The Hydrogen-Bonding Network of Water Molecules and the Peptide Backbone in the Region Connecting Asp83, Gly120, and Glu113 in Bovine Rhodopsin†

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Received May 4, 1998; Revised Manuscript Received October 5, 1998

ABSTRACT: Difference Fourier transform infrared spectra were recorded between mutants of rhodopsin and their batho products. The pigments studied were single and combined mutants of intramembrane residues of bovine rhodopsin: Asp83, Glu113, Gly120, Gly121, and Glu122. Previous studies [Nagata, T., Terakita, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1997) *Biochemistry* 36, 6164−6170] showed that one of the water molecules which undergoes structural changes in this process forms hydrogen bonds with Glu113 and the Schiff base, and that another water molecule is linked to this structure through the peptide backbone. The present results show that this water molecule is located at the place that is affected by the replacements of Asp83 and Gly120 but only slightly by Gly121 and not at all by Glu122. Asp83 and Gly120 are close to each other, in view of the observations that the carboxylic C═O stretching vibration of Asp83 is affected by the G120A replacement and that each replacement affects the common peptide carbonyl groups. Our results suggest that these residues in the middle of helices B and C are linked—through a hydrogen-bonding network composed of water and the peptide backbone—with the region around Glu113.

Rhodopsin is a heptahelical membrane protein which traverses the disk membrane in the outer segments of rod photoreceptors (*1*, *2*); it faces both the cytoplasmic and intradiscal media. An 11-cis retinal chromophore attached to Lys296 through a protonated Schiff base linkage is located roughly in the middle of the membrane. Light absorbed by the chromophore causes an 11-cis to all-trans isomerization. Subsequent thermal reactions through a series of intermediates finally lead to the transfer of the proton on the Schiff base to Glu113, located toward the intradiscal surface, producing metarhodopsin II (Meta II).[1] Meta II's formation is accompanied by the movement of helix C relative to helix F on the cytoplasmic surface (*3*, *4*) and proton uptake via or to Glu134 (*5*). Recently, the importance of structural changes in this region for the activation of transducin was shown (*6*). Thus, the proton transfer to Glu113 eventually results in structural changes at the cytoplasmic surface where the altered interhelical loops of Meta II interact with and activate transducin (*7*, *8*).

Compared to a hydrated film, an unhydrated dry film of rhodopsin does not form Meta II upon illumination and shows different features for the hydrogen-bonding changes of Glu122 and Asp83 (*9*, *10*). Glu122 also helps determine the lifetime of Meta II (*11*). These observations indicate that, besides helices C and F, Asp83 in helix B is involved in establishing the structure of Meta II and in propagating the changes from the Schiff base region to the cytoplasmic surface.

Our FTIR studies of bovine rhodopsin have revealed that the photochemical reactions leading to various intermediate states are accompanied by structural changes of internal water molecules (*10*, *12*−*14*). An active role for water is also suggested by the inhibition of Meta II formation in water-depleted bovine rhodopsin (*9*, *15*). Site-directed mutation studies by Nagata et al. (*14*) have identified a water molecule that is close to Glu113, the counterion of the protonated Schiff base (*16*−*18*). They suggested that this water moves together with the protonated Schiff base in the isomerization of 11-cis retinal to the all-trans form. Nagata et al. (*14*) also observed structural changes of other internal water molecules, one of which was connected through the peptide backbone. Water molecules along with the carbonyl groups of the peptide backbone in helices B and C seem to be involved in a hydrogen-bonding network, as was also observed for bacteriorhodopsin (*19*, *20*).

Recent X-ray crystallographic studies on bacteriorhodopsin (*21*) identified three ordered internal water molecules; two form hydrogen bonds to Asp85, one of which is further bonded to the Schiff base and Asp212, and the third to both Arg82 and Glu204, which are linked to Asp85 through hydrogen bonds. These locations correlate precisely with water locations inferred from FTIR measurement (see the references cited in ref *22*). A water O−H stretching vibration at 3643 cm^{-1} which undergoes frequency shifts in the

† This work is supported by grants from the Japanese Ministry of Education, Culture, Sport and Science to A.M. and Y.S. (08308038), H.K. (09833002), and A.T. (09740626).

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[1] Abbreviations: Meta II, metarhodopsin II; FTIR, Fourier transform infrared; Rho, rhodopsin; Iso, isorhodopsin; Batho, bathorhodopsin; λ_{max}, the wavelength for the maximum absorbance; HOOP, hydrogen out-of-plane.

10.1021/bi9810149 CCC: $15.00
Published on Web 11/12/1998

photochemical reactions is abolished in a mutant of Asp85 and perturbed in mutants of Asp212, Arg82, and Glu204. Another water O−H stretching vibration at 3625 cm^{-1} is also perturbed in mutants of Arg82. FTIR spectral analysis also showed two water molecules close to the functionally important residues, Asp96 and Thr46, in the cytoplasmic region (*22*). The X-ray crystallographic study revealed a cavity near these residues that may accommodate one or two relatively disordered water molecules (*21*). At present, structural studies of rhodopsin are unable to detect water molecules (*1*); however, the success of the FTIR studies of bacteriorhodopsin in locating waters makes it look promising to carry out similar studies of mutant rhodopsins in order to associate internal water molecules with specific residues, even if their exact locations are not specified. FTIR spectroscopy for the formation of various intermediates can further reveal disordered water molecules and their dynamics.

The present study aims at revealing the possible relation of internal water molecules to functional residues in the transmembrane region of bovine rhodopsin. Attention will be focused on the intramembrane residues, Asp83, Gly120, Gly121, and Glu122, on the cytoplasmic side of the chromophore. Residues with protonated carboxylic acids, Asp83 and Glu122 (*23*), are affected differently in water-depleted membranes from those in the hydrated membranes (*9*, *10*), so these two residues in rhodopsin have been chosen for further study. Two consecutive glycine residues close to Glu122, Gly120 and Gly121, are expected to form a flexible site that could accommodate water. Gly121 is also known to interact with the retinal side chain in a complementary way to Phe261 in helix F (*24*, *25*). Glu122 is located close to the β-ionone ring of the retinal and affects the visible spectrum of rhodopsin (*16*). FTIR spectra were recorded using the reversible photoreactions between rhodopsin (Rho), isorhodopsin (Iso), and bathorhodopsin (Batho) at 78 K. Our results suggest that the environment around the site of interaction between Asp83 in helix B and Gly120 in helix C is regulated by the site of interaction of Glu113 with the Schiff base. Regulation occurs through hydrogen-bonding linkages of internal water molecules and the peptide backbone.

MATERIALS AND METHODS

The methods for the construction and expression of the rhodopsin mutant genes of D83N, G120A, G121A, E122Q, D83N/G120A, D83N/E113Q, and E113Q/G120A in 293S cells were described in an earlier paper (*14*). The visual pigments were extracted in 1% dodecylmaltoside in a buffer composed of 0.05 M HEPES and 0.14 M NaCl (pH 6.5), and purified first by their absorption to the rhodopsin antibody 1D4 (*26*) and then by their elution using the epitope for the antibody, a C-terminus nonapeptide (*27*, *28*) in 0.02% dodecylmaltoside, 0.05 M HEPES buffer, and 0.14 M NaCl (pH 6.5). See also Nagata et al. (*14*) for details.

The visible spectra were recorded at this stage with a Shimadzu recording spectrophotometer MPS-2000. The wavelengths for the maximum absorbance (λ_{max}) of the wild type, the mutants E122Q, G121A, and D83N are at 499, 482, 496, and 497 nm, respectively; these values are almost identical with those previously reported for these pigments (*16*, *18*, *24*). The spectra of our new mutants, G120A, D83N/G120A, D83N/E113Q, and E113Q/G120A, show their λ_{max} at 498, 494, 499, and 499 nm, respectively. The ratios of the absorbance at 280 nm to that at the λ_{max} of 1.84 and 1.94 for G120A and D83N/G120A, respectively, are almost identical with that of 1.81 for the wild type. Both D83N/E113Q and E113Q/G120A mutant proteins may have similar purity in view of their yields being comparable to others, though both had larger 380 nm bands, due to a species with an unprotonated Schiff base characteristic of E113Q mutants (*17*). The rhodopsin was then reconstituted into liposomes with 100-fold molar excess of egg yolk phosphatidylcholine (Sigma type XI-E) (*14*). A film was made by drying a 40 μL aliquot of the liposome suspension in 5 mM NaCl and 1 mM phosphate buffer (pH 5.7), on a BaF_2 window (18 mm in diameter). Wild type rhodopsin was also prepared in the same way. Its spectral properties are identical with that of native rhodopsin (*14*). The 380 nm band of D83N/E113Q and E113Q/G120A protein disappears in the film because the concentration of chloride is enough to protonate the Schiff base. The film was immediately covered by another BaF_2 window and a spacer after about ~1 μL of H_2O or D_2O was put aside the film. Then the sample was installed into a holder in an Oxford DN1704 cryostat and cooled to 78 K. The difference FTIR spectra were recorded for the hydrated films by illumination at 78 K as described previously (*13*, *14*).

RESULTS

Photoreactions of the Mutants. At 78 K the difference spectrum upon illumination of wild type rhodopsin with 500 nm light and the back photoreaction with >610 nm light to attain the photosteady state gives mainly the Batho minus Rho spectra, but there is still a contribution from the Batho minus Iso spectrum (*13*, *14*). The difference spectrum of the latter was obtained by alternately illuminating with 500 and >520 nm light. To obtain the pure Batho minus Rho spectrum, the latter was subtracted from the former after scaling the Iso-specific bands at 959 and 3479 cm^{-1}. Figure 1 shows the Batho minus Rho spectra in the 1800−800 cm^{-1} region of the mutant proteins of E122Q (b), G121A (c), G120A (d), D83N (e), and D83N/G120A (f), which were also obtained using the same method. The C−C stretching bands in the 1270−1170 cm^{-1} region are nearly identical in shape with those of the wild type (a). The positive band at 1536 cm^{-1} of the wild type (a) can be assigned to the C═C stretching vibration because the same band appears in the resonance Raman spectrum of Batho (*29*). This band coincides with the corresponding band in the mutants (c−e) except for the 1543 cm^{-1} band of E122Q (b) and the 1537 cm^{-1} band for D83N/E122Q (f). Hydrogen out-of-plane (HOOP) bands emerging at 920−922 cm^{-1} and disappearing at 966−968 cm^{-1} are also common for these mutants. As a whole, these replacements did not give gross structural changes in the protein. These HOOP bands are also observed in the resonance Raman spectra of the Batho and Rho, respectively, of the wild type (*30*), ensuring that the normal photoreaction to Batho in the suspensions was retained in the films.

The negative band at 1558 cm^{-1} of the wild type (a), which is persistent through these mutant proteins (b−f), is accompanied by bands at slightly different frequencies in the varioius mutants. These bands could be composed of either

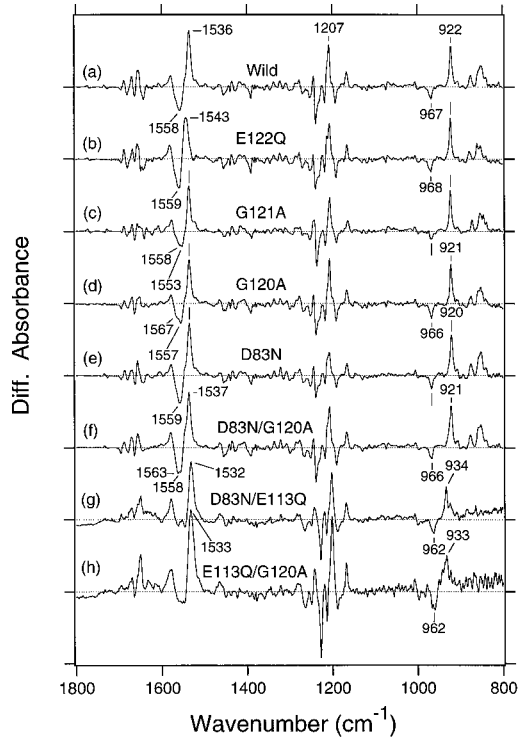

FIGURE 1: Batho minus Rho FTIR spectra in the 1800−800 cm^{-1} region for the wild type (a), E122Q (b), G121A (c), G120A (d), D83N (e), D83N/G120A (f), D83N/E113Q (g), and E113Q/G120A (h). The spectrum of the wild type was from Nagata et al. (*14*). The frequencies which are the same in the wild type were omitted. One division in the ordinate is 4.0, 3.4, 3.4, 1.2, 2.2, 3.3, 2.0, and 1.0 milliabsorbance units for (a), (b), (c), (d), (e), (f), (g), and (h), respectively.

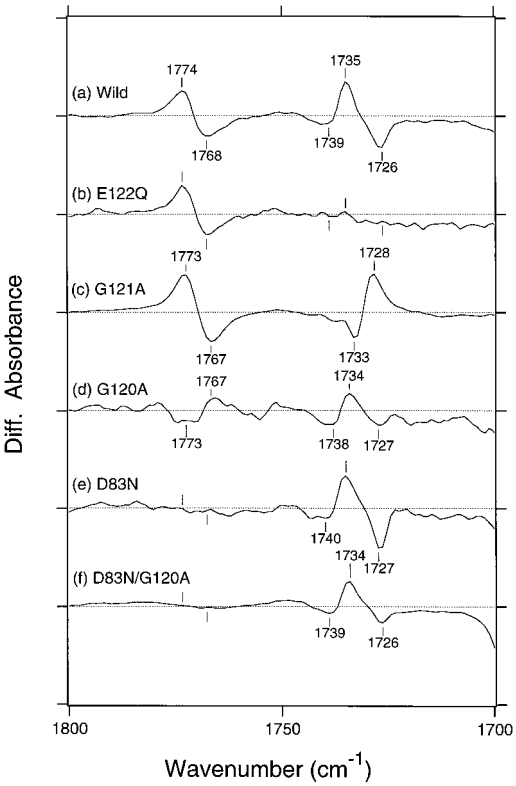

FIGURE 2: Batho minus Rho FTIR spectra in the 1800−1700 cm^{-1} region for the wild type (a), E122Q (b), G121A (c), G120A (d), D83N (e), and D83N/G120A (f). The frequencies which are the same in the wild type were omitted. One division in the ordinate is 0.30, 0.26, 0.25, 0.09, 0.17, and 0.25 milliabsorbance units for (a), (b), (c), (d), (e), and (f), respectively.

or both of the C═C stretching vibrations of the retinal and the amide II. If they are due to amide II, the intensity of ∼1.4 milliabsorbance units in the wild type (a) must correspond to the intensity of at least two residues in view of about 0.25 absorbance units of the whole amide II band in the absolute spectrum (not shown) and the total 348 residues in rhodopsin. This is along with the fact that an intensity larger than that of the amide I band makes it unlikely that these bands are totally due to amide II. The 1558 cm^{-1} band is located at a frequency considerably different from that of the C═C stretching vibration frequency at 1546 cm^{-1} observed in the resonance Raman spectra (*29*), which in general gives almost the same vibrational bands due to the chromophore as in the FTIR spectra (*31*). Their λ_{max}'s, between 499 and 494 nm (except for E122Q at 482 nm, not shown), are close to that of the wild type at 499 nm, suggesting that their corresponding C═C stretching vibrations should be located close to 1546 cm^{-1} of the wild type, on the basis of a linear relation between the λ_{max} and the C═C stretching frequency. The C═C stretching vibration of the retinal, however, is not confined to only a single band, and resonance Raman and infrared spectra could give the different C═C stretching bands with different intensities. Nevertheless, the other variable shoulders at 1553 cm^{-1} for G121A (c), 1567 cm^{-1} for G120A (d), and 1563 cm^{-1} for D83N/G120A (f) could be more reasonably assigned to the amide II band (a coupled mode of the C−N stretching and N−H bending vibrations of a peptide bond). These bands did not shift under the present conditions with a short exposure to D_2O (not shown). This behavior of amide II was shown for peptide bonds in the intramembrane region (*32*−*34*). It is most likely that the 1558 cm^{-1} band of the wild type contains both the C═C stretching vibration and amide II bands. In this case, amide II band is located at 1558 cm^{-1} in the wild type (a) and D83N (b), is shifted toward higher frequencies at 1567 cm^{-1} in G120A (d), and returns toward the lower frequency at 1563 cm^{-1} in D83N/G120A (f). A shift opposite to 1553 cm^{-1} was observed for G121A (c). Thus, the amide II band in the 1558 cm^{-1} band of the Rho state is ascribable to the peptide N−H around Gly120 and Gly121.

The spectra for the Batho-like products of D83N/E113Q (g) and E113Q/G120A (h) were obtained at an early stage of the illumination protocol (2 s vs 2 min for the photosteady state) at 78 K. Since further illumination to attain photosteady states and the subsequent illumination with red light to restore the Rho state as was done for other mutant proteins yielded unidentified side products, the Batho minus Rho spectrum was recorded only once. A positive HOOP band at 933−934 cm^{-1} and a downshifted C═C stretching vibrational band at 1532−1533 cm^{-1} relative to the wild type in these double mutants are also observed in the Batho minus Rho spectrum of E113Q (*14*). Other features are not markedly different from those of the wild type.

Below, we first discuss the 1800−1700 and 1700−1600 cm^{-1} spectral regions, and then the region above 3500 cm^{-1}, which is not included in the spectra of Figure 1.

Effects of the Mutations on the Carboxylic Acids. Spectra in the 1800−1700 cm^{-1} region (Figure 2) show the C═O stretching vibrations of protonated carboxylic acids. Two

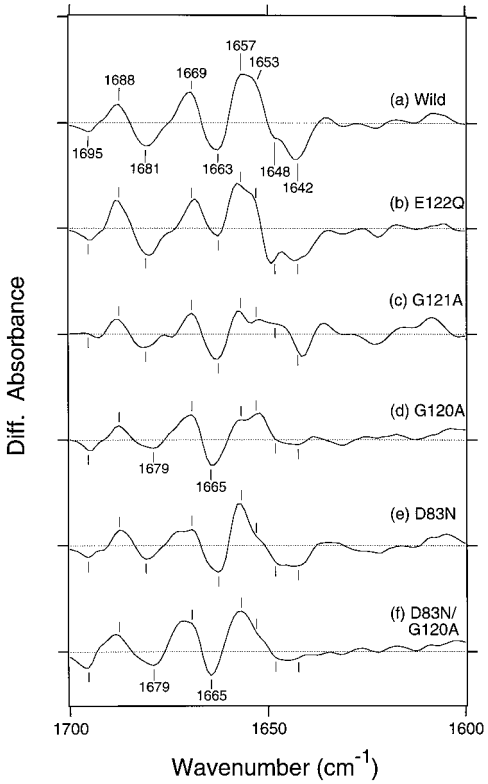

FIGURE 3: Batho minus Rho FTIR spectra in the 1700−1600 cm^{-1} region for the wild type (a), E122Q (b), G121A (c), G120A (d), D83N (e), and D83N/G120A (f). The frequencies which are the same in the wild type were omitted. One division in the ordinate is 2.0, 1.7, 1.7, 0.6, 1.1, and 1.7 milliabsorbance units for (a), (b), (c), (d), (e), and (f), respectively.

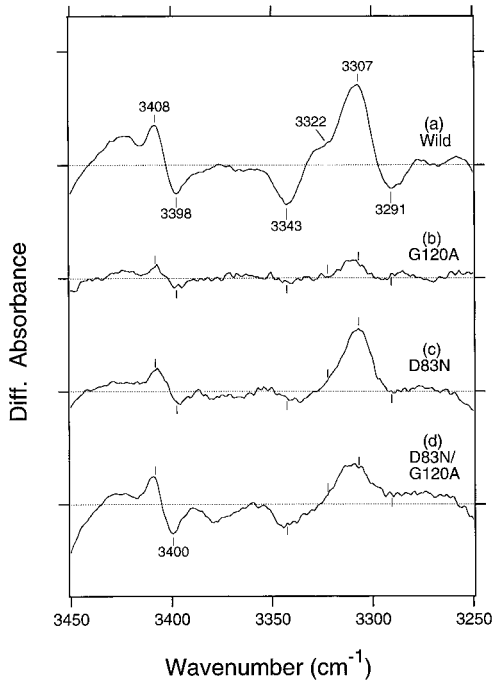

FIGURE 4: Batho minus Rho FTIR spectra in the 3450−3250 cm^{-1} region for the wild type (a), G120A (b), D83N (c), and D83N/G120A (d). The frequencies which are the same in the wild type were omitted. One division in the ordinate is 1.00, 0.29, 0.56, and 0.83 milliabsorbance units for (a), (b), (c), and (d), respectively.

negative bands at 1739 and 1726 cm^{-1} with a positive band at 1735 cm^{-1} in the wild type (a) disappear in E122Q (b). These along with the previous results on the spectrum for Meta II formation (*23*, *35*) suggest that these bands are mainly composed of the C═O stretching vibration due to the carboxylic carbonyl of Glu122. The G121A mutation affects these bands, indicating that Gly121 is located at a position that can affect the environment of the C═O of Glu122. G120A (d) and D83N (e) do not affect these bands.

A bilobe with 1774 (+) and 1768 (−) cm^{-1} bands in the wild type (a) disappears completely in D83N (e) and D83N/G120A (f). Hence, these bands are ascribed to Asp83 as was inferred from the bilobe with 1767 (−) and 1745 (+) cm^{-1} bands in the Meta II minus Rho spectrum (*23*). These bands of Asp83 persist in E122Q (b) and G121A (c) but are replaced in G120A (c) by 1773 (−) and 1767 (+) cm^{-1} bands, suggesting an interaction between Asp83 and Gly120.

Effect of the Mutations on the Peptide Backbone. Figure 3 shows the spectral changes in the 1700−1600 cm^{-1} region for the amide I bands (mainly due to the stretching vibration of the peptide carbonyl which is coupled with the bending vibration of peptide amide). The wild type (a) exhibits bands on the negative side at 1695, 1681, 1663, 1648, and 1642 cm^{-1} and on the positive side at 1688, 1669, 1657, and 1653 cm^{-1}. These are all abolished in E113Q (*14*). In the G120A and D83N/G120A replacements (d and f), both of the two negative bands at 1648 and 1642 cm^{-1} decrease extensively and the negative band at 1681 cm^{-1} is shifted to 1679 cm^{-1}. D83N affects the negative band at 1642 cm^{-1} (e), as is also seen in the G120A (d) and D83N/G120A (f) replacements. Thus, the negative band of the Rho state at 1642 cm^{-1} is ascribable to the peptide carbonyl around Gly120 and Asp83. The 1663 cm^{-1} band decreases in intensity in E122Q (b) and shifts to 1665 cm^{-1} in G120A (d) and D83N/G120A (f), suggesting that it is due to the peptide carbonyl around Glu122 and Gly120. The missing negative band at 1648 cm^{-1} in G121A may be due to its cancellation by a positive band at a similar frequency (c).

The spectra in the 3450−3250 cm^{-1} region (Figure 4) contain amide A bands (almost exclusively due to the stretching vibration of the peptide N−H). All of these bands did not shift after a short exposure to D_2O (not shown), as was also seen for the amide II bands that undergo frequency shifts upon Batho formation (Figure 1). The wild type (a) exhibits negative bands at 3398, 3343, and 3291 cm^{-1} and positive bands at 3408, 3322, and 3307 cm^{-1}. These bands, which are all abolished in E113Q (*14*), are diminished extensively in G120A (b). A large part of these intensities are restored with the additional replacement of D83N (d). The 3322 (+) and 3291 (−) cm^{-1} bands are extinguished also in D83N (c) and D83N/G120A (d), suggesting that these are due to the peptide backbone close to the Asp83 and Gly120.

Effects of the Mutations on Internal Water Molecules. The spectra in the 3650−3500 cm^{-1} region of the Batho minus Rho spectra contain the O−H stretching vibrations of water. A small fraction of the O−H stretching vibrations which does not undergo deuterium exchange with a short exposure to D_2O is also included in this region (*13*, *14*). These vibrations are due to either water or the O−H of protein side chains. In the present study, the measurements were done after a short exposure to D_2O at room temperature with immediate cooling to 78 K. Thus, the analysis was confined to water molecules, whose hydrogens exchange readily with deuter-

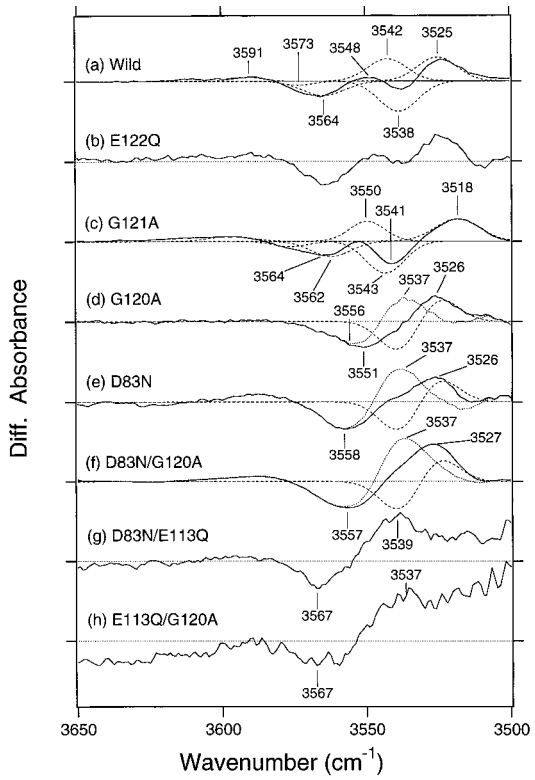

FIGURE 5: Batho minus Rho FTIR spectra in the 3650−3500 cm^{-1} region for the wild type (a), E122Q (b), G121A (c), G120A (d), D83N (e), D83N/G120A (f), D83N/E113Q (g), and E113Q/G120A (h). Dotted lines in (a) and (c) show the best fitted Gaussian curves. The spectral contribution of Wat-1 in (a) was converted to the sum of the two Gaussian curves and shown by dashed lines in (d−f). The spectra after its subtraction from the recorded spectra are shown by dotted lines. The spectrum of the wild type was duplicated from Nagata et al. (*14*). The frequencies which are the same in the wild type were omitted. One division in the ordinate is 1.00, 0.85, 0.85, 0.29, 0.56, 0.83, 0.50, and 0.25 milliabsorbance units for (a), (b), (c), (d), (e), (f), (g), and (h), respectively.

ons. The spectra after the subtraction of the corresponding spectra in D_2O are shown in Figure 5 except for D83N/E113Q (g) and E113Q/G120A (h). This gives the spectra for the O−H stretching vibrations of the water whose hydrogens rapidly exchange with deuterium of D_2O. By applying a Gaussian approximation, the recorded spectrum of the wild type (a, solid line) was fitted by three bilobes (a, dotted line). The bilobe due to the small bands at 3591 (+) and 3573 (−) cm^{-1} will not be discussed. The larger one with 3538 (−) and 3525 (+) cm^{-1} bands was previously ascribed to the water molecule present close to Glu113 (Wat-1), and another with 3564 (−) and 3542 (+) cm^{-1} bands to another water molecule (Wat-2), which is also affected by E113Q substitution (*14*). The spectrum of these water molecules remains unchanged in E122Q (b). In G121A (c, solid line), a positive band at 3518 cm^{-1} ascribable to Wat-1 seems to form a bilobe with a negative band at 3541 cm^{-1}. A negative band at 3564 cm^{-1} due to Wat-2 may have a corresponding positive band around 3550 cm^{-1} in the trough region of the two negative bands, though it would be unseen due to cancellation. The spectrum could be fit by two bilobes: Wat-1 at 3543 (−) and 3518 (+) cm^{-1} and Wat-2 at 3562 (−) and 3550 (+) cm^{-1} (c, dotted line). Thus, the 3538 cm^{-1} band of Wat-1 in the Rho state of the wild type is shifted toward higher frequencies by 5 cm^{-1}, while the corresponding 3525 cm^{-1} band in the Batho state is shifted considerably toward a lower frequency (7 cm^{-1}). This may be related to the fact that Gly121 is in proximity to the 9-methyl group of the retinal, which should move during Batho formation (*24*, *25*). On the other hand, Wat-2 at 3562 cm^{-1} in the Rho state is affected only slightly.

The spectra of G120A (d), D83N (e), and D83N/G120A (f) show apparently only a single pair with negative bands in the Rho state at 3551, 3558, and 3557 cm^{-1} for G120A (d), D83N (e), and D83N/G120A (f), respectively. The frequency of the positive band at 3526−3527 cm^{-1} is presumably due to Wat-1 because it is similar to that at 3525 cm^{-1} for the wild type. It is also highly probable that these mutants, which keep Glu113 unaltered, retain Wat-1 because of the absence of unusual spectral properties such as those found for E113Q (*14*). It is also hard to imagine that, if Wat-1 is close to Glu113, its O−H band would be abolished by the replacement of Gly120, which is located two helix turns away from Glu113. The possibility that this pair is due to Wat-1 could be further examined in the spectra of D83N/E113Q (g) and E113Q/G120A (h), which should not exhibit the bands due to Wat-1 (*14*). In these cases, the contribution of the spectra in D_2O was not subtracted because the sample allowed only a single recording and the amount of the sample was not enough to allow a second experiment in D_2O. Fortunately, the contribution from the D_2O-insensitive fraction in this region is almost negligible and qualitatively correct results are expected without subtracting the spectra in D_2O. Despite the low signal-to-noise ratio due to a single recording and the short duration for the photoreaction (see above), the spectra clearly showed a single pair with bands at frequencies similar to those of Wat-2 observed for E113Q at 3571 (−) and 3545 (+) cm^{-1} (*14*). It is hard to regard these bands as being due to a water molecule other than Wat-1 and Wat-2, because the structural changes induced by the mutations of Asp83 and Gly120 are not extensive enough to result in such a drastic change.

These arguments lead to the supposition that the water O−H stretching bands in the G120A (d), D83N (e), and D83N/G120A (f) must be composed of both Wat-1 and Wat-2. There is no systematic way to reveal two pairs, as was done for the wild type (a) and G121A (c). We examined a situation where Wat-1 is not affected at all in these mutants. Subtraction of the spectrum of Wat-1 of the wild type (dashed line) revealed other bilobes (dotted lines) with a negative band at 3556−3558 cm^{-1}. Thus, the replacement of either Asp83 or Gly120 causes a shift in the negative Rho bands of Wat-2 toward lower frequencies. This is evident even in the raw spectra, and this conclusion will not be affected even if Wat-1 is altered by these replacements. On the other hand, the negative bands of Wat-2 in these double mutants (g and h) are found at higher frequencies than for the corresponding G120A and D83N single mutants (d and e), respectively, as observed for E113Q (3571 cm^{-1}) relative to the wild type (3564 cm^{-1}) (*14*). The E113Q mutation, or a replacement of the counterion by chloride, causes a higher frequency shift for the negative band of Wat-2. It is not clear whether the negative band of Wat-1 is affected by the replacements of Gly120 and Asp83. It is a reasonable assumption that the negative band of Wat-1 is not affected so much by the replacement of Gly120 and Asp83 because a bilobe ascribable to Wat-2 after the subtraction of the bilobe of Wat-1 in

the wild type from the spectra of these mutants exhibits a reasonable shape. The positive band of Wat-1 in the Batho state is certainly unaffected.

DISCUSSION

Our previous studies (*14*) have shown that a bilobe due to the water O−H stretching vibration bands at 3538 (−) and 3525 (+) cm^{-1} in the Batho minus Rho spectrum of the wild type disappears in E113Q, while another bilobe with 3564 (−) and 3542 (+) cm^{-1} bands remains with a slight shift. The $N-H^+$ stretching vibration of the protonated Schiff base of E113Q undergoes structural changes, while that of the wild type does not. With the E113Q mutation in the presence of chloride, almost all of the changes in the peptide carbonyl and amide bands that occur upon Batho formation are abolished. From these facts, Nagata et al. (*14*) proposed that a water molecule with a 3538 cm^{-1} O−H band (Wat-1) forms hydrogen bonds with the Schiff base and Glu113, and that another water molecule with a 3564 cm^{-1} band (Wat-2) is located in a different place but is linked to Glu113 through the peptide backbone.

The present results show that Wat-2 is affected by the replacement of either Gly120 or Asp83 (Figure 5). A change of the $N-H^+$ stretching vibration as was observed for E113Q is not detected for D83N and G120A (not shown). This suggests that Wat-1 is closer to the pair of Glu113 and the Schiff base than Wat-2. Glu113 links to Wat-2 close to Asp83 and Gly120 through a network of hydrogen bonds and covalent bonds, and possibly even by helix tilting. Similar long-range interactions through a network of hydrogen bonds were shown for bacteriorhodopsin between Asp85 and Asp96 that are separated by ∼1 nm (*20*), and a change of interaction at a distant site was suggested to be caused by helix tilting upon the formation of the N intermediate of bacteriorhodopsin (*36*).

The G120A mutation greatly reduces the intensities of the C═O stretching bands of the carboxylic acids of Glu122 and Asp83 (Figure 2) and of the amide A band (Figure 4) of the peptide backbone. Similar reductions were also observed for amide I and amide A bands in E113Q (*14*). Intensity decreases in the difference spectra could result from either smaller frequency shifts or less-polarized environments for these portions of the peptide backbone. The peptide backbone as well as the interactions involving water molecules probably is part of a complex allowing long-range interaction from the site of Glu113 to Wat-2. More restricted changes which were observed for the peptide carbonyls (amide I) by the replacements of Gly120, Gly121, and Asp83 (Figure 3) suggest the local distortion of the peptide backbone around these residues. The two consecutive glycine residues in this region would lead to a distorted structure differing from an α-helix.

Wat-2 is located at a position not influenced by Glu122 and only slightly influenced by Gly121; it is in the region surrounded by Asp83, Gly120, and Glu113 and not beyond the region containing Gly121 and Glu122. Wat-1 whose O−H band is abolished in E113Q is most likely located in the proximity of Glu113, similar to the water molecule in bacteriorhodopsin whose vibration disappears after the replacement of Asp85 (*37*). Wat-2 is not abolished but perturbed by the replacements of either Asp83 or Gly120, suggesting that it is connected to these residues. The C═O stretching frequency of the carboxylic C═O of Asp83 is affected only in G120A (Figure 2), suggesting the proximal location of Asp83 to Gly120. Also, some abolition of the changes in the peptide carbonyls (Figure 3) and amides (Figure 4) occurs in common for D83N and G120A, suggesting a local distortion of the α-helix around Gly120 and Asp83. It is likely that these changes occur with an accompanying structural change of a water molecule, Wat-2. Even direct hydrogen-bonding interactions of Wat-2 to the peptide carbonyl of Gly120 and the carboxylic carbonyl of Asp83 are likely; relatively similar replacements of side chains such as glycine to alanine and aspartic acid to asparagine may keep these hydrogen bonds, though changing their strength.

The carboxylic C═O band of Asp83 appears at unusually high frequencies, 1768 cm^{-1} for Rho and at 1774 cm^{-1} for Batho, compared to carboxylic acids in solution. According to a proposal by Dioumaev and Braiman (*38*), a carboxylic acid with a high frequency must be in a quite apolar environment with a dielectric constant of ∼2. The change in frequency upon the formation of Batho might be caused by a change in either the hydrogen bond of the O−H bond whose bending vibration couples with the C═O stretching vibration or the coupling between them. The shift of the C═O stretching band of Asp83 from 1768 to 1774 cm^{-1} upon formation of Batho could be due to a change of the interaction of the O−H bond. Its further shift to 1748 cm^{-1} in Meta II (*23*) may be due to the movement of Wat-2 toward the C═O of Asp83. The D_2O-insensitive peptide backbone whose amide I and II vibrations undergo a frequency shift upon Batho formation would be located in the transmembrane region, and its N−H does not readily undergo D_2O exchange. On the other hand, the O−Hs of water and Asp83 form relatively weak hydrogen bonds and are able to exchange, even if the O−H of either water or Asp83 forms a hydrogen bond with the peptide carbonyl. In unhydrated bovine rhodopsin, the C═O stretching vibration of Asp83 and the peptide backbone did not show any structural changes upon Batho formation (*10*). Probably, a water molecule is involved in hydrogen bonds between the carboxylic O−H of Asp83 and the peptide C═O of Gly120.

What are the roles of Asp83 and Gly120? Asp83 is conserved in almost all G-protein coupled receptors (*39*), except for some visual pigments in cone cells (*40*). The mutation of this residue enhances agonist binding in the absence of bound G-protein in an α_2-adrenergic receptor (*41*). In the case of rhodopsin, an all-trans retinal in Meta II can be regarded as an agonist (*42*). The D83N mutation increases the fraction of Meta II at the expense of its equilibrium partner, Meta I, even at alkaline pH (*43*), where extensive formation of Meta II normally requires the presence of transducin. These suggest that the reversion of Meta II to Meta I upon release of transducin is regulated by Asp83. Thus the events for the interaction with transducin at the cytoplasmic surface are related to the protonation state of the Schiff base relatively close to the extracellular side and also to the intramembrane Asp83. Though the linkages between these regions are unknown, the present study suggested that Gly120 and a water molecule close to Asp83 may be mediators.

ACKNOWLEDGMENT

The authors thank to Professor Thomas G. Ebrey for critically reading the manuscript and improving its style.

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BI9810149