Water-Mediated Proton Transfer in Proteins: An FTIR Study of Bacteriorhodopsin

Hideki Kandori,† Yoichi Yamazaki,† Jun Sasaki,†
Richard Needleman,‡ Janos K. Lanyi,§ and Akio Maeda*,†

Department of Biophysics, Faculty of Science
Kyoto University, Sakyo-ku, Kyoto 606-01, Japan
The Department of Biochemistry
Wayne State University, Detroit, Michigan 48201
Department of Physiology and Biophysics
University of California, Irvine, California 92717

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Fourier transform infrared (FTIR) spectroscopy was used to investigate structural changes of water that precede the initial proton transfer reaction in bacteriorhodopsin. The Asp212 → Asn (D212N) protein under alkaline conditions where M is not formed (i.e., there is no proton transfer) was compared with the wild-type and Asp85 → Asn (D85N) proteins at 170 K for the O−H stretching mode of water. The observed increase in the H-bonding strength of water is very small in D212N and absent in D85N, in contrast to the wild-type bacteriorhodopsin at the intermediate stage before proton transfer (L intermediate). On the basis of these results, we conclude that, besides H-bonding of a water molecule between the Schiff base and Asp85, the formation of H-bonding with Asp212 of the originally unbound O−H is required for the proton transfer.

Proton transfer reactions play important roles in many biological systems, and the molecular mechanism of such a fundamental chemical reaction in the complex protein matrix has been of great interest.[1] Bacteriorhodopsin (BR) is a light-driven proton pump in the purple membrane of *Halobacterium salinarium*.[2] Since BR is one of the simplest pump systems and is activated by light, extensive studies have been done to explore its mechanism.[3] Illumination of BR causes the isomerization of the retinal chromophore from the all-trans to the 13-cis form. This is followed by a series of thermal steps that result in intermediate states (J, K, KL, L, M, N, and O), distinguished by visible spectroscopy.[3] The initial proton transfer occurs from the protonated Schiff base (C=NH$^+$) to Asp85 (COO$^-$) in the L-to-M conversion process in the microsecond time domain. This is followed by proton release from a hitherto unidentified site to the extracellular aqueous phase.[3]

Our previous Fourier transform infrared (FTIR) analysis with D85N in the O−H stretching region[4,5] revealed that the H-bonding of a specific water molecule localized in the proximity of Asp85 becomes stronger in L. Water coordinated to Asp85 was proposed to participate in the formation of L with a distorted 13-cis retinal conformation,[6] which may be prerequisite for the proton transfer from the Schiff base to Asp85.[5] Thus, FTIR studies in the O−H stretching region have opened a way to resolve structural changes at the active site of BR.

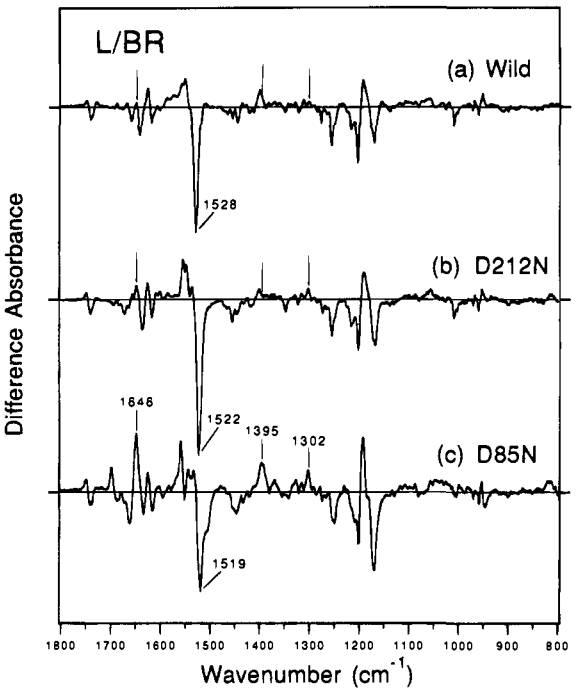

Figure 1. L/BR spectra of (a) wild-type BR, (b) D212N, and (c) D85N in the 1800−800 cm^{-1} region. Spectra a and c are reproduced from ref 5 for comparison.

The replacement of another negatively charged group, Asp212 (COO$^-$), with a neutral residue causes a red shift in the absorption spectrum,[7a] though to a much smaller extent (17 nm) than the 48 nm for Asp85. Replacement of Asp85 lowers the pK_a value of the Schiff base while that of Asp212 does not. Asp212 remains unprotonated in the photocycle.[8] An interesting issue in relation to Asp212 is that at pH > 7 the proton transfer does not occur in D212N, i.e., M is not formed, even though there are a proton donor (the Schiff base) and an acceptor (Asp85).[7] This suggests a role for Asp212 in the proton transfer, and we expected that changes in the microscopic structure in D212N could be detected by FTIR spectroscopy.

The BR mutant, D212N, was described previously.[7] The purple membranes containing this protein were suspended in 10 mM borate buffer (pH 10), where the λ_{max} is at 585 nm after light adaptation.[7a] The air-dried BR film on a BaF_2 window was humidified by H_2O or $H_2{}^{18}O$. FTIR spectroscopy was with a Nicolet FTIR spectrometer, SX-60. The film sample was cooled to 170 K in an Oxford DN-1704 cryostat and irradiated with >600 nm light for 2 min, giving the L/BR difference spectra.

First, the L/BR difference spectrum of D212N in the 1800−800 cm^{-1} region was compared with those of the wild type and D85N (Figure 1). As described previously, L of D85N exhibits a considerably different spectrum from the wild type. The unique positive bands related to the Schiff base modes at 1395 and 1302 cm^{-1} and the amide I at 1648 cm^{-1} (Figure 1) are more characteristic of N of the wild type than L.[5] On the other hand, the L/BR spectrum of D212N, which essentially reproduces the previously reported one,[7b] resembles more the spectrum of the wild type than D85N. This indicates that Asp212 has less influence on the protonated Schiff base than Asp85, and Asp85 plays a primary role in interacting with the

* To whom correspondence should be addressed.
† Kyoto University.
‡ Wayne State University.
§ University of California.

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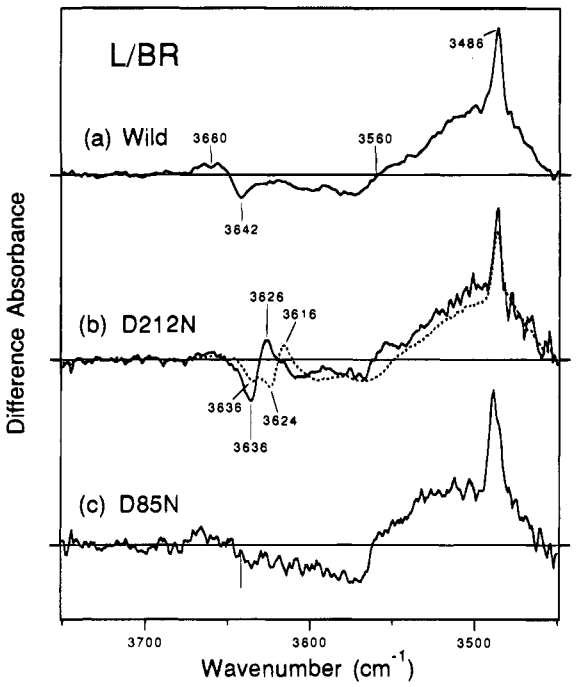

Figure 2. The L/BR spectra of (a) wild-type BR, (b) D212N, and (c) D85N in the 3750−3450 cm^{-1} region. The dotted line is the spectrum of D212N hydrated with $H_2{}^{18}O$. Spectra a and c are reproduced from ref 5 for comparison.

Schiff base in L. This is in good agreement with the fact that Asp85 is the proton acceptor. However, it raises the question as to why no proton transfer to Asp85 occurs in D212N[7] in spite of the fact that L of D212N has a similar structure to that of the wild type and possesses both proton donor (protonated Schiff base) and acceptor (Asp85). There must be some influence of the substitution of Asp212.

We then inspected the L/BR spectra in the 3750−3450 cm^{-1} region (Figure 2). The sharp positive peak around 3486 cm^{-1} is the L-specific indole N−H stretching vibration of Trp182.[9,10] In the spectrum of the wild type, the negative band at 3642 cm^{-1} is the O−H stretching vibration of a water molecule.[4] It originates from the "monomer" or "free" O−H, whose H-bonding is very weak (or even absent). Upon L formation, the O−H stretching band at 3642 cm^{-1} experiences a down shift of more than 80 cm^{-1} (<3560 cm^{-1}).[4] The shift to a lower frequency indicates stronger H-bonding formation.[4,11] The disappearance of the negative water band in D85N was regarded as evidence that this water is located between the Schiff base and Asp85.[5] In D212N a slightly down-shifted negative band appears at 3636 cm^{-1} with an accompanying positive band at 3626 cm^{-1}. Upon replacement by $H_2{}^{18}O$, shifted bands appear at 3624 and 3616 cm^{-1}, respectively. Thus, these bands are due to the O−H stretching vibration of water molecule(s). The remaining band at 3636 cm^{-1} could be a protein O−H vibration, but its assignment is a future issue. A shift of only 10 cm^{-1} in D212N strongly implies that the COO^- of Asp212 is responsible for the stronger H-bonding formation of the water in L.

The $H_2{}^{18}O$ shift of the band between 3560 and 3450 cm^{-1} may be a result of the contribution of water in a region other than near Asp85. The decrease in the intensity of the small band around 3660 cm^{-1} in D212N relative to the wild type may be due to a high-frequency shift of another water close to Asp212 or a cancellation with the more intense negative band at 3636 cm^{-1}. These alternatives will be examined in future studies.

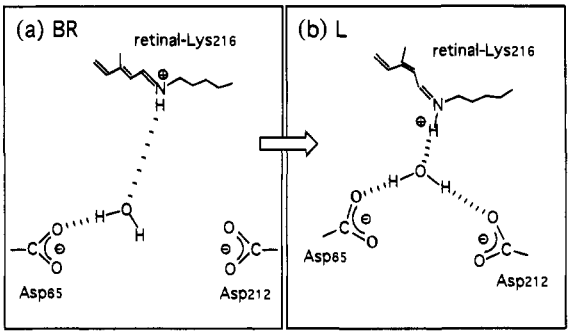

Figure 3. Schematic drawing of the Schiff base moiety of (a) bacteriorhodopsin and (b) the L intermediate, containing Asp85, Asp212, and water molecule(s). Shaded lines represent hydrogen bonding, whose strength is proportional to the density of the lines.

On the basis of these results, we propose a scheme of the structural change of a water molecule coordinated to Asp85 in a system containing the protonated Schiff base, Asp85, and Asp212 (Figure 3). The number of water molecules involved could be more than one, but for simplicity only one molecule is depicted. In the unphotolyzed state of BR, one of the two water O−H groups forms a H-bond with the unprotonated Asp85, and the other O−H is free. The latter provides the O−H stretching frequency at 3642 cm^{-1} (Figure 3a). Upon L-formation, the H-bonding of the Schiff base (N−H) to the water coordinated to Asp85 becomes stronger (Figure 3b),[5,6] and Asp212 forms a new H-bond with the "free" O−H of the water. Thus, the formation of intensified H-bonding of the "free" O−H of water linked to three charged groups must be necessary for constructing the distorted structure in the retinal moiety and a correct alignment of the N−H bond[12] for lowering the pK_a of the Schiff base, and hence the ensuing proton transfer in the protein. The impaired proton transfer in D212N is ascribed to the deficient H-bonding of the water with Asp212 in L. A recent molecular dynamics study shows two water molecules between Asp85 and Asp212, which form full H-bonding.[13] The present FTIR study, however, shows that at least one O−H of these waters is in a non-H-bonding state, which may play a key role for the proton transfer in the protein.

It is likely that the H-bonding network is composed of more residues, such as Tyr57, Arg82, Trp86, Thr89, Tyr185, the peptide backbone (C=O and N−H), and the water(s).[13−15] An effect of replacing Tyr57 with aspartate on water was presented recently.[16] The presence of the non-water O−H stretching band at 3636 cm^{-1} suggests a further extended network. The structural change of the complex cluster in BR will be fully understood when the contributions of these groups are known. In conclusion, the present FTIR study of BR revealed a structural role of a water molecule in the proton transfer process in the protein matrix. The ordered structure associated with Asp212 is necessary for the proton transfer.

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