Direct Observation of the Bridged Water Stretching Vibrations Inside a Protein

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Proton pumps can actively translocate protons through hydrophobic regions inside the protein. Therefore, internal water molecules are considered to play a crucial role in active proton transport.[1] While little is known about the structure and function of internal water molecules in such proteins generally, the most progress has been made in bacteriorhodopsin (BR).[2] Fourier transform infrared (FTIR) spectroscopy has observed changes in the hydrogen bonding of water molecules during the protonmotive photocycle of BR, where specific isotope shifts of water O−H groups were obtained by use of $H_2{}^{18}O$.[1] Subsequently, studies on BR mutants implicated specific locations of water molecules in the proton pathway.[1] In addition, the presence of a "switch water" has been suggested, with an O−H stretching frequency at 3643 cm^{-1}.[3,4] Since the frequency indicates the O−H group is not hydrogen bonded, we proposed that the observed frequency corresponds to the free O−H of the bridged water between the Schiff base and Asp85.[1,4,5]

Recent high-resolution X-ray crystallography has identified locations of several internal water molecules (Figure 1).[6,7] The crystal structure indicated the presence of a water molecule between the Schiff base and Asp85, as proposed by our FTIR study.[1,4,5] However, this water was found to be part of a complex of three water molecules in a pentagonal structure. Thus, it is possible that the switch for vectorial proton transport involves the entire cluster of water molecules in the Schiff base region. The frequency at 3643 cm^{-1} may not originate from water402, because it tends to form full hydrogen bonds (Figure 1).

The water cluster requires more detailed FTIR study, particularly regarding the hydrogen bonds of the bridged water molecules (Figure 1). This requires investigation in a very crowded part of the spectrum. A water molecule has two O−H groups, and their frequencies distribute in the wide 3650−2800-cm^{-1} region depending on their coupling and hydrogen bonding strength.[8] The previous FTIR study observed water bands at >3450 cm^{-1}, the narrow region for relatively free O−H groups.[1] The reason for the difficulty in identifying water O−H bands in the 3450−2800-cm^{-1} region is mainly owing to the spectral overlap of many bands from the protein and bulk waters. In addition, strongly hydrogen bonded water possesses broad O−H stretches,[8] which disturbs the observation of clear isotope shifts. In fact, an isotope shift between O−H and ^{18}O−H is about 10 cm^{-1}, and such a small shift could be hidden in the complex spectral features in the 3450−2800-cm^{-1} region.

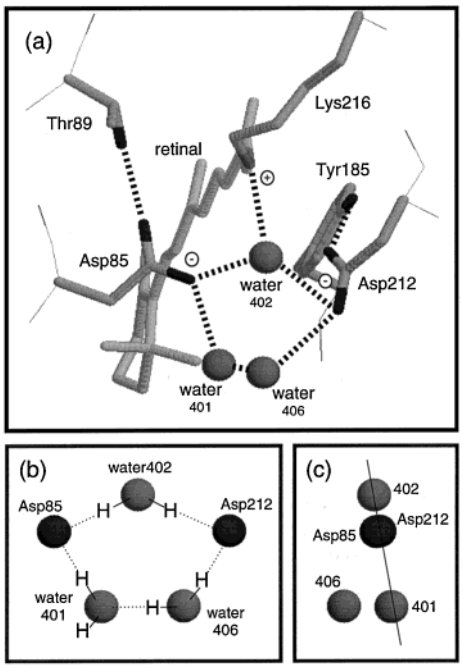

Figure 1. Diffraction structure of the Schiff base region in bacteriorhodopsin. (a) The side view of 1C3W.[6] The membrane normal is approximately in the vertical direction of this figure. Upper and lower regions correspond to the cytoplasmic and extracellular sides, respectively. Circles (401, 402, and 406) represent water molecules in the Schiff base region. Dotted lines represent supposed hydrogen bonds. In addition, the side chain of Arg82 is a possible hydrogen bonding donor to water406 (not shown). (b and c) Detailed views of the water-containing pentagonal structure in the Schiff base region. Hydrogen atoms and hydrogen bonds are inferred from the crystal structure. Two oxygen atoms of Asp85 and Asp212 are overlapped in part c.

Recently we have found that the discriminating power of polarized FTIR spectroscopy can be applied advantageously to this region.[9] Optimization of our polarized FTIR spectrometer allowed the detection of a single vibration in the whole mid-infrared region (4000−700 cm^{-1}) of the K minus BR difference spectrum with dipole moment parallel to the membrane normal. Encouraged by these highly accurate measurements, we have now attempted to observe the stretching vibration of the bridged water molecules in D_2O. Purple membrane was prepared and polarized FTIR spectroscopy was applied as described previously.[9,10] The BR film prepared in 2 mM phosphate buffer (pH 7.0) was hydrated by 1 μL of D_2O or $D_2{}^{18}O$. The hydrated film was kept at 77 K after light adaptation, and the K minus BR difference spectra (an average of 24 spectra of 128 interferograms) were obtained at various window tilting angles.[9]

Examination of water stretching modes in D_2O has two advantages. One is that the D_2O-insensitive stretching vibrations are separated in frequency. The other is an expected isotope shift between O−D and ^{18}O−D of about 17 cm^{-1}, which is greater

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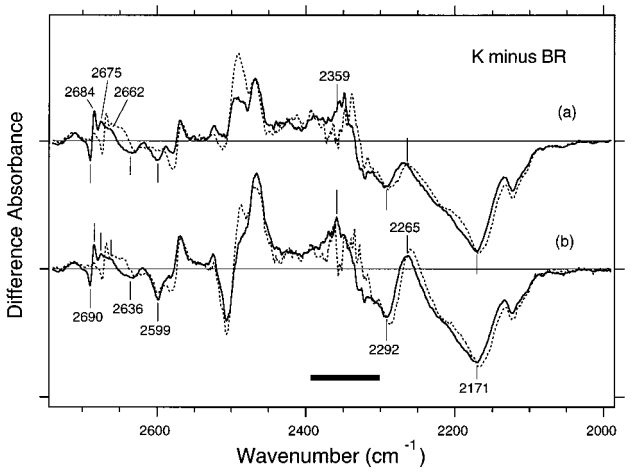

Figure 2. The K minus BR spectra are compared between hydration with D_2O (solid lines) and $D_2{}^{18}O$ (dotted lines) in the 2740−1990-cm^{-1} region. The window tilting angles are 0° (a) and 53.5° (b). One division of the *y* axis corresponds to 0.002 absorbance unit. The thick bar represents the frequency region, where CO_2 gas present in the spectrometer perturbs the signal. Labeled frequencies correspond to those identified as water stretching vibrations.

than that between O−H and ^{18}O−H (10 cm^{-1}). Therefore, we expect to observe the isotope shift even for the bridged water stretches. Figure 2 shows the spectral comparison between hydration with D_2O (solid lines) and $D_2{}^{18}O$ (dotted lines) in the 2740−1990-cm^{-1} region, for window tilting angles of 0° (a) and 53.5° (b). Spectral comparison clearly demonstrates the isotope shift of water molecules in the wide region. The negative 2690-cm^{-1} and the positive 2684-cm^{-1} bands are shifted to 2673 and 2667 cm^{-1}, respectively, consistent with the expected isotope shift (17 cm^{-1}). Broad positive features with peaks at 2675 and 2662 cm^{-1} also downshift by about 17 cm^{-1}, and a negative feature at about 2636 cm^{-1} shifts to lower frequency. Thus, the spectral changes in the 2740−2620-cm^{-1} region originate predominantly from the O−D stretches of weakly hydrogen bonded water molecules.

In contrast, a complex spectral feature is visible in the 2620−2400-cm^{-1} region. The negative 2599-cm^{-1} band with the tilting angle 0° (Figure 2a) exhibits a downshift to 2579 cm^{-1}. Nevertheless, the same band with the window tilting angle of 53.5° (Figure 2b) appeared at 2599 cm^{-1}. These results indicate that the 2599-cm^{-1} band is composed of at least two vibrations and only one is due to the water O−D stretch. The water O−D stretch is independent of window tilting, implying that the dipole moment of the O−D stretch is close to the magic angle of the membrane normal. In contrast, the other 2599-cm^{-1} band is parallel to the membrane normal as shown in the spectra with $D_2{}^{18}O$ hydration (Figure 2a,b). The 2506 (−)/2466 (+)-cm^{-1} bands were assigned as the O−D stretch of Thr89.[10c] Nevertheless, it seems that water O−D stretch is involved in this frequency region, because the isotope-induced spectral deviation is reproducible. Detailed analysis is in progress for its identification.

Peaks at 2359 (+), 2292 (−), 2265 (+), and 2171 (−) cm^{-1} all exhibited downshifts by 5−10 cm^{-1}, indicating that spectral changes in the 2400−2000-cm^{-1} region include water O−D stretches. The exception in this region is the negative band at 2123 cm^{-1}. The smaller isotope shifts may originate from the presence of vibrations other than those of water. It is noted that the 2171-cm^{-1} band corresponds to the water O−H stretch at 2796 cm^{-1}.[9] Walrafen et al. attempted to subdivide the broad absorption band of liquid water in the O−H stretching vibration region into four peaks at 3620, 3540, 3435, and 3240 cm^{-1},[11a,b] and assigned the 3540- and 3240-cm^{-1} bands to vibrations of the fully hydrated tetrahedral water molecules.[11b,c] The O−H stretch of the water at about 2800 cm^{-1} is much lower than these references, indicating that the hydrogen bond of the water in BR is much stronger than that of the fully hydrated tetrahedral water. This fact suggests that the hydrogen bond acceptor of this water molecule is negatively charged, such as oxygens of Asp85 and Asp212 (Figure 1).

Upon K formation, it seems that the water O−D stretches generally shift to higher frequency, indicating that the hydrogen bond is weakened (Figure 2). This is consistent with perturbation of the hydrogen bonding network in the active center by retinal isomerization. An exception is the band at 2690 cm^{-1} in BR, which corresponds to the O−H stretch at 3643 cm^{-1} previously attributed to the switch water.[1,3,4] The 2690-cm^{-1} band exhibits a spectral downshift upon K-formation, implying that the "free" O−D group forms a hydrogen bonding partner. Figure 2 shows that the amplitude of water stretching vibrations tends to decrease upon tilting of the window, which indicates that the angles of the dipole moments of the O−D stretching modes to the membrane normal are larger than the magic angle (54.7°). The only exception is the 2265-cm^{-1} band of the K intermediate (Figure 2), whose O−D stretch mode is parallel to the membrane normal. This implies that photoisomerization of the retinal chromophore causes rotation of one water molecule so that the O−D group changes its angle. The low frequency (2265 cm^{-1}) suggests that the O−D group forms a hydrogen bond with either Asp85 or Asp212. Thus, the rotational motion presumably occurs for one of three water molecules in Figure 1.

The present results are summarized as follows. (i) At least five water O−D stretching vibrations are observed in K minus BR polarized FTIR difference spectra. While their locations have to be assigned in the future, O−D stretches of water molecules in the pentagonal cluster (Figure 1) are likely to be involved. (ii) The frequencies are widely distributed over the possible stretching vibrations of water. (iii) The water stretching vibrations of K tend to be higher in frequency, implying that the overall hydrogen bonding becomes weaker upon photoisomerization. The initial stable pentagonal structure is probably forced to restructure. (iv) The angles of the dipole moments of the O−D stretching modes to the membrane normal are generally larger than the magic angle (54.7°). The only exception is the 2265-cm^{-1} band of the K intermediate, suggesting that the photoisomerization is accompanied by rotation of a water molecule. The present study successfully extends FTIR analysis through the entire water stretching region. Further experimental and theoretical efforts in this area will provide a better understanding of the role of internal water molecules in the light-driven proton pump of BR.

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