Structural Changes of Water Molecules during the Photoactivation Processes in Bovine Rhodopsin†

Yuji Furutani,‡,§,|| Yoshinori Shichida,§,|| and Hideki Kandori*,‡,||

Department of Applied Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan, Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kyoto 606-8502, Japan

Received April 14, 2003; Revised Manuscript Received June 8, 2003

ABSTRACT: Internal water molecules of rhodopsins play an important role in stabilizing the crucial ion pair comprised by the protonated retinal Schiff base and its counterion. Previous low-temperature FTIR spectroscopy of archaeal rhodopsins observed water O−D stretching vibrations at 2400−2100 cm^{-1} in D_2O, corresponding to strong hydrogen bonds. Since a water molecule bridges the protonated Schiff base and an aspartate in archaeal rhodopsins, the observed water molecules presumably hydrate the negative charges in the Schiff base region. In contrast, the FTIR spectroscopy data of bovine rhodopsin presented here revealed that there are no spectral changes of water molecules under strongly hydrogen-bonding conditions (in the range <2400 cm^{-1} for O−D stretch) during the photoactivation processes. The only observed water bands were located in the >2500 cm^{-1} region that corresponds to weak hydrogen bonding. These results imply that the ion pair state in vertebrate visual rhodopsins is stabilized in a manner different from that in archaeal rhodopsins. In addition, the internal water molecules that hydrate the negative charges do not play important role in the photoactivation processes of rhodopsin that involve proton transfer from the Schiff base to Glu113 upon formation of Meta II. Structural changes of the H−D exchangeable peptide amide of a β-sheet are observed upon formation of metarhodopsin II, suggesting that motion of a β-sheet is coupled to the proton transfer reaction from the Schiff base to its counterion.

Visual rhodopsin is a member of G protein-coupled receptor family that has evolved into a photoreceptive protein in visual cells (*1*−*4*). It is a membrane protein consisting of a single polypeptide opsin and a light-absorbing chromophore, 11-*cis*-retinal. The opsin contains seven transmembrane α-helices, the structural motif typical of the G protein-coupled receptors, and the 11-*cis*-retinal is bound to it through the protonated Schiff base linkage ($C{=}NH^+$) with Lys296 (numbering for bovine rhodopsin) in transmembrane helix 7. Absorption of a photon by the chromophore causes isomerization to the all-*trans* form, followed by conformational changes of the protein (*5*). Several intermediate states in the bleaching process are identified as photorhodopsin, bathorhodopsin (Batho),[1] lumirhodopsin (Lumi), metarhodopsin I (Meta I), and metarhodopsin II (Meta II) (*6*). Meta II activates the GDP−GTP exchange reaction in the trimeric G protein transducin (*7*).

It is known that the protonation state of the Schiff base is stabilized by its counterion, Glu113 (*8*, *9*), so that the pK_a of the Schiff base is being kept high. Internal water molecules may stabilize the ion pair state inside the protein. That is indeed the case in archaeal rhodopsins such as bacteriorhodopsin (BR) and *pharaonis* phoborhodopsin (*p*pR), where internal water molecules participate in the Schiff base stabilization (*10*, *11*). One water molecule bridges the Schiff base and the counterion in BR and *p*pR, and its motion presumably controls the proton transfer from the Schiff base to an aspartate (*12*). Previous FTIR studies of bovine rhodopsin similarly reported on the presence of internal water molecules near the retinal chromophore (*13*, *14*). Then, solid-state NMR spectroscopy estimated the distance between the Schiff base nitrogen and Glu113 to be >4 Å (*15*, *16*), suggesting that there should be a bridging water molecule as in archaeal rhodopsins.

In contrast, the first X-ray crystal structure of bovine rhodopsin with 2.8 Å resolution reported no water molecules in the Schiff base region (*17*). As the resolution was improved and the structure was refined, the number of detected internal water molecules has gradually increased (*18*, *19*). Figure 1a shows the two water molecules, Wat2a and Wat2b, in the Schiff base region of bovine rhodopsin (*19*). These water molecules would be important in stabilizing the ion pair state, whereas their locations are entirely different from those in archaeal rhodopsins. The Schiff base region of BR, a light-driven proton pump, is shown in Figure 1b, where three water molecules constitute

† This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to H.K. and by research fellowships from the Japan Society for the Promotion of Science for Young Scientists to Y.F.

* To whom correspondence should be addressed. Phone/Fax: 81-52-735-5207. E-mail: kandori@ach.nitech.ac.jp.

‡ Nagoya Institute of Technology.

§ Kyoto University.

|| CREST.

[1] Abbreviations: Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; BR, bacteriorhodopsin; *p*pR, *pharaonis* phoborhodopsin; FTIR, Fourier transform infrared; Iso, isorhodopsin; ROS, rod outer segments; HOOP, hydrogen out of plane.

10.1021/bi034592k CCC: $25.00
Published on Web 07/23/2003

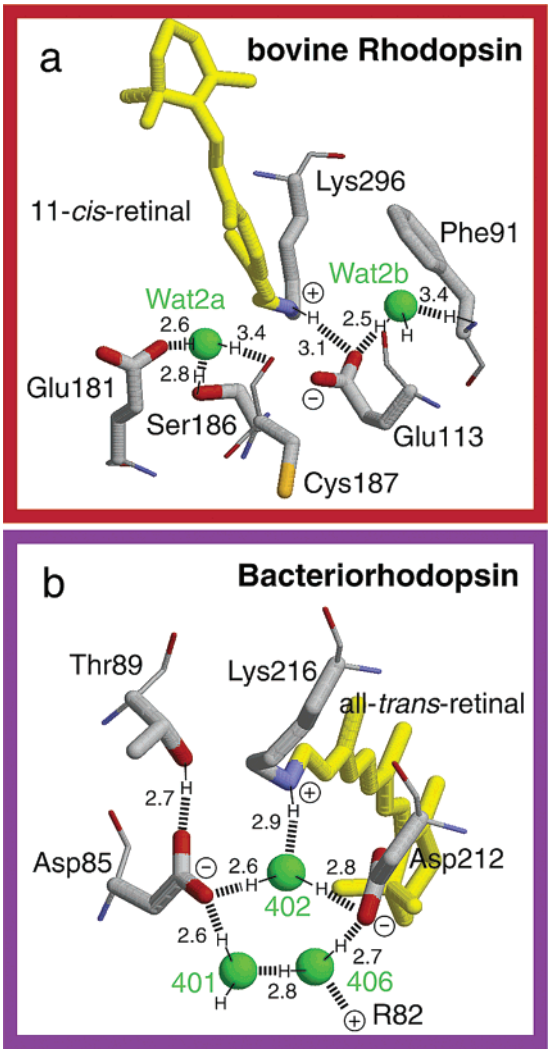

FIGURE 1: Schiff base structure of bovine rhodopsin (a) (1L9H; ref *19*) and bacteriorhodopsin (b) (1C3W; ref *34*). Water molecules in the Schiff base region (Wat2a and Wat2b in rhodopsin and water 401, 402, and 406 in bacteriorhodopsin) are shown together with several amino acids involved in the hydrogen-bonding network. Dotted lines represent putative hydrogen bonds, and numbers are the distances in angstroms between hydrogen-bonding acceptors and donors.

a roughly planar pentagonal cluster structure (*10*, *11*). The water molecules stabilize the electric quadrupole [the Schiff base (+), Arg82 (+), Asp85 (−), and Asp212 (−)] in the Schiff base region. It appears that they also work for the primary proton transfer reaction from the Schiff base to Asp85 (*12*), which is essential for the vectorial proton transport of BR. Different locations of internal water molecules (Figure 1) suggest their different static and dynamic roles in visual and archaeal rhodopsins.

FTIR spectroscopy is a powerful tool to investigate the structural and functional role of internal water molecules (*10*). Although the frequency region was limited to that of the water bands with weak hydrogen bonds in previous FTIR studies, highly accurate spectral comparison in D_2O and $D_2{}^{18}O$ at cryogenic temperatures allowed information to be obtained on strongly hydrogen-bonded waters in archaeal rhodopsins (*10*, *11*). As a consequence, we have observed frequency changes of an extremely strongly hydrogen-bonded water before and after retinal photoisomerization in BR (*20*) and *p*pR (*21*). On the basis of the measurements of late intermediates in BR, we have proposed a proton transfer mechanism in the Schiff base region. According to the "hydration switch model", the hydration by the bridging water (water 402; Figure 1b) is switched from Asp85 in the L intermediate to Asp212 in the M intermediate, which drives proton transfer from the Schiff base to Asp85 (*12*). Thus, low-temperature FTIR spectroscopy has provided useful information on internal water molecules at work in archaeal rhodopsins.

In the present study, we extended highly accurate FTIR measurements of water molecules to bovine rhodopsin, and water structural changes were examined during the photoactivation processes. Unlike for archaeal rhodopsins, water bands were not observed in the <2400 cm^{-1} region at 77 K for rhodopsin (11-*cis*-retinal), Iso (9-*cis*-retinal), and Batho (all-*trans*-retinal). The same results were obtained for the subsequent intermediates such as Lumi, Meta I, and Meta II. The only observed water bands were located in the >2500 cm^{-1} region that corresponds to weak hydrogen bonding. These results imply that the ion pair state in vertebrate visual rhodopsins is stabilized in a manner different from that in archaeal rhodopsins. In addition, internal water molecules that hydrate negative charges do not play an important role in the photoactivation processes of rhodopsin that involve proton transfer from the Schiff base to Glu113 upon formation of Meta II.

MATERIALS AND METHODS

Frozen bovine retinas were purchased from J. A. & W. L. Lawson Co. (Lincoln, NE). Crude rod outer segments (ROS) were isolated from the retinas in ROS buffer (10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mg/L aprotinin, 1 mg/L leupeptin, 1 mM DTT, pH 7.3) by a sucrose flotation method as described previously (*22*). A discontinuous sucrose gradient was used to purify ROS. ROS were then washed five times with distilled water by centrifugation at 80000*g*, followed by suspension in 2 mM phosphate buffer (pH 7.0) for the measurements of Iso, Batho, Lumi, and Meta I or in 2 mM citrate buffer (pH 5.0) for the measurement of Meta II. The final concentration of the rhodopsin molecule in the ROS suspension was controlled to 3 mg/mL.

A 90 μL aliquot of the ROS suspension was deposited on a BaF_2 window with a diameter of 18 mm and dried in the glass vessel that was evacuated by an aspirator. The dry film was then hydrated by placing <1 μL of heavy water (D_2O or $D_2{}^{18}O$) next to the film. The sample was sealed by use of another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The film sample was cooled about 10 min after hydration.[2] The experimental setup was the same as described previously (*13*, *23*), where the cryostat was mounted in a Bio-Rad FTS40 FTIR spectrometer. The cryostat was connected with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The FTIR spectra were recorded with 2 cm^{-1} resolution and constructed from 128 interferograms.

[2] Under the present experimental condition, absorbance at about 2500 cm^{-1} for X−D stretching vibrations was comparable to that at 1650 cm^{-1} for amide I vibration. In addition, the analysis of amide II vibration upon D_2O hydration showed that about 25% of the rhodopsin peptide groups undergo H/D exchange, which is consistent with the previous report (*35*).

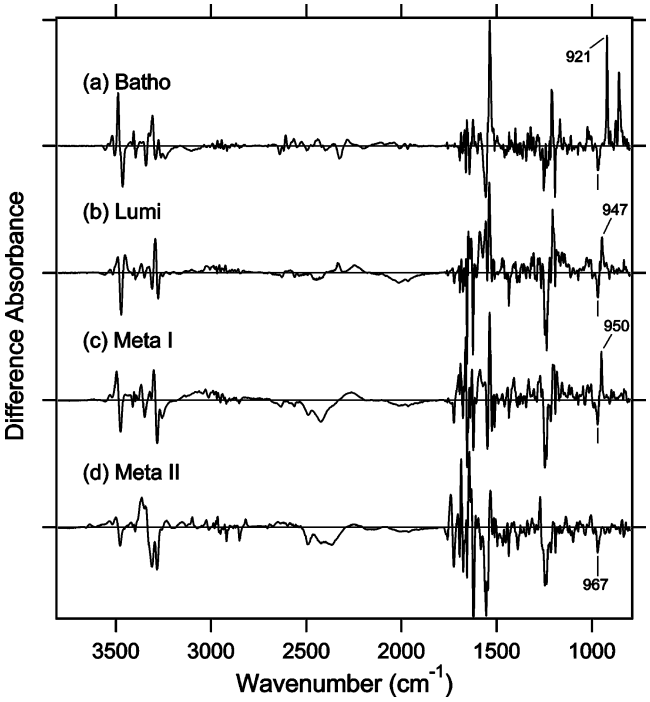

FIGURE 2: Difference IR spectra between the original rhodopsin and several intermediate states measured at low temperature in the whole mid-infrared region (4000−800 cm^{-1}). The Batho (a), Lumi (b), Meta I (c), and Meta II (d) minus rhodopsin spectra are measured at 77, 200, 240, and 270 K, respectively, where 24 (a) or 4 (b−d) independent measurements with 128 scans were averaged. These spectra are normalized at the negative 967 cm^{-1} band, indicating that the same number of rhodopsin molecules is converted to each of the intermediates. One division of the *y*-axis corresponds to 0.01 absorbance unit.

The Batho minus rhodopsin and Batho minus Iso spectra were recorded at 77 K as described previously, where Iso was produced by light illumination (*13*). Conversion of rhodopsin or Iso to Batho was achieved by illumination with 501 nm light (by use of an interference filter) for 2 min, while reversion of Batho to rhodopsin or Iso was done by illumination with >610 or >530 nm light for 1 min, respectively. Twenty-four independent measurements were averaged for D_2O and $D_2{}^{18}O$ hydrations. The Lumi minus rhodopsin, Meta I minus rhodopsin, and Meta II minus rhodopsin spectra were recorded at 200, 240, and 270 K, respectively, where rhodopsin was illuminated with >560 nm light for 1 min. Under the present illumination conditions, formation of Iso was negligible, as judged from the Iso-specific bands at 959 and 1207 cm^{-1} (*13*). Because of the bleaching of the samples, a fresh ROS sample was prepared for each recording, and four independent measurements were averaged for obtaining difference spectra for Lumi, Meta I, and Meta II in D_2O and $D_2{}^{18}O$. Linear dichroism experiments revealed random orientation of the rhodopsin molecules in the film, so we have not applied polarized FTIR measurements.

RESULTS

Acquisition of the FTIR Difference Spectra in the Whole Mid-Infrared Region. Figure 2 shows the Batho minus rhodopsin (a), Lumi minus rhodopsin (b), Meta I minus rhodopsin (c), and Meta II minus rhodopsin (d) spectra measured at 77, 200, 240, and 270 K, respectively, in D_2O. These spectra were normalized at the negative 967 cm^{-1} band, a characteristic HOOP mode of rhodopsin. This suggests that the same number of rhodopsin molecules is converted in the case of each intermediate (*23*). The positive HOOP bands at 921, 947, and 950 cm^{-1} are characteristic for Batho, Lumi, and Meta I, respectively, while there are no HOOP bands in Meta II. It is known that the Schiff base nitrogen is deuterated in D_2O (*13*, *24*). Therefore, the water molecules in the Schiff base region are presumably exchanged to D_2O or $D_2{}^{18}O$, whose stretching vibrations appear in the 2700−2000 cm^{-1} region. Below we present the isotope effect of ^{18}O of heavy water. Spectra were identical in other frequency regions for D_2O and $D_2{}^{18}O$ hydrations (data not shown).

Water-Stretching Vibrations in the Batho minus Rhodopsin or Iso Spectra. The red curves in Figure 3a,b show the Batho minus rhodopsin and Batho minus Iso difference spectra in D_2O at 77 K, respectively. They look similar to each other except for the 2000−1900 cm^{-1} region, indicating that the hydrogen-bonding network around the retinal chromophore is similar in 11-cis (rhodopsin) and 9-cis (Iso) forms but is altered in the all-*trans* form (Batho). This observation is consistent with the previous results for the X−H stretch (*13*). The spectra are identical between D_2O (red curve) and $D_2{}^{18}O$ (blue curve) in the <2400 cm^{-1} region of Figure 3a,b, and the isotope effect of water was observed in the 2700−2500 cm^{-1} region. Since the detected water bands are located at the high-frequency side of liquid D_2O (shaded curve in Figure 3), they correspond to weakly hydrogen-bonded water molecules.

The observation for bovine rhodopsin is in high contrast to the case of archaeal rhodopsins. Figure 3c shows the K minus BR spectra, which exhibit isotope shift of water bands in the 2400−2100 cm^{-1} region as well. We have interpreted these water molecules as hydrating the negative charges such as Asp85 and Asp212 (Figure 1b) (*11*, *20*), which is consistent with the normal mode analysis on the basis of quantum mechanical and molecular dynamics calculations (*25*). Water O−D stretches of *p*pR were observed in the 2400−2200 cm^{-1} region (*11*, *21*). Strongly hydrogen-bonded water molecules are thus characteristic for the bridging water structures in the crucial ion pair of archaeal rhodopsins. The present result suggests that bovine rhodopsin does not have the BR-like bridging water molecule, being consistent with the X-ray crystal structure (Figure 1) (*19*).

Both Batho minus rhodopsin (Figure 3a) and Batho minus Iso (Figure 3b) spectra show six negative and six positive water bands in the 2700−2500 cm^{-1} region. The observed isotope shifts in $D_2{}^{18}O$ ranged from 6 to17 cm^{-1}. If the normal mode of water contains only a single O−D group, the expected isotope shift is 17 cm^{-1} as for the bands at 2702 (−)/2698 (+) cm^{-1}. Coupling with other vibrations reduces the isotope shift, so that the observed shifts show large variation. Contribution of other vibrations could also affect the apparently small isotope shifts. Previous site-directed mutagenesis of bovine rhodopsin revealed that the bands at 3536 (−)/3522 (+) cm^{-1} disappeared in the spectrum of E113Q (*14*), which correspond to those at 2618 (−)/2609 (+) cm^{-1} in D_2O (Figure 3a). Similarly, the negative band at 3562 cm^{-1} disappeared in the spectra of G120A and D83N (*26*), which corresponds to the 2640 cm^{-1} band in D_2O (Figure 3a). These results suggested the

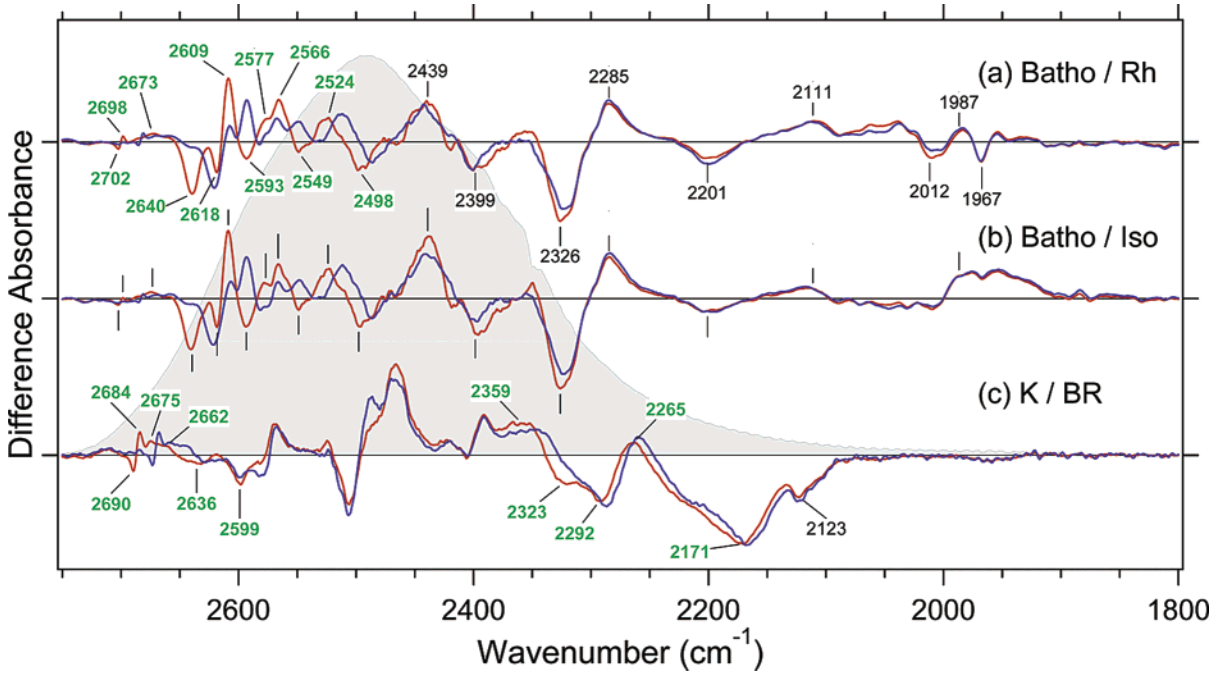

FIGURE 3: Difference IR spectra of rhodopsin and bacteriorhodopsin at 77 K. The Batho minus rhodopsin (a) and Batho minus Iso (b) spectra in the 2750−1800 cm^{-1} region are shown together with the K minus BR (c) spectrum reproduced from ref *12*. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively. Green labeled frequencies correspond to those identified as water-stretching vibrations. The grayish curve in the 2700−2000 cm^{-1} region represents O−D stretching vibrations of D_2O at room temperature. One division of the *y*-axis corresponds to 0.002 absorbance unit.

locations of the water molecules possessing the O−D stretches at 2618 or 2640 cm^{-1} near Glu113 or Gly120 and Asp83, respectively (*14*, *26*). The former water molecule may be one of the waters in Figure 1a (Wat2a or Wat2b), and its O−D group is almost free from hydrogen bonding, as indicated by its stretching frequency.

The Batho minus Iso spectrum is nearly identical to the Batho minus rhodopsin spectrum in the >2100 cm^{-1} region. This fact implies that water structures are identical between rhodopsin and Iso. The only difference between the two spectra can be seen at around 2000 cm^{-1}. If we calculate the Iso minus rhodopsin spectrum, it possesses a sharp negative band at 1967 cm^{-1} and a broad positive band at about 2050 cm^{-1} (data not shown). From the vibrational analysis of C═NH and C═ND stretches of the Schiff base, it is known that its hydrogen-bonding strength is comparable in rhodopsin and Batho and is weaker in Iso (*24*). Since the higher frequency of the N−D stretch corresponds to a weaker hydrogen bond, it is likely that the N−D stretch of the Schiff base is located in this frequency region.

Water-Stretching Vibrations in the Lumi, Meta I, and Meta II minus Rhodopsin Spectra. Figure 4 shows the Lumi minus rhodopsin (a), Meta I minus rhodopsin (b), and Meta II minus rhodopsin (c) spectra in the 2750−1800 cm^{-1} region. The bands at 2704 (−), 2673 (−), 2629 (−),and 2610 (+) cm^{-1} clearly exhibit an isotope shift of water in the Lumi minus rhodopsin spectrum (Figure 4a). The 2629 (−)/2610 (+) cm^{-1} bands correspond to the 3563 (−)/3534 (+) cm^{-1} bands previously assigned to the O−H stretch of water (*23*). Water bands are reduced in number for the Lumi minus rhodopsin spectrum as compared to the Batho minus rhodopsin spectrum (Figure 3a), suggesting that local structural perturbation of internal water molecules upon retinal photoisomerization is considerably reduced in Lumi. Absence of water bands in the <2400 cm^{-1} region implies that there is no hydrogen-bonding alterations of the water molecules hydrating the negative charges. It is known that hydrogen bonding of the Schiff base is significantly weakened in Lumi, while it is comparable between rhodopsin and Batho (*27*). This suggests that the ion pair interaction (possibly involving water molecules) has not changed much in Batho but has significantly changed in Lumi. On the other hand, absence of water bands in the <2400 cm^{-1} region of Figure 4a indicates that there are no structural changes of strongly hydrogen-bonded waters even in Lumi.

The Meta I minus rhodopsin spectrum (Figure 4b) exhibits water bands similar to those of the Lumi minus rhodopsin spectrum (Figure 4a). Namely, water bands appear at 2704 (−), 2673 (−), 2629 (−), and 2616 (+) cm^{-1}, and no water bands were observed in the <2400 cm^{-1} region. Thus, the water structure is nearly identical between Lumi and Meta I, and its hydrogen bonds are persistent during these intermediate states. Figure 4a,b also provides information on the N−D stretching vibrations of Lumi and Meta I. As described above, previous vibrational analysis of the C═NH and C═ND stretches showed that the hydrogen-bonding strength of the Schiff base in Lumi or Meta I is much weaker or slightly weaker, respectively, than in rhodopsin (*24*, *27*). Thus, we can tentatively assign the bands at 2337 and 2262 cm^{-1} as the N−D stretching vibrations of the Schiff base in Lumi and Meta I, respectively. The negative bands at 2012 and 1966 cm^{-1} are presumably assignable to the N−D stretches of rhodopsin.

Meta II formation is accompanied by a proton transfer in the Schiff base region, probably from the Schiff base to Glu113 (Figure 1a). Thus, if water molecules strongly interact with Glu113, we might expect the water bands to appear in the <2400 cm^{-1} region. Nevertheless, there are no water bands in the region, and the Meta II minus rhodopsin spectrum (Figure 4c) exhibits water bands at 2704 (−), 2694

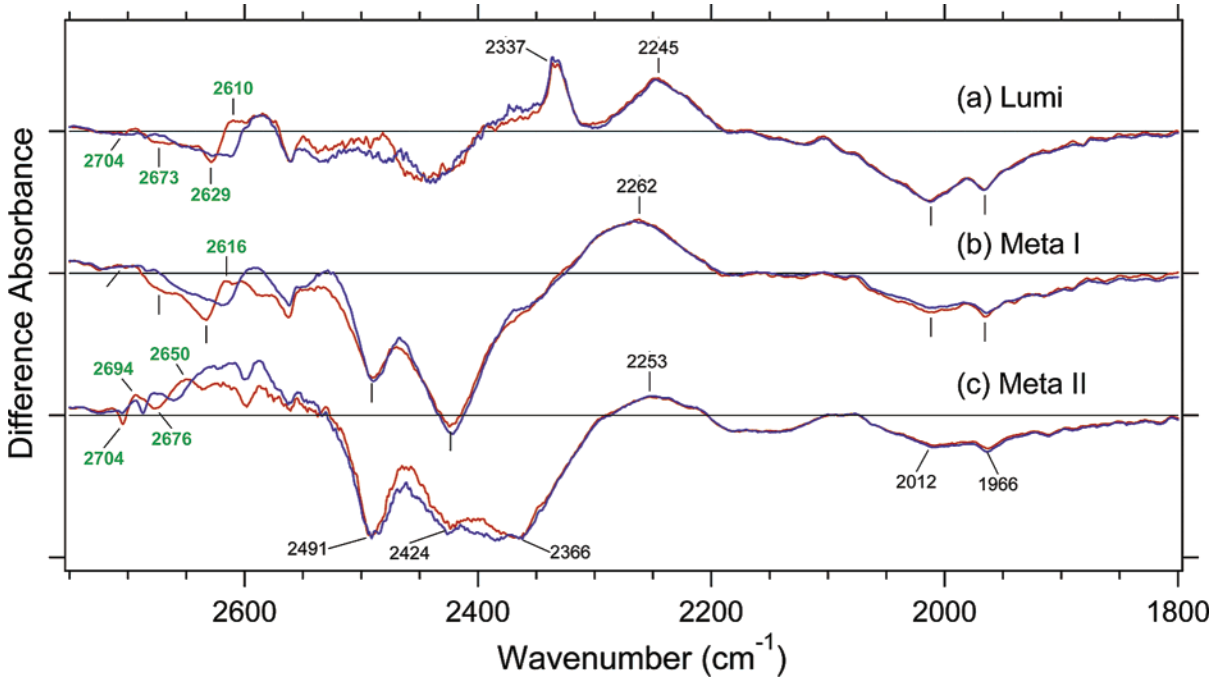

FIGURE 4: The Lumi minus rhodopsin (a), Meta I minus rhodopsin (b), and Meta II minus rhodopsin (c) spectra in the 2750−1800 cm^{-1} region measured at 200, 240, and 270 K, respectively. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively. Green labeled frequencies correspond to those identified as water-stretching vibrations. One division of the *y*-axis corresponds to 0.0016 absorbance unit.

(+), 2676 (−), and 2650 (+) cm^{-1}. Since the negative 2673 cm^{-1} band in Figure 4a,b is close in its frequency to that at 2676 cm^{-1} in Figure 4c, the same water molecule in rhodopsin presumably changes the frequency in the late intermediates. The corresponding positive bands may be at 2610 cm^{-1} in Lumi, at 2616 cm^{-1} in Meta I, and at 2650 cm^{-1} in Meta II, respectively. Since there is no negative band at 2673 cm^{-1} in the Batho minus rhodopsin spectrum (Figure 3a), it is likely that the water molecule has the O−D stretch at 2673 cm^{-1} in rhodopsin and Batho, at 2610 cm^{-1} in Lumi, at 2616 cm^{-1} in Meta I, and at 2650 cm^{-1} in Meta II.

The Meta I minus rhodopsin and Meta II minus rhodopsin spectra exhibit strong negative bands at 2491 and 2424 cm^{-1} (Figure 4b,c). In addition, the latter spectrum shows the negative band at 2366 cm^{-1} (Figure 4c). These bands do not originate from water molecules. Since the N−D stretch of the Schiff base is probably different in frequency, they originate from protein vibrations such as O−D or N−D stretches of side chains and N−D stretch of the peptide backbone (amide A). It is known that amide A (N−H stretching) vibrations of the α-helix and *β*-sheet appear at 3279 and 3242 cm^{-1}, respectively (*28*).[3] The calculated N−D stretches are 2389 cm^{-1} for α-helix and 2367 cm^{-1} for *β*-sheet; the latter coincides well with the negative band in the Meta II minus rhodopsin spectrum. The observed 2424 cm^{-1} band is also assignable as the amide A mode of the α-helix. Therefore, it is likely that formation of Meta I accompanies the structural changes in the H−D exchangeable peptide amide groups of an α-helix, while formation of Meta II accompanies additional structural changes in the H−D exchangeable peptide amide groups of a *β*-sheet. It is known that the extracellular side of the retinal chromophore is covered by the antiparallel *β*-sheet composed of Arg177−Glu181 and Ser186−Asp190 (*17*). Transition from Meta I to Meta II may be coupled to the motion in this region, so that the environment of the Schiff base is dramatically changed.

DISCUSSION

The present FTIR spectroscopic study identified the frequencies of the O−D stretching vibrations of internal water molecules in rhodopsin, Iso, Batho, Lumi, Meta I, and Meta II. All in all, seven O−D stretches with frequencies located at >2400 cm^{-1} are changed during photoactivation processes of bovine rhodopsin. The recently refined X-ray crystal structure reported on the presence of seven water molecules in the transmembrane region of bovine rhodopsin (*19*). The present FTIR study presumably probes some of these water molecules. In this study, the largest number of water bands was observed for the Batho minus rhodopsin spectrum. This result needs an explanation, since the protein structural changes are generally expected for the late intermediates only. The present observation suggests that the formation of Batho is accompanied by structural changes of most of the internal water molecules of bovine rhodopsin. In fact, the previous FTIR study of rhodopsin mutants demonstrated structural changes of a water molecule close to Gly120 and Asp83 (*26*). According to the X-ray structure, the distances from the candidates (Wat1a and Wat1b) to the nearest atom of the retinal chromophore are 8.7 and 11.5 Å, respectively. Thus, the formation of Batho may involve some protein structural changes far from the retinal. In addition, the smaller number of water bands in the late intermediates may suggest that only limited protein structural changes take place there.

[3] Rath et al. showed the presence of the negative band at about 3286 cm^{-1} in the Meta I minus rhodopsin and Meta II minus rhodopsin spectra (*36*), which corresponds to amide A vibration of the α-helix.

The most unexpected and surprising observation in the present study is the lack of water bands under strong hydrogen-bonding conditions (Figures 3 and 4). This is entirely different from the case of archaeal rhodopsins. BR and *p*pR possess an electric quadrupole in the Schiff base region (Figure 1b), and three water molecules participate in a roughly planar pentagonal cluster structure that must stabilize the quadrupole (*10*, *11*). FTIR spectroscopy of BR and *p*pR detected water O−D stretches in the 2400−2100 and 2400−2200 cm^{-1} region, respectively, which correspond to strong hydrogen bonding. Unlike in archaeal rhodopsins, water bands were not observed in the <2400 cm^{-1} region for the photobleaching intermediates as well as for Iso. These results imply that the ion pair state in vertebrate visual rhodopsins is stabilized in a manner different from that for archaeal rhodopsins. It is likely that the ion pair state of the Schiff base and Glu113 does not have its bridging water molecule, as suggested by the recent X-ray crystal structure (*19*).

Figure 1a shows the presence of a water molecule (Wat2b) in a hydrogen-bonding distance (2.5 Å) from a side-chain oxygen of Glu113. If Wat2b forms a strong hydrogen bond with Glu113, the O−D stretch should appear in the <2400 cm^{-1} region. Nevertheless, we did not observe water bands in the region. How is this observation explained? No change in interaction between Wat2b and Glu113 is unlikely, because formation of Meta II is accompanied by the protonation of Glu113 (*29*). Lack of a negative charge must significantly change the interaction. The reason is unclear at present. One possibility is that the oxygen atom of Glu113 interacting with Wat2b is much less negatively charged, because the other oxygen possesses a negative charge. Electrostatic interaction without a bridging water molecule may result in such charge distribution. The second possibility is that Wat2b is not exchangeable for isotope waters because of a strong hydrogen bond, though the Schiff base is easily deuterated. The third possibility is that the local structure of the Schiff base region is somewhat different between ROS and the crystal. Further experimental and theoretical efforts will give an answer.

The new spectra for the X−D stretching vibrations provided information on groups other than water molecules, such as the N−D stretch of the Schiff base and H−D exchangeable amide A vibrations. The hydrogen-bonding strength of the Schiff base is an important marker in the structure−function study of rhodopsins. The frequency difference of the C═N stretch in H_2O and D_2O has been regarded as the marker of hydrogen-bonding strength of the Schiff base, where a large difference corresponds to a strong hydrogen bond (*30*, *31*). The differences in rhodopsin, Iso, Batho, Lumi, and Meta I were reported to be 36, 28, 36, 4, and 22 cm^{-1}, respectively (*27*). Another and more direct probe of the hydrogen-bonding strength is the N−H (N−D) stretch of the Schiff base. The frequencies of the N−D stretch of the Schiff base are tentatively assigned as 2012 and 1967 cm^{-1} for rhodopsin. These values are much lower than those of BR at 2171 and 2123 cm^{-1} (*32*), being consistent with the fact that the hydrogen bond of the Schiff base is stronger in rhodopsin than in BR (*30*). The frequency of Batho is located at the same frequency region, while the spectral feature is somewhat different (Figure 3a). The frequency of Iso is upshifted, which is also consistent with the weakened hydrogen bond (*27*). We also assigned the N−D stretches of Lumi and Meta I tentatively at 2337 and 2262 cm^{-1}, respectively. Thus, hydrogen-bonding alterations of the Schiff base are essentially the same whether deduced from the analysis of the N−D stretch or from the difference of C═NH and C═ND stretches.

The Meta I to Meta II transition is accompanied by the proton transfer from the Schiff base to Glu113, as well as the opening of the cytoplasmic domain to activate transducin (*33*). In archaeal rhodopsins, the bridging water molecule seems to play an essential role for the proton transfer (*12*). In rhodopsin, however, the role of internal water molecules appears to be less important for the proton transfer from the Schiff base to Glu113, because no water bands were observed in the <2400 cm^{-1} region for the Meta II minus rhodopsin spectrum. Thus, the mechanism of proton transfer from the Schiff base to Glu113 in rhodopsin is still an open question. It is known that the extracellular side of the retinal chromophore is covered by an antiparallel β-sheet composed of Arg177−Glu181 and Ser186−Asp190 (*17*), and the present FTIR study observed the H−D exchangeable peptide amide vibrations of a β-sheet (2366 cm^{-1}) only in the Meta II minus rhodopsin spectrum (Figure 4c) but not in the Meta I minus rhodopsin spectrum (Figure 4b). Meta II formation might involve destabilization of the β-sheet structure, so that the Schiff base environment becomes more polar and/or less ordered.

REFERENCES

1. Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1−4.
2. Hofmann, K.-P., and Helmreich, E. J. M. (1996) *Biochim. Biophys. Acta 1286*, 285−322.
3. Shichida, Y., and Imai, H. (1998) *CMLS, Cell. Mol. Life Sci. 54*, 1299−1315.
4. Sakmar, T. P. (1998) *Prog. Nucleic Acid Res. Mol. Biol. 59*, 1−33.
5. Kandori, H., Shichida, Y., and Yoshizawa, T. (2001) *Biochemistry (Moscow) 66*, 1197−1209.
6. Shichida, Y. (1986) *Photobiochem. Photobiophys. 13*, 287−307.
7. Hofmann, K. P. (1986) *Photobiochem. Photobiophys. 13*, 309−327.
8. Zhukovsky, E. A., and Oprian, D. D. (1989) *Science 246*, 928−930.
9. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.
10. Kandori, H. (2000) *Biochim. Biophys. Acta 1460*, 177−191.
11. Furutani, Y., and Kandori, H. (2002) *Mol. Membr. Biol. 19*, 257−265.
12. Tanimoto, T., Furutani, Y., and Kandori, H. (2003) *Biochemistry 42*, 2300−2306.
13. Kandori, H., and Maeda, A. (1995) *Biochemistry 34*, 14220−14229.
14. Nagata, T., Terakita, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1997) *Biochemistry 36*, 6164−6170.
15. Eilers, M., Reeves, P. J., Ying, W., Khorana, H. G., and Smith, S. O. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 487−492.
16. Creemers, A. F. L., Klaassen, C. H. W., Bovee-Geurts, P. H. M., Kelle, R., Kragl, R., Raap, J., De Grip, W. J., Lugtenburg, J., and de Groot, H. J. M. (1999) *Biochemistry 38*, 7195−7199.
17. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745.
18. Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., and Stenkamp, R. E. (2001) *Biochemistry 40*, 7761−7772.
19. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−5987.
20. Kandori, H., and Shichida, Y. (2000) *J. Am. Chem. Soc. 122*, 11745−11746.
21. Kandori, H., Furutani, Y., Shimono, K., Shichida, Y., and Kamo, N. (2001) *Biochemistry 40*, 15693−15698.

22. Kandori, H., Matuoka, S., Shichida, Y., and Yoshizawa, T. (1989) *Photochem. Photobiol. 49*, 181−184.
23. Nishimura, S., Kandori, H., and Maeda, A. (1997) *Photochem. Photobiol. 66*, 796−801.
24. Siebert, F. (1995) *Isr. J. Chem. 35*, 309−323.
25. Hayashi, S., and Ohmine, I. (2000) *J. Phys. Chem. B 104*, 10678−10691.
26. Nagata, T., Terakita, A., Kandori, H., Shichida, Y., and Maeda, A. (1998) *Biochemistry 37*, 17216−17222.
27. Ganter, U. M., Gärtner, W., and Siebert, F. (1988) *Biochemistry 27*, 7480−7488.
28. Krimm, S., and Bandekar, J. (1986) *Adv. Protein Chem. 38*, 183−364.
29. Fahmy, K., Jäger, F., Beck, M., Zvyaga, T. A., Sakmar, T. P., and Siebert, F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10206−10210.
30. Baasov, T., Friedman, N., and Sheves, M. (1987) *Biochemistry 26*, 3210−3217.
31. Rodman-Gilson, H. S., Honig, B. H., Croteau, A., Zarrilli, G., and Nakanishi, K. (1988) *Biophys. J. 53*, 261−269.
32. Kandori, H., Belenky, M., and Herzfeld, J. (2002) *Biochemistry 41*, 6026−6031.
33. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science 274*, 768−770.
34. Luecke, H., Schobert, B., Richter, H.-T., Cartailler, J. P., and Lanyi, J. K. (1999) *J. Mol. Biol. 291*, 899−911.
35. Rath, P., De Grip, W. J., and Rothschild, K. J. (1998) *Biophys. J. 74*, 192−198.
36. Rath, P., Delange, F., De Grip, W. J., and Rothschild, K. J. (1998) *Biochem. J. 329*, 713−717.

BI034592K