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Water Structural Changes in the Bacteriorhodopsin Photocycle: Analysis by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The Fourier transform infrared difference spectra between light-adapted bacteriorhodopsin (BR) and its photointermediates, L and M, were analyzed for the 3750-3450-cm^{-1} region. The O-H stretching vibrational bands were identified from spectra upon substitution with ^2H_2O. Among them, the 3642-cm^{-1} band of BR was assigned to water by substitution with $H_2{}^{18}O$. By a comparison with the published infrared spectra of the water in model systems [Mohr, S. C., Wilk, W. D., & Barrow, G. M. (1965) *J. Am. Chem. Soc. 87*, 3048-3052], it is shown that the O-H bonds of the water in BR interact very weakly. Upon formation of L, the interaction becomes stronger. The O-H bonds of the protein side chain undergo similar changes. On the other hand, M formation further weakens the interaction of the same water molecules in BR. The appearance of a sharp band at 3486 cm^{-1}, which was assigned tentatively to the N-H stretching vibration of the peptide bond, is unique to L. The results suggest that the water molecules are involved in the perturbation of Asp-96 in the L intermediate and that they are exerted from the protonated Schiff base which changes position upon the light-induced reaction.

Bacteriorhodopsin (bR)[1] is an intrinsic membrane protein which transports protons upon absorption of light by its retinylidene chromophore (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1984; Mathies et al., 1991). In the transport process, protons are released to the exterior side of the membrane at the same time or just after the conversion of an L intermediate to an M intermediate, in which the Schiff base is unprotonated (Drachev et al., 1984; Grzesiek & Dencher, 1986; Vârô & Lanyi, 1990; Liu, 1990; Liu et al., 1990). The deprotonation of the Schiff base facilitates the uptake of a proton from the opposite side of the membrane via Asp-96 (Holz et al., 1989; Otto et al., 1989). Thus, the structural analysis of the L intermediate is crucial for elucidating the mechanism of proton pumping.

Water has been proposed to be a molecule involved in the hydrogen-bonding network surrounding the Schiff base in the resting *all-trans*-bR (Hildebrandt & Stockburger, 1984; Baasov et al, 1987; de Groot et al., 1989; Papadopoulos et al., 1990), whose Schiff base N-H bond points toward Asp-85 and Asp-212 (Lin & Mathies, 1989). Upon isomerization, the N-H bond turns to the opposite side, toward Asp-96, because Asp-96 is perturbed for L and N but not for M, suggesting a strong interaction between the protonated Schiff base of the L or N intermediate with Asp-96 (Pfefferlê et al., 1991). However, the 1.0-1.2-nm distance between the Schiff base and Asp-96 (Henderson et al., 1990) is too far to account for the perturbation of Asp-96 by direct interaction. Serine or threonine residues (Marti et al., 1991) or water between the Schiff base and Asp-96 may form a hydrogen-bonding network, which facilitates their interaction. Henderson et al. (1990) have suggested the presence of Thr-89 and -90 and at least one or two water molecules in the hydrophobic channel between the Schiff base and Asp-96.

Except for the N-H stretching vibration of the Schiff base by resonance Raman spectroscopy (Hildebrandt & Stockburger, 1984) and a very recent study on the O-H stretching vibration of the protein side chains of bR (Chang et al., 1991), exploration of the vibrational bands in the region above 3000 cm^{-1} has never been reported for retinoid proteins. Changes in the water band in the O-H bending vibrational region have previously been observed upon formation of the bathorhodopsin form of the visual pigment (Ganter et al., 1988).

The present study aims at analyzing the O-H stretching vibrational bands in the 3800-3000-cm^{-1} region of the FTIR difference spectra of intermediates L and M to examine the vibrational bands related to the water molecules. Changes in the water bands were observed for these intermediates. The

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[1] Abbreviations: bR, bacteriorhodopsin; BR, light-adapted bR; FTIR, Fourier transform infrared.

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relation with the changes in the hydrogen-bonding properties of the water molecules are discussed on the basis of previous model spectra of the O–H stretching vibrational modes of water in various solvents (Mohr et al., 1965; Walrafen, 1967; Glew & Rath, 1971; Monosmith & Walrafen, 1984; Conrad & Strauss, 1985; Walrafen & Fisher, 1986). It is suggested that water structural changes upon L formation are important to understand the mechanism for the initiation of the proton transfer reaction in bR.

Materials and Methods

bR in the purple membrane was obtained from *Halobacterium halobium* ET1001 (provided by Dr. T. Kouyama) by using the procedure described by Oesterhelt and Stoeckenius (1974). To examine the isotope effect of the water, bR was lyophilized and then dissolved in normal water (H_2O) or its isotope derivative, ^2H_2O (>99.75%; Wako, Japan) or $H_2{}^{18}O$ (97.8 atom %; obtained from MSD Isotopes, Canada), three days before the experiments. The film of bR for the FTIR measurements was made by depositing 40 μL of bR (total 0.2 absorbance unit) on a BaF_2 window (10 mm in diameter) and leaving it in a desiccator for 5 h over $CaCl_2$. The sample was then rehydrated just before the experiment by normal water or its isotope derivative in a specially designed copper block as described previously (Maeda et al., 1991). The water content is about 50% by weight as judged from the intensity of the 3400-cm^{-1} band (Braiman et al., 1987).

FTIR spectra were recorded in a Nicolet 60-SX Spectrometer. An Oxford cryostat CF-1204 was used with liquid nitrogen as a coolant. The temperature was maintained within 0.1 K with a Oxford ITC-4 temperature controller. The light source for the irradiation was a 1-kW halogen tungsten lamp in a slide projector.

The procedures used to obtain the difference spectrum between light- and dark-adapted states and that for either L, M, or N to the light-adapted bR were same in principle as described previously (Maeda et al., 1991; Pfefferlé et al., 1991). Before being cooled to subfreezing temperatures, the bR sample was light-adapted at 274 K by irradiation with light of wavelengths longer than 490 nm (>490-nm light) for 10 min. This sample will be called BR[1] hereafter. The difference between the spectra before and after the light adaptation was then obtained. The BR was kept at the same temperature for a further 10 min before cooling. For the experiments at 170 K, where the L intermediate is a main photoproduct, the irradiation was with light of wavelengths longer than 600 nm (≥600-nm light) for 2 min, and the difference between the spectra before and after the irradiation was calculated. Repeated recordings with the same sample were done after warming to 274 K and subsequent recooling to 170 K. Experiments at 200 K were also done in the same way.

The difference spectrum at 230 K was recorded as the difference before and after irradiating BR with light of wavelengths longer than 500 nm (>500-nm light) for 30 s. For the repeated recordings, the M intermediate thus formed was converted to BR at the same temperature by irradiating with 436-nm light for 8 min. Irradiation of BR at 260 K for 20 s with >500-nm light yielded a mixture of M and N. Under these conditions, all the photoproducts decayed during 1 min.

The control without light irradiation was obtained as a difference between two recordings with an intermittent pause in the dark of the same duration as that for the irradiation. Spectra in Figures 2 and 3 were presented after subtracting the control curves. Recordings were done at a 2-cm^{-1} resolution. The short horizontal lines of the ordinate of all the figures represents zero absorbance. The vertically aligned broken lines in each figure represent the same wavenumber, as labeled.

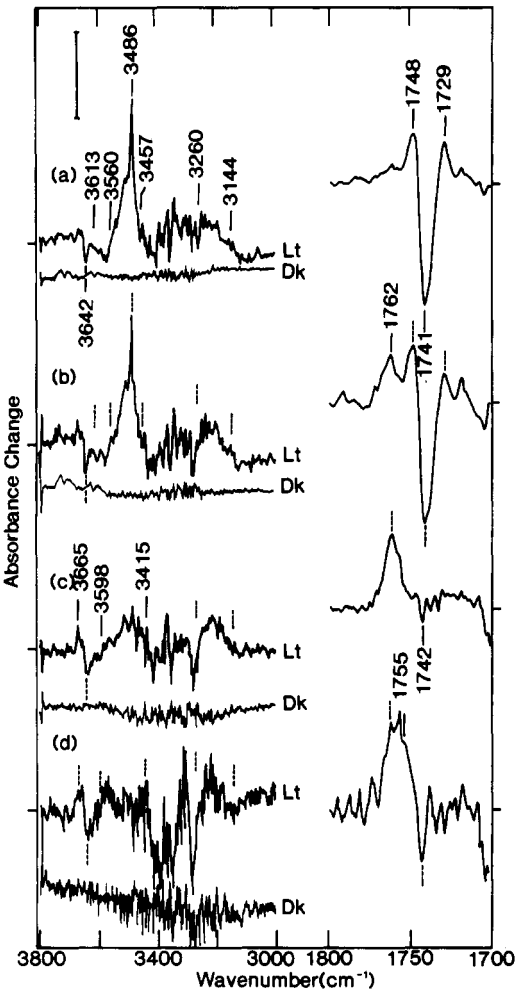

FIGURE 1: FTIR difference spectra in the 3800–3000-cm^{-1} region (left panel) and the 1800–1700-cm^{-1} region (right panel) upon irradiation of BR at 170 (a), 200 (b), 230 (c), and 260 K (d) are shown with the label of Lt in the upper part. The differences obtained in the same manner in the dark were shown with the label of Dk in the lower part. The scale bar in the figure represents 0.0035, 0.0032, 0.0024, and 0.00058 for the figures in the left panel (3800–3000 cm^{-1}) of spectra a, b, c, and d, respectively. The ordinate in the right panel was expanded twice. All the figures in the left panel were smoothed. Interferograms coadded were 1024 for spectra a, b, and c and 128 for spectrum d.

Results

Spectrum in the 3800–3000-cm^{-1} Region. FTIR spectra were measured before and after irradiation of BR, respectively, at 170, 200, 230, and 260 K. The difference between these spectra in the 3800–3000-cm^{-1} region is shown with a label of Lt in the left panel of Figure 1. The control measurement without irradiation is shown with a label of Dk under each spectrum labeled with Lt. The difference at 274 K between dark- and light-adapted bR does not give any difference (not shown in the figures). The C=O stretching vibrational bands of the protonated carboxylic acid in the 1800–1700-cm^{-1} region in the right panel of Figure 1 are useful for the assignment of the intermediate species (Engelhard et al., 1985; Braiman et al., 1988; Pfefferlé et al., 1991).

Irradiation with >600-nm light at 170 K forms an intermediate L, as shown by positive bands at 1748 and 1729 cm^{-1}, and a deep negative band at 1741 cm^{-1} due to BR (Figure 1a). The L/BR spectrum thus obtained exhibits a sharp peak at 3486 cm^{-1} over a broad positive band ranging from 3560 to 3457 cm^{-1}. A negative band was observed at 3642 cm^{-1} in

the higher frequency side of a broad negative band extending from 3613 to 3560 cm^{-1}. Structural positive bands also emerge between the 3260- and 3144-cm^{-1} and between the 3457- and 3260-cm^{-1} regions. However, these will not be discussed below because of greater noise due to the large absorption of water.

Difference spectra were measured at other temperatures. Water condensation on the windows of the cryostate made measurements below 150 K impossible for the 3800–3000-cm^{-1} region. The spectrum measured at 200 K (Figure 1b) shows a large negative band at 1741 cm^{-1} and positive bands at 1748 and 1729 cm^{-1}, all of which are characteristic of the L/BR spectrum, though with an additional small band at 1762 cm^{-1} due to M. The corresponding spectrum in the 3800–3000-cm^{-1} region was shown by adjusting the intensity of the L-specific band at 1741 cm^{-1} to the same height as that measured at 170 K. This normalization on the basis of the molar amount of L is rational, because in this way other L-specific bands at 1400 and 1192 cm^{-1} exhibit the same intensities as those obtained at 170 K [not shown in figures; see Maeda et al. (1991)]. Intensities in the 3260–3144-cm^{-1} region increase slightly, probably due to the presence of M (see below). On the other hand, a whole feature in the 3560–3457-cm^{-1} region is almost the same as that at 170 K, including the sharp band at 3486 cm^{-1}.

The spectrum obtained upon irradiation at 230 K with >500-nm light (Figure 1c) shows a positive band at 1762 cm^{-1}, which is an indication of M. The 1742-cm^{-1} band in the negative side, which would appear if N is present (Pfefferlé et al., 1991), is very small. The spectrum of M in Figure 1c was depicted by adjusting the intensity of the negative band at 1202 cm^{-1} due to BR [not shown in the figures; see Maeda et al. (1991)] to the same height as that of L in Figure 1a. The positive sharp band at 3486 cm^{-1} does not appear. The broad band of M extends over a wider range (3598–3415 cm^{-1}) than that of L, though it is less intense. A negative band at 3642 cm^{-1} and a positive band at 3665 cm^{-1} appear distinctively. Another broad set of bands in the 3260–3144-cm^{-1} region is mainly due to M. As shown previously (Pfefferlé et al., 1991), the difference spectrum at 260 K (Figure 1d) is contributed by both M and N. A positive 1755-cm^{-1} band and a negative 1742-cm^{-1} band belong to N and a 1762-cm^{-1} band to M. The small signal-to-noise ratio of the spectrum in the 3800–3000-cm^{-1} region makes it difficult to assess the contribution of N in this spectrum.

Effects of ^2H_2O and $H_2{}^{18}O$ Substitutions for L. The light-induced difference spectrum at 170 K for the 3750–3450-cm^{-1} region is enlarged (Figure 2a). This spectrum was compared with that of the sample hydrated with ^2H_2O (Figure 2b) by adjusting the intensities of the negative band at 1202 cm^{-1} (not shown in figures; see above) to the same height. It completely retains the sharp band at 3486 cm^{-1}, while the negative band at 3642 cm^{-1}, the broad features in the negative side between 3613 and 3560 cm^{-1}, and those in the positive side between 3560 and 3457 cm^{-1} are removed completely and shift to the region between 2700 and 2440 cm^{-1} (not shown in figures). These ^2H_2O-sensitive bands should arise from the O–H groups in the protein and water. This deuteriated sample was obtained by keeping the bR in ^2H_2O for 3 days. The results were the same when bR was exposed to ^2H_2O only just before the start of the experiment. Thus, the 3486-cm^{-1} band is completely insensitive to ^2H_2O. Intensities in the 3260–3144-cm^{-1} region largely extinguish upon substitution by ^2H_2O (not shown in Figure 2; see Figure 1).

Hydration by $H_2{}^{18}O$ can distinguish the water bands in this region. The negative band at 3642 cm^{-1} of BR was replaced by a negative band at 3630 cm^{-1} (Figure 2c, full line). To show the effect of $H_2{}^{18}O$ substitution more clearly, the spectrum in H_2O was overlaid by a dotted line. The intensities of a set of the positive bands between 3560 and 3490 cm^{-1} decrease, and those below 3486 cm^{-1} increase, indicating the presence of water bands. The remaining positive part, except for the sharp band at 3486 cm^{-1}, is due to the non-water O–H stretching vibration.

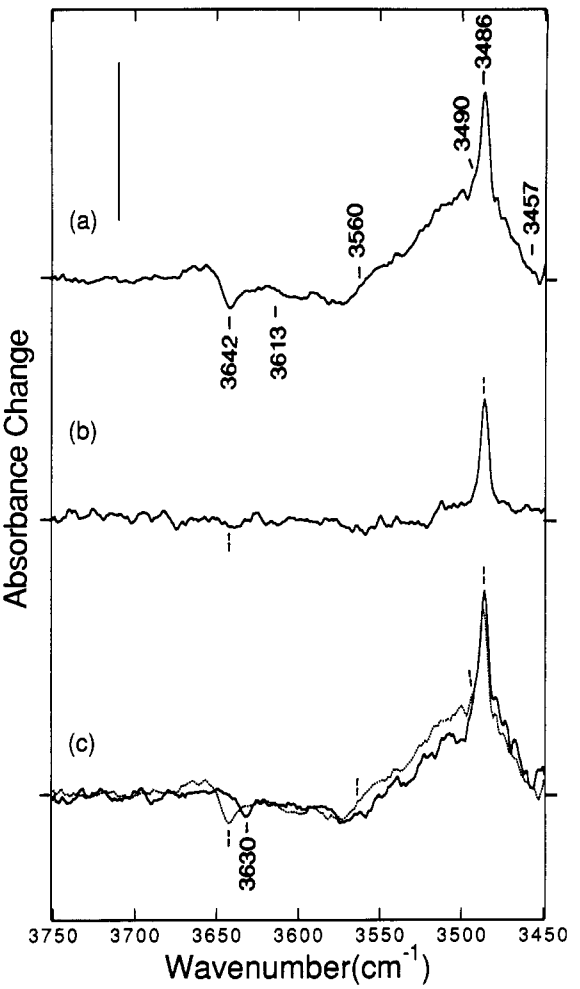

FIGURE 2: L/BR spectra in the 3750–3450-cm^{-1} region recorded at 170 K after irradiation with >600 nm light for 2 min for the BR hydrated with H_2O (a), ^2H_2O (b), and $H_2{}^{18}O$ (c). The dotted line in spectrum c is the trace of spectrum a. The scale bar represents 0.0060, 0.0066, and 0.0039 absorbance unit for spectra a, b, and c, respectively. Each spectrum was presented by averaging the data of two independent samples, each of which is also an average of four recordings.

Effects of ^2H_2O and $H_2{}^{18}O$ Substitutions for M. The M/BR difference spectrum is shown for the 3750–3450-cm^{-1} region (Figure 3a). For a comparison, the L/BR spectrum of Figure 2a was overlaid (dashed line) by adjusting the intensity of the negative 1202-cm^{-1} band (see above). A negative band at 3642 cm^{-1} of BR appears at the same frequency as the same BR band in the L/BR spectrum. Thus, probably the same molecules of water in BR undergo a frequency change in the reactions, resulting in either L or M. A positive band at 3665 cm^{-1} for M (Figure 3a) is virtually unobservable in the L/BR spectrum. These bands disappear upon ^2H_2O substitution, together with a set of positive bands below 3598 cm^{-1} (Figure 3b), and shift to the region between 2700 and 2440 cm^{-1} (not shown in figures). The positive band at 3486 cm^{-1}, which is the ^2H_2O-insensitive band of L, was seen only weakly.

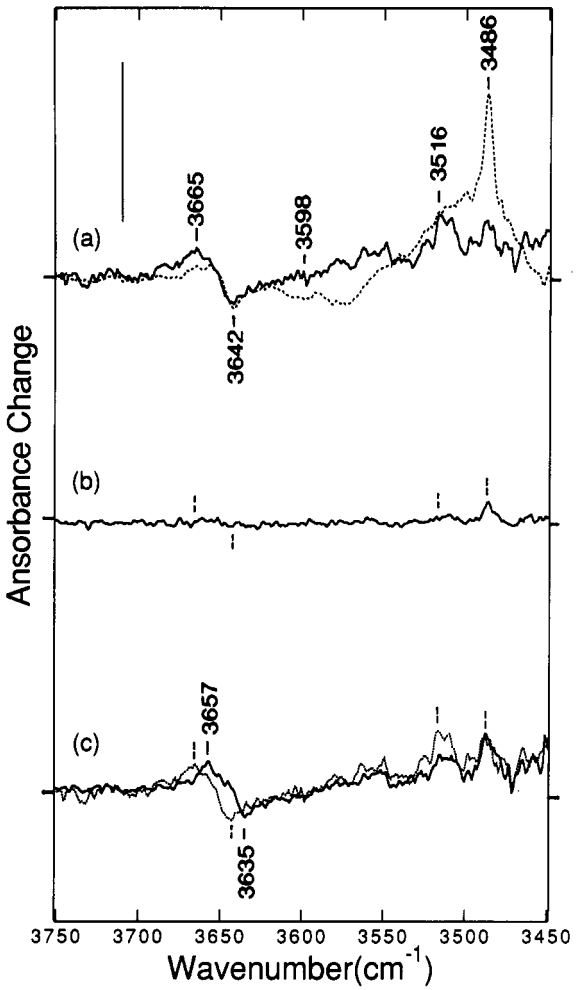

FIGURE 3: Comparison of the M/BR spectra in the 3750–3450-cm^{-1} region for the BR hydrated with H_2O (a), ^2H_2O (b), and $H_2{}^{18}O$ (c). The dashed line in spectrum a is a trace of Figure 2a by adjusting the both intensities of the 1202-cm^{-1} band, and the dotted line in spectrum c is the trace of spectrum a. The scale bar represents 0.0039, 0.0036, and 0.0025, and 0.0060 absorbance unit for the full line spectra (a, b, and c), and the dashed line spectrum (a), respectively. Interferograms coadded were 128. A total of 16 recordings were averaged.

The spectrum in $H_2{}^{18}O$ (Figure 3c, full line) was compared with the overlaid spectrum in H_2O (dotted line). The negative 3642-cm^{-1} band shifts to 3635 cm^{-1}, and the positive 3665-cm^{-1} band shifts to 3657 cm^{-1}. The shift due to $H_2{}^{18}O$ substitution in the 3560–3490-cm^{-1} region for L is almost unobservable except for a depletion around 3516 cm^{-1}.

Discussion

The O–H stretching vibration bands on the formation of the intermediates L and M between 3750 and 3450 cm^{-1} were analyzed upon substitution with ^2H_2O or $H_2{}^{18}O$ out of the peak region of the intense water band around 3300 cm^{-1}. The water involved in the light–dark reactions (Korenstein & Hess, 1979; Hildebrandt & Stockburger 1984) does not affect the observed spectral changes upon light-adaptation. A water dependence for the N formation observed by Váró and Lanyi (1991) and Cao et al. (1991) cannot be correlated with the present spectrum containing both M and N (Figure 1d).

Among the ^2H_2O-sensitive bands, a negative band at 3642 cm^{-1}, which arises from its disappearance from BR upon formation of either L or M, the small part of the positive band distributed between 3560 and 3490 cm^{-1} of L, a positive band of M at 3665 cm^{-1}, and a positive band of M around 3510 cm^{-1} are all sensitive to $H_2{}^{18}O$ substitution. These bands are thus assigned to water. This is the first description of the water bands in the dynamic process of bR. The recent studies by Chang et al. (1991) attributed the positive and negative bands at 3655 and 3642 cm^{-1} in the M/BR spectrum to the O–H stretching vibrations.

Below, we will use previous data for the assignment of the water band observed in the present paper. Water has C_{2v} symmetry and shows two bands of asymmetric and symmetric O–H stretching vibrational modes in the 3-μm region. For the water which interacts only with one of two O–H bonds to the hydrogen-bonding acceptor (C_s symmetry), the higher frequency band in the two vibrational bands is assigned to the stretching modes of the unbonded O–H and the lower frequency band to that of the bonded O–H (Glew & Rath, 1971). The water bands described above for BR and its photointermediates must therefore be attributed to the asymmetric stretching mode for C_{2v} water or to the unbonded mode for C_s water, because no other bands were observed in the higher frequency side.

Monomeric water in a nitrogen matrix shows the asymmetric and symmetric modes at 3725 and 3627 cm^{-1}, respectively (van Thiel et al., 1957). The frequency reduces progressively with the polymerization of the water molecules; 3691 and 3546 cm^{-1} for the dimer, 3510 and 3355 cm^{-1} for trimer, and so on. The broad absorption band of liquid water in the O–H stretching vibration region has been subdivided into four peaks at 3620, 3540, 3435, and 3240 cm^{-1} (Walrafen, 1967; Monosmith & Walrafen, 1984). The 3620- and 3540-cm^{-1} bands are assigned to the stretching modes of the unbonded and bonded O–H of the C_s water, respectively (three-bonded water molecule) (Monosmith & Walrafen, 1984; Walrafen & Fisher, 1986). The 3540- and 3250-cm^{-1} bands are assigned to the vibrations of the fully hydrated tetrahedral water molecules (Monosmith & Walrafen, 1984; Walrafen & Fisher, 1986). Thus, the water bands for bR described above are not attributed to any water clusters which may be present on the protein surface (Blake 1983; Teeter, 1984, 1991), and those bands which are due to the strongly hydrogen-bonded waters are hidden in the noisy region where the characterization was not done in the present work.

Mohr et al. (1965) and Glew and Rath (1971) have studied the interaction of the water monomer with various hydrogen-bonding acceptors by dissolving these molecules together with water in a relatively inert solvent like carbon tetrachloride. In a weakly hydrogen-bonded solvent like ethyl acetate, symmetric and asymmetric bands are located at 3640 and 3550 cm^{-1}, respectively (Mohr et al., 1965). Upon further increases in basicity, the two bands become indistinguishable from each other, owing to the broadening of the band width and the smaller frequency difference between the two modes. In *N*,*N*-dimethylformamide, those bands appear at 3550 and 3490 cm^{-1}, respectively. For C_s water, these two bands, which also undergo downward shifts with the increase of basicity, are located in the 3684–3655- and 3500–3400-cm^{-1} regions, respectively (Glew & Rath, 1971). No data are available for the case where only oxygen is involved in the hydrogen-bonding interaction. It may absorb above 3700 cm^{-1} if one applies the data for the O–H bond of ethanol (Coburn & Grunwald, 1958; Nakanishi et al., 1978).

The frequency at 3642 cm^{-1} of BR is therefore close to the asymmetric stretching mode of the water which is hydrogen bonded with a quite weak base like ethyl acetate (Mohr et al., 1965). The 3665-cm^{-1} band of M is a result of a further weakening of the hydrogen-bonding interaction. However, this

frequency can also be explained as due to the nonbonding O–H of the C_s water with a single relatively strong hydrogen-bonding interaction (see above). In either case, an accompanying band must be detected in the lower frequency side, but it may be hidden under overlapping intense positive bands.

The broad water bands in the region between 3560 and 3490 cm^{-1} for L (Figure 2c) probably result from the shift of the 3642-cm^{-1} band of BR, though we have no analysis in the further lower frequency region. It could be accounted for by the formation of the stronger hydrogen-bonding interaction in the process from BR to L. Model experiments by Mohr et al. (1965) show that the frequencies between 3560 and 3490 cm^{-1} are close to those with *N,N*-dimethylformamide. We made a similar experiment with *N,N*-dimethylacetamide. It also shows a broad band between 3567 and 3347 cm^{-1} with a peak at 3449 cm^{-1}. Their most likely counterpart in the protein is the carbonyl oxygen of the amide and peptide bonds. It is a general tendency that the band width and the molar extinction increase with increasing the hydrogen-bonding strength (Glew & Rath, 1971; Nakanishi et al., 1978). The wider distribution of the $H_2{}^{18}O$-sensitive band upon L formation is thus consonant with the model system with increasing hydrogen-bonding strength.

The molar extinction of water has been estimated to be 100–200 (Glew & Rath, 1971). The absorption change at 3642 cm^{-1} of 0.001 for the BR film (Figure 2) with A_{570nm} of about 0.5 [the molar extinction of 63 000 (Oesterhelt & Hess, 1973)] indicates that one or a few water molecule at most may be responsible for the observed intensity change.

As a model for the hydrogen-bonding interaction of the side chain O–H, the frequency change in the O–H stretching vibrational bands of methanol depending on the solvent basicity is useful (Glew & Rath, 1971). The frequency of the O–H stretching bands of methanol is smaller than that of the asymmetric stretching vibration of water by 60–120 cm^{-1} (Glew & Rath, 1971). The non-water O–H stretching vibrations for BR in the 3613–3560-cm^{-1} region is in accordance with this relation. Therefore, those O–H groups in BR have very weak interactions and their hydrogen bonding increases upon L formation, like the water. The residues responsible for these O–H stretching vibrations are serine, threonine, tyrosine, and aspartic acid. The assignments are now in progress.

Water–protein interaction has so far been studied by X-ray and neutron diffraction methods (Sakabe et al., 1980, 1981, 1985; Blake et al., 1983; Teeter, 1984, 1991; Wlodawer et al., 1987; Saenger, 1987; Thanki et al., 1988). Accumulated data indicate that almost all of the ordered water molecules within a single layer of the protein surface, or buried in the protein, are located in hydrogen-bonding distance from polar side-chain groups or peptide bonds. Even water at van der Waals distance from an apolar residue forms an ordered structure with a combination of other water molecules or polar residues (Blake et al., 1983; Teeter, 1984, 1991). It is hard to find waters having only van der Waals contact with the protein, even for the case of the buried water molecules in the photosynthetic reaction center (Deisenhofer & Michel, 1989). Water molecules in the second layer of the protein surface in the narrow channel between the protein monomer in insulin hexamer crystals have been identified as partially occupied water (Sakabe et al., 1985). It remains as a candidate for the water molecules with very weak interaction in the protein. It is interesting to note that serine, threonine, and tyrosine in the hydration shell are hydrated by less than one water molecule on average (Saenger, 1987).

The contribution of water absorption in bR around 3642 cm^{-1} is quite small at 170 K and located in the edge region of the slope of the strong absorption due to the hydrogen-bonded waters (about 4% of the maximum absorbance at 3280 cm^{-1}; not shown in figures). The absolute spectrum itself is quite featureless with only a broad peak around 3300 cm^{-1}. A similar spectral shape was drawn for lysozyme and bovine pancreatic trypsin inhibitor, whose water structures are well characterized by X-ray analysis (Blake et al., 1983; Wlodawer et al., 1987). In comparison with these proteins, no special features appear in the spectrum of hydrated bR. Further analysis of the water in comparison with the protein analyzed well by X-ray diffraction is impossible at the present stage.

Although the sharp band at 3486 cm^{-1} of L was completely insensitive to ^2H_2O, it is likely to be an O–H or N–H stretching vibration, because a C–H stretching vibration is not expected to be located in this frequency region. A similar insensitivity to ^2H_2O substitution was also found for transmembrane peptide bonds of bR (Earnest et al., 1990). By extrapolating the relation of the N–H stretching vibrational frequency to the N···O distance (Krimm & Bandekar, 1986), the frequency of 3486 cm^{-1} can be attained if it is increased to 0.36 nm. The band sharpening is a general phenomenon associated with the loss of the hydrogen bonding. In this respect, the 3486-cm^{-1} band seems to be due more to N–H stretching with a very weak hydrogen bonding than to O–H stretching vibration with strong hydrogen bondings. N–H stretching vibrations appear at 3520 and 3430 cm^{-1} for *o*-toluidine having no hydrogen bondings (Nakanishi et al., 1978). The 3486-cm^{-1} band of L could not be, however, due to the Schiff base, because it must be sensitive to ^2H_2O. Rather some peptide N–H bond may be responsible. The assignment of this interesting band must be done in a future study.

The most prominent changes occurring upon L formation are the strong interaction of the Schiff base N–H (Maeda et al., 1991), the introduction of a twist in the retinal moiety close to the Schiff base (Fahmy et al., 1989; Maeda et al., 1991), and the perturbation of Asp-96 (Engelhard et al., 1985; Braiman et al., 1988; Gerwert et al., 1989). Also observed here are stronger hydrogen bonding of the water and O–H side chains of the protein. A specific change for L is also detected in the peptide N–H interaction, though assigned tentatively. This may be correlated with the frequency and dichroic ratio changes in amide I bands observed by Fahmy et al. (1989). On the other hand, for M, Asp-96 is just in the same state as that in BR, and also the water molecule returns to a state similar to that in BR. The origin of a small band around 3510 cm^{-1} of M may not be the water showing the 3642-cm^{-1} band in BR. This has to be determined in a future study.

The most likely site for the water molecules in BR is a narrow channel between Asp-96 and the protonated Schiff base. Alternatively, the water molecules present close to the Schiff base (Hildebrandt & Stockburger, 1984) in the hydrogen-bonding network may be a candidate for the water band of BR if it is interacting very weakly with the Schiff base proton. In either case, the water molecules may be involved in the perturbation of Asp-96 in the L intermediate, and they are exerted from the protonated Schiff base which changes position upon the light-induced reaction (Pfefferlē et al., 1991).

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Registry No. L-Aspartic acid, 56-84-8; L-serine, 56-45-1; L-threonine, 72-19-5; L-tyrosine, 60-18-4.

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