Structural Dynamics of Water and the Peptide Backbone around the Schiff Base Associated with the Light-Activated Process of Octopus Rhodopsin†

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ABSTRACT: Difference Fourier transform infrared spectra were recorded for the formation of the photointermediates and isorhodopsin from octopus rhodopsin at low temperatures. Analysis was done for H bonding of the Schiff base, internal water molecules, and the peptide backbone. The imine hydrogen of the Schiff base was in the same H bonding state throughout the photointermediates and the unphotolyzed state. In contrast, H bonding of the hydrogen of the water molecule whose oxygen might be complexed with the imine hydrogen of the Schiff base was altered upon the formation of bathorhodopsin. The same water molecule was in a different H bonding state in the subsequent intermediates, lumirhodopsin and mesorhodopsin. These intermediates were also characterized by a decrease in the C═N bond order of the Schiff base as a reflection of distorted structure around the Schiff base. The polar N−H bond in these intermediates could be also ascribed to the Schiff base. Some changes in H bonding of water and the perturbation of the polyene chain in lumirhodopsin and mesorhodopsin were also observed in isorhodopsin. Acid metarhodopsin exhibited extensive changes in the H bonding states of the peptide backbone and internal water molecules. A large part of these changes was extinguished in alkaline metarhodopsin with the unprotonated Schiff base, suggesting interaction of the protonated Schiff base with the peptide backbone and intramembrane water molecules in acid metarhodopsin.

Octopus rhodopsin is present in the microvillar membranes of the photoreceptor cells (Tsuda, 1987). It contains 11-*cis*-retinal bound to the lysine residue via a protonated Schiff base as the chromophore, which undergoes the isomerization to the *all-trans* form upon illumination. Photointermediates called bathorhodopsin (Batho),[1] lumirhodopsin (Lumi), mesorhodopsin (Meso), and acid metarhodopsin (acid-Meta) (Tsuda, 1979; Cooper et al., 1986) form consecutively afterward. In contrast to vertebrate rhodopsin, the final photoproduct, acid-Meta, has the protonated Schiff base at physiological pH, and the deprotonation of the Schiff base does not occur in the light-induced process in a physiological pH range. Acid-Meta is in a pH-dependent equilibrium with alkaline metarhodopsin (alkali-Meta) with the unprotonated Schiff base (Kitagawa & Tsuda, 1980; Koutalos et al., 1990). Acid-Meta would be responsible for the activation of G-protein as in other invertebrate rhodopsins (Terakita et al., 1993; Kiselev & Subramaniam, 1994; Suzuki et al., 1995).

The protonated Schiff base of bovine rhodopsin is electrostatically interacting with the negatively charged Glu-113 (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). It is replaced by Tyr-112 in octopus rhodopsin (Ovchinnikov et al., 1988). Tyrosine at this position is conserved throughout invertebrate rhodopsins with an exception for phenylalanine in place of tyrosine in *Drosophila* UV receptors (Fryxell & Meyerowitz, 1987; Montel et al., 1987). A recent UV-Raman study (Hashimoto et al., 1996) has indicated the absence of a tyrosinate residue in octopus rhodopsin. The lack of interaction with the negative charge would result in a decrease in the pK_a value of the Schiff base. The titration experiments with octopus rhodopsin indicate that its pK_a value is about 10.65 in 4.0 M KCl (Koutalos et al., 1990). This value is much lower than >16 estimated for bovine rhodopsin (Steinberg et al., 1993) but still greater than the pK_a value of the protonated Schiff base in solution (∼7.0).

The involvement of water molecules for the pK_a increase of the Schiff base has been suggested in model studies (Gat & Sheves, 1993; Sampogna & Honig, 1994). Indeed, water molecules are suggested to be present close to the Schiff base for bovine rhodopsin (Deng et al., 1994). Structural changes of intramembrane water molecules upon formation of the photointermediates of bovine rhodopsin have been detected by Fourier transform infrared (FTIR) spectroscopy as changes in frequency of the O−H stretching vibrations (Maeda et al., 1993; Kandori & Maeda, 1995). Extensive depletion of water from bovine rhodopsin results in the deprotonation of the Schiff base (Rafferty & Shichi, 1981). Relaxation of distortion close to the Schiff base in conversion from bovine lumirhodopsin to metarhodopsin I (Ohkita et al., 1995) is blocked even by moderate depletion of water (Nishimura et al., 1995). Changes in peptide carbonyls (amide I) were also detected upon formation of metarhodop-

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[1] Abbreviations: Rho, rhodopsin; Batho, bathorhodopsin; Lumi, lumirhodopsin; Meso, mesorhodopsin; acid-Meta, acid metarhodopsin; alkali-Meta, alkaline metarhodopsin; Iso, isorhodopsin (these were used only for octopus rhodopsin); FTIR, Fourier transform infrared; HOOP, hydrogen-out-of-plane.

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sin II of bovine rhodopsin (Klinger & Braiman, 1992; Maeda et al., 1993). Peptide carbonyl groups close to the Schiff base are involved in the structural change in the photointermediates of bacteriorhodopsin (Takei et al., 1994; Yamazaki et al., 1996). The delocalized charges in water and peptide bonds are thus supposed to stabilize the protonated Schiff base in octopus rhodopsin and its final product, acid-Meta.

In the present study, we recorded difference FTIR spectra of the photochemical intermediates of octopus rhodopsin at temperatures where they are stable. The spectrum of alkali-Meta with unprotonated Schiff base was also recorded. Structural changes in intramembrane water molecules and peptide backbone were examined for all the intermediates in the process from Rho to acid-Meta.

MATERIALS AND METHODS

Octopus (Mizudako, *Paroctopus defleini*) microvillar membranes were purified as described previously (Tsuda, 1979). The rhodopsin content in the total membrane proteins in the purified microvillar membranes was estimated to be more than 90%, judging from the SDS−PAGE analysis (not shown). The hydrated film was prepared by placing a 50 μL aliquot of the suspensions of microvillar membranes ($A_{476nm} = 2.0$) in 5.7 mM MOPS (pH 7.4) or 5 mM borate buffer containing 5 mM NaCl (pH 10) on a BaF_2 window and dried under room air in the dark. The alkaline sample was dried under vacuum just before use. About 1 μL of H_2O, D_2O, or $H_2{}^{18}O$ was put aside for the air-dried film for the hydration. The film was sealed by another window and mounted in an Oxford cryostat, DN1704 and CF1204 models for FTIR and visible measurements, respectively. The temperature of the sample was controlled in a 0.1 K range with an Oxford temperature controller (ITC4).

Visible spectra were recorded in a Shimadzu MPS-2000 recording spectrophotometer. FTIR spectra were recorded in a Bio-Rad FTS-40 spectrometer. One hundred twenty-eight interferograms at 2 cm^{-1} resolution were recorded in about 85 s except for alkali-Meta and converted to an absorption spectrum. In the case of alkali-Meta, the spectrum was obtained from 64 interferograms with 8 measurements. The light source was a 1 kW halogen−tungsten lamp. The wavelength for the illumination was selected by Toshiba filters: a KL47 interference filter (maximum at 471 nm with a full width at half-maximum of 15 nm), a KL40 interference filter (the maximum at 401 nm with a full width at half-maximum of 17 nm), an R61 cutoff filter ($T_{1/2}$ at 613 nm and $T_{1/100}$ at 590 nm), an R62 cutoff filter ($T_{1/2}$ at 622 nm and $T_{1/100}$ at 600 nm), a Y52 cutoff filter ($T_{1/2}$ at 520 nm and $T_{1/100}$ at 499 nm), and a band filter V44 (the maximum at 430 nm with the a full width at half-maxima of 114 nm).

Illumination for recordings of the FTIR spectra on the formation of isorhodopsin (Iso) and Batho at 80 K was done in principle by the same procedures as those used for bovine rhodopsin (Kandori & Maeda, 1995) but taking into consideration the difference in the λ_{max} value of octopus rhodopsin at 476 nm from that of bovine rhodopsin at 498 nm, namely (1) illumination with 471 nm light for 20 s for the conversion from rhodopsin (Rho) to Batho, (2) illumination with 471 nm light for 60 s for the conversion from Iso to Batho, (3) illumination with >590 nm light for 30 s for the conversion from Batho to Rho, and (4) illumination with >499 nm light for 30 s for the conversion from Batho to Iso. The cycle was repeated, and 36−40 recordings were averaged. These conditions, however, did not provide complete conversions between the photoproducts. Batho produced by procedure 1 was accompanied by 21% of Iso as estimated from the intensity of an Iso-specific band at 957 cm^{-1}, and Iso by procedure 4 was accompanied by 3% of Rho from the intensity of a Rho-specific band at 973−970 cm^{-1}. The pure Batho minus Rho and Iso minus Rho spectra were then calculated by subtracting the contribution of the other spectra. The Iso minus Rho spectrum was calculated from Batho minus Rho and Iso minus Rho spectra by canceling the Batho-specific band at 942 cm^{-1}.

For the conversion from Rho to Lumi and Meso, the hydrated film was illuminated with 471 nm light for 20 s at 168 and 215 K, respectively. The spectra presented were averages of four or five recordings with the films without prior illumination. The absence of Iso in the photoproducts was assured by the absence of the 1322 cm^{-1} band characteristic of Iso (see below, Figure 2). The acid-Meta minus Rho spectrum was obtained by illumination with 471 nm light for 20 s at 280 K. About 50% Rho was regenerated by >600 nm light for 4 min. The cycle was repeated, and 10 recordings were averaged. Any Iso with the 957 cm^{-1} band was not observed.

All the difference FTIR spectra were presented by subtracting the base line fluctuations, which were recorded in the same way without the illumination as was done for the photoreaction.

RESULTS

Photochemical Intermediates at Low Temperatures. The hydrated rhodopsin film was illuminated at 80 K with light passed from the band filter V44 for 5 s. Changes in λ_{max} values were examined by warming the photoproducts (Figure 1a). The λ_{max} at 505 nm at 80 K is a weighted average of that of Batho (λ_{max} at 540 nm) and Rho (λ_{max} at 486 nm at 80 K[2]) with the ratio produced depending on the particular illumination conditions. An increase in the temperature of these photoproducts to the 160−170 and 210−220 K regions resulted in blue-shifted states containing Lumi and Meso with apparent λ_{max} values at 491 and 487 nm, respectively. Figure 1b shows the difference between the two states containing Lumi and Meso in the visible spectra. The state containing Lumi (curve 2) was produced by illumination of Rho (curve 1) at 168 K. The spectrum was shifted to the blue with the formation of Meso upon warming to 215 K (curve 3). It was conserved by recooling to 168 K (curve 4), distinct from that of Lumi at the same temperature (curve 2). These two states correspond to the LM and Lumi intermediates of squid rhodopsin (Tokunaga et al., 1975). Subsequently, acid-Meta was identified in the 270−280 K region with an apparent λ_{max} at 492 nm. This sequence is identical with that previously described (Tsuda, 1979; Kusumi et al., 1983; Cooper et al., 1986).

On the basis of the above results, the Iso minus Rho (a), Batho minus Rho (b), Lumi minus Rho (c), Meso minus Rho

[2] This value is red-shifted by 10 nm to 476 nm at 280 K. A similar effect was observed in bovine rhodopsin (Yoshizawa & Wald, 1963).

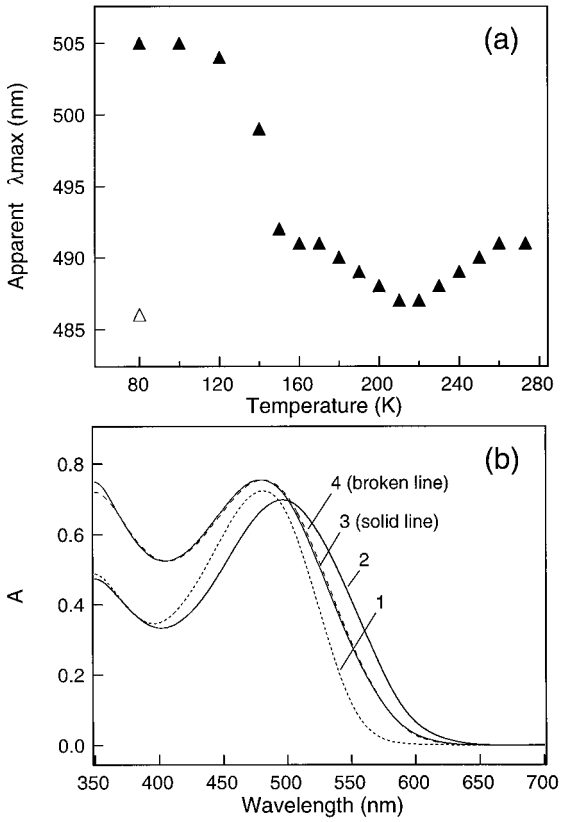

FIGURE 1: (a) Changes in measured λ_{max} values versus temperature of the photoproducts in the course for elevating temperature after illumination of the hydrated film with light passed from the V44 filter for 5 s at 80 K (closed triangles). Notice that each λ_{max} value is determined in the presence of unphotolyzed Rho, which exhibited a λ_{max} of 486 nm at 80 K (an open triangle). (b) The difference in the visible spectra between Lumi and Meso. Curves 1 and 2 are the spectra before and after the illumination, respectively, with light passed from the V44 filter for 5 s at 168 K. Curve 3 was recorded after warming the illuminated film to 215 K and curve 4 after recooling to 168 K.

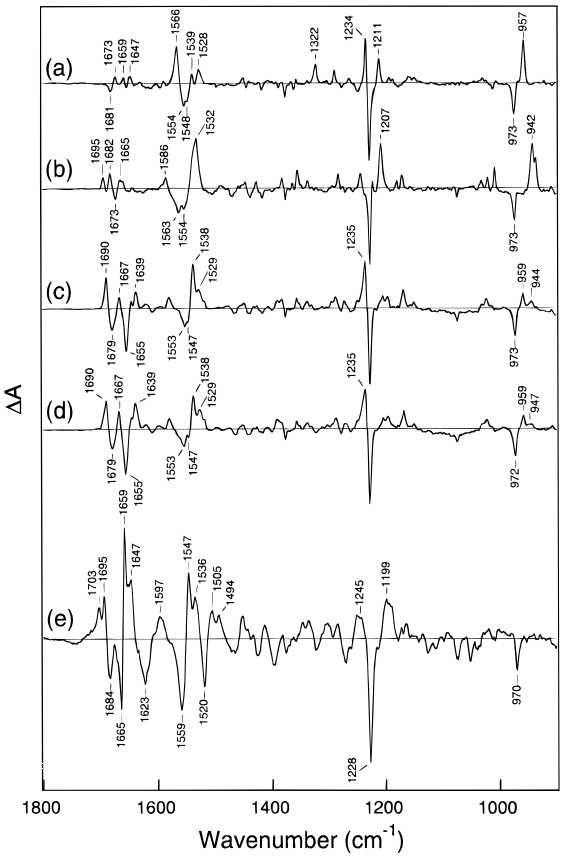

FIGURE 2: Difference FTIR spectra in the 1800−900 cm^{-1} region on the photoconversion of (a) Rho to Iso, (b) Rho to Batho, (c) Rho to Lumi, (d) Rho to Meso, and (e) Rho to acid-Meta. The full height of the ordinate corresponds to 0.035 (a), 0.035 (b), 0.033 (c), 0.029 (d), and 0.012 (e) absorbance unit.

(d), and acid-Meta minus Rho (e) difference FTIR spectra were recorded at 80, 80, 168, 215, and 280 K, respectively, as described in Materials and Methods. The amplitudes of all these spectra were adjusted by the intensity of the negative hydrogen-out-of-plane (HOOP) band of the unphotolyzed chromophore band at 973−970 cm^{-1}. This allows us to compare all the spectra under the same extent of the photoreaction as seen with rhodopsin. The spectra in the 1800−900 (Figure 2), 3450−3200 (Figure 3), and 3720−3490 (Figure 4) cm^{-1} regions are presented below separately. The spectra in the 3720−3490 and 3450−3200 cm^{-1} regions were first recorded. Even the whole 1800−900 cm^{-1} regions for the Lumi minus Rho (c), Meso minus Rho (d), and acid-Meta minus Rho (e) spectra have not been presented previously. On the basis of these spectra, we will discuss below particular features of these intermediates from various aspects.

FTIR Spectra in the 1800−900 cm⁻¹ Region. The 1800−900 cm^{-1} region of the Iso minus Rho (a) and Batho minus Rho (b) spectra at 80 K is identical with those previously calculated on the basis of the isomer ratios in the extracted retinals (Bagley et al., 1989). A previous FTIR difference spectrum after illumination with 420−430 nm light at 210 K (Masuda et al., 1993a), corresponding to the Meso minus Rho spectrum, showed a negative band at 1564 cm^{-1}, which is not present in the present spectrum. This might be due to the depletion of Iso. It would be produced together with Rho by illumination of acid-Meta with >500 nm light, which was converted from the photoproduct at 210 K by warming. The Lumi minus Rho (c) and Meso minus Rho (d) spectra in the present study were always recorded from new samples without any prior illumination to avoid possible side reactions.

The Lumi minus Rho (c) and Meso minus Rho (d) spectra are similar in shape to each other, though with differences in intensity. The bands reflecting the structure of the chromophore of Lumi and Meso, the C_{11}═C_{12} HOOP vibration at 959 cm^{-1} and the C−C stretch at 1235 cm^{-1}, resemble further those of Iso (a), even though the other parts are different from each other. The intense HOOP bands in the infrared spectra have been ascribed to the twisted polyene chain (Rothschild et al., 1984). Only acid-Meta did not display HOOP bands, suggesting the complete relaxation of the twist in the chromophore.

The C═N stretching vibrational band of Rho at 1656 cm^{-1} is common to those of Batho and acid-Meta in resonance Raman spectra (Pande et al., 1987). Hence, it is canceled in the difference FTIR spectra of these intermediates (b and e). The absence of the corresponding band in the Iso minus Rho spectrum (a) further indicates the same frequency of the C═N stretching vibration in Iso. A negative band at 1655 cm^{-1} appears, however, in the Lumi minus Rho (c) and Meso minus Rho (d) spectra with a concomitant positive band at 1639−1638 cm^{-1} as described by Masuda et al. (1993a). These are assigned to the C═N stretching vibra-

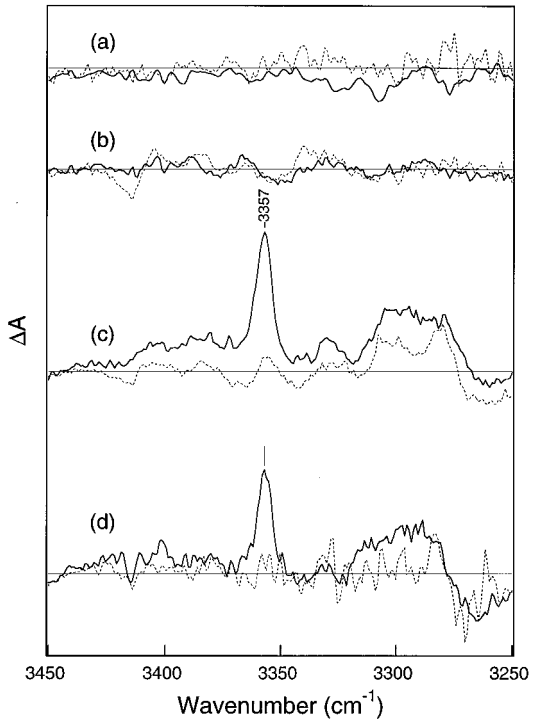

FIGURE 3: Difference FTIR spectra in H_2O (solid lines) and D_2O (dotted lines) in the 3450−3250 cm^{-1} region on the photoconversion of (a) Rho to Iso, (b) Rho to Batho, (c) Rho to Lumi, and (d) Rho to Meso. The full height of the ordinate corresponds to 0.0044 (a), 0.0044 (b), 0.0040 (c), and 0.0035 (d) absorbance unit for the film in H_2O and 0.0025 (a), 0.0025 (b), 0.0028 (c), and 0.0019 (d) absorbance unit for the film in D_2O.

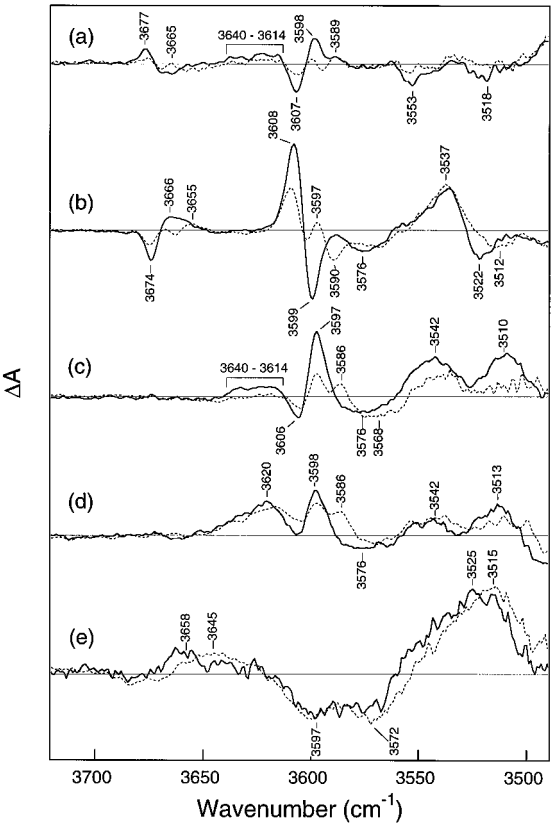

FIGURE 4: Difference FTIR spectra in H_2O (solid lines) and $H_2{}^{18}O$ (dotted lines) in the 3720−3490 cm^{-1} region on the photoconversion of (a) Rho to Iso, (b) Rho to Batho, (c) Rho to Lumi, (d) Rho to Meso, and (e) Rho to acid-Meta. The full height of the ordinate corresponds to 0.0037 (a), 0.0037 (b), 0.0034 (c), 0.0030 (d), and 0.0013 (e) absorbance unit for the film in H_2O and 0.0040 (a), 0.0040 (b), 0.0026 (c), 0.0038 (d), and 0.0038 (e) absorbance unit for the film in $H_2{}^{18}O$.

tions because of their sensitivity to D_2O substitution (not shown). The extent of the shift upon deuteration has been regarded as indicating the H bonding strength of the protonated Schiff base (Rodman-Gilson et al., 1988). This value of 22−24 cm^{-1} for Lumi and Meso is close to 25−26 cm^{-1} for Rho (not shown) and Batho (Deng et al., 1991). Thus, the strength of H bonding of the imine hydrogen of the Schiff base remains almost unchanged throughout the intermediates. The decrease of the intrinsic C═N stretching vibration frequency in D_2O from 1631 cm^{-1} in Batho (Deng et al., 1991) to 1618−1616 cm^{-1} in Lumi and Meso (data not shown) is interesting. A similar value of 1620 cm^{-1} was obtained for Meso in D_2O by Masuda et al. (1993a). Such a low frequency arises from a decrease in the force constant of the C═N bond, probably due to the bond order decreasing.

Another D_2O-insensitive band in the 1700−1600 cm^{-1} region is amide I, mainly due to the peptide carbonyl. The Lumi minus Rho (c) and Meso minus Rho (d) spectra show bands at 1690 (+), 1679 (−), and 1667 (+) cm^{-1} relatively more intense than similar bands in the Batho minus Rho spectrum (b). The acid-Meta minus Rho spectrum exhibits further intense bands in the lower-frequency region at 1659 (+), 1647 (+), and 1623 (−) cm^{-1} besides high-frequency bands at 1695 (+), 1684 (−), and 1665 (−) cm^{-1}.

N−H Stretching Vibration. Figure 3 shows a sharp positive band at 3357 cm^{-1} in Lumi (c) and Meso (d) due to N−H stretching vibrations. The absence of the corresponding negative band indicates that it does not arise from a frequency shift but from an intensity increase. This band is entirely sensitive to D_2O (dotted lines). It is due to the N−H stretching vibrations of either the Schiff base or the peptide amide. It is not seen in Iso (a) and Batho (b). A band at 3361 cm^{-1} in Meta (see Figure 7) is rather broad even if it is due to the same mode.

O−H Stretching Vibration of Water. Figure 4 compares the H_2O spectra (solid lines) in the 3720−3490 cm^{-1} region with those in $H_2{}^{18}O$ (dotted lines). The Batho minus Rho spectrum (b) shows a small depletion at 3674 cm^{-1} with an accompanying broad positive feature at 3666 cm^{-1}. Intensities and frequencies of this band are comparable with that of a similar positive band at 3653 cm^{-1} in the formation of bovine bathorhodopsin (Kandori & Maeda, 1995). There are more intense negative and positive bands at 3599 and 3608 cm^{-1}, respectively. These are characteristic of Batho formation and are not observed in the formation of the later intermediates, Lumi (c), Meso (d), and acid-Meta (e). Those bands contain the O−H stretching vibrations of water because they shifted by about 10 cm^{-1} in $H_2{}^{18}O$. The Iso minus Rho spectrum (a) exhibits a negative band at 3607 cm^{-1} with a sharp positive band at 3598 cm^{-1} and a broad positive feature in the 3640−3614 cm^{-1} range. These are similar to those in the Lumi minus Rho spectrum (c). The Meso minus Rho spectrum (d) is also similar in shape, but the positive band with a peak at 3620 cm^{-1} is more intense, probably due to the depletion of the negative band at 3606 cm^{-1} in the Lumi minus Rho spectrum.

These bands in the Batho minus Rho and Iso minus Rho spectra were well fitted as the difference of two Gaussian curves with the maxima at 3605, 3601, and 3600 cm^{-1} for Batho, Rho, and Iso, respectively (not shown). Thus, the

apparent shifts from 3599 cm^{-1} of Rho to 3608 cm^{-1} of Batho (b) and from 3607 cm^{-1} of Rho to 3598 cm^{-1} of Iso (c) are actually the shifts from 3601 cm^{-1} of Rho to 3605 cm^{-1} of Batho and 3600 cm^{-1} of Iso, respectively. Intensities of these bands of about 6×10^{-4} absorbance unit correspond to those of the O−H stretching vibration of one or a few water molecules, on the basis of a rough calculation taking the molar extinction of water as about 100 (Glew & Rath, 1971). In other words, one water O−H with the stretching vibration at 3600 cm^{-1} undergoes a frequency shift to 3605 cm^{-1} in Batho and to 3600 cm^{-1} in Iso.

Two positive bands at 3542 and 3510 cm^{-1} in the Lumi minus Rho spectrum (c) are preserved in the Meso minus Rho (d) spectrum. The acid-Meta minus Rho spectrum further exhibits an intense broad positive feature with a center at 3525 cm^{-1}, which may be comprised of the 3542 and 3513 cm^{-1} bands of the preceding intermediates and a new band between them. A negative band at 3576 cm^{-1} arises in the Batho minus Rho spectrum (b). It persists up to the Meso minus Rho spectrum and increases in intensity in the acid-Meta minus Rho spectrum (e). All these bands are clearly shifted toward lower frequencies in $H_2{}^{18}O$ and hence can be assigned to water. The negative band at 3597 cm^{-1} of the acid-Meta minus Rho spectrum seems not to be water, being different from the 3599 cm^{-1} band observed in the Batho minus Rho spectrum.

Deprotonation of the Schiff Base. Since acid-Meta and alkali-Meta are in a pH-dependent equilibrium with respect to the protonated state of the Schiff base, it is informative to compare these spectra. Illumination of octopus rhodopsin in the film at pH 10 (Figure 5a, curve 1) with 471 nm light for 30 s at 280 K produced a blue-shifted peak as well as a red-shifted one (Figure 5a, curve 2) in the visible spectrum. The blue-shifted product is certainly alkali-Meta with the unprotonated Schiff base but did not arise from hydrolysis of the Schiff base because it was reversed to Rho when illuminated with 401 nm light (not shown). The incomplete conversion (about 50%) can be ascribed to a photosteady state. Lognormal fitting procedures (Metzler & Harris, 1978) were applied to the spectrum after the photoreaction (Figure 5b). Subtraction of the contribution of the remaining Rho (curve 3; 26% of curve 1 in a) from the spectrum after the photoreaction (curve 2 in b) yields a blue-shifted product with the λ_{max} at 376 nm (curve 5) and a red-shifted product with the λ_{max} at 514 nm (curve 4). By use of the ratio of the molar extinctions between acid-Meta and alkali-Meta (Koutalos et al., 1990), the molar content of acid-Meta was estimated to be between 13 and 19%, depending on the assumed intensity of the β-band relative to the α-band between 0 and 50%.

A difference FTIR spectrum containing both acid-Meta and alkali-Meta was then recorded at pH 10 after illumination at 280 K with the same method that was used for recording visible spectra (Figure 6a). The pure alkali-Meta minus Rho spectrum was calculated by subtracting the contribution of acid-Meta of 16% as an average between 13 and 19% (solid line in Figure 6b). The overall spectral shape is not so much different from that in Figure 6b, if one calculates with the content of either 13 or 19%. The spectrum in the 1800−900 cm^{-1} region thus obtained was overlaid with the acid-Meta minus Rho spectrum (dotted line; reproduced from Figure 2e). Striking differences were detected in the 1800−1480 cm^{-1} region. The peptide carbonyl bands in the acid-

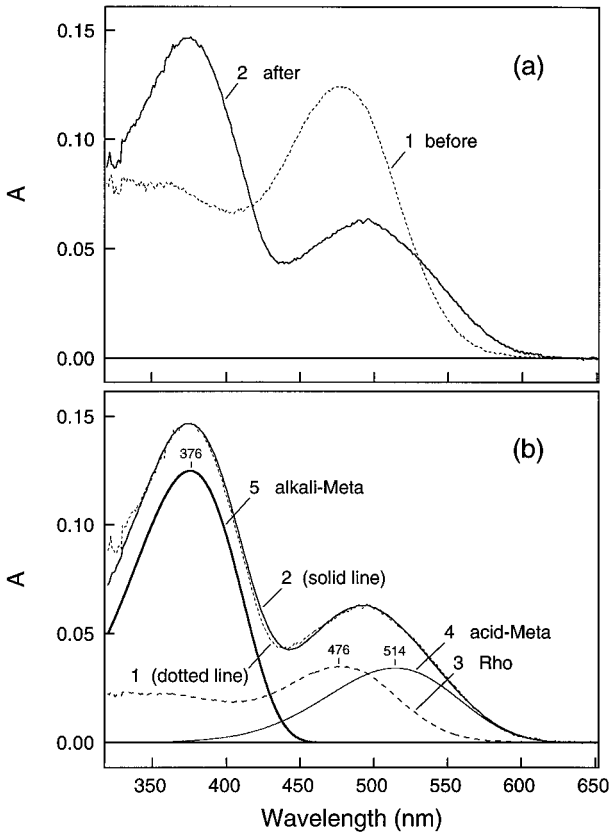

FIGURE 5: (a) Visible spectra before (curve 1) and after (curve 2) illumination of the hydrated film at pH 10 with 470 nm light at 280 K. (b) The amounts of acid-Meta and alkali-Meta in the photoproducts were estimated by spectral decomposition. The spectrum after illumination (curve 1 reproduced from curve 2 in panel a) was divided into three spectra by lognormal fitting procedures (see details in the text). The spectra of Rho, acid-Meta, and alkali-Meta were shown by curves 3−5, respectively. The sum of these three spectra (curve 2) is nearly identical with curve 1.

Meta minus Rho spectrum (dotted line) at 1684 (−), 1659 (+), 1647 (+), and 1623 (−) cm^{-1} almost disappear in the alkali-Meta minus Rho spectrum (solid line). Thus, the deprotonation of the Schiff base exerts large effects on those peptide bonds. The negative band at 1665 cm^{-1} decreases partly. The positive bands at 1703 and 1695 cm^{-1} are common to both metarhodopsins with the protonated and unprotonated Schiff base. A negative band at 1742 cm^{-1} due to a carboxylic acid appears in the alkali-Meta minus Rho spectrum with a concomitant increase in intensity around 1720 cm^{-1}. It may be ascribed to Asp-81, which is inside the membrane according to the model of Ovchinnikov et al. (1988). The perturbation of the same band was previously observed by repeated alternative illuminations with blue and orange lights at 282 K (Masuda et al., 1993b). The C═N stretching vibration of the unprotonated Schiff base of alkali-Meta, which should appear at 1625 cm^{-1} according to the previous resonance Raman study (Kitagawa & Tsuda, 1980), was not observed probably as a general property of the chromophore bands with the unprotonated Schiff base. As stated above, the C═N stretching vibration of acid-Meta was undetected due to the cancellation with that of Rho at the same frequency.

Figure 7 shows the pure alkali-Meta minus Rho spectrum in the 3750−3200 cm^{-1} region, which includes the water O−H and peptide N−H stretching vibrations (amide A). The positive band of the O−H stretching vibration of water at

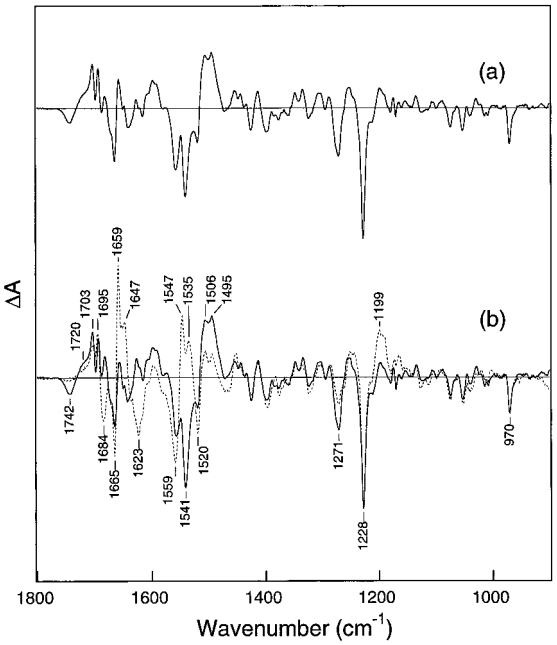

FIGURE 6: (a) Difference FTIR spectrum in the 1800−900 cm^{-1} region composed of acid-Meta minus Rho (13−19%) and alkali-Meta minus Rho (87−81%) spectra upon illumination at the same conditions described in the legend of Figure 5. (b) The spectrum of the alkali-Meta minus Rho (solid line) obtained after subtracting the contribution of acid-Meta and the acid-Meta minus Rho (dotted line; reproduced from Figure 2e) spectra were overlaid by adjusting intensities at 970 cm^{-1}. The full height of the ordinate corresponds to 0.015 and 0.008 absorbance unit for the alkali-Meta and acid-Meta spectra, respectively.

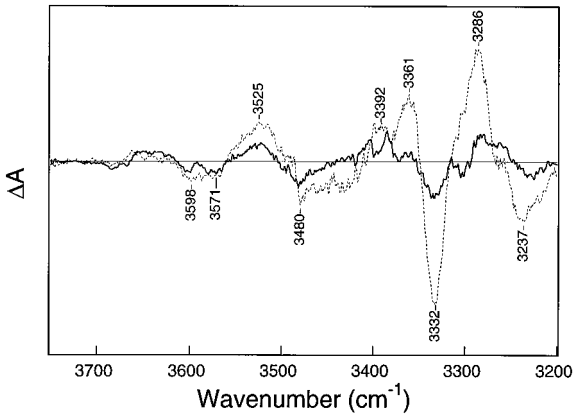

FIGURE 7: Difference FTIR spectrum in the 3750−3200 cm^{-1} region upon formation of alkali-Meta from Rho (solid line) was overlaid with the corresponding spectrum for the formation of acid-Meta (dotted line; reproduced from Figure 2e). The full height of the ordinate corresponds to 0.0040 and 0.0021 absorbance unit for alkali-Meta and acid-Meta, respectively.

3525 cm^{-1}, characteristic for acid-Meta (dotted line, see also Figure 3e), clearly decreases in intensity to below half in alkali-Meta (solid line). The acid-Meta minus Rho spectrum (dotted line) displays intense bands in the amide A region at 3361 (+), 3332 (−), 3286 (+), and 3237 (−) cm^{-1}. These were also almost extinguished in the alkali-Meta minus Rho spectrum (solid line).

DISCUSSION

Dynamics around the Schiff Base. The H bonding strength of the Schiff base N−H of octopus rhodopsin did not change upon formation of Batho despite the isomerization of the chromophore, which should move the imine hydrogen of the Schiff base. A similar conservation for bovine bathorhodopsin was explained by postulating that twists in the chromophore fix the relative position of the Schiff base to the countercharge (Palings et al., 1989). However, as discussed also by Deng et al. (1994), if the Schiff base complexes with a water oxygen by H bonding and they move together in the initial step for the isomerization, the strength of H bonding of the Schiff base could remain unchanged. In such a situation, the water hydrogens change the relative positions with respect to the H bonding acceptors, oxygens of another water, peptide carbonyl, or tyrosine, and then the O−H stretching vibrations are shifted. The bilobes with the negative band at 3674 and 3599 cm^{-1} in the Batho minus Rho spectrum (Figure 3b) can be ascribed to such a water molecule complexed with the Schiff base. Though other possibilities cannot be excluded, the most likely site to be changed in Batho is the Schiff base and the water molecule directly linked to it.

Bovine rhodopsin exhibits changes of the water O−H stretching vibration (Kandori & Maeda, 1995) at lower frequencies than octopus rhodopsin. This probably is because the Schiff base-linked water molecule is in a stronger H bonding state with the localized negative charge of Glu-113. In contrast, the negative charge in octopus rhodopsin might be delocalized in oxygens of residues surrounding the Schiff base. The higher frequency of the C_{11}═C_{12} HOOP band of Batho at 942 cm^{-1} compared to that in bovine rhodopsin at 921 cm^{-1} has already been noted (Deng et al., 1991). The lower frequency of the C_{11}═C_{12} HOOP band in bovine bathorhodopsin has been ascribed to a strong electrostatic interaction between C_{12}−H and one of the carbonyl oxygens of Glu-113 as the countercharge (Han & Smith, 1995). A smaller perturbation of the Batho of octopus compared to that of bovine might be explained also by the absence of the carboxylate in octopus as the localized countercharge.

In the later intermediates, Lumi or Meso, it is reasonably supposed that the water hydrogens find new acceptors of H bonding and change the H bonding state. This may be reflected in a shift of the O-H stretching vibrational band of water diffeent from that in Batho. A decrease in the force constant of the C═N bond in Lumi and Meso may be due to a decrease in the bond order of the Schiff base (closer to the single-bond state). This could be a reflection of the structure of the transitional state. The 3357 cm^{-1} band appears with an intensity increase only in Lumi and Meso (Figure 3c, d) in parallel with the decreased C═N force constant of the Schiff base and hence could be attributed to the N−H stretching vibration of the Schiff base. A similar intensity increase has also been noticed in the N−H stretching vibration of the indole in Trp-182 in the L intermediate of bacteriorhodopsin (Yamazaki et al., 1995). These intensity increments are caused by an increase in the polarity in the environment. Distortion of the retinal close to the Schiff base has also been proposed for the L intermediate of bacteriorhodopsin [Humphrey et al., 1995; for a review, see Maeda (1996)] and bovine lumirhodopsin (Ohkita et al., 1995).

The chromophore in acid-Meta is completely relaxed as envisaged by the abolition of the HOOP bands. Its formation is accompanied by considerable changes in the H bonding of the peptide backbone as well as of the internal water

molecules. Some part of these changes did not occur in alkali-Meta with the unprotonated Schiff base, suggesting the rearrangements of the peptide backbone and water in close proximity to the protonated Schiff base. The oxygens of the water molecules and some of the peptide carbonyls form stronger H bonds, indicating that their structural role is to accommodate the protonated Schiff base. The newly acquired structure of acid-Meta may be compensated for by an alteration of the electrostatic interaction of the Schiff base with the surrounding delocalized charges, so as to cause the decrease in the pK_a value of the Schiff base by about 2 pH units from that of rhodopsin compared to that to metarhodopsin (Koutalos et al., 1990).

Characteristic Features of Acid-Meta. Water structural changes observed in acid-Meta are also very similar to those in bovine rhodopsin upon its conversion to the lumirhodopsin or metarhodopsin I (Maeda et al., 1993). This occurs before to the proton transfer from the Schiff base to Glu-113 to form metarhodopsin II, which is the state to activate the G-protein. On the other hand, the greatest changes in the peptide backbone, like those in acid-Meta of octopus rhodopsin, appear in bovine metarhodopsin II with the unprotonated Schiff base (Klinger & Braiman, 1992; Maeda et al., 1993) and in the N and M_N intermediates after the deprotonation of the Schiff base for bacteriorhodopsin (Sasaki et al., 1992).

A mutant of bovine rhodopsin whose countercharge was replaced by Glu-117 (Fahmy et al., 1994) has a metarhodopsin II-like protein structure which can activate the G-protein, even though the Schiff base remains protonated. Fahmy et al. (1994) have concluded that G-protein activation in the bovine system can occur in the absence of the negative charge at position 113 with the isomerization of the chromophore. The acid-Meta intermediate of octopus rhodopsin fulfills these requirements. One of the consequences of the isomerization and the formation of acid-Meta of octopus rhodopsin is the induction of structural changes in the peptide bonds, water, and the Schiff base.

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REFERENCES

Bagley, K., Eisenstein, L., Ebrey, T. G., & Tsuda, M. (1989) *Biochemistry 28*, 3366−3373.
Cooper, A., Dixon, S. F., & Tsuda, M. (1986) *Eur. Biophys. J. 13*, 195−201.
Deng, H., Manor, D., Weng, G., Rath, P., Koutalos, Y., Ebrey, T., Gebhard, R., Lugtenburg, J., Tsuda, M., & Callender, R. H. (1991) *Photochem. Photobiol. 54*, 1001−1007.
Deng, H., Huang, L., Callender, R., & Ebrey, T. (1994) *Biophys. J. 66*, 1129−1136.
Fahmy, K., Siebert, F., & Sakmar, T. P. (1994) *Biochemistry 33*, 13700−13705.
Fryxell, K. J., & Meyerowitz, E. M. (1987) *EMBO J. 6*, 443−451.
Gat, Y., & Sheves, M. (1993) *J. Am. Chem. Soc. 115*, 3772−3773.
Glew, D. N., & Rath, N. S. (1971) *Can. J. Chem. 49*, 837−856.
Han, M., & Smith, S. O. (1995) *Biochemistry 34*, 1425−1432.
Hashimoto, S., Takeuchi, H., Nakagawa, M., & Tsuda, M. (1996) *FEBS Lett. 398*, 239−242.
Humphrey, W., Xu, D., Sheves, M., & Schulten, K. (1995) *J. Phys. Chem. 99*, 14549−14560.
Kandori, H., & Maeda, A. (1995) *Biochemistry 34*, 14220−14229.
Kiselev, A., & Subramaniam, S. (1994) *Science 266*, 1369−1373.
Kitagawa, T., & Tsuda, M. (1980) *Biochim. Biophys. Acta 624*, 211−217.
Klinger, A., & Braiman, M. S. (1992) *Biophys. J. 63*, 1244−1255.
Koutalos, Y., Ebrey, T. G., Gilson, H. R., & Honig, B. (1990) *Biophys. J. 58*, 493−501.
Kusumi, A., Tsuda, M., Akino, T., Ohnishi, S., & Terayama, Y. (1983) *Biochemistry 22*, 1165−1170.
Maeda, A. (1996) *Isr. J. Chem. 35*, 387−400.
Maeda, A., Ohkita, Y. J., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 12033−12038.
Masuda, S., Morita, E. H., Tasumi, M., Iwasa, T., & Tsuda, M. (1993a) *J. Mol. Struct. 297*, 29−34.
Masuda, S., Morita, E. H., Tasumi, M., Iwasa, T., & Tsuda, M. (1993b) *FEBS Lett. 317*, 223−227.
Metzler, D. E., & Harris, C. M. (1978) *Vision Res. 18*, 1417−1430.
Montell, C., Jones, K., Zuker, C., & Rubin, G. (1987) *J. Neurosci. 7*, 1558−1566.
Nathans, N. (1990) *Biochemistry 29*, 9746−9752.
Nishimura, S., Sasaki, J., Kandori, H., Lugtenburg, J., & Maeda, A. (1995) *Biochemistry 34*, 16758−16763.
Ohkita, Y. J., Sasaki, J., Maeda, A., Yoshizawa, T., Groesbeek, M., Verdegem, P., & Lugtenburg, J. (1995) *Biophys. Chem. 56*, 71−78.
Ovchinnikov, Y. A., Abdulaev, N. G., Zorotalaev, A. S., Artamonov, I. D., Bespalov, I. A., Dergachev, A. E., & Tsuda, M. (1988) *FEBS Lett. 232*, 69−72.
Palings, I., van den Berg, E. M. M., Lugtenburg, J., & Mathies, R. A. (1989) *Biochemistry 28*, 1498−1507.
Pande, C., Pande, A., Yue, W. T., & Callender, R. (1987) *Biochemistry 26*, 4941−4947.
Rafferty, C. N., & Shichi, H. (1981) *Photochem. Photobiol. 33*, 229−234.
Rodman-Gilson, H. S., Honig, B., Vroteau, A., Zarrilli, G., & Nakanishi, K. (1988) *Biophys. J. 53*, 261−269.
Rothschild, K. J., Marrero, H., Braiman, M., & Mathies, R. (1984) *Photochem. Photobiol. 40*, 675−679.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.
Sampogna, R. V., & Honig, B. (1994) *Biophys. J. 66*, 1341−1352.
Steinberg, G., Ottolenghi, M., & Sheves, M. (1993) *Biophys. J. 64*, 1499−1502.
Suzuki, T., Narita, K., Yoshihara, K., Nagai, K., & Kito, Y. (1995) *Vision Res. 35*, 1011−1017.
Takei, H., Gat, Y., Rothman, Z., Lewis, A., & Sheves, M. (1994) *J. Biol. Chem. 269*, 7387−7389.
Terakita, A., Tsukahara, Y., Hariyama, T., Seki, T., & Tashiro, H. (1993) *Vision Res. 33*, 2421−2426.
Tokunaga, F., Shichida, Y., & Yoshizawa, T. (1975) *FEBS Lett. 55*, 229−232.
Tsuda, M. (1979) *Biochim. Biophys. Acta 545*, 537−546.
Tsuda, M. (1987) *Photochem. Photobiol. 45*, 915−931.
Yamazaki, Y., Sasaki, J., Hatanaka, M., Kandori, H., Maeda, A., Needleman, R., Sinada, T., Yoshihara, K., Brown, L. S., & Lanyi, J. K. (1995) *Biochemistry 34*, 577−582.
Yamazaki, Y., Tuzi, S., Saitô, H., Kandori, H., Needleman, R., Lanyi, J. K., & Maeda, A. (1996) *Biochemistry 35*, 4063−4068.
Yoshizawa, T., & Wald, G. (1963) *Nature 197*, 1279−1286.
Zhukovsky, E. A., & Oprian, D. D. (1989) *Science 246*, 928−930.

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