Water Structural Changes in Lumirhodopsin, Metarhodopsin I, and Metarhodopsin II upon Photolysis of Bovine Rhodopsin: Analysis by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: Difference Fourier transform infrared spectra of lumirhodopsin, metarhodopsin I, and metarhodopsin II versus rhodopsin were recorded with hydrated films of bovine rod outer segments at 200, 240, and 270 K. In the region between 3700 and 3450 cm^{-1}, the O–H stretching vibrational bands of water were identified by $H_2{}^{18}O$ and ^2H_2O shifts. Lumirhodopsin and metarhodopsin I exhibit almost identical spectral shape in this region. The O–H stretching vibration band of water was detected at 3533 cm^{-1} upon formation of lumirhodopsin and metarhodopsin I and at 3641 cm^{-1} upon formation of metarhodopsin II. The results suggest that hydrogen bonding of water molecules in the protein is stronger in lumirhodopsin and metarhodopsin I, intermediates with a protonated Schiff base, than in metarhodopsin II with an unprotonated Schiff base. This is similar to the case of photoreaction of bacteriorhodopsin, in which stronger hydrogen bonding of water is formed in the L intermediate than the M intermediate [Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462–467].

Retinal proteins, which contain retinal bound to a lysine residue of the protein through a protonated Schiff base, convert light energy into chemical signals. The light-induced changes could be elucidated by structural analysis of a series of intermediates distinguished by spectroscopic methods. Fourier transform infrared (FTIR)[1] spectroscopy has proved to be one of the most useful techniques for elucidating changes in the functional residues in this process for bacteriorhodopsin (Braiman & Rothschild, 1988; Kitagawa & Maeda, 1989).

By inspection of FTIR spectra in a frequency region above 3450 cm^{-1}, transient changes in water structure were observed for the L and M intermediates of bacteriorhodopsin. Strong hydrogen bonding of water is formed in the L intermediate, and it disappears upon conversion to the M intermediate with an unprotonated Schiff base (Maeda et al., 1992a). It was suggested that water is involved in a strong interaction with the Schiff base in L, and this induces deprotonation of the Schiff base (Hildebrandt & Stockburger, 1984; Maeda et al., 1991). The latter is obligatory for the subsequent reactions which result in proton uptake.

Absorption of a photon by bovine rhodopsin results in isomerization of the retinal chromophore to form a highly distorted all-trans chromophore in photorhodopsin (Shichida et al., 1984; Kandori et al., 1989; Schoenlein et al., 1991). Its partly relaxed form, bathorhodopsin (Mizukami et al., 1993), induces a change in the opsin–chromophore interaction near the ring portion of the chromophore upon conversion to lumirhodopsin (Okada et al., 1991). Specific interaction of the protonated Schiff base with the protein may be induced in some of the intermediates, leading to deprotonation of the Schiff base with accompanying proton transfer into the protein moiety. The ultimate result of these events is activation of the GTP–GDP exchange reaction in transducin (Hofmann, 1986; Stryer, 1991).

Several lines of evidence for the involvement of water in the photochemical reactions of rhodopsin have been presented (Rafferty & Shichi, 1981; Ganter et al., 1988b). Thus, it appears that photolyzed bovine rhodopsin might experience a reaction pathway involving water molecules similar to that observed for bacteriorhodopsin. The conversion of metarhodopsin I to metarhodopsin II corresponds to the L-to-M conversion of bacteriorhodopsin, since both involve deprotonation of the Schiff base. Also, lumirhodopsin in the bovine rhodopsin system is similar to L: Lumirhodopsin is produced from the bathochromic product, its absorption maximum is shifted slightly to the shorter wavelength side than those of bathochromic products, and it is present over a time range similar to L (Yoshizawa, 1984; Shichida, 1986).

In the present work, we first established the conditions and procedures to obtain authentic FTIR difference spectra of lumirhodopsin, metarhodopsin I, and metarhodopsin II versus rhodopsin, and then analyzed specific changes in the O–H stretching vibrations of water in the 3700–3450-cm^{-1} region upon formation of these intermediates with hydrated films of bovine rhodopsin in the rod outer segments. The results are discussed by comparing them with the corresponding changes in bacteriorhodopsin.

MATERIALS AND METHODS

Bovine rhodopsin in rod outer segments was prepared as described previously (Sasaki et al., 1992). A 40-μL aliquot of the sample (A_{500nm} = 2) in water (pH 7) was dried on a BaF_2 window. The sample hydrated with 0.5 μL of either H_2O, $H_2{}^{18}O$ (97.8 atom %; obtained from MSD Isotopes,

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[1] Abbreviation: FTIR, Fourier transform infrared.

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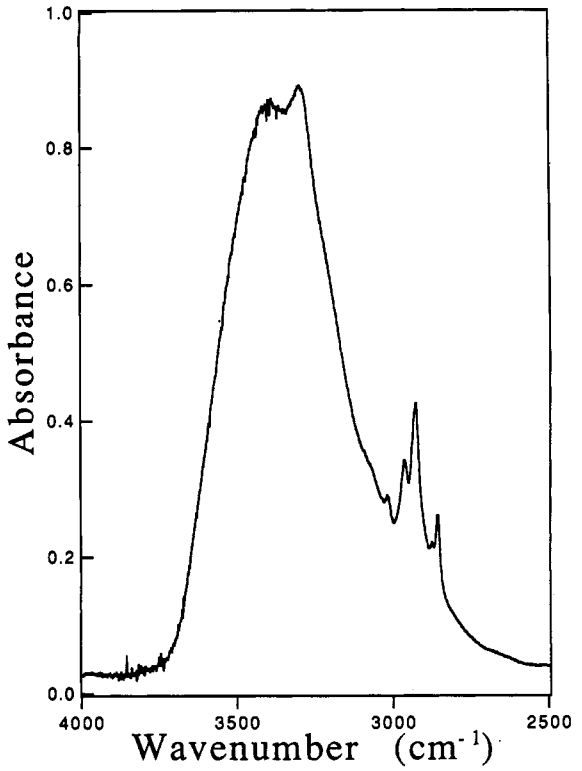

FIGURE 1: Absolute spectra in the 4000–2500-cm^{-1} region of the hydrated film of rod outer segments recorded at 294 K.

Canada) or ^2H_2O (>99.75%; Wako Chemicals, Japan) was mounted in an Oxford CF1204 cryostat as described previously (Maeda et al., 1992a). The temperature was maintained in the range of 0.1 K with an Oxford ITC-4 temperature controller. Liquid nitrogen was used as a coolant.

Absorption spectra in the visible region were recorded in a Shimadzu MPS-2000 recording spectrophotometer. FTIR spectra were recorded by collecting 512 double-sided interferograms in a Nicolet SX60 spectrometer with 2-cm^{-1} resolution. Difference FTIR spectra were calculated as differences between spectra taken after and before irradiation of the sample.

The water content was estimated to be about 50% from the absolute absorption spectrum recorded at 294 K (Figure 1) by use of the molar extinction of water of about 100 at 3500 cm^{-1} (Glew & Rath, 1971; Braiman et al., 1987).

The light source for irradiation was a 1-kW halogen–tungsten lamp in a slide projector. The wavelength was selected by passing a cutoff filter of Toshiba VR58 (>560 nm) or Y52 (>500 nm), or a 501-nm interference filter. Infrared recordings were repeated 8–10 times after changing the sample for each recording.

RESULTS

Photoreactions at Low Temperature by Means of Visible Spectroscopy. Before the FTIR spectra were recorded, we examined the photochemical reactions of rhodopsin of the hydrated film of bovine rod outer segments by visible absorption spectroscopy. Bathorhodopsin produced by irradiation of rhodopsin with 501-nm light at 77 K was warmed gradually (rate of increase about 2 K/min), and the spectra were recorded at an interval of 10 K. The successive conversions of bathorhodopsin to lumirhodopsin, metarhodopsin I, and metarhodopsin II took place above 130, 220, and 260 K, respectively (not shown in figures). The spectra of these intermediates and the temperatures for the conversions

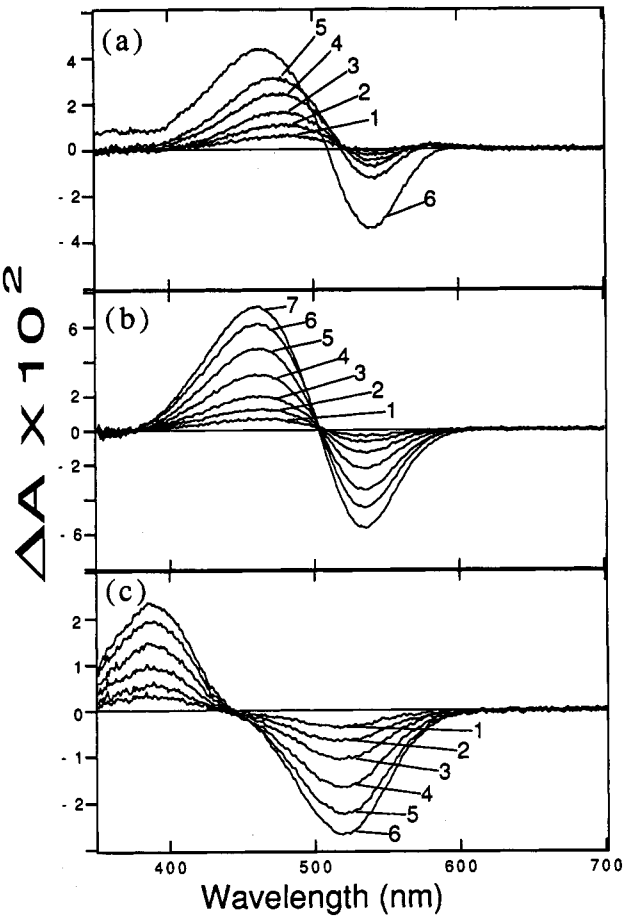

FIGURE 2: Changes in the difference absorption spectra in the visible region after irradiation of rhodopsin in a hydrated film with >560-nm light (a) at 200 K for 2, 4, 8, 16, and 32 s (curves 1–5) and 2 min (curve 6); (b) at 240 K for 2, 4, 8, 16, and 32 s (curves 1–5) and 1 and 4 min (curves 6 and 7); and (c) at 270 K for 2, 4, 8, 16, and 32 s (curves 1–5) and 1 min (curve 6). The absorbance at 500 nm before the irradiation was 0.16, 0.25, and 0.06 absorbance unit for (a), (b), and (c), respectively.

between them were identical to those for the digitonin solution (Yoshizawa, 1984). For measurements of the difference between the FTIR spectra of the intermediates vs rhodopsin, however, the conversion between intermediates by changing temperature was unsuited, because it caused large base-line distortions. Therefore, the hydrated film of rhodopsin was irradiated at a fixed temperature at which lumirhodopsin, metarhodopsin I, or metarhodopsin II is stable. It is known that irradiation of rhodopsin at these temperatures causes formation of 7-*cis*- and 9-*cis*-retinal pigments also (Maeda et al., 1978). Therefore, the contribution of these pigments was checked in both the visible and FTIR spectra.

Lumirhodopsin. Rhodopsin in the hydrated film was irradiated with >560-nm light at 200 K where lumirhodopsin was stable but bathorhodopsin was unstable. The difference spectra upon irradiation up to 32 s (curves 1–5 in Figure 2a) formed an isosbestic point at 523 nm. Further irradiation for a total of 2 min (curve 6) moved the curve intersection point slightly to the shorter wavelength side. This is due to the formation of isorhodopsin as previously shown by Maeda et al. (1978).

The corresponding FTIR spectra were recorded as differences between after and before irradiation at 200 K for 30 s (Figure 3a), 2 min (Figure 3b), and 8 min (Figure 3c). While the intensity increased from 30 s to 2 min, the spectrum with 2-min irradiation (Figure 3b) exhibited the same shape as the

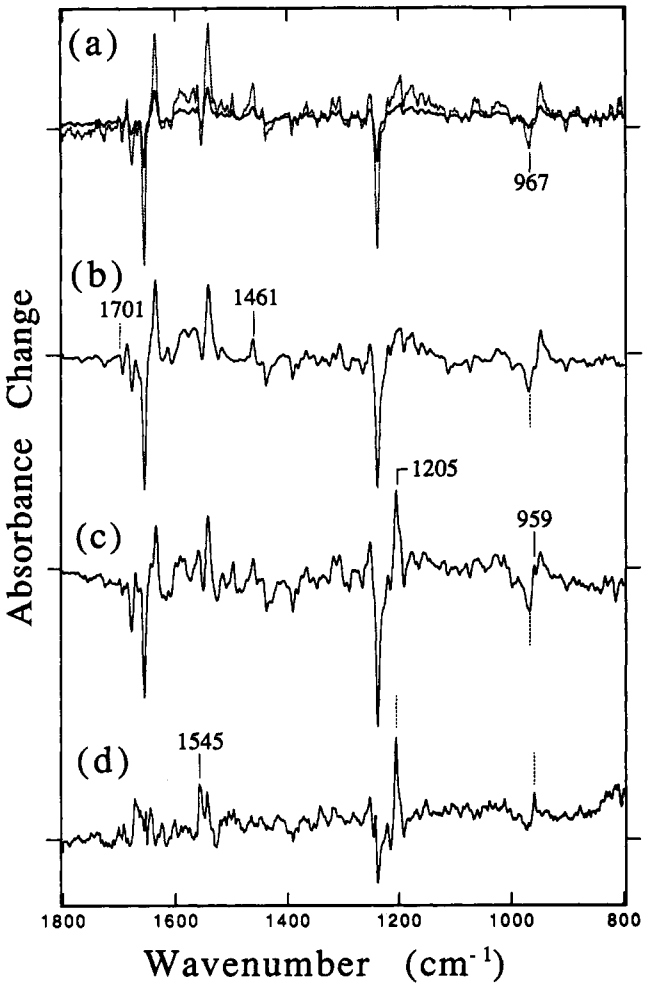

FIGURE 3: Difference FTIR spectra of the products vs rhodopsin in the 1800–800-cm^{-1} region upon irradiation at 200 K with >560-nm light for 30 s (a), 2 min (b), and 8 min (c). The spectrum shown by the dotted line in (a) was depicted after multiplication by a factor of 3 to adjust the intensities to the same amplitude as those of the spectrum in (b) to make comparison between these spectra easier. (d) is the spectrum obtained by subtraction of (a) from (c) by a factor of 2.7 to adjust the intensity of the 967-cm^{-1} band. The vertical dotted lines in (b) and (c) show the same frequency as the corresponding band in (a), and those in (d) show the same frequencies as the corresponding bands in (c). The full height of the vertical axis is 0.040 absorbance unit. (b) is the lumi/rho spectrum in H_2O in this paper. The label of 1701 with a vertical dotted line indicates the absence of a metarhodopsin I band at 1701 cm^{-1}.

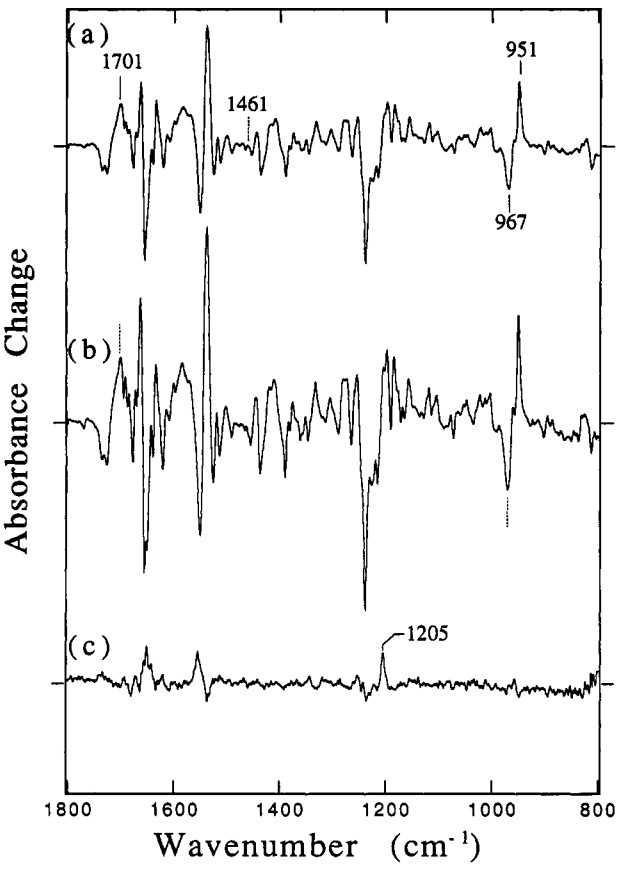

FIGURE 4: Difference FTIR spectra of the products vs rhodopsin in the 1800–800-cm^{-1} region upon irradiation at 240 K with >560-nm light for 1 min (a) and 4 min (b). (c) is the difference spectrum of the products by further irradiation with >500-nm light for 1 min vs the photolyzed mixture after 4-min irradiation with >560-nm light (b). The vertical dotted line in (b) shows the corresponding band in (a). The full height of the vertical axis is 0.022 absorbance unit. (b) is the metaI/rho spectrum in H_2O in this paper. The label of 1461 with a vertical dotted line indicates the absence of a lumirhodopsin band at 1461 cm^{-1}.

spectrum with 30-s irradiation (dotted line in Figure 3a). Further irradiation for 8 min induced additional bands at 1205 and 959 cm^{-1} (Figure 3c) without appreciable changes in the amplitude of the rest. The spectrum of the 30-s irradiation (Figure 3a) was subtracted from that of the 8-min irradiation (Figure 3c) by scaling to the 967-cm^{-1} band. The spectrum thus obtained shows positive bands at 1545, 1205, and 959 cm^{-1} (Figure 3d), all of which are characteristic of the difference spectrum of isorhodopsin vs rhodopsin (Sasaki et al., 1992; Siebert et al., 1983). These bands of isorhodopsin were almost invisible in the 2-min spectrum (Figure 3b). The inconsistency of the irradiation times before the appearance of isorhodopsin between the visible and FTIR measurements might be due to the different rates of the photoreaction induced by the difference in light intensity at the site of the sample in the two different instruments.

Thus, the first two FTIR spectra (Figure 3a,b) refer to pure lumi/rho, and the third (Figure 3c) contains the contribution of isorhodopsin. Irradiation with >560-nm light for 2 min at 200 K will thus be used as the conditions for obtaining the lumi/rho spectrum (Figure 3b). The lack of contribution of metarhodopsin I was assured by the absence of its characteristic band at 1701 cm^{-1} (see Figure 4a).

Metarhodopsin I. Metarhodopsin I was produced by irradiation with >560-nm light at 240 K where lumirhodopsin was unstable. The difference spectra in the photoreaction up to 4 min (Figure 2b) show a positive peak at 460 nm and a negative peak at 534 nm, and a fixed curve intersection point at 506 nm.

The corresponding difference FTIR spectra are shown in Figure 4. The spectral shape by 4-min irradiation (Figure 4b) is identical with that by 1-min irradiation (Figure 4a), indicating that isorhodopsin is not produced under these irradiation conditions. An additional 1205-cm^{-1} band due to isorhodopsin appeared only upon further irradiation with >500-nm light for 1 min (Figure 4c). Through these spectra, the lack of a contribution of lumirhodopsin was assured by the absence of the 1461-cm^{-1} band which was characteristic of lumirhodopsin (Figure 3b). Thus, the spectrum obtained by irradiation with >560-nm light for 1 min at 240 K will be referred to as the metaI/rho spectrum (Figure 4a).

Metarhodopsin II. Visible spectral analysis of the warmed products of bathorhodopsin showed that metarhodopsin II appeared above 260 K. It decayed gradually and converted to metarhodopsin III above 280 K. The half-time for the decay of metarhodopsin II at 294 K was about 5 min for the

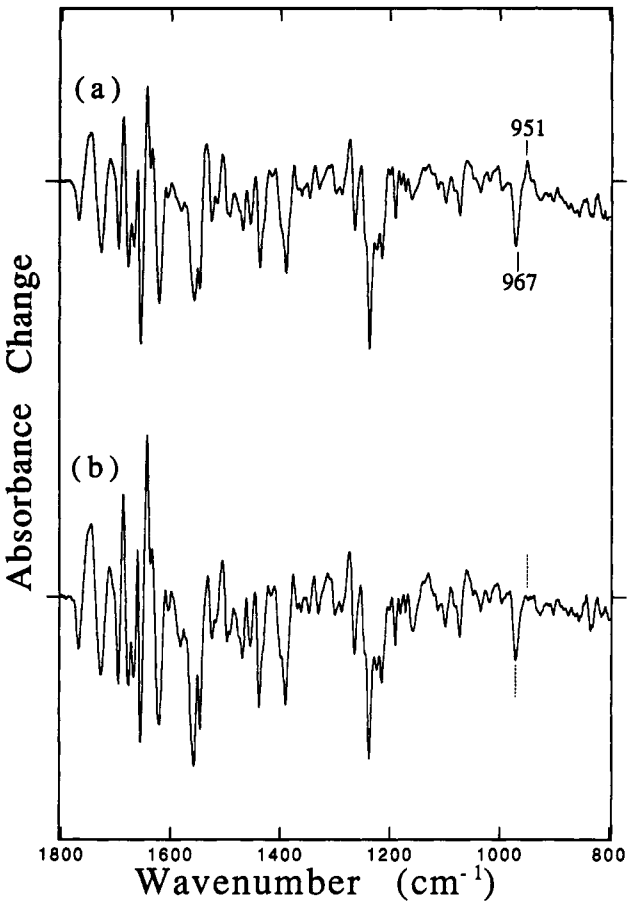

FIGURE 5: Difference FTIR spectra in the 1800–800-cm^{-1} region before and after irradiation of rhodopsin at 270 K with >560-nm light for 1 min (a). (b) is the metaII/rho spectrum obtained by subtraction of the metaI/rho spectrum in Figure 3a by adjusting the amplitude of the 967-cm^{-1} band. The vertical dotted lines in (b) show the same frequency as the corresponding band in (a). The full height of the vertical axis is 0.020 absorbance unit.

film of rod outer segments. This value agrees with those for suspensions under the same conditions (Benett et al., 1982; Bornancin et al., 1989; Kibelbek et al. 1991).

The absorption increase at 380 nm upon irradiation with >560-nm light at 270 K for up to 1 min is due to the formation of metarhodopsin II (Figure 2c). Flattening around 460 nm (Figure 2c) might be caused by the metarhodopsin I band which partially canceled the depletion of the rhodopsin band, and no formation of metarhodopsin III was observed.

The difference FTIR spectrum upon 1-min irradiation is shown in Figure 5a. The 951-cm^{-1} band was characteristic of metarhodopsin I (see Figure 4) because it was not found for metarhodopsin II in the spectrum at 294 K (data not shown in figures), or at 270 K with rhodopsin at pH 5.5 (Ganter et al., 1989). By subtraction of the metaI/rho spectrum in Figure 4a, a metaII/rho spectrum (Figure 5b) was obtained in a shape which is almost identical with the corresponding spectrum at room temperature (Klinger & Braiman, 1992; our data not shown in figures).

Stretching Vibrational Bands at 3700–3450 cm^{-1}. The lumi/rho, metaI/rho, and metaII/rho spectra at 3700–3450 cm^{-1} are shown in panels I, II, and III, respectively, of Figure 6. For assignment of the O–H stretching vibrational mode of water and protein residues, the film was hydrated with H_2O (a), $H_2{}^{18}O$ (b), and ^2H_2O (c), respectively. All the FTIR spectra in each figure can be compared on the basis of the same molar amount of the photoproduct by adjusting the

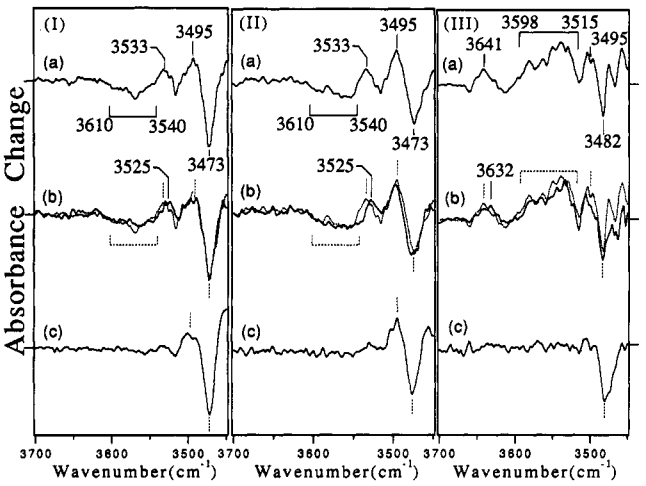

FIGURE 6: Lumi/rho (I), metaI/rho (II), and metaII/rho (III) spectra in the 3700–3450-cm^{-1} region for rhodopsin samples humidified with H_2O (a), $H_2{}^{18}O$ (b), and ^2H_2O (c). In order to show the shifts by $H_2{}^{18}O$ more clearly, the spectrum in H_2O (a) is superimposed with dotted lines on the spectrum in $H_2{}^{18}O$ (b). The amplitudes were adjusted by scaling the 967-cm^{-1} band. The vertical dotted lines in (b) and (c) show the same frequency as the corresponding bands in (a). The full height of the vertical axis is 0.013 absorbance unit for (Ia), with the others almost the same.

intensity of the hydrogen out-of-plane vibrational band of rhodopsin at 967 cm^{-1} (see Figures 3–5), which is unaffected by ^2H_2O substitution (not shown in figures).

The positive band at 3533 cm^{-1} of the lumi/rho spectrum (Figure 6Ia) shifts by about 8 cm^{-1} upon $H_2{}^{18}O$ exchange (Figure 6Ib). This band was assigned to water. The broad negative feature at 3610–3540 cm^{-1} shows no clear shift upon $H_2{}^{18}O$ exchange (Figure 6Ib) but disappears upon ^2H_2O substitution (Figure 6Ic). A negative band at 3473 cm^{-1} and a positive band at 3495 cm^{-1} remain unaffected in ^2H_2O (Figure 6Ic).

The metaI/rho spectra (Figure 6II) are very similar to the lumi/rho spectra (Figure 6I) in their general shape. The positive band at 3533 cm^{-1} (Figure 6IIa), which shifts to 3525 cm^{-1} upon $H_2{}^{18}O$ substitution (Figure 6IIb), was clearly assigned to water. A broad negative feature at 3610–3540 cm^{-1} (Figure 6IIa), which shows no appreciable shift upon $H_2{}^{18}O$ substitution (Figure 6IIb), disappears upon ^2H_2O exchange (Figure 6IIc). A positive band at 3495 cm^{-1} and a negative band at 3473 cm^{-1} (Figure 6IIa) remain unchanged upon ^2H_2O substitution (Figure 6IIc). However, these bands in the metaI/rho spectra (Figure 6II) are different in some respects from the corresponding bands in the lumi/rho spectra (Figure 6I). The metaI/rho spectrum is more intense in the positive band and less intense in the negative band than the lumi/rho spectrum.

The metaII/rho spectra (Figure 6III) show completely different features from those of the lumi/rho and metaI/rho spectra (Figure 6I,II). A positive band at 3641 cm^{-1} (Figure 6IIIa) undergoes a $H_2{}^{18}O$ shift to 3632 cm^{-1} (Figure 6IIIb) and disappears upon ^2H_2O exchange (Figure 6IIIc). This band was thus assigned to water. A broad positive feature between 3598 and 3515 cm^{-1} (Figure 6IIIa) is sensitive to ^2H_2O exchange (Figure 6IIIc) but not to $H_2{}^{18}O$ (Figure 6IIIb). A negative band at 3482 cm^{-1} (Figure 6IIIa) which is insensitive to ^2H_2O substitution (Figure 6IIIc) corresponds to the negative band at 3473 cm^{-1} in the lumi/rho (Figure 6Ia) and metaI/rho (Figure 6IIa) spectra. The positive band at 3495 cm^{-1} (Figure 6IIIa) also may correspond to the positive band at 3495 cm^{-1} in the lumi/rho (Figure 6Ia) and metaI/

rho (Figure 6IIa) spectra, though the band of metarhodopsin II is sensitive to ^2H_2O exchange (Figure 6IIIc).

DISCUSSION

The lumi/rho, metaI/rho, and metaII/rho FTIR spectra of bovine rhodopsin were recorded by irradiation with >560-nm light at 200, 240, and 270 K, respectively. The contribution of isorhodopsin in view of the obvious presence in the previously published lumi/rho and metaI/rho spectra (de Grip et al., 1988; Ganter et al., 1989) was eliminated by carefully controlling the conditions for irradiation. By $H_2{}^{18}O$ substitution, the 3700–3450-cm^{-1} region of these refined spectra was examined to assign the O–H stretching vibration bands of water, as done previously for bacteriorhodopsin (Maeda et al., 1992a).

There is either one or only a few molecules of water involved in the reaction of rhodopsin, on the basis of the observed absorbance change of 0.0003 at 3641 cm^{-1} compared with the molar extinction of about 100 for water at a similar frequency (Glew & Rath, 1971). Thus, the water molecule detected in the FTIR difference spectrum may be an isolated one in the interior of the protein and does not constitute a cluster on the protein surface (Teeter, 1991).

The vibrational band of water appearing in the region between 3700 and 3450 cm^{-1} is the higher frequency band in the two O–H stretching modes. If the two O–H bonds of water form hydrogen bonds, the one at the higher frequency side is an antisymmetric stretching vibration. If only one of its two O–H bonds forms hydrogen bonds, the vibration of the O–H bond without hydrogen bonding (Monosmith & Walrafen, 1984) is higher in frequency. Mohr et al. (1965) have examined the O–H stretching vibration frequencies of water interacting with various hydrogen-bonding acceptors in carbon tetrachloride, for both one and two bonding complexes.

The water band for metarhodopsin II observed at 3641 cm^{-1} is close to the antisymmetric vibrational band at 3640 cm^{-1} of water when its two O–H bonds interact with a weak base like ethyl acetate (Mohr et al., 1965). These bands are also located close in frequency to the vibrational band at 3650 cm^{-1} of the unbonded O–H in liquid water (Walrafen & Fisher, 1986). Thus, at least one of the O–H bonds of the water molecule in metarhodopsin II makes no interaction or interacts weakly. A similar observation was made for the M intermediate of bacteriorhodopsin (Maeda et al., 1992a).

The 3533-cm^{-1} band of either lumirhodopsin or metarhodopsin I is located at a frequency close to the 3550-cm^{-1} band of the antisymmetric O–H stretching vibration of water which interacts with a stronger base like *N*,*N*′-dimethylformamide in carbon tetrachloride (Mohr et al., 1965) or to the 3540-cm^{-1} band of the same mode of the fully hydrated tetrahedral water (Walrafen & Fisher, 1986). Such a low-frequency value was not found for the unbonded O–H of the one-bonding water complex. Thus, the O–H bond of a specific water molecule in lumirhodopsin or metarhodopsin I makes stronger hydrogen bonding. The situation is very similar to the L intermediate of bacteriorhodopsin (Maeda et al., 1992a).

Negative water bands originating from unphotolyzed rhodopsin are not clearly observed in the region above 3450 cm^{-1} (Figure 6). A small $H_2{}^{18}O$ shift (about 10 cm^{-1}) would be obscured under broad bands in the 3610–3540-cm^{-1} region for the lumi/rho and metaI/rho spectra and in the 3598–3515-cm^{-1} region for the metaII/rho spectrum. Water bands with strong hydrogen bonding would also be present in the region below 3450 cm^{-1} (not shown in figures), because water in ice is known to exhibit the antisymmetric stretching vibration at 3300 cm^{-1} (Glew & Rath, 1971). The detection of possible water bands in the region between 3450 and 3300 cm^{-1} would be disturbed by many other bands. In any event, further intensive studies are required to attain a clear conclusion for the negative bands. At the present stage, we have discussed only the water bands of the photolyzed intermediates in the positive side, which was clearly distinguishable.

The results for the light-induced intermediates of both rhodopsin and bacteriorhodopsin could be converged into a notion that water exhibits a stronger hydrogen-bonding interaction in intermediates with a protonated Schiff base than in intermediates with an unprotonated Schiff base. Thus, water may be involved in the stabilization of the protonated Schiff base, as was suggested by an *ab initio* molecular orbital method (Beppu et al., 1992) for a model system, or argued for various other reasons (Hildebrandt & Stockburger, 1984; de Groot et al., 1989, 1990; Teeter, 1991; Warshel & Åqvist, 1991; Maeda et al., 1992a).

Though not assigned by means of mutation or isotope substitution, the other vibrational bands in the 3700–3450-cm^{-1} region can be explained on the basis of their similarity to results with bacteriorhodopsin. The O–H stretching protein band between 3610 and 3540 cm^{-1} which disappears upon formation of lumirhodopsin and metarhodopsin I may be due to the O–H stretching vibration of carboxylic acid by the analogy of *all-trans*-bacteriorhodopsin (Maeda et al., 1992b). These frequencies are close to 3565 cm^{-1} for gaseous acetic acid (Haurie & Novak, 1965), indicating very weak interaction in rhodopsin.

The 3495-cm^{-1} band of lumirhodopsin and metarhodopsin I and the negative band at 3473 cm^{-1} of rhodopsin are probably due to N–H stretching vibrations. For bacteriorhodopsin, the 3486-cm^{-1} band in the L intermediate was assigned to the indole N–H stretching vibration without hydrogen bonding (Maeda et al., 1992c). Since the frequency of the N–H stretching vibration decreases with an increase in H-bonding strength (Fuson et al., 1952), these results suggest that hydrogen bonding of an N–H bond in rhodopsin is weakened upon conversion to lumirhodopsin and metarhodopsin I. In the unphotolyzed rhodopsin, lumirhodopsin, and metarhodopsin I, the tentatively assigned N–H bond is insensitive to ^2H_2O exchange, while it becomes sensitive to metarhodopsin II. This means that this N–H becomes accessible to water upon metarhodopsin II formation. These protein residues must be identified by using mutated proteins in future studies.

The FTIR spectrum of lumirhodopsin in the 3700–3450-cm^{-1} region is very similar to that of metarhodopsin I. Nevertheless, lumirhodopsin is different from metarhodopsin I, in view of various structural parameters, as revealed by the FTIR spectrum in the 1800–800-cm^{-1} region (see Figures 3–5) (de Grip et al., 1988; Ganter et al., 1988a, 1989), and an environment difference of the 9-methyl group of the retinal moiety, as revealed by visible spectral analysis of the photoproducts of 7-*cis*-rhodopsin (Shichida et al., 1991).

The present studies show that in the photoreaction process of bovine rhodopsin, water in the interior of the protein enters into a hydrogen-bonding system upon formation of lumirhodopsin and metarhodopsin I, and becomes released from it upon subsequent deprotonation of the Schiff base. These events are similar to those occurring in the L-to-M conversion of bacteriorhodopsin, and confirm the involvement of water in the deprotonation process of the Schiff base.

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