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Review

Hydrogen-bonding changes of internal water molecules upon the actions of microbial rhodopsins studied by FTIR spectroscopy☆

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ABSTRACT

Microbial rhodopsins are classified into type-I rhodopsins, which utilize light energy to perform wide varieties of function, such as proton pumping, ion pumping, light sensing, cation channels, and so on. The crystal structures of several type-I rhodopsins were solved and the molecular mechanisms have been investigated based on the atomic structures. However, the crystal structures of proteins of interest are not always available and the basic architectures are sometimes quite similar, which obscures how the proteins achieve different functions. Stimulus-induced difference FTIR spectroscopy is a powerful tool to detect minute structural changes providing a clue for elucidating the molecular mechanisms. In this review, the studies on type-I rhodopsins from fungi and marine bacteria, whose crystal structures have not been solved yet, were summarized. *Neurospora* rhodopsin and *Leptosphaeria* rhodopsin found from Fungi have sequence similarity. The former has no proton pumping function, while the latter has. Proteorhodopsin is another example, whose proton pumping machinery is altered at alkaline and acidic conditions. We described how the structural changes of protein were different and how water molecules were involved in them. We reviewed the results on dynamics of the internal water molecules in *pharaonis* halorhodopsin as well. This article is part of a Special Issue entitled: Retinal Proteins — You can teach an old dog new tricks.

1. Introduction

Ion pump proteins are membrane proteins that actively transport ions. The generated electrochemical potential is utilized for ATP synthesis, motility of bacterial cells, electric signals in nerve systems. Bacteriorhodopsin (BR), a light-driven proton pump protein in *Halobacterium salinarum*, is the best characterized molecular pump, whose atomic structure [1–3] and proton pathway have been determined (Fig. 1) [2,4–7]. Light absorption induces photoisomerization of the retinal chromophore from the all-*trans* to 13-*cis* form in the subpico-second time scale. The primary proton transfer from the protonated retinal Schiff base to Asp85 takes place in the submillisecond time scale and is followed by sequential proton transfer reactions in BR. As a consequence, one proton is transported by each photo-isomerization reaction. It is generally accepted that a negatively charged aspartate (Asp85 in case of BR) and its transient protonation are necessary for the proton pumping function of BR, because the mutation of the residue into threonine or serine converts the function to the chloride ion pump like as halorhodopsin [8].

Microbial rhodopsins have been also discovered in eucaryotes [9–13] and eubacteria [14–16] recently. They are called type-I rhodopsins to discriminate phylogenetically from type-II rhodopsins (e.g. visual rhodopsin, melanopsin, and so-called non-visual rhodopsins). The discovery of type-I rhodopsins in many organisms provided a good opportunity to test the molecular mechanism of proton pumping. However, the accumulated knowledge on these proteins questions the common mechanism for light-driven proton pumping in type-I rhodopsins. *Neurospora* rhodopsin (NR) and *Leptosphaeria* rhodopsin (LR) found in fungi, possess very similar amino acids (the identities of amino acids between LR and BR, and between LR and NR are 25.7% and 55.8%, respectively) and preserve the amino acid residues involved in the retinal binding site and proton pathway of BR. Some exceptions are as follows: Val49 in BR is replaced by Ile in LR and NR, Asp96 is replaced by Glu in NR, and Glu194 is replaced by Asp in LR, while it is deleted in NR. Nevertheless, it was found that NR does not pump protons [17], while LR does [18].

In the other kingdom, various species of marine γ-proteo-bacteria possess archaeal-type rhodopsins that function as light-driven proton pumps, which are called proteorhodopsins (PR) [14,19]. Proteorhodopsins are

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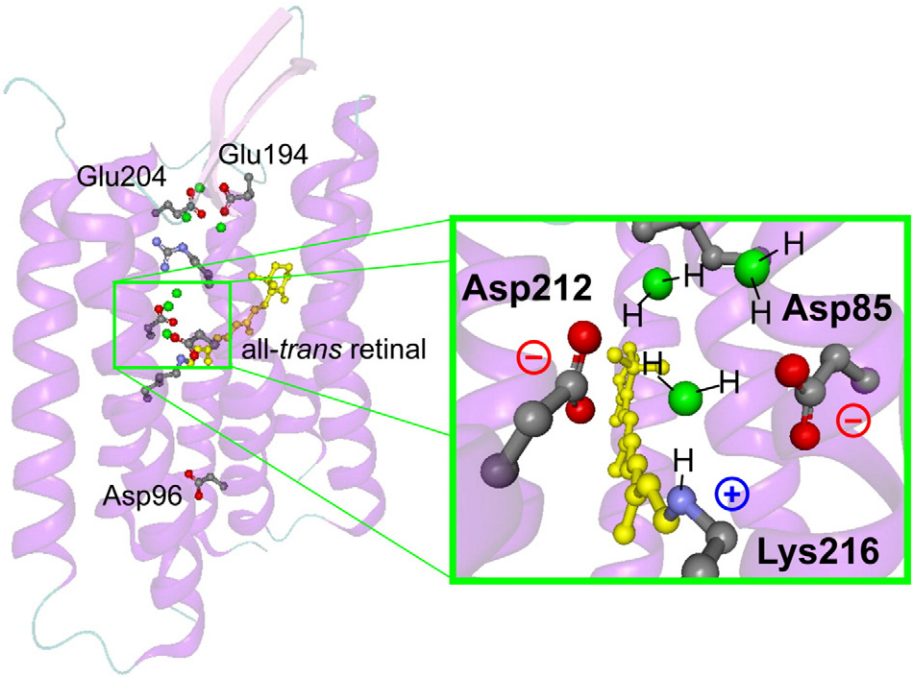

Fig. 1. The X-ray crystal structure of bacteriorhodopsin (BR) (PDB code; 1C3W) [77]. The Schiff base region is expanded in the right hand side. The oxygen atoms of water molecules are shown as green spheres. Asp96 is the internal proton donor for the Schiff base. Glu204 and Glu194 constitute the proton releasing group.

diverged in accordance with the light environment which changes depending on the depth of the ocean and mainly classified into green- (λ_{max} ~ 525 nm) and blue-absorbing (λ_{max} ~ 490 nm) PRs [19]. Among thousands of PRs, green-absorbing PR is better characterized than others (below, we call this protein PR). The counterion of the Schiff base in PR is Asp97, and its pKa is considerably higher in PR (~7) than that of homologous Asp85 in BR (2–3) [20,21]. This indicates that Asp97 has a negative charge only at alkaline pH. There has been a debate on the pH dependence of the proton-pumping activity of PR. One group reported that only the alkaline form of PR pumps protons, while the acid PR does not [20,22]. In contrast, another group reported that the alkaline and acid PR pump protons by two-photon and single-photon absorption mechanisms, but the direction of transportation of protons is outward and inward, respectively [21]. Thus, the proton-pumping mechanism of PR is still not understood well.

The prediction of protein function from the amino acid sequence information partly succeeded in the phylogenetic analysis. The proteins exhibiting similar function constitute a cluster in the phylogenetic tree. As has been seen in fungal rhodopsins and proteorhodopsin, it has been still difficult to explain the protein function from the sequence similarity. X-ray crystal structures may help us to understand the molecular mechanisms of the proteins. However, it is still generally difficult to crystallize membrane proteins, although many improvements in crystallography have been developed [1–3,23]. The basic architecture of type-I rhodopsins are quite similar and sometimes does not tell us how the protein acquires different functions. It may be due to the limited resolution in the crystal structural data or lack of information about protein dynamics. Vibrational spectroscopy can be used as a complementary method covering the pit hole of the crystallographic study which has generally difficulty in monitoring hydrogen atoms and protonation state of carboxyl residues in protein.

Fourier-transform infrared (FTIR) spectroscopy produces highly accurate and reproducible spectra, which allows us to calculate the difference spectrum with minute absorbance changes (up to ~10^{-6}) caused by photo reaction in type-I rhodopsins. The technique has been established with progress in the study of the molecular mechanism of bacteriorhodopsin by various pioneer researchers [5–7,24,25]. By using attenuated total reflection (ATR) method, the stimulus-induced difference infrared spectroscopy is applicable for various stimuli, such as electric potential changes, ligand binding, ion binding, and so on [26–28]. Among them, light stimulus can be utilized in various conditions, initiate photoreaction even at cryogenic temperature, and is easily controlled by electronics. Time-resolved measurement is readily applicable by combining with modern laser technique.

Therefore, not only before but also after obtaining the precise crystal structures of proteins, the stimulus-induced difference infrared spectroscopy is a powerful tool to investigate the molecular mechanisms of the proteins. In this review, we have summarized the FTIR spectroscopic investigation of the hydrogen-bonding network around the Schiff base region of fungal rhodopsins and proteorhodopsin, whose crystal structures have not been solved yet. *pharaonis* Halorhodopsin (*p*HR) from *Natronomonas pharaonis* is another good example. The crystal structure has been already solved in atomic resolution. *p*HR function as light-driven chloride ion pump and has been studied by several physicochemical methods, while it is less known how the internal water molecules are involved in the anion pumping mechanism.

2. The hydrogen-bonding network around the Schiff base region is a key structural element for proton pumping function in type-I rhodopsins

The initial proton transfer reaction in BR occurs from the protonated Schiff base to Asp85 upon the formation of M intermediate. The X-ray crystal structure revealed the pentagonal cluster structure including three water molecules and two aspartate residues (Fig. 1, right). Previously, we have studied protein structure and structural changes of several archaeal rhodopsins by means of low-temperature Fourier-transform infrared (FTIR) spectroscopy. Newly developed measurements in a frequency region detecting X–H and X–D (X = O, N) stretching vibrations (4000−1800 cm^{-1}) provided new information about hydrogen-bonding network including internal water molecules [29]. In fact, comparing the K intermediate (BR_K) minus BR difference

spectra recorded with D_2O or $D_2^{18}O$ in the X–D stretching region (2700—1800 cm^{-1}) enabled us to assign the O–D stretching vibrations of water molecules not only under weak hydrogen-bonding (at >2500 cm^{-1}) but also under strong hydrogen-bonding (at <2400 cm^{-1}) conditions (Fig. 2a) [30]. Several mutational studies showed that one of the O–D stretches (2171 cm^{-1}) originates from a water molecule bridging between the Schiff base and its counterion (Asp85) [31]. Such observation is fully consistent with the QM/MM calculation of the Schiff base region of BR [32]. Hydration switch of the water plays an important role in the proton transfer reactions in BR [33]. Another QM/MM calculation also predicted that the existence of the bridging water affects the proton-transfer pathway from the Schiff base to Asp85 [34]. Interestingly, comprehensive studies of BR mutants and other rhodopsins have revealed that strongly hydrogen-bonded water molecules are found only in the proteins exhibiting proton-pumping activity [30,35–38]. This suggests that a strongly hydrogen-bonded water molecule that bridges the Schiff base and its counterion is essential for the proton-pumping function. What is the case for fungal rhodopsins, NR and LR? The former has no proton pumping activity, but the latter has.

2.1. Fungal rhodopsins

Biophysical studies on heterologously expressed fungal rhodopsins provided interesting and suggestive information on their putative functions even if the physiological data are not available. The first heterologously expressed rhodopsin from *Neurospora crassa* (NR) [9], exhibits slow photocyclic reaction, which is characteristic to a photosensory rhodopsin and not to a BR-like proton pump. It contradicts the expectations based on its high sequence similarity to BR [17,39]. The replacement of the cytoplasmic Glu142, which corresponds to Asp96 in BR and potentially works as proton donor of the Schiff base, with Gln does not affect the rate of the Schiff base reprotonation [17]. The structure of the hydrogen-bonding network around the retinal Schiff base was suggested to be very different between NR and BR [40], which may explain the lack of the reprotonation switch (the reorientation of the Schiff base into the cytoplasmic direction) in NR. In particular, as shown later, there is no O–D stretching vibration due to a strongly hydrogen-bonded water molecule near the Schiff base of NR which shows the frequency shift upon the photoisomerization [40]. The feature is known to correlate with the proton-pumping ability in many rhodopsins [41].

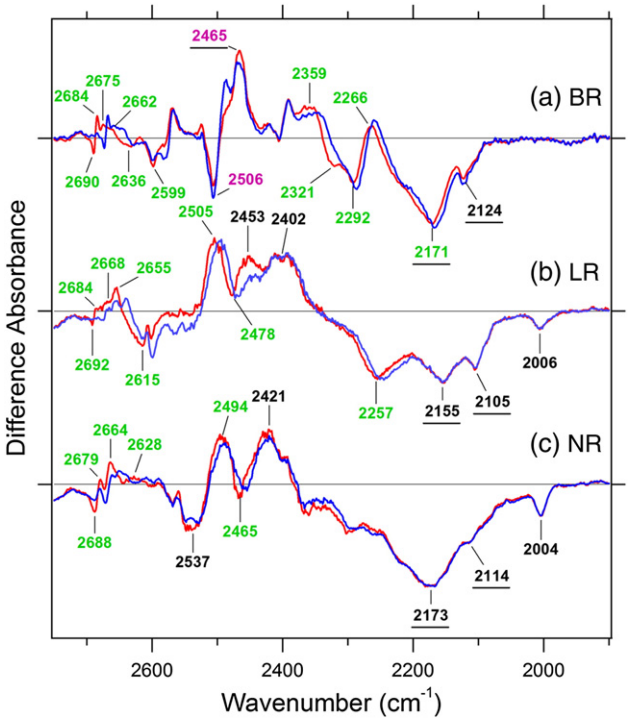

Fig. 2. The BR_K minus BR (a), LR_K minus LR (b), and NR_K minus NR (c) spectra in the 2750—1900 cm^{-1} region. The sample was hydrated with D_2O (red lines) or $D_2^{18}O$ (blue lines). The spectra are reproduced from Sumii et al. [44]. Green-labeled frequencies correspond to those identified as water stretching vibrations. In BR (a), purple-labeled frequencies are O–D stretches of Thr89 [56,78], while the underlined frequencies are N–D stretches of the Schiff base [54]. One division of the y-axis corresponds to 0.00065 absorbance units.

The second fungal rhodopsin analyzed physicochemically was found in the genome of *Leptosphaeria maculans* [42]. *Leptosphaeria* rhodopsin (LR) showed even higher sequence similarity with BR, suggesting that it may work as a proton pump. Indeed, the protein sample heterologously expressed in *Pichia pastoris* showed very fast photocycle as similarly to BR, and exhibited light-dependent proton pumping activity in liposomes [18]. The replacement of Asp150, the homolog of Asp96 in BR, with Asn resulted in dramatic slowdown of the reprotonation of the Schiff base, which suggests that Asp150 is an internal proton donor for the Schiff base like Asp96 in BR. These findings demonstrated that LR is the first eucaryotic retinal-based light-driven proton pump, although its physiological role in the fungus is still mysterious.

Fig. 2 shows the light-induced difference infrared spectra in the 2750—1900 cm^{-1} region. In NR, only two negative peaks assignable to the O–D stretching vibrations of water at 2688 and 2465 cm^{-1} were observed [40] (Fig. 2c). The water stretching vibrations of NR_K were assigned to the bands at 2679, 2664, 2628, and 2494 cm^{-1}. In the case of LR, four negative bands were observed at 2692, 2615, 2478, and 2257 cm^{-1} (Fig. 2b). Corresponding positive bands for LR_K are located at 2684, 2668, 2655 and 2505 cm^{-1}. It should be emphasized that there are no water bands in the <2400 cm^{-1} region for NR (Fig. 2c), whereas LR possesses the O–D stretch of water at 2257 cm^{-1} (Fig. 2b). Therefore, in contrast to NR, LR contains a strongly hydrogen-bonded water molecule.

The differences in the primary structures of LR and NR were analyzed before [42–44]. There are just two outstanding differences in the conserved residues important for the functioning of BR; one is the homologs of Glu194 and the other is the homologs of Asp96. Carboxylic sidechain of Glu194, known to be important for the proton release in BR [45], is missing in NR, but present as Asp248 in LR. While the Schiff base proton donor of BR (Asp96) is conserved as Asp150 in LR, it is conservatively replaced in NR, being present as Glu142 (see Fig. 1 in ref. [44]). It is hard to imagine that a replacement as conservative as Asp->Glu would abolish the proton transport completely. Indeed, a glutamate residue at this position serves as a Schiff base proton donor in eubacterial proton pump proteorhodopsin [20] and the D96E mutant of BR was reported to be fully active in terms of proton pumping [46,47], at least in the reconstituted systems. Nevertheless, the same BR mutant in the native purple membrane shows very slow reprotonation of the Schiff base [48]. Namely, the M-decay time constant of the D96E mutant in aqueous suspension is 8 times larger than that of the wild-type BR, but 6.5 times smaller than that of the D96N mutant. It suggests that the geometry of the proton donor may be important for the optimal rate of the proton translocation.

We applied low-temperature FT-IR to analyze perturbations of the molecular structure around the retinal chromophore in LR induced by the introduction of Glu or Asn at the position 150 [49]. Asp150 is homologous to Asp96 in BR, which is located 12 Å from the retinal Schiff base nitrogen, suggesting that the structure around the Schiff base should not be changed by these mutations. Thus, it is remarkable that the LR_K minus LR difference spectra of the mutants, especially D150E, become more similar to that of NR, which has glutamate at the corresponding position. From our FT-IR results, the alteration of amide I vibrations suggests that the packing of helices B, C, and G is affected by the mutations. The bands observed in the C–N stretching region of proline suggest that the structural change of one of the proline residues may become larger

than that of the wild-type LR and similar to that of NR [49]. One possible interpretation is that the helix C may tilt at the flexible kink at Pro145 (Pro91 in BR) as a result of the side-chain elongation or modification at the position 150. Because Pro50 on helix B in BR is replaced by threonine in LR and NR, the flexibility of helix B is probably reduced. Thus, it is a plausible assumption that the structural perturbation of the helix B is smaller than that of the helix C. This structural hypothesis is illustrated in Fig. 3. Such structural change would cause a perturbation of the hydrogen bonds between Thr143 and Asp139 (Thr89 and Asp85 in BR), and between Thr144 and Asp169 (Thr90 and Asp115 in BR). These effects were confirmed by the observed changes in the vibrational bands of Asp169 and the water molecule (2257 cm^{-1}), which was assumed to bridge Asp139 and the Schiff base nitrogen [49].

At a later time, we observed a strongly hydrogen-bonded water molecule in another fungal rhodopsin, *Phaeosphaeria* rhodopsin 2 (PhaeoRD2) (water O–D stretch in D_2O at 2258 cm^{-1}) [50]. The amino acid sequence of PhaeoRD2 is more similar to LR than to BR, but the difference is small. The identities between PhaeoRD2 and LR, and between PhaeoRD2 and BR are 34.5% and 30.2%, respectively. In addition, the amino acids uniquely identical for the fungal rhodopsins are located rather far from the retinal chromophore. In fact, the amino acid identities within 4 Å from the retinal chromophore are the same among PhaeoRD2, LR, and BR. For more than 100 amino acids located within 12 Å from the retinal chromophore, the identities are 48.7% between PhaeoRD2 and LR, 46.0% between PhaeoRD2 and BR, and 47.8% between LR and BR. These results suggest that protein core structures are equally different among the three rhodopsins. However, the light-induced FTIR spectra of PhaeoRD2 recorded at various temperatures are almost identical to those of LR, but considerably different from those of BR [50].

The FTIR results suggest that fungal rhodopsins possess some common structural motif and dynamics not obvious from the amino acid sequences.

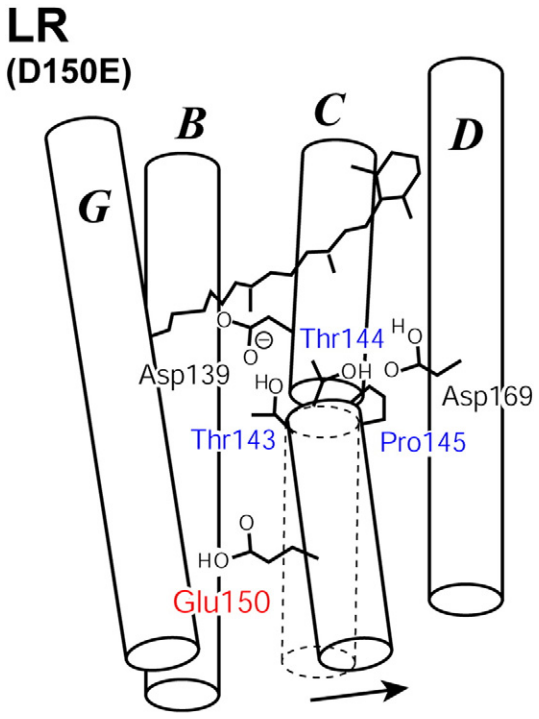

Fig. 3. Schematic representation of a possible structural alteration caused by the D150E mutation in LR. The arrangement of the helices is based on the crystal structure of BR. We proposed that tilt of the helix C at the kink at Pro145 (Pro91 in BR) may perturb the hydrogen-bonds of Asp139 and Thr143 (Asp85 and Thr89 in BR), and Thr144 and Asp169 (Thr90 and Asp115 in BR).

2.2. Proteorhodopsin

Proteorhodopsin (PR) has been found in various species of marine γ-proteobacteria [14,19,51]. The counterion of the Schiff base in PR is Asp97, and its pKa is higher in PR (~7) than that of homologous Asp85 in BR (2–3) [20,21]. This indicates that Asp97 has a negative charge only at alkaline pH. As already mentioned, the protonation state of Asp97 affects the proton pumping machinery of PR. How is the protein structure altered in each condition?

By comparing light-induced difference spectra of PR recorded at acidic and alkaline conditions, we elucidated highly pH-dependent spectra in the O–D and N–D stretching frequency region (in the 2700 – 2000 cm^{-1}) in D_2O, though the FTIR spectra in the 1800 – 900 cm^{-1} region were similar to each other [52]. Fig. 4 shows the difference spectra at pH 10 (a) and pH 5 (b), where spectral differences between pH 10 and 5 are more prominent than in the 1800 – 900 cm^{-1} region. Negative bands at 2094 cm^{-1} (a) and 2120 cm^{-1} (b) were assigned to the N–D stretch of the Schiff base [53]. Lower frequency at pH 10 corresponds to the stronger hydrogen bond of the Schiff base. Observation of the positive bands at 2199 and 2180 cm^{-1} at pH 10, but not at pH 5, indicates that hydrogen-bonding alteration upon photoisomerization is different at pH 10 and 5 [52].

Fig. 4 also compares the water bands. Four positive and four negative bands of water were observed at pH 10 and 5. Negative bands at 2683, 2667, and 2463 cm^{-1} and positive bands at 2676, 2634, and 2530 cm^{-1} were similarly observed both at pH 10 and at pH 5. In contrast, the water bands at 2385 (+)/2315 (−) cm^{-1} and at 2482 (+)/2582 (−) cm^{-1} were specifically observed at pH 10 and 5, respectively. This fact suggests that the water molecule responsible for these bands is located near Asp97. It is particularly notable that the water O–D stretch at low frequency region (<2400 cm^{-1}) was observed only for the alkaline PR (2315 cm^{-1}), which probably shifted to 2385 cm^{-1} in the K intermediate (Fig. 4a). Thus, we concluded that only the alkaline form of PR possesses a strongly hydrogen-bonded water molecule which changes its hydrogen bond upon retinal photo-isomerization [52]. The corresponding O–D stretch of PR at pH 5 (2582 cm^{-1}) is remarkably upshifted, suggesting that its hydrogen bond is weakened.

The N–D stretch(es) of the alkaline PR at 2152 and 2094 cm^{-1} are comparable to those in BR (2173 and 2123 cm^{-1}) [54], and upshift of

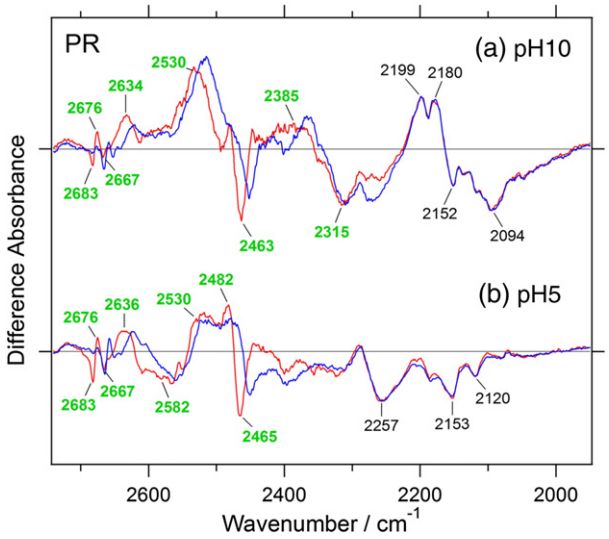

Fig. 4. PR_K minus PR spectra in the 2740−1950 cm^{-1} region measured at pH 10 (a) or pH 5 (b). The spectra are reproduced from Furutani et al. [52]. The samples were hydrated with D_2O (red lines) or $D_2^{18}O$ (blue lines). Green-labeled frequencies correspond to those identified as water O–D stretching vibrations. One division of the y-axis corresponds to 0.00055 absorbance units.

the N – D stretch upon PR_K formation indicates weakened hydrogen bond of the Schiff base after retinal isomerization as is the case in BR. It should be however noted that the frequency changes are much smaller in PR_K (2199 and 2180 cm^{-1}) [53] than in BR_K (2495 and 2468 cm^{-1}) [54]. This indicates that the Schiff base still forms a hydrogen bond in PR_K. The spectral upshift of the N – D stretch is further reduced at acidic pH in the wild-type.

We also found that spectral features in D97N were similar to those of the wild-type at pH 5 [53]. Spectral features in D97E were also similar to those of the wild-type at pH 10 [53]. Therefore, protonation of Asp97 affects the hydrogen-bonding alteration of the Schiff base upon isomerization. Nevertheless, similar spectra of D97E and the wild-type (pH 10) suggest that Asp97 plays a minor role in the hydrogen-bonding network after retinal isomerization.

On the other hand, entirely different spectra were obtained for the mutants of Asp227 [53]. In the case of D227N mutant, the hydrogen bond of the Schiff base is considerably weakened upon PR_K formation, which is consistent with the interpretation from the C=N stretch of the Schiff base. Weakened hydrogen bond of the Schiff base in D227N suggests that the Schiff base somehow interacts with Asp227 in PR_K. Interestingly, the N – D stretch exhibited downshift upon the PR_K formation in the D227E mutant [53]. The results on the mutants of Asp97 and Asp227 strongly suggest that the Schiff base mainly interacts with Asp227 in the PR_K intermediate. It means that the photoisomerization should move the protonated Schiff base toward Asp227 in PR. Such motion has been reported for the MD simulation of the primary photoreaction in BR [55]. Therefore, similar motion of the Schiff base probably takes place in PR, though the Schiff base keeps the hydrogen bond after such motion in the initial intermediate state.

Similarity of the N – D stretches of the Schiff base in PR to those in BR strongly suggests that the protonated Schiff base in PR forms a strong hydrogen bond, presumably with a water molecule. Strongly hydrogen-bonded water molecule, whose O – D stretch is located at 2315 cm^{-1}, disappears upon protonation of Asp97 (pH 5) [52] and upon mutation of Asp97 into asparagine [53]. From these facts, we proposed that PR possesses a bridged water molecule between the Schiff base and Asp97 similar to BR (Fig. 5). While the hydrogen-bonding strength of the Schiff base with the water is similar between BR and PR, that of the water with Asp97 is weaker in PR than that of the water hydrogen-bonded with Asp85 in BR as seen from the frequency of water O – D stretches (2315 vs 2171 cm^{-1}). Weaker association of the water with Asp97 probably contributes to its higher pKa (~7), along with weaker association of Thr101 to Asp97 compared to that of Thr89 in BR [56,57]. The NMR studies suggested that His75 interacts with Asp95, which may also contribute to the increase of the pKa.

3. Time-resolved FTIR spectroscopy for revealing dynamics of internal water molecules in type-I rhodopsins

Low temperature technique enables us to freeze photointermediate states and to measure the highly precise light-induced difference spectra by taking enough time which we need for averaging the data sufficiently. As seen in the previous section, the technique is very powerful to elucidate the hydrogen-bonded structure around the Schiff base region of rhodopsins including the internal water molecules, but it is generally hard to apply it on the later intermediate states which decay kinetically at the observable temperature. Time-resolved FTIR technique is another powerful tool to analyze the hydrogen-bonding changes of protein and water molecules [58–63], which monitors O – H stretching modes of not only an individual water molecule and also protonated water cluster [59–62]. The technical details were described in the literature. Here, the recent study on a light-driven chloride ion pump, halorhodopsin, is summarized.

3.1. pharaonis Halorhodopsin

Halorhodopsin (HR) is a light-driven chloride ion pump protein discovered in *Haloarchea* [64]. The absorption maximum (λ_{max} = 575 nm) is close to that of BR in the salinity condition [65]. Several intermediate states [e.g., K, L_1, L_2, N, and O] are formed during the

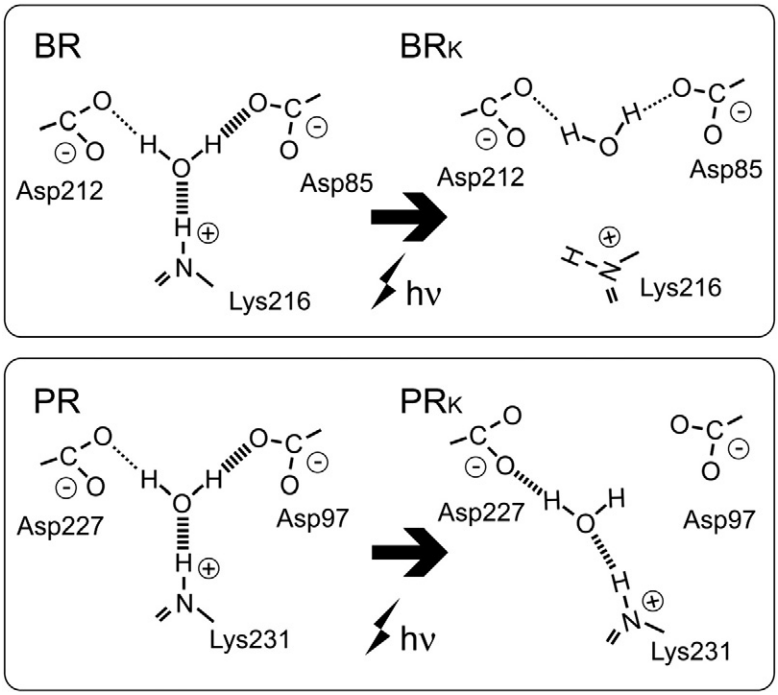

Fig. 5. Schematic drawing of the hydrogen-bonding interactions of the Schiff base and water molecule with counterion based on the FTIR results. The stacked bars indicate strong hydrogen bonds. The bridged water molecule interacts with D227 in PR_K.

photocyclic reaction [66–68]. *pharaonis* Halorhodopsin (*p*HR) was found in *Natronomonas pharaonis*, which is stable in the low salinity conditions and is highly expressed in *Escherichia* coli. Therefore, the photoreaction and structure of *p*HR has been extensively studied by various physicochemical methods; however, the dynamics of water molecules during the ion pumping process is little understood.

HR has an initial anion binding site near the protonated Schiff base. The anion is stabilized by the hydroxyl groups of Thr126 and Ser130 and the positively charged guanidium group of Arg123 [69,70]. It is considered that the chloride ion release and uptake occur in the transition from N to O and from O to *p*HR, respectively [67,71,72]. It is proposed that the anion in the initial binding site is likely to be translocated to the cytoplasmic side around Lys215 and Thr218 [70,73]. The cytoplasmic side of *p*HR is hydrophobic environment, and only one water molecule near Thr218 was found in the X-ray crystal structure [74]. Previous low-temperature FTIR spectroscopy revealed that the molecules near the protonated Schiff base are rearranged in the early intermediate states, K and L_1 [38,75]. How do the water molecules move in the later intermediates?

Time-resolved FTIR (TR-FTIR) spectroscopy was applied on *p*HR at pH 7 and 288 K with 12.5-μs intervals [63]. The intermediate spectra of *p*HR in the 3750–3500-cm^{-1} region after the removal of the transiently heated bulk water bands are shown in Fig. 6. The 3605 (+)/3626 (−) cm^{-1} bands were observed in the N intermediate (Fig. 6b). A relatively intense positive band was observed at 3608 cm^{-1} in the O intermediate with a negative band at 3626 cm^{-1} (Fig. 6c). These bands were downshifted in the $H_2^{18}O$ condition and assigned to O–H stretching vibrations of water molecules, which locate in the frequency region of a dangling bond of a water molecule [63]. Water has a broad O–H stretching vibration in the 3650–2800-cm^{-1} region, and the frequency depends on the hydrogen-bonding structure of water molecules. In the hydrogen-bonded network of water, a water molecule with a free O–H group, called a dangling bond, exhibits a frequency higher than 3600 cm^{-1} [24,60]. These bands are considered to be a useful probe of water molecules inside the protein because of their peculiarly higher frequency with a narrower bandwidth than ordinary water O–H stretching bands. In the L_1 and L_2 intermediate states, these bands are less obvious than in the later intermediates as seen in Fig. 6a.

The time courses of the peak-to-peak height at 3605 and 3626 cm^{-1} for the lower (magenta) and higher (green) salt conditions are plotted (Fig. 6d). The accumulation of the O intermediate was considerably reduced at higher salt condition. The maximum amplitude of the lower salt condition is 1.5 times that of the higher salt condition because of the relatively intense peak at 3608 cm^{-1}, as already shown in the difference spectrum of the O intermediate. In addition to the intermediate spectra in Fig. 6 (left panel), we concluded that the intensity of the water dangling mode increases when N and O intermediate states are formed.

How do the internal water molecules change the hydrogen-bonded network during the *p*HR photocycle? A dangling bond of a water molecule is free from a hydrogen-bonded network. Amino acid residues inside the protein provide a hydrogen bond donor and acceptor for stabilizing water molecules. The limited numbers of acceptor groups and spaces for water molecules inevitably yield a water molecule without a hydrogen bond. Therefore, the intensity of the O–H stretching vibrations of dangling bonds probably correlate well with the number of water molecules which have a free O-H group inside the protein. In the N intermediate spectrum, the spectral shape of the bands at 3605 (+)/3626 (−) cm^{-1} is symmetrical with respect to the zero line (Fig. 6b), implying that the number of dangling bonds does not change when the N intermediate is formed. The origin of the 3626-cm^{-1} band is assumed to be a water molecule near the initial anion binding site. Therefore, the environmental change of the water molecule may occur in N. On the other hand, the positive band at 3608 cm^{-1} is considerably larger than the negative band at 3626 cm^{-1} in the O intermediate spectrum. The disappearance of the chloride ion from the initial binding site may force some water molecules to cut their hydrogen bonds, which increases the number of the water dangling bonds in O. Such rearrangement of the hydrogen-bonded network may facilitate transportation of a chloride ion in *p*HR (Fig. 7).

4. Conclusions

Light-induced difference FTIR spectroscopy can give an insight on the motion of the internal water molecules in rhodopsins, which are frozen in the static crystal structures. The low-temperature FTIR spectroscopy still has an advantage on the characterization of the initial intermediate state, which is stabilized at liquid nitrogen temperature. The strongly hydrogen-bonded water near the protonated Schiff base was found to be commonly preserved in the proton pumping

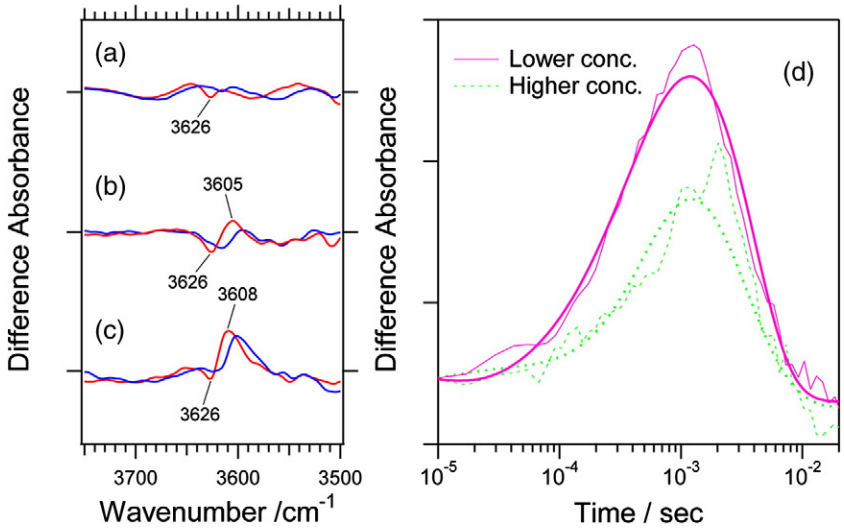

Fig. 6. (a–c) The intermediate spectra in the 3750–3500-cm^{-1} region. The blue and red spectra were recorded under H_2O and $H_2^{18}O$ hydration, respectively. (d) Time courses of dangling water O–H stretching bands. Peak-to-peak absorbance at 3605 and 3226 cm^{-1} are plotted for lower (magenta) and higher (green) salt conditions. The spectra and time traces are reproduced from Furutani et al. [63].

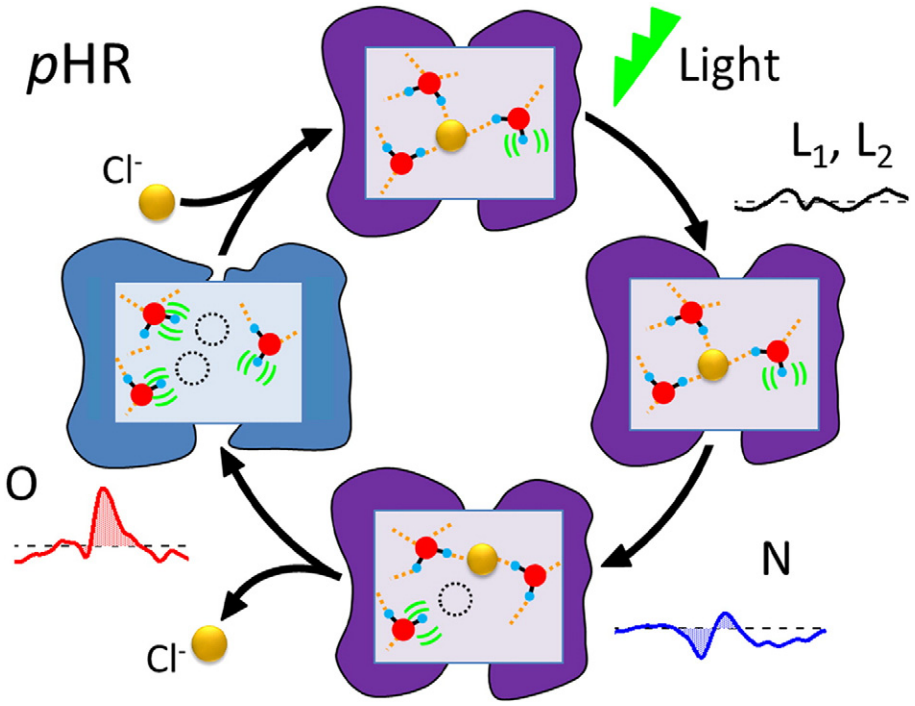

Fig. 7. Schematic illustration of the hydrogen-bonding alteration during the photocyle of *p*HR. Chloride ion is shown as yellow filled circle. The green parentheses indicate the dangling bonds of the water molecules inside *p*HR.

rhodopsins, which is probably involved in triggering the initial proton transfer reaction from the Schiff base to the counter-ion carboxylate group. The recent progress in genome sequence technology reveals tremendous amount of microbial rhodopsins in various species. The systematic analysis on these rhodopsins is prerequisite to elucidating the basic architecture for the proton-pumping machinery. The dynamics of water molecules in the various hydrogen-bonding conditions are basically monitored by time-resolved FTIR spectroscopy. Here, we haven't mentioned the protonated water cluster which takes an important role in the proton ejection process and focused on water molecules with an O–H group free from a hydrogen bond. The dangling bond of water is a useful probe for detecting water motion inside protein. We elucidated that the increase of dangling bonds correlates well with the release of a chloride ion from a light-driven proton pump, *p*HR. The importance of water molecules inside protein in their function has been discussed in terms of hydration free energy [76]. It is considered that the presence of water molecules make the protein more flexible. The anion transportation may be facilitated by involvement of such water molecules. In this way, the technique added cutting-edge aspects on the molecular mechanisms of halobacterial and microbacterial rhodopsins.

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