Review

Role of internal water molecules in bacteriorhodopsin

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Abstract

Internal water molecules are considered to play a crucial role in the functional processes of proton pump proteins. They may participate in hydrogen-bonding networks inside proteins that constitute proton pathways. In addition, they could participate in the switch reaction by mediating an essential proton transfer at the active site. Nevertheless, little has been known about the structure and function of internal water molecules in such proteins. Recent progress in infrared spectroscopy and X-ray crystallography provided new information on water molecules inside bacteriorhodopsin, the light-driven proton pump. The accumulated knowledge on bacteriorhodopsin in the last decade of the 20th century will lead to a realistic picture of internal water molecules at work in the 21st century. In this review, I describe how the role of water molecules has been studied in bacteriorhodopsin, and what should be known about the role of water molecules in the future. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

1.1. General description of internal water molecules in proton pump proteins

Biological systems contain various proton pumps, because the driving force of ATP synthase is the proton gradient and thus ATP synthesis is coupled to proton movement through the membrane [1]. Proton pump proteins are characterized by their ability to actively transport protons, i.e. they translocate protons even when the proton concentration is higher at the other side of the membrane. How can these proteins produce vectorial transport? For proton pumping, two elements are required: (i) proton conduction pathways and (ii) switching machinery.

Proton pump proteins are membrane proteins, and membrane proteins are generally hydrophobic. This means that the positively charged protons have to be translocated through the hydrophobic membrane spanning region of the protein. Therefore, one can imagine that water molecules are present inside and possibly assist the transport. Water molecules may participate in the proton pathways by forming hydrogen-bonding network. The presence of water in such channels will raise a dielectric constant and provide a more favorable environment for protons [2,3]. Such a 'proton wire' of water molecules has been discussed mainly from a theoretical point of view [4,5], while structural determination allowed to extend the discussion to actual systems such as the gramicidin A channel [6] and the photosynthetic reaction center from Rhodobacter sphaeroides [7]. Ad-
ditionally to constituting ‘proton wires’ for conducting protons, water could be a direct carrier of protons. Protons do not exist as isolated particles, but are covalently bound to some groups in protein. Unlike the positionally fixed amino acid side chains and backbones, water molecules are movable so that they are candidates for carriers of protons. Thus proton translocation involving release and uptake could be achieved as H$_3$O$^+$ transport.

Proton pathways are necessary for pumps, but proton pathways cannot be fully connected between the two sides of the membrane, because the proton gradient formed will be collapsed. This is an important aspect in distinguishing pumps from channels. The former needs a ‘switch’, which ensures the vectoriality of the pumping. As the heart of pumps, the switching machinery is interesting, though little is known about it. Besides participating in hydrogen-bonding networks, water molecules may be elements of the switch. The switch presumably mediates an essential proton transfer, where an actual charge moves inside proteins. Therefore, water molecules could mediate such an essential proton transfer. Fig. 1 shows a schematic drawing of water-mediated proton transfer in the switch. In this simple scheme, a water molecule stabilizes an ion pair state between proton donor and acceptor in the active center (switch). Energy input displaces the position of the water, which yields protonation changes in one side according to the dielectric properties of the environment. Clockwise rotation of the switch with the water is thus coupled with vectorial transport of a proton. This kind of ‘flip-flop’ mechanism with water is also essential for functional processes of enzymes [2].

While the scheme shown in Fig. 1 is possible, these transient states (b–e) as well as the initial state (a) have to be captured as evidence for it. It is safe to say, however, that such a simple mechanism has never been regarded as realistic for most pump proteins. The complete description of functional processes of proton pump proteins will be achieved by (1) determination of the tertiary structure, (2) determination of the proton pathways, and (3) determination of the origin of vectoriality in the switch. Structural determination of terminal oxidases [8,9] and ATP synthase [10] opened the possibility to reveal their mechanisms, whereas proton pathways and switching machinery are remained to be revealed. In this sense, the best understood proton pump is bacteriorhodopsin.

### 1.2. Bacteriorhodopsin as an ideal model of proton pump proteins

Bacteriorhodopsin (BR) is a light-driven proton pump in *Halobacterium salinarum* that contains all-trans retinal as chromophore (reviewed in [11–14]). Its tertiary structure has been recently reported from several groups by cryo-electron microscopy [15–17] and X-ray crystallography [18–23]. The resolution has now reached those to describe the positions of water molecules [19,21] as shown below. The retinal binds covalently to Lys216 through a protonated Schiff base linkage. Absorption of light triggers a cyclic reaction that comprises a series of intermediates, designated as the J, K, L, M, N and O states. Protein structural changes in these intermediate states cause proton translocation across the protein. These intermediate states were first identified by visible absorption spectroscopy (reviewed in [24]), while resonance Raman spectroscopy revealed the structure of the retinal chromophore (reviewed in [25,26]). They showed that the all-trans to 13-cis isomerization occurs upon photoexcitation of BR, and the Schiff base is deprotonated in the M intermediate and reprotonated in the N intermediate, indicating that the Schiff base proton is conducted into the extracellular side upon M formation, and the cytoplasmic side supplies a proton to the Schiff base upon M decay.

Extensive studies by Fourier transform infrared (FTIR) spectroscopy then revealed how protons are captured by specific amino acids during the pumping process (reviewed in [27–30]). For this analysis, the C = O stretching vibrations of protonated carboxylic acids in the 1800–1700 cm$^{-1}$ region provided fruitful information [31–34], though the analysis of deprotonated C-O stretching vibrations (antisymmetric mode at about 1550–1610 cm$^{-1}$ and symmetric mode at about 1300–1420 cm$^{-1}$) were complicated by many other vibrations in these frequency regions. Protonation of Asp96 in BR [35–38], its transient deprotonation in N [39–43], and transient protonation of Asp85 in M, N, and O [36–47] were observed. These facts indicate that a proton is translocated via Asp96,
Schi base, and Asp85. Namely, in the case of BR, the upper carboxylate, XH\(^+\), and the lower carboxylate in Fig. 1a correspond to Asp85, protonated Schi base, and Asp96, respectively. Reprotonation of Asp96 upon N decay \([42,43,46]\) is essentially coincident with the proton uptake of BR, though more complex kinetics were shown dependent on pH \([48,49]\). Asp96 is thus the proton uptake group from aqueous phase. Surface aspartates at the cytoplasmic side may act as collectors of protons \([50]\), though the replacement of these aspartates to asparagines does not alter the photocycle kinetics much \([51]\). On the other hand, protonation of Asp85 after proton release upon M formation shows that there is a proton release group near the aqueous phase \([52–55]\). Mutation studies revealed that Glu204 plays a central role in the release process, in association with Glu194 \([56–59]\). However, the infrared band of Glu204 was less intense than other carboxylic groups (Asp85 and Asp96) \([56]\), and time-resolved FTIR measurement questioned about the specific assignment to Glu204 \([60]\). Currently, it is believed by many researchers that the released proton is delocalized in the hydrogen-bonding network possibly involving water molecules.

2. Role of internal water molecules in the proton pathway of bacteriorhodopsin

Since the Schi base is positively charged and Asp85 is negatively charged in BR, the presence of water molecule between them is a reasonable postulation. In addition to theoretical predictions \([61,62]\), several experimental results suggested the presence of internal water molecules before a high resolution X-ray crystallographic structure was available. Resonance Raman spectroscopy proposed energy transfer from a water molecule to the Schi base \([63]\). Neutron diffraction showed the presence of water molecules inside BR \([64]\). An anomalous chemical shift by \(^{15}\)N NMR spectroscopy was interpreted in terms of the presence of water molecules in the Schi base.

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Fig. 1. Schematic drawing of water-mediated proton transport in the switch. In this scheme, a single water molecule constitutes the switch with a positively charged X-H\(^+\) group (a). Two carboxylates are present at both upper and lower sides as a possible proton acceptor and donor, respectively. I assume that the upper side is in more polar environment than the lower side, as described by higher dielectric constant (\(\varepsilon_1 > \varepsilon_2\)). The water molecule bridges the protonated XH\(^+\) group and the negatively charged upper carboxylate, while the lower carboxylate does not have a charge (protonated) (a). Energy is consumed for pulling the bridged water out of the hydrogen-bonding network. This event drops the dielectric constant of the upper side, so that the proton bound for the group X is transferred to the upper carboxylate (b). Then the water finds new hydrogen bonding between group X and the lower carboxylate by moving clockwise in the figure. Raised dielectric constant at the lower side allows proton transfer from the lower carboxylate to group X, so that the water-bridged hydrogen-bonding network is formed as a metastable state (c). Since this state (c) is higher in energy than in (a), relaxation occurs toward the initial state. This process accompanies clockwise rotational motion of XH\(^+\) with the water, leaving the lower carboxylate negatively charged (d). Finally the initial conformation of the switch requires deprotonation of the upper carboxylate (proton release) and protonation of the lower carboxylate (proton uptake) (e). In this way, unidirectional proton movement is possible through water-mediated proton transfers.
region [65]. $pK_a$ measurements of a model system suggested the presence of the water molecule close to the Schiff base in BR [66]. Among them, FTIR spectroscopy has provided strong evidence on the presence of internal water molecules and their structural changes upon the functional process of BR.

2.1. Detection of water by FTIR spectroscopy

FTIR spectroscopy of biological molecules is usually applied in the frequency region $<1800$ cm$^{-1}$, giving secondary structural information. Strong absorption of water molecules in the $3700$–$2700$ cm$^{-1}$ region (Fig. 2) prevents obtaining accurate spectra in the region. That is, information of internal water molecules and their structural changes upon the functional process of BR.

Fig. 2. Infrared difference spectra between the original BR and several photointermediate states measured at low temperature. A hydrated film sample at pH 7 or 10 was used for producing K and L, or M and N, respectively. These spectra were normalized at the negative $1202$ cm$^{-1}$ band, indicating that the same numbers of BR molecules are converted to intermediates. See each reference for experimental details of K minus BR [102], L minus BR [73], M minus BR [79], and N minus BR [79]. The light blue curve in the $3700$–$2700$ cm$^{-1}$ region represents O-H stretching vibrations of water molecules at room temperature, which normally prevents from accurate signal detection in this region. Technical progress involving the hydration conditions enabled us to obtain accurate spectra in the higher frequency region, which led to observe water structural changes.

In their pioneering studies on water molecules, Maeda et al. found in 1992 some vibrations in the $3750$–$3450$ cm$^{-1}$ region of the L minus BR and M minus BR spectra exhibiting isotope shifts upon hydration with H$_2^{18}$O [70]. This fact clearly indicated that water structural changes occur upon formation of L and M, and opened the possibility to experimentally reveal the role of water molecules in the functional process of BR. Fig. 3a reproduces the spectra of the L minus BR with better signal-to-noise ratio than the original data [70]. Three negative bands at 3643, 3607, and 3577 cm$^{-1}$ and broad positive bands in the 3550–3450 cm$^{-1}$ region exhibit
isotope shift upon hydration of $H_{18}^2O$, indicating that these bands originate from the O-H stretching vibration of water molecules.

These water molecules can be internal to the protein, but possibly at the surface or somewhere else. Unlike amino acids, water molecules cannot be specifically labeled. Our strategy was to use mutants, by which we expected to obtain information from the local structural modifications. This approach, including reports by other groups [56,71–81], provided useful information on the location of internal water molecules and their changes upon photocycle of BR (reviewed in [29,82]). Fig. 3b–f shows an example. The negative band at 3643 cm$^{-1}$ of the L minus BR spectrum disappears in the mutant of Asp85 (D85N), while no change is observed in other mutants, such as T46V, V49M, D96N, and W182F. Therefore, it was concluded that the water possessing the O-H stretching frequency at 3643 cm$^{-1}$ is present near Asp85 (Fig. 4) [71]. Since the positive signal decreases in the 3520–3450 cm$^{-1}$ region in D85N (Fig. 3b), the O-H stretching band of the water in the L intermediate of the wild type seems to be in this region. In contrast, the two negative bands at 3607 and 3577 cm$^{-1}$ are preserved in D85N, but affected in other mutants [73,75,83]. The 3607 cm$^{-1}$ band disappears in all mutants in the cytoplasmic

Fig. 3. L minus BR difference spectra in the 3750–3450 cm$^{-1}$ region. (a) Red and green curves represent spectra upon hydration of $H_2O$ and $H_{18}^2O$, respectively. (b–f) Blue curves represent the spectra of mutants, while red dotted curves are the spectra of the wild type. Published spectra in (a), (c), (e) [73], and (d) [75] are reproduced without change. The published spectra for D85N [71] and W182F [83] are reproduced but after measuring with better signal-to-noise ratio.

Fig. 4. Proposed location of internal water molecules from FTIR spectroscopy of BR in the literature. Positions of water molecules are shown in the side view of the tertiary structure of BR (2BRD) [15]. Four water molecules have been suggested, with their locations based on the FTIR data.
side. On the other hand, the 3577 cm\(^{-1}\) band disappeared in T46V and V49M, while it was preserved in D96N and shifted in W182F. On the basis of these results, we proposed these water molecules are located at the cytoplasmic side (Fig. 4), where the water possessing the O-H stretching vibration at 3577 cm\(^{-1}\) is localized more towards the Thr46-Val49 region [82].

Similarly, the small negative band at 3625 cm\(^{-1}\) in the M minus BR spectrum disappears in R82A, R82K, and E204Q, but is preserved in R82Q and E204D [56,76]. On the basis of these results, we inferred that the water is located in the Arg82-Glu204 region (Fig. 4) that is important in controlling the proton release. In particular, a similar feature with glutamine at position 82 (R82Q) to arginine (wild-type), but not to lysine (R82K) strongly suggests that the N-H at the \(\text{O}^\text{-}\)-position is important and the water possibly forms a hydrogen bond with this group [76].

We have thus assigned the location of four water molecules. Disappearance of the band in the difference IR spectra (Fig. 3) has three possibilities for their interpretations. First, the water indeed disappears upon the mutation. Second, the water is positionally preserved but its frequency is changed by mutation. Third, the water position and frequency are preserved, whereas hydrogen-bonding alteration upon formation of intermediates disappears upon the mutation.

The new findings on internal water molecules of BR by FTIR spectroscopy raised various questions. One of the most important of them is: where is the other stretching vibration of the observed O-H stretch of water? This is closely related to the origin of the stretching vibrational modes of water molecules. A water molecule has two O-H groups, and their frequencies distribute in the wide 3700–2700 cm\(^{-1}\) region dependent on their coupling and hydrogen-bonding strength [84]. Gaseous water exhibits asymmetric and symmetric stretching modes at 3756 and 3657 cm\(^{-1}\), respectively [85], and the stretching frequency is lowered as its hydrogen bonding becomes stronger [84]. It is, however, noted that the hydrogen-bonding strengths of the two O-H groups are probably not equivalent in the restricted protein environment, which breaks the \(C_2\) type symmetry. In such \(C_s\) type symmetry, one O-H is hydrogen bonded and the other O-H is unbonded, and their frequencies are widely split [85]. The symmetry of actual internal water molecules would be between the two extremes, \(C_2\) and \(C_s\) types.

We have discussed water structural changes from the data in the 3750–3450 cm\(^{-1}\) region, which is the higher frequency tail of water stretching vibration (Fig. 2). Therefore, our detection of water stretching vibrations was for the O-H groups under weak hydrogen-bonding conditions. So far, we have considered that stronger hydrogen bonds of water molecules lie in the frequency region lower than 3450 cm\(^{-1}\), on the basis of \(C_s\)-like symmetry in the restricted protein environment. However, it has not been experimentally observed. The mode coupling of internal water molecules is important for the proton transfer reaction.

On the other hand, a water molecule has bending vibration at about 1640 cm\(^{-1}\), which could be a good marker band too [84]. However, in my experience, the isotope shift by H\(^{18}\)O is not clear for the modes, possibly because of many other protein vibrations involved in the region. Since N-H stretching frequency appears in the <3500 cm\(^{-1}\) region, the vibrations in the >3500 cm\(^{-1}\) are ascribable for only O-H stretches, and the candidates are threonine, serine, tyrosine, protonated carboxylic acids, and water molecules. Thus, detection of the stretching vibrations in the 3750–3450 cm\(^{-1}\) was advantageous in the assignment of water molecules.

Among the various water vibrations of BR, the band at 3643 cm\(^{-1}\) is particularly noted, because it is closely related to the switch of BR. As shown in Fig. 4, we regard the water as locating between Schiff base and Asp85 [72], namely the ‘switch’ water in Fig. 1. Interestingly, this frequency is the highest among water vibrations of BR, indicating that the hydrogen bonding of the water is very weak (or absent). In the scheme of Fig. 1a, it may correspond to the free O-H group of the intervening water molecule in the \(C_s\)-like symmetry. The role of the water is discussed in detail in Section 3.1 after taking into account the atomic structure of water from X-ray crystallography in the following section.

2.2. Positional water by X-ray crystallography and correlation with FTIR data

The recent high resolution X-ray crystallography

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provided another breakthrough on internal water molecules of BR by giving their positions. However, the first report in 1997 [18] was rather surprising. The structure by Pebay-Peyroula et al. showed neither a water molecule between the Schiff base and Asp85, nor any water molecules within hydrogen-bonding distance of Asp85 [18]. In contrast, Luecke et al. showed the different water positions on the basis of the same crystallization procedure, in which a water molecule is present between the Schiff base and Asp85 (water 402) as well as another hydrogen-bonded water with Asp85 (water 401) [20]. This complicated situation became clarified in 1999, when the higher resolution structures from the two groups coincided well [19,21]. Fig. 5 shows internal water molecules of BR in the structure by Luecke et al. [21]. Belrhali et al. reported identical water molecules at positions 401, 402, and 406 (numbered as 400) [19], though there were still some differences in other regions.

The water structures in BR are characterized by various aspects. One is non-symmetric numbers of water molecules between two sides. In fact, seven internal water molecules are at the extracellular side, and only two are at the cytoplasmic side (Fig. 5). This may be reasonable because charged and polar residues are present at the extracellular side, while more hydrophobic residues lie at the cytoplasmic side. Such a non-symmetric nature of the BR structure is presumably correlated with the functional processes of BR; namely proton release first at the extracellular side, followed by proton uptake at the other side.

Another intriguing issue is the water-containing pentagonal structure at the Schiff base region. Three water oxygens and two carboxylate oxygens (Asp85 and Asp212) (Fig. 5) constitute a pentagonal hydrogen-bonding cluster accessible to the protonated Schiff base. The pentagon is considerably planar, and the positions of two oxygens of Asp85 and Asp212 look symmetrical (Fig. 5). Nevertheless, the Schiff base proton is transferred only to Asp85 in the photocycle of BR. Luecke et al. attempted to explain the reason in terms of hydrogen bonds of Asp212 with two sterically restricted tyrosine residues (Tyr57 and Tyr185) [21]. Regarding such apparently symmetric structure, the author feels the similarity to those of bacterial photosynthetic reaction centers,

![Diagram of BR structure](image)
where a highly symmetrical structure provides two possible electron transfer pathways, but actual electron transfer occurs by use of only one side [86]. Nature seems to use apparently symmetric structure and antisymmetric chemical reaction in proteins.

Since the positions of the water molecules are now at hand, comparison with the FTIR data is interesting. Two waters of stretching frequencies at 3607 and 3577 cm$^{-1}$ probably correspond to waters 501 and 502, both of which are involved in a π-bulge that causes a non-proline kink of helix G [21]. Water 501 bridges helices F and G (hydrogen bonds with indole N-H of Trp182 and peptide carbonyl of Ala215), while water 502 bridges helices B and G (hydrogen bonds with peptide carboxyls of Thr46 and Lys216). Since the 3577 cm$^{-1}$ water is more localized in the Thr46-Val49 region (Fig. 3), it is likely to correspond to water 502. On the other hand, the 3607 cm$^{-1}$ water presumably corresponds to water 501. Only one hydrogen-bonding acceptor for water 501 (peptide carbonyl of Ala215) yields a free O-H, explaining why the frequency (3607 cm$^{-1}$) is higher than that of water 502 (3577 cm$^{-1}$).

Since water 403 forms a hydrogen bond with the O-nitrogen of Arg82, this water may correspond to that possessing the O-H stretch at 3625 cm$^{-1}$ (Fig. 4) [76]. The most important correlation between X-ray structure and FTIR data is regarding the water with an O-H stretching vibration at 3643 cm$^{-1}$, because this water is closely correlated with the switch. Previously, we argued the hydrogen-bonding structure of this water molecule on the basis of a simple model containing the Schiff base, the water, and Asp85 [72]. However, the X-ray structure shows a pentagonal cluster containing three water molecules in the Schiff base region. This issue is discussed in Section 3.1 in relation to the switching machinery of BR.

3. Role of internal water molecules in the switch of bacteriorhodopsin

The switch is the heart of pump proteins. To explain the cause of the vectoriality in BR, some models have been proposed, such as the ‘IST’ model [87,88] and ‘local access’ model [89,90]. Since we have high-resolution structures of BR (see Section 2.2) and possibly of photointermediates (see Section 4.1), switching machinery will (should) be discussed from the structural basis. Two factors are concerned with the switch: (i) energy input, and (ii) protein conformations. In the case of BR, the energy source is light and isomerization of the retinal chromophore triggers protein conformational changes. For the switch, two protein conformations are considered, whose accessibilities alter from one side to the other of the membrane [87–91]. Having the BR structure at hand, the origin of the switch in BR is intriguing. How large is the switch? Does the switching process accompany local or global structure changes? In this regard, extensive studies have been carried out from the structural background [92–95].

Regarding the switching machinery, mutation studies have provided important information. It is particularly noted that the proton pumping ability is retained by replacement of any charged or polar residues of BR with neutral residues except for Asp85 [96]. The mutation involves Asp96 [96,97] and Glu204 [96,98]. As mentioned, transported protons are trapped to Asp96 and Glu204 (or release group containing Glu204). This indicates that Asp96 and Glu204 constitute the proton pathway, but are not involved in the switch. Thus, it is likely that the switch is a rather local machinery.

There is no doubt that Asp85 is involved in the switch, because its replacement results in the loss of proton pumping ability [96]. Not only losing the proton pump function, the replacement of aspartate with threonine at position 85 can convert into a chloride pump like halorhodopsin [99]. The water-containing pentagonal cluster (Fig. 5) presumably also constitutes the switch. In this section, I like to discuss the role of water molecules in the switch of BR based on the FTIR results. The 3643 cm$^{-1}$ band of BR is mainly discussed as the ‘switch water’.

3.1. Water-mediated proton transfer in bacteriorhodopsin

The water O-H stretching band at 3643 cm$^{-1}$ disappears in the L minus BR spectrum of the D85N...
mutant (Fig. 3b). It is well known that this mutant lacks the ability of proton translocation because proton acceptor is not present [96]. Maeda et al. proposed that water coordinated to Asp85 participates in the formation of L with distorted 13-cis retinal conformation, which may be a prerequisite for the proton transfer from the Schiff base to Asp85 [71]. How is the primary proton transfer mediated by water molecule(s)? According to the scheme in Fig. 1a, the switch contains a water molecule bridging between proton donor and acceptor, and one of the water O-Hs is strongly hydrogen bonded and the other is free. The frequency at 3643 cm$^{-1}$ indicates that the hydrogen bonding is very weak, and originates possibly from the free O-H of the bridged water.

Then mutant D212N was examined [72]. There is no proton transfer for the mutant under alkaline conditions, even when there are proton donor (protonated Schiff base) and acceptor (Asp85) [100]. The results showed that, unlike D85N, the frequency change of the water band in the switch was observed for D212N, although the frequency change (10 cm$^{-1}$) was much smaller than in the wild type (> 80 cm$^{-1}$) [72]. On the basis of these results, we concluded that, besides a hydrogen bond of a water molecule between the Schiff base and Asp85, the formation of a hydrogen bond with Asp212 of the originally unbound O-H is required for proton transfer [72].

On the assumption that a single water molecule is present between the Schiff base and Asp85, two mechanisms could be considered (Fig. 6). In both cases, one of the two water O-Hs forms a hydrogen bond with the unprotonated Asp85, and the other O-H is free in BR (Fig. 6a,c). The latter provides the O-H stretching frequency at 3643 cm$^{-1}$ and this free O-H forms a new hydrogen bond with Asp212 upon L formation in both schemes. One scheme (Fig. 6b) explains that the formation of intensified hydrogen bonding of the free water O-H linked to three charged groups is necessary for constructing the distorted structure in the retinal moiety and a correct alignment of the N-H bond of the Schiff base for lowering its $pK_a$, and hence the ensuing proton transfer in the protein. We proposed this scheme in a previous article [72]. On the other hand, the other scheme (Fig. 6c,d) is closer to that of Fig. 1. Namely, Asp212 pulls out the water from the hydrogen-bonding bridge between the Schiff base and Asp85, which makes the distance of the ion pair close enough for proton transfer.

Structural determination [19,21] raised a question on the water-mediated proton transfer mechanism, because the switch water may not be a single water. Rather at least three water molecules are involved in the pentagonal cluster (Fig. 5), being considerably different from those in Fig. 6. Which water possesses the O-H stretching vibration at 3643 cm$^{-1}$? Importantly, water 402 seems to form hydrogen bonds not only with Asp85, but also with Asp212. If we simply deduce the 3643 cm$^{-1}$ water from the picture in Fig. 5, the free O-H of water 401 is most likely. Then, newly formed hydrogen bonding of the free O-H in L may not be with Asp212, but with other groups.

More information was obtained from polarized FTIR spectroscopy. Using a highly oriented BR film sample, this technique reveals the angle of the dipole moment of vibration in question to the membrane normal [101–105]. We applied to the 3643 cm$^{-1}$ band, and determined the angle of the dipole moment to be 60$^\circ$ to the membrane normal [106]. The O-H group of water 401 in the structure appears to be parallel with the membrane normal (Fig. 5), being inconsistent with the polarized FTIR data. However, it should be noted that further analysis is necessary for appropriate interpretation. In the case of the structural study, positions of the hydrogen atoms (Fig. 5) are all supposed and not experimentally shown. X-Ray analysis with much higher resolution or high resolution neutron diffraction is necessary for further information. In the case of FTIR study, the angle of the dipole moment does not necessarily coincide with the actual bonds, because of mode coupling. Normal mode analysis based on the structure is necessary. Since charges are possibly delocalized on water molecules, quantum chemical calculation may be required for obtaining accurate normal modes.

3.2. Two domain structures divided by the switch, as probed by vibrational modes of waters

Two protein conformations have been considered that allow alternative access to the two membrane surfaces in pump proteins [87–91], and more generally in other membrane proteins such as receptors.
Section 1, I described that proton pump proteins possess conduction pathways of protons, which are divided by switch into two halves (Fig. 1). Does it really happen in BR? Are there two domain structures divided by switch? In this section, I show our study on such domain structures of BR using water O-H stretching vibrations as the probe. Particular water vibrations were used for BR and M, and we tested how mutation affects the bands. M is a key intermediate in the proton pump [87–91], where the molecular accessibility is changed to the cytoplasmic side.

We tested ten amino acids: five (Tyr57, Arg82, Asp85, Glu204, Asp212) located to the extracellular side of the retinal, and the other five (Thr46, Val49, Leu93, Asp96, Phe219) to the cytoplasmic side. All residues are located along the proton pathway. If the retinal at the Schiff base moiety is the switch, the...
The effect of the mutation would emerge differently at the two sides. This is indeed the case. Fig. 7 depicts the frequency change in water O-H stretching vibration upon conversion of BR to M. Both negative 3643 cm\(^{-1}\) and positive 3671 cm\(^{-1}\) bands are observable in the wild type, where each band was assigned to water O-H stretch by means of the isotope shift with H\(^{18}\)O [106].

The negative 3643 cm\(^{-1}\) band is not influenced by mutation at the cytoplasmic side (red), whereas it shifts by mutation at the extracellular side (blue) (Fig. 7). This indicates that this water is involved in the hydrogen-bonding network at the extracellular side of BR, as mentioned. As described, the disappearance of this band in D85N (Fig. 3), as well as the fact that no M is formed, strongly suggests that this water is located close to the Schiff base-Asp85 region [71]. D212N also has a different frequency at 3636 cm\(^{-1}\) in BR [72], though here also M is not formed. Note that Asp85 is much closer to Val49 than Glu204, implicating two domain structures. The mutation effect of Glu204 probably originates from an extended hydrogen-bonding network [19,21]. In contrast, the positive 3671 cm\(^{-1}\) band is not influenced by mutation at the extracellular side (blue), whereas it significantly alters by mutation at the cytoplasmic side (red) (Fig. 7). This indicates that the water is involved in the cytoplasmic domain in M.

The BR molecule, as a proton pump, thus possesses two domains that are strictly divided in time and space by the retinal chromophore. A hydrogen-bonding network is present at the extracellular side (Fig. 5), and mutation at this side changes the hydrogen-bonding structure, which is not changed by mutation at the cytoplasmic side. In contrast, the hydrophobic cytoplasmic region does not have an extended hydrogen-bonding network [21], but a water with free O-H (3671 cm\(^{-1}\)) appears in M. This water may donate a proton to the Schiff base by use of the other bridged O-H. Coherent proton transfer could occur in a proton wire from Asp96 to the Schiff base through water molecules [4]. The O-H stretching vibration at 3671 cm\(^{-1}\) has an angle of dipole moment of 87° to the membrane normal [106], and shifts to 3654 cm\(^{-1}\) in N [79]. The water is likely to correspond to waters 501 or 502 in BR, but it is not denied that a water at the extracellular side moves toward the cytoplasmic side as shown in Fig. 1.

4. Perspectives in the study of water in bacteriorhodopsin

4.1. Positional water molecules by X-ray crystallography of photointermediates

In the same year of the high resolution X-ray crystallography [19,21], X-ray crystallographic structures...
of photointermediates trapped at low temperature were reported by two groups [107,108]. Since this method, as well as time-resolved X-ray crystallography, visualizes the structure of photointermediates, it will strongly contribute in understanding the role of water molecules in proton pumping of BR.

Edman et al. [107] reported the X-ray crystallographic structure of the K intermediate. They observed water 402 disappearing and water 401 moving (Fig. 5). Dislocation of water 402 allows Asp85 to move up closer to the Schiff base. As a result, the water-containing pentagonal cluster structure is changed. Rearrangement of the hydrogen-bonding network in L could somehow lead to the primary proton transfer. Luecke et al. [108] reported the X-ray crystallographic structure of the D96N mutant and its M photointermediate. They observed virtually no differences in the BR structure between D96N and wild-type proteins except at position 96. They found that the hydrogen-bonding network at the extracellular side is less extensive in M than in BR. In fact, the number of water molecules decreased in M. It may suggest that proton release accompanies the release of water molecules as well.

However, a detailed discussion based on the current reports may be too early, because the resolutions in the X-ray analysis of the intermediates are lower than those of BR [107,108]. A decrease in the number of water molecules may originate from lower resolution, or positional heterogeneity in intermediates. It is unlikely that water 402 moves largely out of the original position just after photoisomerization (the K intermediate) [107]. The structure by Luecke et al. in 1998 [20] showed the position of waters 401 and 402, while lacking water 406 probably because of resolution.

Detection of positional water molecules by X-ray crystallography of photointermediates is promising, and will provide information for the proton pumping in atomic detail. However, as mentioned, hydrogen bonding based on X-ray crystallography shows water molecules as points, and positions of hydrogen atoms need much higher resolution. The understanding of chemical reactions in atomic detail requires accurate drawing of hydrogen bonds.

4.2. Current limitation and possible progress in FTIR spectroscopy

FTIR spectroscopy has played an important role in the water study of BR. Is there any more progress by FTIR? Various mutants have been tested for several intermediate states. Nevertheless, I emphasize the possible progress in FTIR spectroscopy of BR by overcoming the current limitation on the frequency region.

The observed frequency region of water molecules in the previous FTIR studies was limited to the $\nu_{3450} - 1$ region (Figs. 2 and 3) [70–81,106]. A water has two O-Hs, and their frequencies are distributed over the wide $\nu_{3700} - 2700 \text{ cm}^{-1}$ region (Fig. 2), dependent of their coupling and hydrogen-bonding strength [85,86]. This means that FTIR spectroscopy has been able to detect only the narrow region for rather free O-H groups (one fourth of the whole frequency region), while information on the bridged water O-H through hydrogen bonding was not possible to obtain. Since the actual proton transfer probably occurs through the hydrogen-bonding network on the bridged O-H, it is important to detect water structural changes in the lower frequency region. It is also needed for revealing the normal modes of water molecules in the protein.

The reason for the difficulty in identifying water O-H bands in the $3450 - 2700 \text{ cm}^{-1}$ region is as fol-

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2 According to the crystallographic structure of the K intermediate [107], the distance of the two side chain oxygens between Thr89 and Asp85 becomes 3.80 Å in K from 2.74 Å in BR, implying that the hydrogen bond between them breaks upon photoisomerization. This result contradicts our recent vibrational analysis. We showed, on the basis of $^{18}$O-threonine labeled BR [105,117], that the hydrogen bond of the O-H group of Thr89 is strengthened upon photoisomerization. The O-H group may form a hydrogen bond with the peptide carbonyl of Asp85 because the distance becomes 2.90 Å in K [107]. However, the angle (55°) between the O-O vector and the membrane normal [107] does not coincide with that (29°) between the dipole moment of the O-H stretch and the membrane normal in the IR study [105]. Thus, it is likely that the hydrogen-bonding acceptor of the O-H group of Thr89 in the K intermediate is also the side chain oxygen of Asp85.

3 A higher resolution structure of M than published (1.8 Å, in preparation), with much better statistics and a very convincing density map, still shows that water 406 is absent (J.K. Lanyi, personal communication).
allows: (i) the spectral accuracy is less because of intense absorption by water, (ii) many protein bands other than water O-H stretches overlap this region, and (iii) strongly hydrogen-bonded water possesses broad O-H stretching bands [85,86]. These facts have disturbed observations of clear isotope shifts. In fact, an isotope shift between O-H and 18O-H is about 10 cm$^{-1}$, and such a small shift could be hidden in complex spectral features in the 3450–2700 cm$^{-1}$ region.

How can such a current limitation be overcome? Recently we optimized the measuring system of polarized FTIR spectroscopy, which was applied to the conversion from BR to the K intermediate [102]. This allowed the detection of single vibrations in the whole mid-infrared region (4000–700 cm$^{-1}$) with angles of dipole moments of vibrations to the membrane normal. Since highly accurate measurements of the difference spectra of BR and the K intermediate are now possible, an attempt for complete identification of water stretching vibration is an important and challenging issue.

5. Concluding remarks

The experimental observation of internal water molecules has allowed to examine their roles in proton pumping by BR. In particular, FTIR spectroscopy has provided useful information on the structural changes of water molecules during the proton pumping process. As expected, internal water molecules are likely to comprise the proton conduction pathways, and also mediate the switch by contributing the essential proton transfer from the Schiff base to Asp85. However, the functional details of water molecules are still unclear. Having the atomic structure of internal water molecules at hand, further experimental and theoretical efforts are expected to describe the mechanism in atomic detail.

As the tool to describe internal water molecules, FTIR spectroscopy has been applied not only for BR, but also for other retinal proteins such as halorhodopsin [109], and visual rhodopsins [110–116]. Specific vibrational features of internal water molecules in these proteins will further help to understand those in BR and vice versa. X-Ray crystallography shows water molecules as points, and the (polarized) FTIR spectroscopy draws lines along water’s hydrogen bonds. A better understanding of the water-mediated proton transfer in BR will be obtained by their systematic analysis.

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