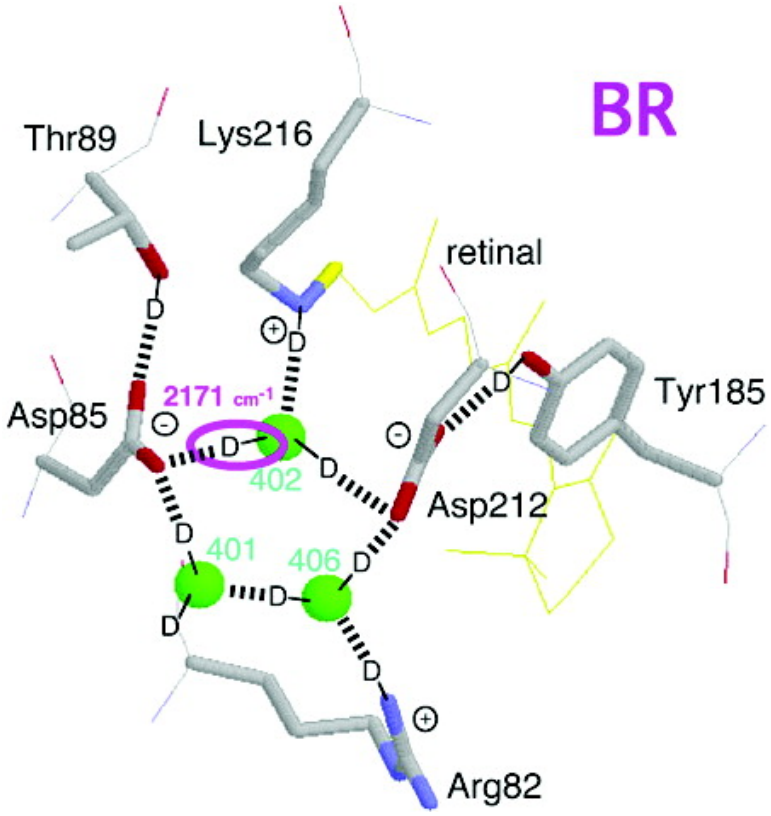
Communication

Water Molecules in the Schiff Base Region of Bacteriorhodopsin

Mikihiro Shibata, Taro Tanimoto, and Hideki Kandori

J. Am. Chem. Soc., **2003**, 125 (44), 13312-13313• DOI: 10.1021/ja037343s • Publication Date (Web): 14 October 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 6 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

J|A|C|S
COMMUNICATIONS

Published on Web 10/14/2003

Water Molecules in the Schiff Base Region of Bacteriorhodopsin

Mikihiro Shibata, Taro Tanimoto, and Hideki Kandori*

Department of Applied Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

Received July 17, 2003; E-mail: kandori@ach.nitech.ac.jp

Bacteriorhodopsin (BR), a membrane protein found in *Halobacterium salinarum*, functions as a light-driven proton pump with a retinylidene chromophore.[1] The Schiff base region of the chromophore 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 1). 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. After light absorption, all-trans to 13-cis photoisomerization takes place, followed by the primary proton transfer from the Schiff base to Asp85 that triggers sequential proton-transfer reactions for the pump.

A notable structural feature is that Asp85 and Asp212 are located at similar distances from the retinal Schiff base, whereas the Schiff base proton is transferred only to Asp85. Is the asymmetric proton transfer programmed in the BR structure? Does the asymmetric proton transfer take place by asymmetric retinal isomerization even in the symmetric structure of BR? Hayashi and Ohmine proposed from their QM/MM calculations that the hydrogen bond of water402 with Asp85 is much stronger than that with Asp212,[2] even though it is not clear from Figure 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.[3] Although observation of water stretching vibrations was initially limited to weak hydrogen bonds, recent accurate spectral comparison between samples in D_2O and $D_2{}^{18}O$ has extended it to water molecules with strong hydrogen bonds. As a consequence, we observed an O−D stretch of a water molecule at 2171 cm^{-1}, which is much lower in frequency than the fully hydrated tetrahedral water molecules.[4] Taking into the account the theoretical result by Hayashi and Ohmine,[2] we concluded that the 2171-cm^{-1} band corresponds to the O−D stretch of water402 interacting with Asp85. On the basis of the measurements for late intermediates, we recently proposed a hydration switch model as the mechanism of the proton transfer from the Schiff base to Asp85.[5]

In this way, FTIR spectroscopy has provided useful information on the hydrogen bonds of internal water molecules. However, it should be noted that the 2171-cm^{-1} band has not been experimentally proven to be the O−D stretch of water402 interacting with Asp85. In the present study, we aimed at revealing the origin of the water bands in the 2400−2100 cm^{-1} region by use of various bacteriorhodopsin mutants. The sample films were prepared in 2 mM phosphate buffer (pH 7.0) except for D212N (pH 10.0)[6] and hydrated by D_2O or $D_2{}^{18}O$. The hydrated films were light-adapted at 273 K except for D85N, and the K minus BR spectra (an average of 40 spectra of 128 interferograms) were measured by photoconversion between K and BR at 77 K.[7] In case of D85N, the hydrated film was illuminated with a 501-nm light at 77 K, where the strong 1194(+)/1202(−) cm^{-1} bands in the fingerprint region (not shown) indicate that photoreaction of all-trans to 13-cis form is predominant. Since orientation of molecules was not good for some mutant samples, we did not use an IR polarizer in the FTIR measurements, and the sample films were tilted by about 30° in the cryostat.

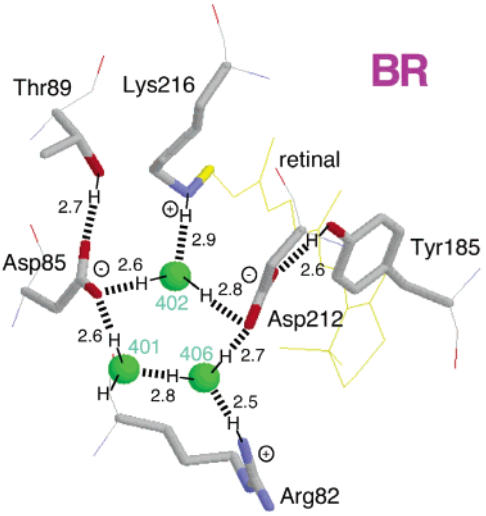


Figure 1. Diffraction structure of the Schiff base region in BR from PDB entry 1C3W.[12] The membrane normal is approximately in the vertical direction of this figure. Upper and lower regions correspond to the cytoplasmic and extracellular sides, respectively. Green spheres (401, 402, and 406) represent water molecules in the Schiff base region. Dotted lines represent supposed hydrogen bonds. Hydrogen atoms and hydrogen bonds are inferred from the crystal structure.

Figure 2a shows that three negative peaks at 2323, 2292, and 2171 cm^{-1}, and a positive peak at 2359 cm^{-1} exhibit ^{18}O isotope shift, indicating that these bands originate from O−D stretches of water in the wild-type BR. In addition, the previous study showed that the 2265-cm^{-1} band of water is highly dichroic and becomes positive when the sample film is further tilted in the polarized measurement.[4] Since water normally has O−D stretching frequencies in the wide 2700−2200 cm^{-1} region, these O−D groups possess unusually strong hydrogen bonds. We previously inferred that hydrogen bond acceptors of these water molecules are negatively charged, such as oxygens of Asp85 and Asp212 (Figure 1). If the 2171-cm^{-1} band originates from the O−D stretch of water402 hydrating Asp85, as predicted by Hayashi and Ohmine,[2] removal of the negative charge at the position 85 by a mutation should significantly affect the band. Figure 2b indeed shows that the broad negative feature in the 2300−2000 cm^{-1} region does not exhibit the isotope shift in D85N. The negative band can be assigned as the N−D stretch of the Schiff base as in the wild-type BR.[8] D85N lacks not only the 2171-cm^{-1} band but also all water bands in this frequency region, suggesting the important role of the negative charge at the position 85. Water bands were observed in the 2600−2500 cm^{-1} region for weak hydrogen bonds (data not shown).

10.1021/ja037343s CCC: $25.00

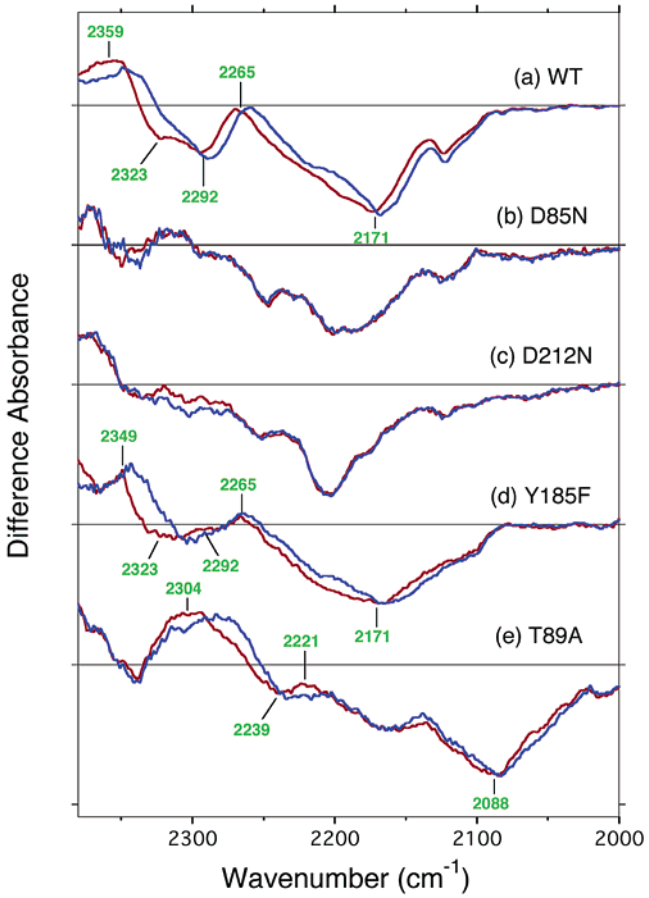


Figure 2. K minus BR difference infrared spectra of the wild type (a), D85N (b), D212N (c), Y185F (d), and T89A (e) BR in the 2380−2000 cm^{-1} region. The samples were hydrated with D_2O (red lines) or $D_2{}^{18}O$ (blue lines), and spectra were measured at 77 K. One division of the *y* axis corresponds to 0.002 absorbance unit. Labeled frequencies correspond to these bands identified as water stretching vibrations.

We also expected that the lack of the negative charge at the position 212 will not significantly affect the 2171-cm^{-1} band according to Hayashi and Ohmine.[2] Nevertheless, Figure 2c shows that all water bands disappear in the <2400 cm^{-1} region in D212N. The results of both D85N and D212N imply 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} as the O−D stretches involved in the pentagonal cluster (Figure 1). On the other hand, we could not establish that the 2171-cm^{-1} band originates from the O−D stretch of water402 hydrating Asp85. It seems that Asp85 and Asp212 are directly involved in the pentagonal cluster structure, so that their mutations greatly change the hydrogen-bonding structure.

Subsequently, we performed additional mutation studies. One oxygen atom of Asp85 and Asp212 is involved in the pentagonal cluster, while another oxygen forms a hydrogen bond with Thr89 and Tyr185, respectively (Figure 1). Therefore, we expected that the cleavage of these hydrogen bonds would localize the negative charges onto the respective oxygens of the pentagonal cluster. As a consequence, the 2171-cm^{-1} band would be further downshifted in a mutant of Thr89, according to Hayashi and Ohmine,[2] while it would not be much influenced in a mutant of Tyr185. That was indeed the case. Figure 2d shows that the 2171-cm^{-1} band of water is preserved in the Y185F mutant, as well as the two positive and the two negative bands of water. On the other hand, a strong negative band appears at 2088 cm^{-1} in the T89A mutant, which exhibits lower frequency shift in $D_2{}^{18}O$. The frequency change by >80 cm^{-1} in T89A, but not in Y185F, strongly supports the proposal by the QM/MM calculation that the 2171-cm^{-1} band originates from the O−D stretch of water402.[2] The negative peak is present at 2166 and 2155 cm^{-1} in T89S and T89C, respectively (data not shown), implying that the hydrogen-bonding interactions with Thr89 and water402 determine negative charge distribution between the side-chain oxygens of Asp85. Earlier, we reported the hydrogen-bonding interaction of Thr89 with Asp85 using [3-^{18}O]-Thr-labeled and mutant BR.[9]

According to the X-ray structure of BR, Asp85 and Asp212 look symmetrical in the pentagonal cluster structure (Figure 1). The present FTIR study of internal water molecules suggests that the hydrogen-bonding interaction of water402 is stronger with Asp85 than with Asp212, being consistent with the QM/MM calculations.[2] Since the bridged water interacts more strongly with Asp85, Asp85 is presumably a stronger counterion than Asp212. The role of the negative charge on Asp212 is also crucial. If it is neutralized, all the bands of strongly hydrogen-bonded water molecules disappear (Figure 2c). Thus, the pentagonal cluster structure composed of the two negative charges may be essential for the function of BR. In fact, D85N and D212N proteins do not pump protons,[10] while mutants of Tyr185 and Thr89 do.[11] In summary, the present FTIR study experimentally established the origin of the 2171-cm^{-1} band. More systematic mutation studies of the other bands will lead to better understanding of the role of internal water molecules, particularly those involved in the pentagonal cluster.

Acknowledgment. We thank Professor R. Needleman for providing the mutant strains. We also thank Y. Furutani for valuable discussion. This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan, Grants 14380316, 14045238 (H.K.).

References

(1) (a) Lanyi, J. K. *J. Struct. Biol.* **1998**, *124*, 164−178. (b) Haupts, U.; Tittor, J.; Oesterhelt, D. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 367−399.
(2) Hayashi, S.; Ohmine, I. *J. Phys. Chem. B* **2000**, *104*, 10678−10691.
(3) Kandori, H. *Biochim. Biophys. Acta* **2000**, *1460*, 177−191.
(4) Kandori, H.; Shichida, Y. *J. Am. Chem. Soc.* **2000**, *122*, 11745−11746.
(5) Tanimoto, T.; Furutani, Y.; Kandori, H. *Biochemistry* **2003**, *42*, 2300−2306.
(6) Kandori, H.; Yamazaki, Y.; Sasaki, J.; Needleman, R.; Lanyi, J. K.; Maeda, A. *J. Am. Chem. Soc.* **1995**, *117*, 2118−2119.
(7) Kandori, H.; Kinoshita, N.; Maeda, A.; Shichida, Y. *J. Phys. Chem. B* **1998**, *102*, 7899−7905.
(8) Kandori, H.; Belenky, M.; Herzfeld, J. *Biochemistry* **2002**, *41*, 6026−6031.
(9) (a) Kandori, H.; Kinoshita, N.; Shichida, Y.; Maeda, A.; Needleman, R.; Lanyi, J. K. *J. Am. Chem. Soc.* **1998**, *120*, 5828−5829. (b) Kandori, H.; Kinoshita, N.; Yamazaki, Y.; Maeda, A.; Shichida, Y.; Needleman, R.; Lanyi, J. K.; Bizounok, M.; Herzfeld, J.; Raap, J.; Lugtenburg, J. *Biochemistry* **1999**, *38*, 9676−9683. (c) Kandori, H.; Kinoshita, N.; Yamazaki, Y.; Maeda, A.; Shichida, Y.; Needleman, R.; Lanyi, J. K.; Bizounok, M.; Herzfeld, J.; Raap, J.; Lugtenburg, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4643−4648. (d) Kandori, H.; Yamazaki, Y.; Shichida, Y.; Raap, J.; Lugtenburg, J.; Belenky, M.; Herzfeld, J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1571−1576.
(10) Mogi, T.; Stern, L. J.; Marti, T.; Chao, B. H.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4148−4152.
(11) (a) Mogi, T.; Stern, L. J.; Hackett, N. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5595−5599. (b) Marti, T.; Otto, H.; Mogi, T.; Rösselet, S. J.; Heyn, M. P.; Khorana, H. G. *J. Biol. Chem.* **1991**, *266*, 6919−6927.
(12) Luecke, H.; Schobert, B.; Richter, H.-T.; Cartailler, J. P.; Lanyi, J. K. *J. Mol. Biol.* **1999**, *291*, 899−911.

JA037343S