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# Internal Water Molecules of the Proton-Pumping Halorhodopsin in the Presence of Azide

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Vectorial ion transport by pump proteins is an essential process in cellular bioenergetics. The light-driven proton and chloride pumps of haloarchaea, bacteriorhodopsin (BR) and halorhodopsin (HR), are among the best-characterized proteins of this functional class (Figure 1).[1−3] Amino acid identity between BR and HR is 25%, indicating that they have evolved into the optimized forms of proton and chloride pumps, respectively. However, previous studies revealed that functional interconversion between proton and chloride pumps is possible under some conditions. Asp85 is the proton acceptor in the primary proton transfer of BR,[4] while the corresponding amino acid is threonine in HR (Figure 1). We found that the D85T mutant of BR pumps chloride ions.[5] On the other hand, the T111D or T126D mutations of HR from *Halobacterium salinarum* (*s*HR) or *Natronomonas pharaonis* (*p*HR), respectively, do not lead to the functional conversion into proton pump.[6,7] Instead, addition of azide converts HR into a proton pump, where azide probably replaces chloride ion in the binding site and controls the p$K_a$ changes of the protonated Schiff base.[7] Despite the accumulated knowledge, little has been understood about the mechanism of ion transport in the functionally converted rhodopsins.

Figure 1 illustrates the presence of internal water molecules in the Schiff base region of *s*HR and BR, suggesting their important roles in function. By means of Fourier-transform infrared (FTIR) spectroscopy, we have directly monitored hydrogen-bonding alterations of internal water molecules of rhodopsins.[8] In the series of studies, we have found a correlation between strongly hydrogen-bonded water molecules and proton-pumping activity of rhodopsins.[9] In $D_2O$, O−D stretching vibrations of water appear at 2200−2700 $cm^{-1}$, dependent on the strength of their hydrogen bonding. We found that strongly hydrogen-bonded water molecules (O−D stretch at <2400 $cm^{-1}$) are only found in the proteins exhibiting proton-pumping activity.[9] This is the case for BR and its mutants,[10−12] *pharaonis* phoborhodopsin (*p*pR),[13] proteorhodopsin (PR),[14] *Anabaena* sensory rhodopsin (ASR),[15] *Neurospora* rhodopsin (NR),[16] *Leptosphaeria* rhodopsin (LR),[17] and bovine rhodopsin.[18] On the basis of these findings, we have now a working hypothesis that "the presence of strongly hydrogen-bonded water molecules is requested for the proton-pumping function of rhodopsins".[9] Such water molecules are absent in HR.[19,20] In the present study, we examined functionally interconverted HR from chloride pump to proton pump with azide to further test this hypothesis.

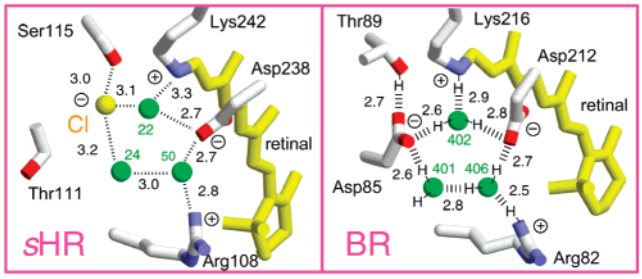


***Figure 1.*** X-ray crystallographic structures of the Schiff base region in halorhodopsin (*s*HR) and bacteriorhodopsin (BR) from *Halobacterium salinarum* (PDB entries are 1E12[3] and 1C3W,[2] respectively). Each membrane normal is approximately in the vertical direction of the figures. Upper and lower regions correspond to the cytoplasmic (CP) and extracellular (EC) sides, respectively. Green spheres represent water molecules. Dotted lines represent supposed hydrogen bonds, and numbers are distances in Å. Positions of hydrogen atoms of water in BR are inferred from the previous FTIR study.[12]

The wild-type *p*HR was expressed in *Escherichia coli*, and the protein, purified through Ni and DEAE column chromatography, was reconstituted into phosphatidylcholine liposomes.[19] The sample film of the azide-bound *p*HR was prepared by drying the protein in 2 mM phosphate buffer (pH 7.0) containing 5 mM $NaN_3$, which was then rehydrated by 1 $\mu$L of $H_2O$. Figure 2 compares light-induced spectral changes of *p*HR($N_3^-$) and *p*HR($Cl^-$) in the UV−visible region. *p*HR($N_3^-$) and *p*HR($Cl^-$) possess $\lambda_{max}$ at 561 and 564 nm, respectively. Illumination with a 500 nm light (through

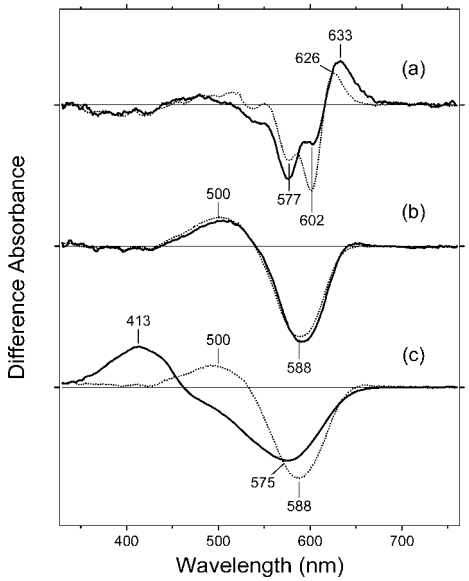


***Figure 2.*** Difference UV−visible spectra between the intermediate and unphotolyzed states of *pharaonis* halorhodopsin (*p*HR). Solid and dotted lines represent the spectra for *p*HR($N_3^-$) and *p*HR($Cl^-$), respectively. (a) The K minus *p*HR difference spectra obtained by illumination with a 500 nm light at 77 K. (b) The L minus *p*HR difference spectra obtained by illumination with a >600 nm light at 170 K. (c) The M minus *p*HR($N_3^-$) (solied line) and L2 minus *p*HR($Cl^-$) (dotted line) difference spectra obtained by illumination with a >600 nm light at 240 K.

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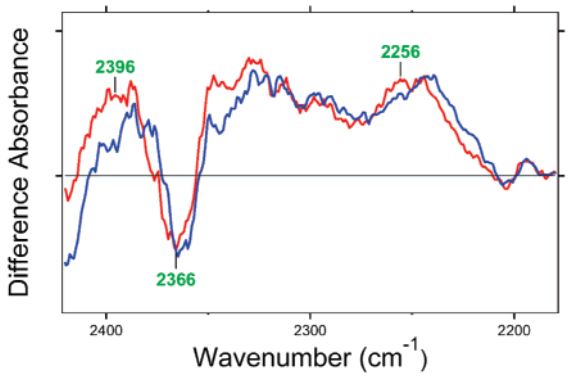


***Figure 3.*** K minus *p*HR difference infrared spectra in the 2420−2180 $cm^{-1}$ region for the azide-bound form (*p*HR($N_3^-$)). The samples were hydrated with $D_2O$ (red line) or $D_2{}^{18}O$ (blue line). Green-labeled frequencies correspond to the bands identified as O−D stretching vibrations of water molecule. One division of the *y* axis corresponds to 0.00015 au.

an interference filter) at 77 K led to formation of the K intermediate in both cases (Figure 2a). On the other hand, different positive peaks at 633 and 626 nm indicate that the K intermediate is more red-shifted for the $N_3^-$-bound form, in contrast to the unphotolyzed state. Very similar difference spectra were obtained for the L intermediate (Figure 2b). These observations strongly suggest that azide acts as the counterion of the protonated Schiff base as well as $Cl^-$ in the early step of photocycles.

On the other hand, different spectral feature was obtained at 240 K. The dotted line in Figure 2c shows the *p*$HR_{L2}$ minus *p*HR spectrum for the Cl-bound form, which is identical to the *p*$HR_{L1}$ minus *p*HR spectrum at 170 K (Figure 2b, dotted line).[20] In contrast, the positive absorption at 413 nm appeared in the presence of azide, indicating that the M intermediate is formed by deprotonation of the Schiff base.[7] This observation is identical to that for *p*HR in DM solution (data not shown). This may suggest that azide acts as a proton acceptor of the Schiff base, which presumably initiates proton translocation of *p*HR in the presence of azide.[7]

We next studied structural changes of *p*HR($N_3^-$) by means of FTIR spectroscopy, particularly focusing O−D stretching vibrations of water molecules. Figure 3 shows the *p*$HR_K$ minus *p*HR spectra in the presence of azide. As is clearly seen, a negative 2366 $cm^{-1}$ band exhibits an isotope shift of $^{18}O$ water by 3 $cm^{-1}$, indicating that this band contains the O−D stretch of water. This observation is in contrast to *p*HR($Cl^-$), which has no water bands at <2400 $cm^{-1}$.[19,20] Thus, it is likely that azide binds to the Schiff base region of *p*HR, where it is hydrated with a water molecule. The water molecule may bridge the Schiff base and azide, an ion pair in the Schiff base region. The bridged water in BR possesses the O−D stretch at 2171 $cm^{-1}$,[12] indicating that the hydrogen bond of water in *p*HR($N_3^-$) is considerably weaker than that in BR. Frequencies in other proton-pumping rhodopsins are 2215 $cm^{-1}$ for *pp*R,[13] 2257 $cm^{-1}$ for LR,[17] 2315 $cm^{-1}$ for PR, and 2339 $cm^{-1}$ for the R82Q/D212N mutant of BR.[12] Figure 3 also shows the presence of positive water bands at 2256 and 2396 $cm^{-1}$, which are also observed at 2263 and 2384 $cm^{-1}$ in *p*HR($Cl^-$).[20] This suggests that the positive band corresponding to the 2366 $cm^{-1}$ band is located at >2400 $cm^{-1}$ by weakening the hydrogen bond upon retinal photoisomerization. The N−D stretch of the Schiff base is also important for ion-pumping, where BR and *p*HR possess the Schiff base mode at ∼2150 $cm^{-1}$ [21] and ∼2500 $cm^{-1}$,[20] respectively. Since there are no negative bands at <2400 $cm^{-1}$ for the N−D stretch in *p*HR($N_3^-$) (Figure 3), the proton-pumping HR does not possess a strong hydrogen bond of the Schiff base like BR. Further FTIR study will reveal the mechanism in more detail, because vibrational bands of retinal (Schiff base), protein, water, and azide can be monitored during the proton-pump processes of *p*HR($N_3^-$). This is our future focus.

The present study clearly showed that the functionally interconverted HR (proton pump) with azide possesses strongly hydrogen-bonded water molecules (O−D stretch at <2400 $cm^{-1}$). This observation is perfectly consistent with the previous data for other rhodopsins,[9] and the correlation between strongly hydrogen-bonded water molecules and proton-pumping activity of rhodopsins is now even more convincing. Then, why does such a correlation exist for rhodopsins? Rhodopsins are molecular machines of nanometer scale, being much larger than a water molecule in size. Is the function of rhodopsins nevertheless determined by a water molecule? Does hydrogen bonding of a water molecule determine the function? It should be noted that photon energy of ∼200 kJ/mol is provided for the retinal chromophore and is stored first in its isomerized form. In addition to the distorted chromophore, the importance of hydrogen-bonding network has been emphasized for light-energy storage.[22] Therefore, it should be possible that a specific hydrogen bond of a water molecule plays a crucial role for the function.

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