Cl⁻ binding site in the vicinity of retinal. Therefore, the rate should increase with an increase in the Cl⁻ concentration. These values increase for the wild type and mutants until about the $K_{d, N \leftrightarrow O}$ value (Fig 16). k_3 should be expressed as

$$k_3 = k^0 \times [O] \times [Cl^-] \quad (13)$$

Here, k_0 is a constant. The O concentration, [O], is proportional to $K_{d, N \leftrightarrow O} / (K_{d, N \leftrightarrow O} + [Cl^-])$, and then

$$k_3 = k_0 \frac{K_{d, N \leftrightarrow O}}{K_{d, N \leftrightarrow O} + [Cl^-]} \times \frac{[Cl^-]}{K_d' + [Cl^-]} \quad (14)$$

k_3 is expected to exhibit Michaelis-Menten behavior. As shown in Fig 16, below the $K_{d, N \leftrightarrow O}$, Eq. 13 seems to hold. However, the values decrease at higher Cl⁻ concentrations. This might be explained if [Cl⁻] is not that of the external medium. There might also be a second Cl⁻ binding site. Mevorat-Kaplan et al. (75) suggested that at the dark state a chloride binding site on the EC channel with a dissociation constant (K_d') of ~100 mM may be present. Recent X-ray crystal structure analysis also showed a weak Cl⁻-binding site locating on the EC side between the BC loop and FG loop (76). During the O-to-NpHR' transition, the Cl⁻ may move from this site to the binding site near the Schiff base. We assume that binding equilibrium of Cl⁻ between this binding site on the EC channel and the external medium is attained and that the Cl⁻ transfer process from this site to the vicinity of retinal is a rate-determining process. Under these assumptions, k_3 is expressed as follows instead of as in Eq. 13:

$$k_3 = k_0 \frac{K_{d, N \leftrightarrow O}}{K_{d, N \leftrightarrow O} + [Cl^-]} \times \frac{[Cl^-]}{K_d' + [Cl^-]} \quad (15)$$

Here, we assume $K_d' < K_{d, N \leftrightarrow O}$. When $[Cl^-]$ is dilute, k_3 increases with the increase of $[Cl^-]$, reflecting the increase of the last term of Eq. 14. With a further increase in $[Cl^-]$, k_3 decreases because the middle term of Eq. 14 decreases while the last term is constant. After the Cl^- movement in the EC channel, a Cl^- uptake should occur from the bulk to the binding site on the EC side when $[Cl^-]$ is larger than K_d' . This probably occurs during the NpHR'-to-NpHR transition. However, the rate constants (k_4) were independent of $[Cl^-]$ for all mutants and for wild-type, this rate inversely slowed down with the increase of $[Cl^-]$ (Fig 16). Thus, there are other factors determining this rate. For the detailed molecular event, further studies are needed.

At lower $[Cl^-]$, some NpHR's do not have the second Cl^- in the dark state. These NpHR's probably undergo the Cl^- transporting photocycle. During the O-to-NpHR' transition, however, they probably capture Cl^- from the bulk. We infer that these NpHR's have very small k_3 due to the slow capture of Cl^- from the bulk. Thus, Eq.9 does not include the contributions of these NpHR's. At lower $[Cl^-]$, some NpHR's probably return to the original dark state without the binding of second Cl^- during NpHR'-to-NpHR transition.

A mutant KM-1 of *N. pharaonis* was isolated by UV radiation. From this cell, a membrane fraction containing large amounts of NpHR can be isolated (77). Using this membrane fraction, $K_{d, N \leftrightarrow O}$ was estimated to be ~ 4 M (data not shown). The value of $K_{d, N \leftrightarrow O}$ may be influenced by the presence of lipids, especially by tightly bound bacterioruberin or the solubilized state. For KM-1 membrane, a decrease in k_3 was not observed at $[Cl^-] < 4$ M, probably because of the large $K_{d, N \leftrightarrow O}$.

Effect of Mutations on Pumping Activity

The measurements of Cl^- -pumping activities, whose results are shown in Figures 18 and 19, are the steady state experiments where *E. coli* cells were illuminated by the constant light. On the other hand, other measurements are the transient state experiments where the NpHR samples were activated by the flash light. Photo-induced HR activity shown in Figures 18 and 19 may be primarily determined by the rate of cycling or the turnover rate (41). Figure 16 shows that the slowest transition is k_4 ($\text{NpHR}' \rightarrow \text{NpHR}$). Therefore, the shift of the $\text{N} \leftrightarrow \text{O}$ equilibrium as well as the change in k_2 and k_3 are not considered to affect the pumping activity. However, Fig 18 and 19A show the variation of the photo-induced proton uptake for the mutants. The uptake of intact cells was measured in the absence of CCCP, i.e., in the presence of membrane potential that was produced by intracellular metabolism. In addition, Fig 19C shows that the relative pumping activity increased with an increase in $K_{\text{d}, \text{N} \leftrightarrow \text{O}}$, indicating that the activity depends on the $\text{N} \leftrightarrow \text{O}$ equilibrium. This means that when the equilibrium shifts to O, the pumping activity is increased. However, Fig 19B shows almost no or smaller variation of mutant pumping activity. The cells used were de-energized cells prepared by gentle shaking for 2 h in the presence of CCCP, and measurements were made in the presence of CCCP, so no interior negative membrane potential being generated. An interior negative membrane potential hinders proton uptake because of the uphill transport, so the scale of Fig 19A is smaller than Fig 19B. Fig 3 shows that the slowest transitions of k_4 are almost equal one another. The data in Fig 19B is understandable from the standpoint of the turnover rate. Of interest, for the de-energized cells, the mutants with immeasurably small $K_{\text{d}, \text{N} \leftrightarrow \text{O}}$ values (Gln, Met, Leu, Phe and Ile) show the large response (Fig 19B), whereas those in the presence of membrane potential are small (Fig 19A). The difference between the energized and non-energized cells is the presence or absence of interior negative membrane potential. Recently, we found that the interior negative membrane potential shifted the $\text{N} \leftrightarrow \text{O}$ equilibrium to N, which decreases the O concentration and thereby decreases k_3 (see. Eq. 13) (in preparation). Particularly for mutants with a small $K_{\text{d}, \text{N} \leftrightarrow \text{O}}$, the slowest process might become k_3 in the presence of membrane potential. The Cl^- release of these mutants might involve larger electrogenecity than the wild type. In the presence of membrane potential, this larger electrogenecity might contribute

to the substantial delay of k_3 through the remarkable shift of $N \leftrightarrow O$ equilibrium toward N. These mutants have low activity in the presence of membrane potential. In other words, a high concentration of O is necessary in order not to decrease the activity in the presence of membrane potential, and in this sense, Thr in the 218th position is suitable. Another possibility is that when the $N \leftrightarrow O$ equilibrium shifts greatly to N, most NpHR returns to the original state through a non-electrogenic pathway (such as $N \rightarrow \text{NpHR}'$ or NpHR). Hackmann et al. (67) previously proposed a similar non-electrogenic pathway based on time-resolved FTIR measurements at a high concentration of Cl^- , although they proposed a $L_2 \rightarrow \text{NpHR}$ pathway. Comparison of HsHR with NpHR. Váró et al. described the photocycle of HsHR as $\text{HsHR} \rightarrow K \leftrightarrow L_1 \leftrightarrow L_2 \leftrightarrow N \rightarrow \text{HsHR}$ and found that the rate constants did not show any or very small Cl^- dependence (42). Comparison with the NpHR cycle indicates that the HsHR cycle lacks the O intermediate, but they considered that the missing of O may have a kinetic cause. The missing O may eliminate the Cl^- -dependent process. Based on the analysis of the present investigation, we infer that the K_d of the $N \leftrightarrow O$ quasi-equilibrium largely shifts to N for HsHR. The residue at the 218th position is not the only factor determining $K_{d, N \leftrightarrow O}$ because the corresponding residue of HsHR is Thr203. There are other factor(s) determining $K_{d, N \leftrightarrow O}$.

CONCLUSIONS

We made various NpHR Thr218 mutants and examined their photochemistry. The photochemically defined intermediate P_3 consists of a quasi-equilibrium between the N and O intermediates. The equilibrium constant $K_{d, N \leftrightarrow O}$ was defined in Eq. 3, and $K_{d, N \leftrightarrow O}$ was the largest for the wild-type NpHR. The partial molar volume changes between N and O states ($\Delta V_{N \rightarrow O}$) were estimated from the pressure dependence of $K_{d, N \leftrightarrow O}$. Except for T218V, the volume of O is larger than N. If the volume change of protein is due to the change in hydration, water could enter through the F-helix opening, which occurs in BR. The largest $K_{d, N \leftrightarrow O}$ and $\Delta V_{N \rightarrow O}$ values were obtained from the wild type, whereas for some mutants, $\Delta V_{N \rightarrow O}$ was large, but $K_{d, N \leftrightarrow O}$ was small. These findings may indicate that water entry is necessary but not sufficient. Special coordination of the water molecule with the OH group of the Thr at the 218th position may be necessary to release Cl⁻ to the CP space. The rate constants k_2 and k_3 are largest for the wild type. The wild-type protein rapidly transfers Cl⁻ from the binding site near the Schiff base in the dark to the CP space. k_3 showed a convex curve with respect to the Cl⁻ concentration, leading to the hypothesis that a second Cl⁻-binding site exists in the EC channel (see Eq. 14). This should be investigated further. The pumping activity in the presence of interior negative membrane potential increased with an increase in $K_{d, N \leftrightarrow O}$. However, for de-energized cells in the presence of CCCP, activities were almost the same for all mutants. Although one possible reason was described above, further investigation is required to determine the true cause. However, the measurements in the presence of CCCP might not be the true activity in some cases.

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