# FTIR Studies of Internal Water Molecules in the Schiff Base Region of Bacteriorhodopsin†

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ABSTRACT: In a light-driven proton pump protein, bacteriorhodopsin (BR), three water molecules participate in a pentagonal cluster that stabilizes an electric quadrupole buried inside the protein. Previously, low-temperature Fourier-transform infrared (FTIR) difference spectra between BR and the K photointermediate in $D_2O$ revealed six O−D stretches of water in BR at 2690, 2636, 2599, 2323, 2292, and 2171 $cm^{-1}$, while five water bands were observed at 2684, 2675, 2662, 2359, and 2265 $cm^{-1}$ for the K intermediate. The frequencies are widely distributed over the possible range of stretching vibrations of water, and water molecules at $<2400$ $cm^{-1}$ were suggested to hydrate negative charges because of their extremely strong hydrogen bonds. In this paper, we aimed to reveal the origin of these water bands in the K minus BR spectra by use of various mutant proteins. The water bands were not affected by the mutations at the cytoplasmic side, such as T46V, D96N, and D115N, implying that the water molecules in the cytoplasmic domain do not change their hydrogen bonds in the BR to K transition. In contrast, significant modifications of the water bands were observed for the mutations in the Schiff base region and at the extracellular side, such as R82Q, D85N, T89A, Y185F, D212N, R82Q/D212N, and E204Q. From these results, we concluded that the six O−D stretches of BR originate from three water molecules, water401, -402, and -406, involved in the pentagonal cluster. Two stretching modes of each water molecule are highly separate (300−470 $cm^{-1}$ for O−D stretches and 500−770 $cm^{-1}$ for O−H stretches), which is consistent with the previous QM/MM calculation. The small amplitudes of vibrational coupling are presumably due to strong association of the waters to negative charges of Asp85 and Asp212. Among various mutant proteins, only D85N and D212N lack strongly hydrogen-bonded water molecules ($<2400$ $cm^{-1}$) and proton pumpimg activity. We thus infer that the presence of a strong hydrogen bond of water is a prerequisite for proton pumping in BR. Internal water molecules in such a specific environment are discussed in terms of functional importance for rhodopsins.

Proteins that actively pump protons across membranes must translocate them through hydrophobic intraprotein regions. Therefore, internal water molecules are presumed to play a crucial role in active proton transport. The water may participate in hydrogen-bonding networks inside proteins that constitute proton pathways and in the switch reaction by mediating an essential proton transfer at the active site (*1*). While little is known about the structure and function of internal water molecules in such proteins generally, the greatest progress has been made in bacteriorhodopsin (BR),[1] a membrane protein found in *Halobacterium salinarum*.

BR functions as a light-driven proton pump with a retinylidene chromophore (*2*, *3*). Absorption of light by the all-trans form of the chromophore triggers a cyclic reaction that comprises a series of intermediates, designated as the J, K, L, M, N, and O states. From the cytoplasmic side to the extracellular side, the proton transport pathway includes Asp96, the Schiff base between the retinal and Lys216, Asp85, and Glu204 (or the Glu204−Glu194 region) (Figure 1). The X-ray crystallographic structure of BR clearly illustrates the presence of 10−15 internal water molecules along the proton pathway (Figure 1a). These internal water molecules must participate in each proton-transfer reaction of BR. Interestingly, previous mutant studies have shown that D96N and E204Q mutants pump protons (*4*), indicating that terminal protonatable groups, such as Asp96 and Glu204, are not essential for the proton pump mechanism. These facts point to the importance of the Schiff base region as the "switch" in the pump function. The crucial role of the Schiff base region is also demonstrated by the conversion of BR into a chloride ion pump by replacement of a single amino acid at position 85 (*5*).

The Schiff base region has a quadrupolar structure with positive charges located at the protonated Schiff base and Arg82, and counterbalancing negative charges located at Asp85 and Asp212 (Figure 1b) (*6*, *7*). The quadrupole inside the protein is stabilized by three water molecules (water401, -402, and -406). Consequently, the Schiff base region contains a roughly planar pentagonal cluster, composed of these waters and one oxygen each of Asp85 and Asp212. A notable structural feature is that Asp85 and Asp212 are located at similar distances from the retinal Schiff base,

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[1] Abbreviations: BR, bacteriorhodopsin; FTIR, Fourier-transform infrared; QM/MM, quantum mechanical/molecular mechanical.

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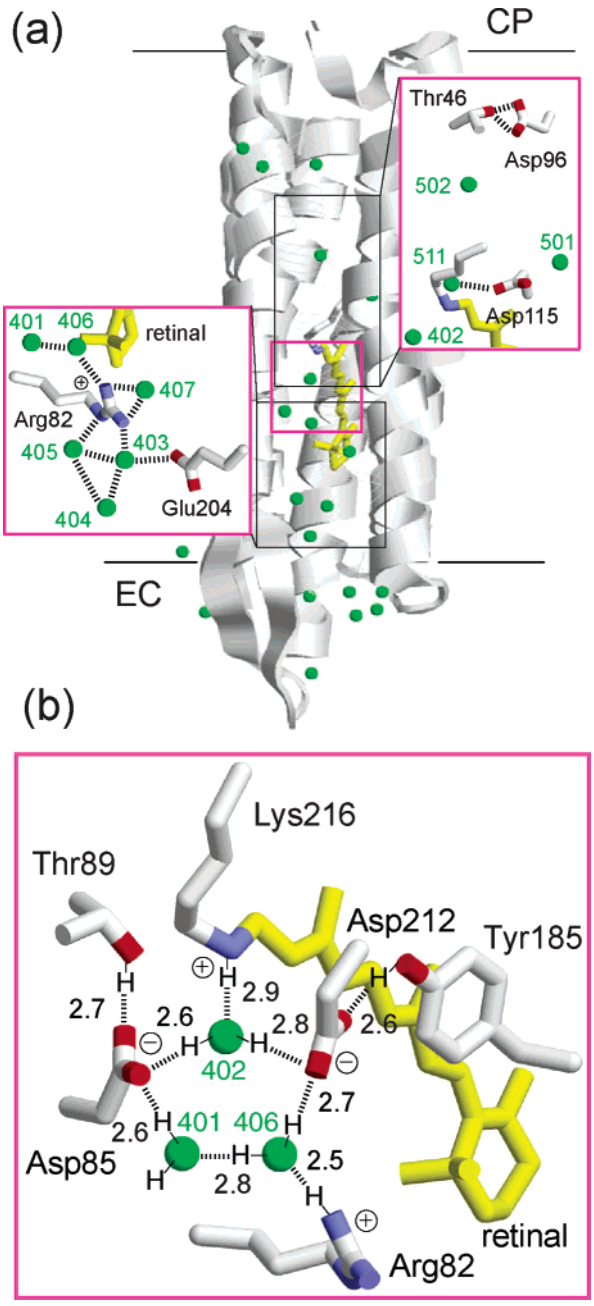


FIGURE 1: (a) X-ray crystallographic structure of BR from PDB entry 1C3W (*6*). The membrane normal is approximately in the vertical direction of this figure. Upper and lower regions correspond to the cytoplasmic (CP) and extracellular (EC) sides, respectively. Green spheres represent water molecules bound inside BR. Internal water molecules at the CP and EC sides are highlighted, while supposed hydrogen bonds with interacting amino acids are shown as dashed lines. (b) X-ray crystallographic structure of the Schiff base region in BR from PDB entry 1C3W (*6*). Water401, -402, and -406 form a roughly planar pentagonal cluster with an oxygen from Asp85 and an oxygen from Asp212. Hydrogen atoms and hydrogen bonds (dashed lines) are supposed from the structure, while the numbers are the hydrogen-bonding distances in angstroms.

whereas the Schiff base proton is transferred to only Asp85 in the L to M transition. The water molecules in the Schiff base region presumably play important roles in the proton-transfer reaction (*1*).

Vibrational analysis is a powerful tool for investigation of hydrogen bonds. In particular, the O−H (O−D) stretching modes are good indicators of hydrogen-bonding strength of water molecules (*1*, *8*). Although observation of water stretching vibrations was initially limited to weak hydrogen bonds in low-temperatrue Fourier-transform infrared (FTIR) spectroscopy of BR (*1*), recent accurate spectral comparison between samples in $D_2O$ and $D_2{}^{18}O$ has extended it to water molecules with strong hydrogen bonds (*9*, *10*). We published the K minus BR difference FTIR spectra (*11*, *12*), where the isotope effect of $^{18}O$ water was observed for five positive and six negative bands. Interestingly, among six water bands of BR, three bands appear in the 2700−2550 $cm^{-1}$ region while the other three bands appear in the 2350−2150 $cm^{-1}$ region. There were no water bands in the middle-frequency range (2550−2350 $cm^{-1}$) (*10*, *11*). The water O−D stretches in the 2700−2550 $cm^{-1}$ region correspond to weak hydrogen bonds. In contrast, the water O−D stretches in the 2350−2150 $cm^{-1}$ region are much lower in frequency than the fully hydrated tetrahedral water molecules, from which we inferred that these water molecules hydrate negative charges (*10*). In fact, the previous QM/MM calculation of the Schiff base region of BR concluded that these water vibrations can be ascribed to the water molecules involved in the pentagonal cluster (*13*).

It should be noted that these water bands have to be experimentally proven to belong to the O−D stretches of water in the pentagonal cluster (Figure 1b). In 2003, we reported that one of the water bands at 2171 $cm^{-1}$ originates from the O−D stretch of water402 hydrating Asp85 (Figure 1b) by use of mutant proteins of BR (*12*). In the study presented here, we aimed to reveal the origin of all water bands involved in the K minus BR spectra by use of various mutant proteins. We tested mutant proteins not only in the Schiff base region but also in the cytoplasmic and extracellular regions. As a result, the water bands were not affected by the mutations at the cytoplasmic side, but significantly altered by the mutations in the Schiff base region and at the extracellular side. We concluded that the six O−D stretches of BR originate from three water molecules, water401, -402, and -406, in the pentagonal cluster (Figure 1b), which is consistent with the previous QM/MM calculation (*13*). Two stretching modes of these waters are highly separate in frequency, and small vibrational couplings are presumably due to strong association of the waters with negative charges of Asp85 and Asp212. We discuss the functional role of these internal water molecules in such a specific environment in terms of proton pumpimg ability.

## MATERIALS AND METHODS

Samples were prepared as described previously (*1*, *12*). A 120 $\mu$L aliquot of the sample in 2 mM phosphate buffer (pH 7.0) except for D212N (pH 10.0) and R82Q (pH 9.5) was dried on a $BaF_2$ window with a diameter of 18 mm. After hydration by 1 $\mu$L of $D_2O$ or $D_2{}^{18}O$, the sample was placed in a cell and then the cell mounted in an Oxford DN-1704 cryostat. The hydrated film was illuminated with >500 nm light for 1 min at 273 K except for D85N (*12*) to obtain the light-adapted state of BR.

Low-temperature FTIR spectroscopy was applied as described previously by use of a Bio-Rad FTS-40 FTIR spectrometer (*9*−*12*, *14*, *15*). Since the orientation of molecules was highly dependent on mutant samples, we did not use an IR polarizer in the FTIR measurements, and the sample films were tilted by ∼30° in the cryostat (*12*). The

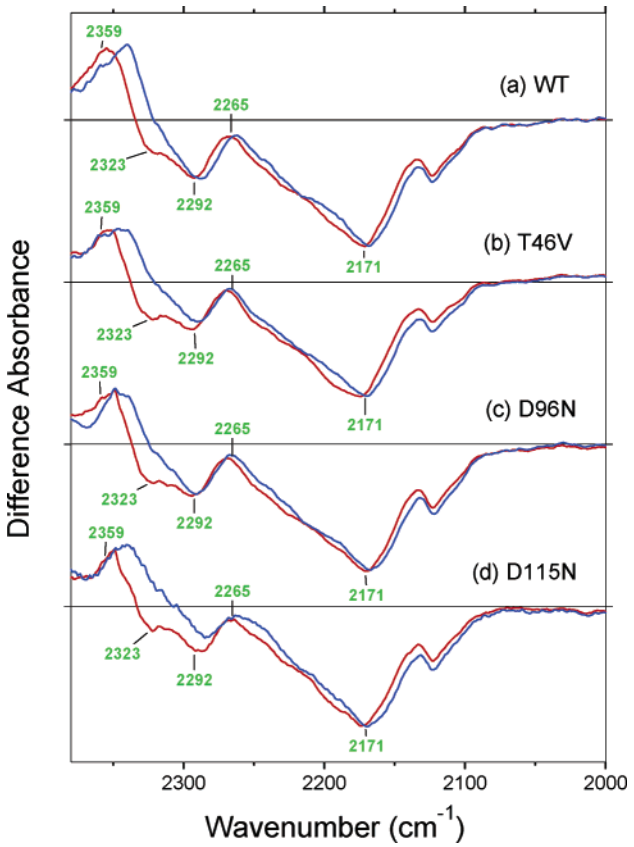


FIGURE 2: K minus BR difference infrared spectra of wild type (a), T46V (b), D96N (c), and D115N (d) BR in the 2380−2000 $cm^{-1}$ region. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

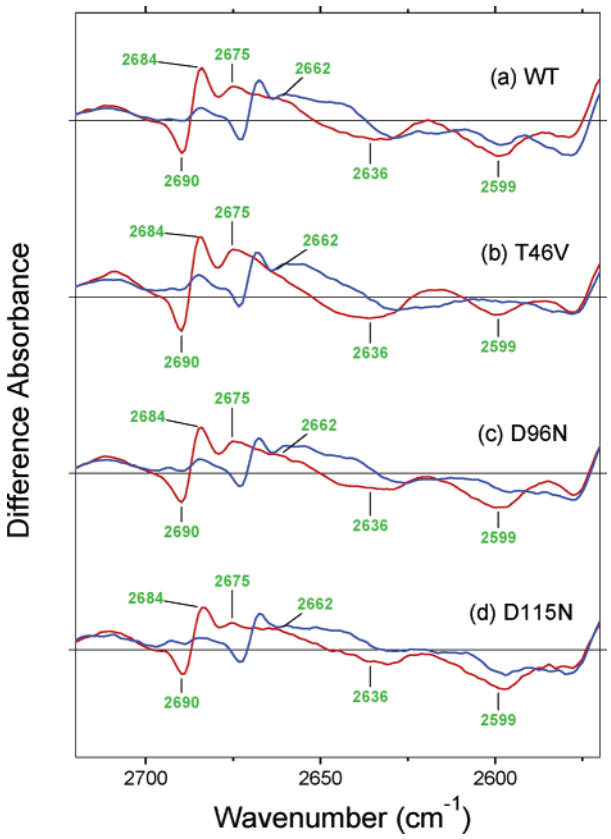


FIGURE 3: K minus BR difference infrared spectra of wild type (a), T46V (b), D96N (c), and D115N (d) BR in the 2720−2570 $cm^{-1}$ region. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

K minus BR spectra (an average of 40 spectra of 128 interferograms) were measured by photoconversion between K and BR at 77 K (*9*, *10*, *12*). All mutant spectra resembled that of the wild type in the 1800−900 $cm^{-1}$ region, indicating the formation of the K intermediate.

## RESULTS

*Water Stretching Vibrations of the BR Mutants at the Cytoplasmic Side.* Figure 2 shows the K minus BR difference spectra of the wild-type and mutant proteins of BR at the cytoplasmic side in the 2380−2000 $cm^{-1}$ region. The spectrum of the wild type was measured again under conditions identical to those of the mutants, which reproduced the reported water bands at 2359 (+), 2323 (−), 2292 (−), 2265 (+), and 2171 (−) $cm^{-1}$ (*11*, *12*). It should be noted that the 2265 $cm^{-1}$ band of water does not appear on the positive side in this measurement, whereas it becomes positive when the sample film is further tilted in the polarized measurement. The reason is that the 2265 $cm^{-1}$ band is highly dichroic. Recent polarized FTIR spectroscopy of the unlabeled and [$\eta_{1,2}$-$^{15}$N]arginine-labeled BR revealed that the 2292 (−)/2265 (+) $cm^{-1}$ bands contain both the N−D stretch of Arg82 and the O−D stretch of water, and the results of spectral fittings showed that the dipole moments of the water bands of BR and K are tilted 49° and 23° from the membrane normal, respectively (*16*). Similarly, the negative 2171 $cm^{-1}$ band of water also contains the N−D stretching vibration of the Schiff base (*14*). These spectral components not associated with the O−D stretches of water presumably yield apparently smaller isotope shifts of the water bands at 2292 (−), 2265 (+), and 2171 (−) $cm^{-1}$ compared with those of the water bands at 2359 (+) and 2323 (−) $cm^{-1}$ (Figure 2a).

The K minus BR difference spectra of T46V (Figure 2b), D96N (Figure 2c), and D115N (Figure 2d) look similar to those of the wild type with some spectral variations. All of these mutants exhibit five water O−D stretches, being identical to the wild type in the 2380−2000 $cm^{-1}$ region. These facts indicate that strongly hydrogen-bonded water molecules are not affected by mutations at the cytoplasmic side. In other words, water molecules in the cytoplasmic side such as water501, -502, and -511 (Figure 1a) do not contribute to the water stretching vibrations in the 2380−2000 $cm^{-1}$ region.

Similar results were obtained for the water bands under weak hydrogen-bonding conditions. Figure 3 shows the K minus BR difference spectra of the wild type and mutant proteins of BR at the cytoplasmic side in the 2720−2570 $cm^{-1}$ region. Spectra of the wild type, T46V, D96N, and D115N all possess O−D stretches of water at 2690 (−), 2684 (+), 2675 (+), 2662 (+), 2636 (−), and 2599 (−) $cm^{-1}$ (Figure 3). Taken together with the results of Figure 2, this means that the mutations at the cytoplasmic side such as T46V, D96N, and D115N do not affect the water bands in the K minus BR difference spectra.

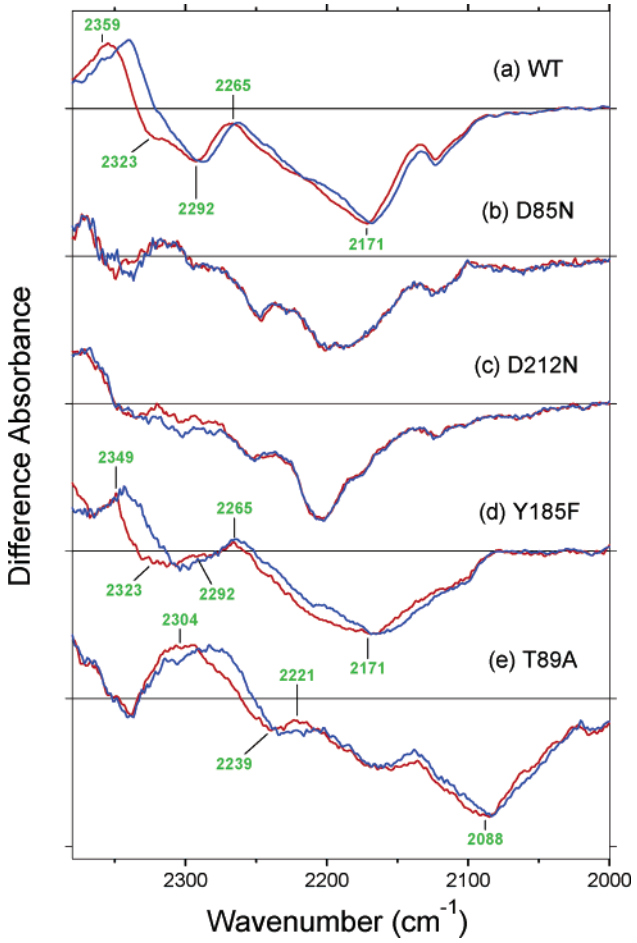


FIGURE 4: K minus BR difference infrared spectra of wild type (a), D85N (b), D212N (c), Y185F (d), and T89A (e) BR in the 2380−2000 $cm^{-1}$ region, which are reproduced from ref *12*. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

*Identification of the Water O−D Stretches under Strong Hydrogen-Bonding Conditions.* Figure 4 shows the K minus BR difference spectra of the wild type and mutant proteins of BR near the Schiff base in the 2380−2000 $cm^{-1}$ region, which is reproduced from our recent paper (*12*). Spectra b and c of Figure 4 show that the broad negative features in the 2300−2000 $cm^{-1}$ region do not exhibit the isotope shift in D85N and D212N, respectively. The negative bands can be assigned as the N−D stretch of the Schiff base like in the wild type BR. The absence of water bands in both D85N and D212N implies the crucial role of each negative charge in the pentagonal cluster, leading to the possible assignment of the water bands at 2323, 2292, and 2171 $cm^{-1}$ to the O−D stretches of water molecules involved in the pentagonal cluster (Figure 1). Since these frequencies are very low for an O−D stretch of water, it is reasonable to suggest that these water molecules associate with negative charges of Asp85 and Asp212. As shown in Figure 1b, there are four O−H groups possibly hydrogen-bonded with them (four O−D groups in $D_2O$).

We next examined the water bands by use of additional mutations. Figure 4d shows that all the water bands were preserved in the Y185F mutant, though the negative 2292 $cm^{-1}$ band loses significant intensity. This observation suggests that the hydrogen bond between Tyr185 and Asp212 does not significantly affect the hydrogen-bonding structure in the water-containing pentagonal cluster. In contrast, a clearly different result was obtained for T89A (Figure 4e), where the water bands of the wild type are almost entirely altered. One oxygen atom of Asp85 and Asp212 is involved in the pentagonal cluster, while another oxygen forms a hydrogen bond with Thr89 and Tyr185 (Figure 1b). Therefore, we expected that the cleavage of these hydrogen bonds would localize the negative charges onto the respective oxygens of the pentagonal cluster, so that water O−D stretches are influenced (*12*). That was indeed the case for T89A (Figure 4e), but not for Y185F (Figure 4d). It was especially surprising that the lowest O−D stretch at 2171 $cm^{-1}$ of the wild type is further downshifted to 2088 $cm^{-1}$ in T89A, which exhibits a lower frequency shift in $D_2{}^{18}O$. According to the QM/MM calculation by Hayashi and Ohmine, the lowest O−D stretch of water is that of water402 interacting with Asp85 [2257 $cm^{-1}$ (Table 1) (*13*)]. The frequency change of >80 $cm^{-1}$ in T89A, but not in Y185F, strongly supports the proposal by the QM/MM calculation that the lowest O−D band originates from the O−D stretch of water402 interacting with Asp85, though the frequency from the experiment (2171 $cm^{-1}$) is considerably different from that from the calculations (2257 $cm^{-1}$) (*12*).

Table 1: Stretching Frequencies of Water401, -402, and -406 in the Schiff Base Region of BR

| | O−D stretch ($cm^{-1}$) | $\Delta\nu_{O-D}$ ($cm^{-1}$) | O−H stretch[a] ($cm^{-1}$) | $\Delta\nu_{O-H}$ ($cm^{-1}$) | O−D calculated[b] ($cm^{-1}$) |
|---|---|---|---|---|---|
| water402 | 2171 | 465 | ∼2800 | ∼770 | 2257 |
| | 2636[c] | | 3570 | | 2618 |
| water401 | 2323 | 367 | 3100 | 543 | 2320 |
| | 2690 | | 3643 | | 2721 |
| water406 | 2292 | 307 | ∼3000 | ∼520 | 2468[d] |
| | 2599[c] | | 3516 | | 2601[d] |

[a] Estimated from ref *6*. [b] From ref *13*. [c] These frequencies were not unequivocally identified, so the alternative assignment is possible. [d] These O−D stretches are coupled modes, where the frequencies at 2468 and 2601 $cm^{-1}$ correspond to symmetric and antisymmetric vibrations of water406, respectively. It should be noted that the quantum chemical calculation was not applied to Arg82, which can strongly influence the frequencies of water406 (*13*).

From where do other vibrations originate? Previous QM/MM calculation predicted that the second lowest O−D stretch is water401 interacting with Asp85 [2320 $cm^{-1}$ (Table 1)], and the third lowest O−D stretch is water406 interacting with Asp212 [2468 $cm^{-1}$ (Table 1)], while the O−D stretch of water402 interacting with Asp212 is at a higher frequency [2618 $cm^{-1}$ (Table 1) (*13*)]. We further attempted to identify these water vibrations experimentally by use of mutants at the extracellular side. Figure 5 shows the K minus BR difference spectra of the wild type and mutant proteins of BR at the extracellular side in the 2380−2000 $cm^{-1}$ region. The 2292 (−)/2265 (+) $cm^{-1}$ bands disappear in R82Q (Figure 5b), whereas the water bands at 2359 (+), 2323 (−), and 2171 (−) $cm^{-1}$ are preserved with small changes in their frequencies. This observation strongly suggests that the water bands at 2292 and 2265 $cm^{-1}$ are highly influenced by the mutation at position 82. The most straightforward interpretation is that the water bands at 2292 and 2265 $cm^{-1}$ originate from water406 interacting with Asp212 in BR and K, respectively. As described above, the 2292 (−)/2265 (+)

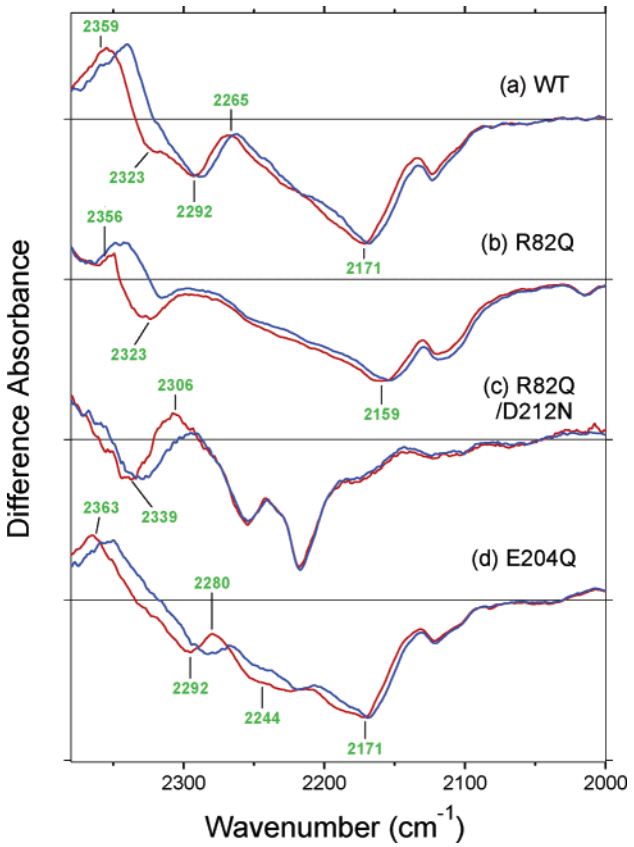


FIGURE 5: K minus BR difference infrared spectra of wild type (a), R82Q (b), R82Q/D212N (c), and E204Q (d) BR in the 2380−2000 $cm^{-1}$ region. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

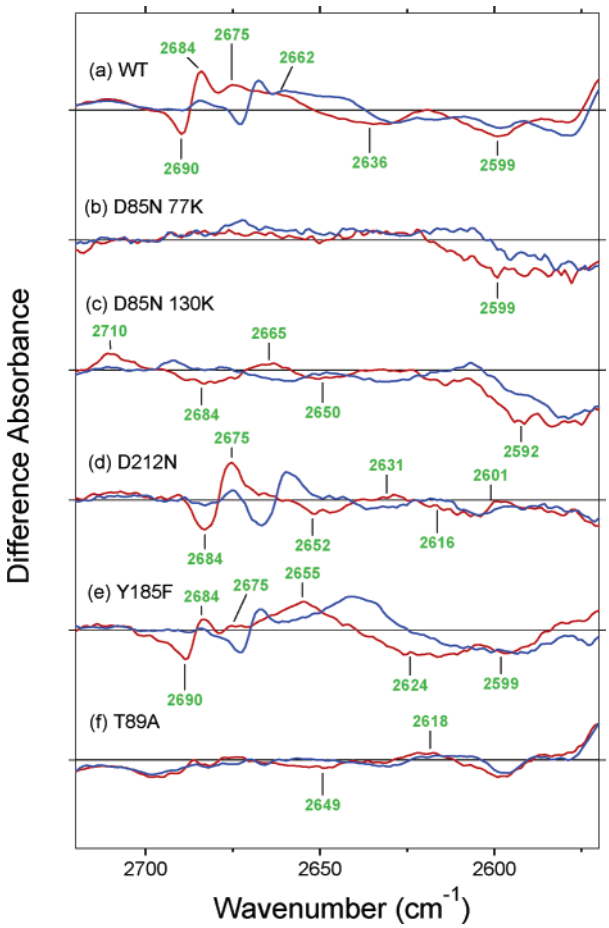


FIGURE 6: K minus BR difference infrared spectra of wild type (a), D85N (b and c), D212N (d), Y185F (e), and T89A (f) BR in the 2720−2570 $cm^{-1}$ region. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K, except for spectrum c. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

$cm^{-1}$ bands also contain the N−D stretch of Arg82 (*16*). Removal of the hydrogen bond between Arg82 and water406 is likely to weaken the hydrogen bond of water406 interacting with Asp212.

The 2292 (−)/2265 (+) $cm^{-1}$ bands disappear in R82Q, whereas the 2359 (+)/2323 (−) $cm^{-1}$ bands of the wild type are likely to appear at 2356 (+)/2323 (−) $cm^{-1}$. The water O−D stretch at 2323 $cm^{-1}$ may originate from (i) water401 interacting with Asp85, or (ii) water402 interacting with Asp212, postulating that this water hydrates a negative charge. According to the QM/MM calculation, however, the latter is under weak hydrogen-bonding conditions [2618 $cm^{-1}$ (Table 1)], while the O−D stretch of water401 is at 2320 $cm^{-1}$, being in good agreement with our FTIR observations (*13*). In fact, Figure 6c shows the presence of the negative 2339 $cm^{-1}$ band in R82Q/D212N, suggesting that the water possessing the O−D stretch at 2323 $cm^{-1}$ does not form a hydrogen bond with Asp212.

In E204Q, water bands were observed 2363 (+), 2292 (−), 2280 (+), 2244 (−), and 2171 (−) $cm^{-1}$ (Figure 5d). The water bands at 2292 (−)/2280 (+) $cm^{-1}$ are likely to be the same as those at 2292 (−)/2265 (+) $cm^{-1}$ for the wild type (Figure 5a), because they are highly dichroic like those of the wild type (data not shown). Thus, among strongly hydrogen-bonded water bands, it seems that only the band at 2323 $cm^{-1}$ in the wild type is downshifted by ∼80 $cm^{-1}$ to 2244 $cm^{-1}$ in E204Q. Glu204 is distant from the Schiff base region, whereas it is known that the p$K_a$ of Asp85 is increased in E204Q, indicating the presence of a long-distance hydrogen-bonding network (*17, 18*). The strengthened hydrogen bond of water401 with Asp85 in E204Q is possibly correlated with the higher p$K_a$ value of Asp85.

On the basis of the results described above, we concluded that the water bands at 2171, 2292, and 2323 $cm^{-1}$ originate from the O−D stretches of water402, -406, and -401, respectively. From the previous results in $H_2O$ (*9*), we also estimated the corresponding O−H stretches to be at ∼2800, ∼3000, and 3100 $cm^{-1}$ for water402, -406, and -401, respectively (Table 1). It should be noted that a water molecule generally possesses coupled stretching vibrations, symmetric and antisymmetric modes, at nearby frequencies (*1*). The absence of the other vibrations in this frequency region strongly suggests that vibrational coupling is small for the water molecules in the pentagonal cluster. It would be reasonable because each water molecule interacts with a negative charge. We expect the other stretching vibrations to appear in a higher-frequency region.

*Identification of the Water O−D Stretches under Weak Hydrogen- Bonding Conditions.* Figure 6a shows the K minus BR difference spectrum of the wild type in the 2720−2570 $cm^{-1}$ region. There are three water bands for both positive and negative sides. The previous QM/MM calculations

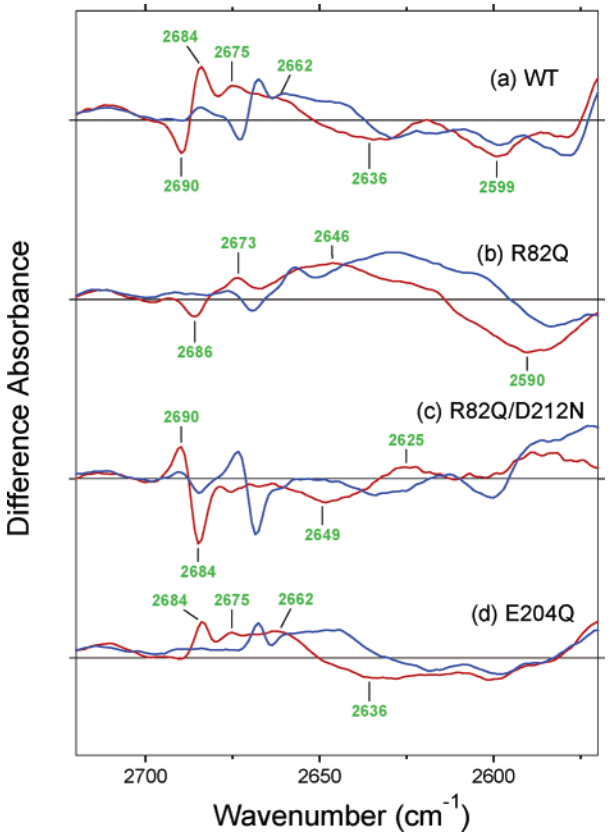


FIGURE 7: K minus BR difference infrared spectra of wild type (a), R82Q (b), R82Q/D212N (c), and E204Q (d) BR in the 2720−2570 $cm^{-1}$ region. The samples were hydrated with $D_2O$ (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to the bands identified as water stretching vibrations.

reproduced the O−D stretches of water molecules in BR rather well (Table 1) (*13*). Here we examine the origin of water bands by use of mutant proteins.

In the case of D85N, only one negative water band (2599 $cm^{-1}$) was observed at 77 K (Figure 6b), which is in high contrast to the wild type. However, the spectra of D85N at 130 K exhibited the presence of three water bands at the negative side (Figure 6c). We found that the mutants of Asp85, such as D85N, D85S, and D85T, exhibit temperature-dependent water bands between 77 and 130 K, whereas other mutants were not influenced by such temperature changes (data not shown). Water bands of D85N were significantly different from those of the wild type even at 130 K. The spectra of D212N possess water bands at 2684 (−), 2675 (+), 2652 (−), 2631 (+), 2616 (−), and 2601 (+) $cm^{-1}$ (Figure 6d), which are different from those of the wild type as well. It must be noted that D85N and D212N entirely lack water bands under strong hydrogen-bonding conditions (spectra b and c of Figure 4, respectively), which might indicate the absence of water molecules in the pentagonal cluster. However, the presence of three negative bands of water for D85N and D212N (spectra c and d of Figure 6, respectively) suggests that these mutants also contain internal water molecules in the Schiff base region like the wild type, whereas the hydrogen-bonding interaction of such water molecules is different.

Spectra e and f of Figure 6 show the difference FTIR spectra for Y185F and T89A, respectively. The spectra of

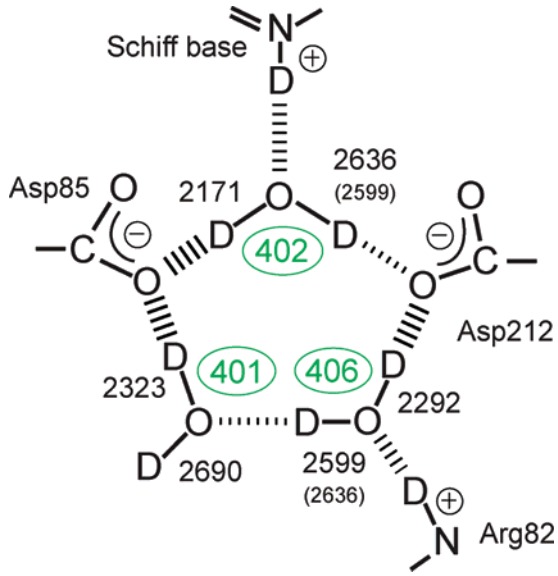


FIGURE 8: Schematic drawing of the deuterated pentagonal cluster structure in the Schiff base region. The numbers are the O−D stretching frequencies of water molecules in inverse centimeters assigned in this FTIR study.

Y185F possess water bands at 2690 (−), 2684 (+), 2675 (+), 2655 (+), 2624 (−), and 2599 (−) $cm^{-1}$, whose spectral features are similar to those of D212N (Figure 6d). In contrast, the spectra of T89A possess water bands at only 2649 (−) and 2618 (+) $cm^{-1}$, whose spectral features are similar to those of D85N (spectra b and c of Figure 6, respectively). These facts suggest the importance of the hydrogen-bonding interactions between Asp85 and Thr89 and between Asp212 and Tyr185.

Figure 7 shows the K minus BR difference spectrum of the wild type and the mutants of amino acids at the extracellular side in the 2720−2570 $cm^{-1}$ region. R82Q possesses water bands at 2686 (−), 2673 (+), 2646 (+), and 2590 (−) $cm^{-1}$ (Figure 7b). Additional mutation at position 212 significantly alters the spectral shape, exhibiting water bands at 2690 (+), 2684 (−), 2649 (−), and 2625 (+) $cm^{-1}$ (Figure 7c). In the case of E204Q, the negative water band of the wild type at 2690 $cm^{-1}$ seems to disappear, while there are three positive bands at 2684, 2675, and 2662 $cm^{-1}$ together with a negative band at 2636 $cm^{-1}$.

From these FTIR spectra of various mutants, it is likely that three water O−D stretches at 2690, 2636, and 2599 $cm^{-1}$ originate from water401, -402, and -406, respectively, in the pentagonal cluster (Figure 8). Because of secondary effects of mutations, however, it is difficult to determine the origin of the water O−D stretches, particularly for weakly hydrogen-bonded water molecules. The model of the pentagonal cluster in BR predicts that one O−D stretch of water401 is free of hydrogen bonds (Figure 8). Therefore, it is a reasonable postulation that this O−D stretch has the highest frequency, namely, at 2690 $cm^{-1}$ (Figures 6 and 7). In fact, the O−D stretch at 2690 $cm^{-1}$ in $D_2O$ corresponds to the O−H stretch at 3643 $cm^{-1}$ in $H_2O$, which is almost free of hydrogen bonds. Such assignment is supported by the fact that the 2690 $cm^{-1}$ band is not significantly affected by the mutations at Asp212 and Tyr185 (spectra d and e of Figure 6), while disappearing in D85N (77 K, Figure 6b), T89A (Figure 6f), and E204Q (Figure 7d). Thus, we conclude that the water O−D stretch at 2690 $cm^{-1}$ originates from water401 that is

free of hydrogen bonds. In contrast, we could not assign the water bands at 2636 and 2599 $cm^{-1}$ uniquely. We follow the assignment of the QM/MM calculations (*13*), and eventually six O−D stretches in BR were identified as shown in Table 1 and Figure 8.

## DISCUSSION

In this article, we attempted to identify the origin of the water O−D stretches observed in the low-temperatrue FTIR difference spectra of BR at 77 K. To accomplish that goal, we extensively studied the K minus BR difference spectra of various mutant proteins, with mutations not only in the Schiff base region but also in the cytoplasmic and extracellular regions. We found that the water bands were not affected by the mutations at the cytoplasmic side, but significantly altered by the mutations in the Schiff base region and at the extracellular side. We concluded that the six O−D stretches of BR originate from three water molecules, water401, -402, and -406, in the pentagonal cluster (Figure 1b), which is consistent with the previous QM/MM calculations (*13*).

It should be noted that the waters in the pentagonal cluster are not necessarily close to the retinal chromophore. The distances from water401, -402, and -406 to the nearest atom of the chromophore, which is the Schiff base nitrogen, are 6.1, 2.9, and 5.9 Å, respectively (*6*). Water501, -502, and -511 are 4.4 Å from the methyl carbon at C13, 7.7 Å from C15, and 9.4 Å from C10, respectively (Figure 1a). Water407, -403, and -405 are 9.6 Å from the Schiff base nitrogen, 9.8 Å from C8, and 8.8 Å from the methyl carbon at C2, respectively (Figure 1a). These facts suggest the importance of the hydrogen-bonding network, where water-containing pentagonal cluster structure must play a crucial role.

While the water bands in the K minus BR spectra were not affected in the mutants at the cytoplasmic side such as T46V, D96N, and D115N (Figures 2 and 3), significant modifications of the water bands were observed for the mutations at the extracellular side such as R82Q, R82Q/D212N, and E204Q (Figures 5 and 7). It is well-known that the p$K_a$ values of Asp85 and the proton release group are correlated with each other, because the mutations of Arg82 and Glu204 increase the p$K_a$ of Asp85 (*17*−*19*). Thus, the importance of an extended hydrogen-bonding network has been discussed. Our FTIR study observed that the 2171 and 2323 $cm^{-1}$ bands downshift to 2159 and 2244 $cm^{-1}$ for R82Q and E204Q, respectively (Figure 5). Since they can be ascribed to the water O−D stretches interacting with Asp85 (Figure 8), the stronger hydrogen bond of water (manifested by the shift to a lower frequency) is presumably correlated with the change in the p$K_a$ of Asp85.

Figure 8 summarizes the O−D stretches of the water molecules in the pentagonal cluster. Two stretching modes of these waters are highly separate in frequency (300−470 $cm^{-1}$ for O−D stretches and 500−770 $cm^{-1}$ for O−H stretches) (Table 1). In general, a water molecule has two O−H groups, and their frequencies are distributed in the wide 3700−2700 $cm^{-1}$ region dependent on their coupling and hydrogen-bonding strength. Gaseous water exhibits asymmetric and symmetric stretching modes at 3755 and 3657 $cm^{-1}$, respectively, and the stretching frequency is lowered as its hydrogen bond becomes stronger (*20*). It must be noted that the hydrogen-bonding strengths of the two O−H groups are probably not equivalent in the restricted protein environment, which breaks the $C_{2v}$-type symmetry. In such $C_S$-type symmetry, one O−H group is hydrogen-bonded and the other O−H group is unbonded, and their frequencies are widely split (*1*). These results can be explained well by such decoupling of the two stretching modes. Small vibrational coupling is presumably due to strong association of the waters with negative charges at Asp85 and Asp212.

The frequencies of water402 are particularly prominent. As we reported previously, the 2171 $cm^{-1}$ band was assigned as the O−D stretch of water402 hydrating Asp85 (*12*). This FTIR study together with the QM/MM calculations strongly suggests that the other O−D stretch of water402 is at 2636 $cm^{-1}$ (or 2599 $cm^{-1}$). Therefore, the frequency difference between the two O−D stretches is as large as 465 $cm^{-1}$, which corresponds to ∼770 $cm^{-1}$ for O−H stretches (Table 1). In the unphotolyzed state of BR, the position of water402 looks symmetrical with respect to Asp85 and Asp212 (Figure 1b). This study, however, showed highly asymmetric association of water402 with the aspartates. Since the hydrogen-bonding strength of water is closely related to the geometry of the O−H (O−D) group, such asymmetric interaction of water402 presumably originates from the orientation of their O−H (O−D) groups. As a consequence, water402 bridges the Schiff base nitrogen and an oxygen of Asp85 ideally.

Formation of the K intermediate is accompanied by hydrogen-bonding alterations of the water molecules in the pentagonal cluster. This FTIR study of R82Q (Figure 5b) suggests that the 2292 $cm^{-1}$ band of water406 in BR corresponds to the 2265 $cm^{-1}$ band in K, which also overlaps with the N−D stretch of Arg82 (*16*). According to the QM/MM calculations by Hayashi et al., the 2690 and 2323 $cm^{-1}$ bands of water401 correspond to the 2684 and 2359 $cm^{-1}$ bands in K, respectively (*21*). Although the QM/MM calculations for the K intermediate explain this FTIR observation well, one exception is the O−D stretch of water402 hydrating Asp85. The QM/MM calculations resulted in the O−D stretches at 2257 and 2423 $cm^{-1}$ for BR and K, respectively (*21*). The observed O−D stretch in BR is at 2171 $cm^{-1}$ (Figure 8), whereas the corresponding O−D stretch in K is not determined in this study. There are no positive water bands around 2423 $cm^{-1}$ in the K minus BR spectra (*10*, *11*). We observed only five positive bands for K (Figures 2 and 3). It is possible that the positive band is present in the 2300−2200 $cm^{-1}$ region, where it clearly cannot be observed. Further experimental and theoretical efforts are necessary to fully understand the water bands in the K intermediate.

Among various mutant proteins we studied here, only D85N and D212N lack strongly hydrogen-bonded water molecules (<2400 $cm^{-1}$) (Figure 4). R82Q/D212N (Figure 5c) lacks water bands in the 2250−2100 $cm^{-1}$ region, like D212N (Figure 4c). Nevertheless, unlike D212N, R82Q/D212N possesses new water bands at 2339 (−)/2306 (+) $cm^{-1}$. It is known that R82Q/D212N pumps protons (*22*), while D212N and D85N do not (*23*). Thus, there is a correlation between proton pumping activity and strongly hydrogen-bonded water molecules. At present, the origin of the band at 2339 $cm^{-1}$ is uncertain for R82Q/D212N. From the similar frequency in the wild type, the 2339 $cm^{-1}$ band

may originate from water401 hydrating Asp85. However, it is also possible that water402 hydrating Asp85 possesses the band at 2339 $cm^{-1}$ in this mutant.

The correlation between proton pumping activity and strongly hydrogen-bonded water molecules is true not only for BR mutants but also for various rhodopsins. We have been examining whether rhodopsins possess strongly hydrogen-bonded water molecules or not systematically. We found that BR and *pharaonis* phoborhodopsin (*24*, *25*), both of which pump protons, possess such water molecules (O−D stretch at $<2400$ $cm^{-1}$ in $D_2O$). In contrast, strongly hydrogen-bonded water molecules were not observed for halorhodopsin (*26*), neurospora rhodopsin (*27*), and bovine rhodopsin (*28*). It is known that none of them pumps protons. Such comprehensive studies of archaeal and visual rhodopsins have thus revealed that strongly hydrogen-bonded water molecules are only found in the proteins exhibiting proton-pumping activities. Taken together with our results for BR mutants, this suggests that strong hydrogen bonds of water molecules and their transient weakening may be essential for the proton pumping function of rhodopsins.

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## REFERENCES

1. Kandori, H. (2000) Role of internal water molecules in bacteriorhodopsin, *Biochim. Biophys. Acta 1460*, 177−191.
2. Lanyi, J. K. (1998) Understanding structure and function in the light-driven proton pump bacteriorhodopsin, *J. Struct. Biol. 124*, 164−178.
3. Haupts, U., Tittor, J., and Oesterhelt, D. (1999) Closing in on bacteriorhodopsin: Progress in understanding the molecules, *Annu. Rev. Biophys. Biomol. Struct. 28*, 367−399.
4. Krebs, M. P., and Khorana, H. G. (1993) Mechanism of light-dependent proton translocation by bacteriorhodopsin, *J. Bacteriol. 175*, 1555−1560.
5. Sasaki, J., Brown, L. S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J. K. (1995) Conversion of bacteriorhodopsin into a chloride ion pump, *Science 269*, 73−75.
6. Luecke, H., Schobert, B., Richter, H.-T., Cartailler, J. P., and Lanyi, J. K. (1999) Structure of bacteriorhodopsin at 1.55 Å resolution, *J. Mol. Biol. 291*, 899−911.
7. Belrhali, H., Nollert, P., Royant, A., Menzel, C., Rosenbusch, J., Landau, E. M., and Pebay-Peyroula, E. (1999) Protein, lipid, and water organization in bacteriorhodopsin crystals: A molecular view of the purple membrane at 1.9 Å resolution, *Structure 7*, 909−917.
8. Kandori, H. (2004) Hydration switch model for the proton transfer in the Schiff base region of bacteriorhodopsin, *Biochim. Biophys. Acta 1658*, 72−79.
9. Kandori, H., Kinoshita, N., Shichida, Y., and Maeda, A. (1998) Protein structural changes in bacteriorhodopsin upon photoisomerization as revealed by polarized FTIR spectroscopy, *J. Phys. Chem. B 102*, 7899−7905.
10. Kandori, H., and Shichida, Y. (2000) Direct observation of the bridged water stretching vibrations inside a protein, *J. Am. Chem. Soc. 122*, 11745−11746.
11. Tanimoto, T., Furutani, Y., and Kandori, H. (2003) Structural changes of water in the Schiff base region of bacteriorhodopsin: Proposal of a hydration switch model, *Biochemistry 42*, 2300−2306.
12. Shibata, M., Tanimoto, T., and Kandori, H. (2003) Water molecules in the Schiff base region of bacteriorhodopsin, *J. Am. Chem. Soc. 125*, 13312−13313.
13. Hayashi, S., and Ohmine, I. (2000) Proton transfer in bacteriorhodopsin: Structure, excitation, IR spectra, and potential energy surface analyses by an ab initio QM/MM method, *J. Phys. Chem. B 104*, 10678−10691.
14. Kandori, H., Belenky, M., and Herzfeld, J. (2002) Vibrational frequency and dipolar orientation of the protonated Schiff base in bacteriorhodopsin before and after photoisomerization, *Biochemistry 41*, 6026−6031.
15. Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y., and Kamo, N. (2001) Structural change of *pharaonis* phoborhodopsin upon photoisomerization of the retinal chromophore: Infrared spectral comparison with bacteriorhodopsin, *Biochemistry 40*, 9238−9246.
16. Tanimoto, T., Shibata, M., Belenky, M., Herzfeld, J., and Kandori, H. (2004) Altered hydrogen-bonding of Arg82 during the proton pump cycle of bacteriorhodopsin: A low-temperature polarized FTIR spectroscopic study, *Biochemistry 43*, 9439−9447.
17. Balashov, S. P., Govindjee, R., Imasheva, E. S., Misra, S., Ebrey, T. G., Feng, Y., Crouch, R. K., and Menick, D. R. (1995) The two p$K_a$'s of aspartate-85 and coutrol of thermal isomerization and proton release in the arginine-82 to lysine mutant of bacteriorhodopsin, *Biochemistry 34*, 8820−8823.
18. Richter, H.-T., Brown, L. S., Needleman, R., and Lanyi, J. K. (1996) A linkage of the p$K_a$'s of asp-85 and glu-204 forms part of the reprotonation switch of bacteriorhodopsin, *Biochemistry 35*, 4054−4062.
19. Subramaniam, S., Marti, T., and Khorana, H. G. (1990) Protonation state of Asp (Glu)-85 regulates the purple-to-blue transition in bacteriorhodopsin mutants Arg-82 → Ala and Asp-85 → Glu: The blue form is inactive in proton translocation, *Proc. Natl. Acad. Sci. U.S.A. 87*, 1013−1017.
20. Eisenberg, D., and Kauzmann, W. (1969) *The structure and properties of water*, Oxford University Press, London.
21. Hayashi, S., Tajkhorshid, E., Kandori, H., and Schulten, K. (2004) Role of hydrogen-bond network in energy storage of bacteriorhodopsin's light-driven proton pump revealed by ab initio normal-mode analysis, *J. Am. Chem. Soc. 126*, 10516−10517.
22. Brown, L. S., Váró, G., Hatanaka, M., Sasaki, J., Kandori, H., Maeda, A., Friedman, N., Sheves, M., Needleman, R., and Lanyi, J. K. (1995) The complex extracellular domain regulates the deprotonation and reprotonation of the retinal Schiff base during the bacteriorhodopsin photocycle, *Biochemistry 34*, 12903−12911.
23. Needleman, R., Chang, M., Ni, B., Váró, G., Fornés, J., White, S. H., and Lanyi, J. K. (1991) Properties of $Asp^{212}$ → Asn bacteriorhodopsin suggest that $Asp^{212}$ and $Asp^{85}$ both participate in a counterion and proton acceptor complex near the Schiff base, *J. Biol. Chem. 266*, 11478−11484.
24. Kandori, H., Furutani, Y., Shimono, K., Shichida, Y., and Kamo, N. (2001) Internal water molecules of *pharaonis* phoborhodopsin studied by low-temperature infrared spectroscopy, *Biochemistry 40*, 15693−15698.
25. Furutani, Y., and Kandori, H. (2002) Internal water molecules of archaeal rhodopsins, *Mol. Membr. Biol. 19*, 257−265.
26. Shibata, M., Muneda, N., Ihara, K., Sasaki, T., Demura, M., and Kandori, H. (2004) Internal water molecules of light-driven chloride pump proteins, *Chem. Phys. Lett. 392*, 330−333.
27. Furutani, Y., Bezerra, A. G., Jr., Waschuk, S., Sumii, M., Brown, L. S., and Kandori, H. (2004) FTIR spectroscopy of the K photointermediate of Neurospore rhodopsin: Structural changes of the retinal, protein, and water molecules after photoisomerization, *Biochemistry 43*, 9636−9646.
28. Furutani, Y., Shichida, Y., and Kandori, H. (2003) Structural changes of water molecules during the photoactivation processes in bovine rhodopsin, *Biochemistry 42*, 9619−9625.

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