Water and Peptide Backbone Structure in the Active Center of Bovine Rhodopsin†

Tomoko Nagata, Akihisa Terakita, Hideki Kandori, Daisuke Kojima,‡ Yoshinori Shichida, and Akio Maeda*

Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-01, Japan

Received November 26, 1996; Revised Manuscript Received February 10, 1997⊗

ABSTRACT: Difference FTIR spectra in the conversion of rhodopsin or isorhodopsin to bathorhodopsin were recorded for recombinant wild-type and E113Q bovine rhodopsins. Differences in various vibrational modes between E113Q and the wild-type proteins whose Schiff bases interact with chloride and Glu113, respectively, were analyzed. Water molecules in rhodopsin that change upon formation of bathorhodopsin are detected by a change in frequency of the O−H stretching vibration from 3538 to 3525 cm^{-1}. This change in the wild-type protein is absent in E113Q. One or a few water molecules are therefore suggested to be located in the proximity of Glu113, the counterion of the Schiff base. Another water vibration at 3564 cm^{-1}, which is shifted to 3542 cm^{-1} in bathorhodopsin in the wild type, persists in E113Q but with ∼5-cm^{-1} shift toward higher frequency. This is due to water molecules that may be located at a site somewhat more remote from Glu113. Structural changes of some peptide carbonyls and amides are also absent in E113Q. On the other hand, the E113Q protein shows shifts of the $N-H^+$ stretching vibrational band, that is probably due to the protonated Schiff base, upon conversion of rhodopsin to bathorhodopsin. No corresponding changes were observed in the wild type. We propose a model in which a water molecule interacts with Glu113, the protonated Schiff base, and peptide carbonyls, and amides. These residues undergo structural changes upon formation of bathorhodopsin.

One of the most important steps in visual transduction is the activation of transducin on the surface of a rhodopsin molecule after light reception. In the bovine system, the activation is carried out by one of the photointermediates, metarhodopsin II (Meta II).[1] Meta II is characterized by two features, proton transfer from the Schiff base to Glu113 (Jäger et al., 1994) at the site close to the intradiscal surface, and structural changes of the cytoplasmic loops that interact with transducin (König et al., 1989; Franke et al., 1990, 1992; Resek et al., 1994; Nishimura et al., 1996). Hence, there should be a pathway to transmit information of the Schiff base deprotonation event across the membrane from the intradiscal side to the cytoplasmic side in Meta II. However, structural changes have already spread in the membrane from the site of the interaction between the Schiff base and its counterion, Glu113, to the region containing Glu122 and Asp83 even in the preceding intermediates with protonated Schiff base, bathorhodopsin (Batho), lumirhodopsin (Lumi), and metarhodopsin I (Meta I) (Ganter et al., 1989; Fahmy et al., 1993; Kandori & Maeda, 1995; Nishimura et al., 1995).

Depletion of water causes disturbance in the process from Meta I to Meta II (Nishimura et al., 1995). The presence of water molecules close to the Schiff base has been inferred from model studies with synthetic compounds (Gat & Sheves, 1993), energetics of a model system (Scheiner & Duan, 1991; Beppu et al., 1992), and theoretical studies based on the NMR data (Han et al., 1993) and on other aspects (Hárosi & Sándorfy, 1995). However, none has provided direct evidence for the presence of water. Recent studies on the kinetics of the deuterium exchange reaction of bovine rhodopsin strongly suggest the presence of water close to the Schiff base (Deng et al., 1994). Fourier transform infrared (FTIR) spectroscopy of bacteriorhodopsin first revealed the presence of the water molecules (Maeda et al., 1992) that undergo structural changes in the functional process. Subsequently, similar water molecules were detected in the process of the formation of Lumi, Meta I, and Meta II in bovine rhodopsin also (Maeda et al., 1993). For bacteriorhodopsin, the locations of these water molecules have been assigned by use of relevant mutant proteins (Maeda et al., 1994; Fischer et al., 1994; Kandori et al., 1995; Yamazaki et al., 1995, 1996; Brown et al., 1995; Hatanaka et al., 1996). This line of experiments has not been simple to apply to rhodopsin because only limited amounts of mutant proteins are available and because of the photobleaching property of rhodopsin. A way to overcome these problems was opened by successful recordings of the water signals in the photochemical reactions for the formation of bathorhodopsin at 77 K (Kandori & Maeda, 1995). In this case, reverse photoreactions from Batho to rhodopsin (Rho) and isorhodopsin (Iso) enabled us to record the spectrum repeatedly as many times as desired. Apart from its technical advantages, Batho is the first stable intermediate after the isomerization of the retinal chromophore that might involve the structural changes around the Schiff base.

The present studies aim at revealing the role of water in the interaction of the Schiff base with the counterion, Glu113, by comparing FTIR spectra upon conversion of Rho or Iso

† This work is supported by grants from the Japanese Ministry of Education, Culture, Sport and Science to A.M. (06404082, 07276218), Y.S. (08308038, 08458210), H.K. (07228231, 07839003), and A.T. (08740652).

* All correspondence should be addressed to this author. Phone and Fax: +81 75 753 4210. E-mail: maeda@ photo2.biophys.kyoto-u.ac.jp.

‡ Present address: Department of Biochemistry and Biophysics, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

[1] Abbreviations: Meta II, metarhodopsin II; Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; Rho, rhodopsin; Iso, isorhodopsin; DM, *n*-dodecyl β-D-maltoside; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

S0006-2960(96)02920-0 CCC: $14.00

to Batho of the wild type with the E113Q mutant protein supplemented with chloride as the counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989, 1991; Nathans 1990). Previous FTIR studies with the mutants of Glu113 focused on the protonated state of Glu113 in the Meta II formation (Jäger et al., 1994; Fahmy et al., 1994). This paper provides the first analysis of the mutant protein of bovine rhodopsin by FTIR spectra in the frequency region, where the stretching vibrations of functionally important O−H and N−H bands appear. The results give information so far unavailable on structural changes of water and N−H bonds, as well as the peptide carbonyls, around the site for the interaction of the Schiff base with the counterion in the photochemical process to form the Batho intermediate.

MATERIALS AND METHODS

DNA. Synthesized bovine rhodopsin cDNA which has *Hin*dIII and *Eco*RI sites at the 5′ and 3′ ends, respectively, was cloned into a pHE vector derived from pBluescript II KS+ (Stratagene).[2] Point mutation (Glu113→Gln) was introduced by a phosphorothioate method using Sculptor (Amersham). The mutant gene was sequenced by the dideoxy termination method with Sequenase (Amersham). The wild-type and mutant genes were recloned into the expression vector pUSRα (Kayada et al., 1995).

Rhodopsin. The recombinant proteins of wild-type bovine opsin and its mutant E113Q were expressed in 293S cells (Nathans et al., 1989), and reconstituted to rhodopsin by mixing the membrane fraction in the disrupted cell lysate with 11-*cis*-retinal (Kojima et al., 1996). All procedures below were carried out under dim red light at 4 °C unless otherwise specified. Rhodopsin was extracted in 1% dodecyl maltoside (DM) in buffer P (0.05 M HEPES, 0.14 M NaCl, pH 6.5). The supernatant fraction was obtained after 2 h incubation by centrifugation at 60000*g* for 30 min in a TL-100.3 rotor (Beckman). It was diluted 2-fold in buffer P to bring the final concentration of DM to 0.5%, and then incubated with rho 1D4 antibody (Molday & MacKenzie, 1983) that was linked to agarose beads (Affigel HZ, Bio-Rad) overnight. The beads were washed with 0.02% DM in buffer P. Rhodopsin was eluted in the same solution containing the C-terminal octadecapeptide of rhodopsin at room temperature (Oprian et al., 1987), supplemented with a 100-fold molar excess of phosphatidylcholine from egg yolk (Sigma type XI-E) dissolved in 0.75% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, and dialyzed to 0.2 M NaCl in 1 mM phosphate buffer (pH 5.7) for 3−4 days. The visible spectrum at this stage shows the maximum absorbance at 499 nm for both the wild-type and E113Q rhodopsins,[3] indicating that the pigments have native conformation. Rhodopsins were collected by centrifugation at 50000*g* for 45 min. The amount of the pigments in the supernatant was less than 10% of the total. The pellets were suspended in 40 μL of 5 mM NaCl in 1 mM phosphate buffer (pH 5.7). The whole suspensions were placed on a BaF_2 window (18 mm in diameter) and dried to a film under vacuum by an aspirator. The films were humidified by ∼1 μL of either H_2O, D_2O, or $H_2{}^{18}O$, sealed by a second window and a silicon rubber O-ring, and mounted in a brass cell holder in an Oxford cryostat (DN1704). The temperature was controlled by an Oxford ITC-4 temperature controller.

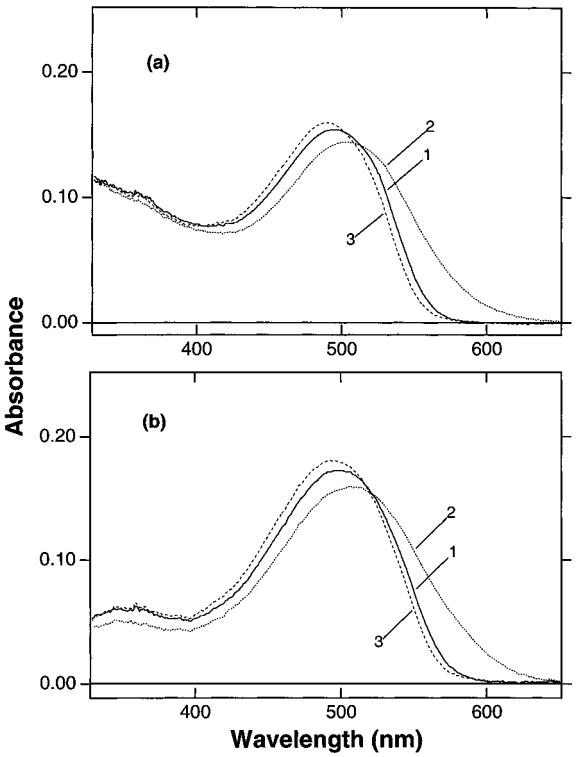

FIGURE 1: Absorption spectra in the visible region of the hydrated films at 77 K for wild type (a) and E113Q (b). The spectra produced by illumination with >610 nm light (1, solid lines) are rich in Rho. The spectra upon illumination with 500 nm light (2, dotted lines) are rich in Batho. The spectra upon illumination with >520 nm light (3, dashed lines) are rich in Iso. Increases toward shorter wavelength in (a) are due to scattering.

Spectroscopy. Difference FTIR spectra in the conversion of Rho and Iso to Batho were recorded in a Bio-Rad FTS60A/896 spectrometer, as described by Kandori and Maeda (1995). The sample film that was hydrated with H_2O was illuminated with 500 nm light to produce the photo-steady-state enriched by Batho. The subsequent illumination with >610 nm light reverts it largely to Rho. These procedures allowed measuring the difference spectrum between the states enriched with Batho and Rho. The difference spectrum between the states enriched with Batho and Iso was obtained by alternate illuminations with 500 nm and >520 nm light. All these procedures were repeated 9 times. Finally, the whole products were converted to Iso by illumination with >520 nm light. The film was then warmed to room temperature. The same procedures were repeated with the same film after the hydration with D_2O or $H_2{}^{18}O$.

Figure 1 shows visible spectra at 77 K recorded for the photo-steady-states enriched by Rho (solid lines), Iso (dashed lines), and Batho (dotted lines), respectively, of the wild type (a) and E113Q (b). These spectral shapes indicate the absence of any species with the absorption band around 380 nm and also that the red shift due to the formation of Batho in E113Q occurs in a similar extent to that in the wild type. They indicate that (1) no unstable intermediates are accumulated in the photo-steady-state enriched with Iso at 77 K, and (2) the sample of E113Q is completely converted to the form with the protonated Schiff base. The concentration

[2] The nucleotide sequences of the synthesized rhodopsin gene and the multicloning site of the vector will be published elsewhere.

[3] Sakmar et al. (1991) observed the maximum absorbance at 496 nm for E113Q in 0.1% DM in the presence of 0.1 M NaCl.

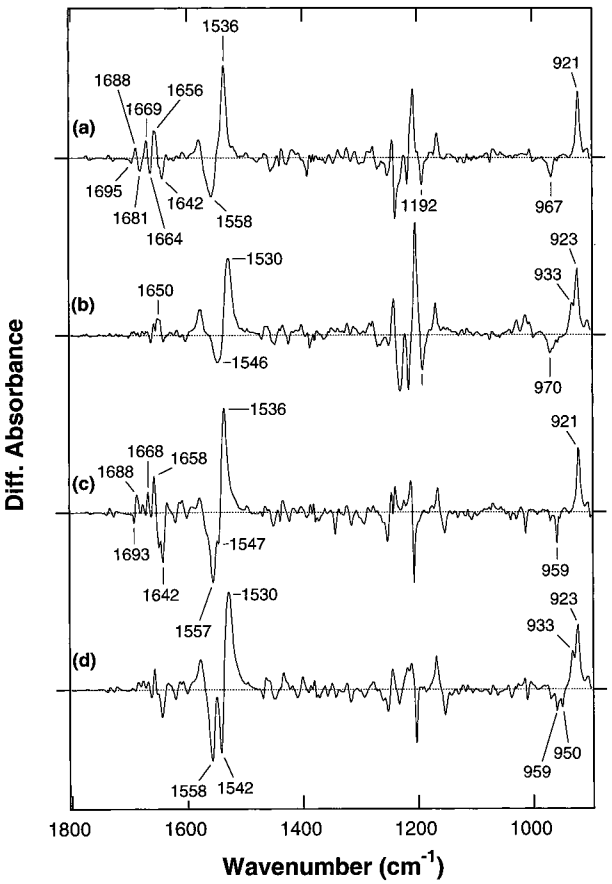

FIGURE 2: Batho minus Rho spectra of the wild type (a) and E113Q (b), and Batho minus Iso spectra of the wild type (c) and E113Q (d) in the 1800−900-cm^{-1} region in H_2O. The amplitudes were adjusted by the HOOP bands at 967−970 cm^{-1} for (a) and (b), and at 921 cm^{-1} for (a) and (c), and at 923 cm^{-1} for (b) and (d). The length of the ordinate from the top border to the bottom corresponds to 0.027 (a), 0.012 (b), 0.029 (c), and 0.021 (d) absorbance units.

of chloride in the films is high enough for the complete protonation of the Schiff base.

RESULTS

Batho minus Rho and Batho minus Iso Spectra. The Batho minus Rho and Batho minus Iso spectra of the recombinant wild-type pigment in chloride were recorded by the previously described procedures (Kandori & Maeda, 1995). These spectra (not shown) were not pure, as judged from the presence of the vibrational bands characteristic for Iso at 959 cm^{-1} and for Rho at 1192 and 967 cm^{-1}. The fraction of the contaminated Batho minus Iso spectrum in the Batho minus Rho spectrum was estimated to be 17%, and the fraction of the Batho minus Rho spectrum in the Batho minus Iso to be 3%, when the amplitudes of these spectra were adjusted by the 921-cm^{-1} band. The spectra containing contaminations were subtracted from each other, so as to eliminate the 959-cm^{-1} band from the Batho minus Rho spectrum, and the 1192-cm^{-1} band from the Batho minus Iso spectrum. Figure 2 shows the resulting pure Batho minus Rho (a) and Batho minus Iso (c) spectra of the wild type. They are identical to those of the recombinant wild-type protein in the absence of chloride except for slight differences in the amplitudes in some chromophore bands (not shown). The spectra are also coincident with those of native rhodopsin prepared by the same extraction and purification procedures from the rod outer segments (not shown), and with published spectra of rhodopsin in the rod outer segments in the 1800−800-cm^{-1} region (Siebert et al., 1983; Bagley et al., 1985; DeGrip et al., 1988; Sasaki et al., 1992) as well as in the 3700−800-cm^{-1} region (Kandori & Maeda, 1995).

The spectrum obtained after illumination of E113Q under the same conditions as used for the Batho minus Rho spectrum of the wild type exhibited two hydrogen out-of-plane (HOOP) bands at 959 and 950 cm^{-1}, in addition to the 970-cm^{-1} band of Rho (not shown). The bands at 959 and 950 cm^{-1} appeared in the Batho minus Iso spectrum of E113Q. The fraction of the Batho minus Iso spectrum to be removed from the Batho minus Rho spectrum was estimated to be 45%[4] by adjusting the amplitudes of both spectra by the 923-cm^{-1} band. A 16% fraction of Batho minus Rho spectrum had also to be removed from the Batho minus Iso spectrum estimated from the intensity of the 1192-cm^{-1} band. The subtractions were done so as to remove these bands, as in the case of the wild type. Figure 2 shows the pure Batho minus Rho (b) and Batho minus Iso (d) spectra.

Characteristic Bands of Batho. The C═C stretching vibrational frequency of Batho of the wild type is at 1536 cm^{-1} (a and c). The corresponding band of E113Q is present at 1530 cm^{-1} (b and d). From an empirical relation to the wavelength for the maximum absorbance in the visible region (Aton et al., 1977), Batho of E113Q is thus more red-shifted than that of the wild type. The C═C stretching bands of Rho and Iso of the wild type at 1558 (a) and 1557 (c) cm^{-1} are clearly different from those at 1546 and 1550 cm^{-1} observed in the resonance Raman spectra (Palings et al., 1987), respectively. Probably, different modes of the C═C stretching vibrations acquired larger intensities in the infrared and Raman spectra. Nevertheless, the C═C stretching band of Rho in E113Q shows a similar lower frequency shift to 1546 cm^{-1} (b) relative to the wild type (a). This tendency was observed in resonance Raman spectra of E113Q in DM (Lin et al., 1992). However, the corresponding bands of Iso at 1557 cm^{-1} for the wild type (c) are almost the same as 1558 cm^{-1} for E113Q (d). The 1542-cm^{-1} band of Iso in E113Q (d) is due to a different mode because it is abolished in D_2O (not shown). The visible spectra of E113Q in Figure 1 are skewed toward longer wavelength more than the wild type.

The HOOP band of the wild-type Batho at a frequency as low as 921 cm^{-1} (a, c) has been attributed to a C_{11}-HOOP mode uncoupled from C_{12}-HOOP (Palings et al., 1989). This uncoupling is assumed to be caused by perturbation from the closely located negative charge of Glu113 (Han & Smith, 1995). The present FTIR spectrum of E113Q exhibits a 923-cm^{-1} band as the main band for Batho and the 933-cm^{-1} band as a shoulder (b, d). A previous resonance Raman study on Batho of E113Q had shown a single band at 935 cm^{-1} (Lin et al., 1992), and attributed it to the C_{11}-HOOP mode. However, the 923-cm^{-1} band can be ascribed to the C_{11}-HOOP mode if this band is inactive in the Raman

[4] A larger fractional amount of Iso in the Batho minus Rho spectra may result from the higher quantum yield in the photoreaction of Batho to Iso for E113Q than in the wild type. A large fraction of Iso was also observed in the pump−probe resonance Raman spectrum of E113Q (Lin et al., 1992). No striking augmentation in the fractional amount of Rho is expected by changing the illumination conditions.

spectrum due to the absence of the twist around C_{11}. The HOOP band in the resonance Raman spectrum has been proposed to arise from a twisting of the chromophore (Eyring et al., 1980). Differences in HOOP bands could be due to differences in conformation between the chromophores of the wild type and E113Q, as envisaged from the C═C stretching vibrational modes. Irrespective of the assignments of HOOP bands, the C_{11}−H and C_{12}−H coupling is very low and the C_{12} is perturbed also in Batho of E113Q. Though outside the present scope, the assignment by isotope labeling is a prerequisite to drawing firm conclusions in these arguments.

While the HOOP band of Rho at 967 cm^{-1} (a) persists nearly intact in E113Q at 970 cm^{-1} (b), the 959-cm^{-1} band of Iso of the wild type (c) is accompanied by the 950-cm^{-1} band in E113Q (d). These two bands present in the same ratio in the photo-steady-states with the illumination by >520 nm (d) and >610 nm light (not shown) suggest that a single species of Iso is responsible for these two bands. The 950-cm^{-1} band was thus completely removed from the Batho minus Rho spectrum in the subtraction process (b). The presence of two bands with the same mode was observed for the 947-cm^{-1} band with its shoulder at the lower frequency in Lumi (Ohkita et al., 1995). The absence of intermediates like Lumi and Meta I in the spectra of Batho or Rho is further assured by the absence of bands around 950 cm^{-1} characteristic for these intermediates (Ohkita et al., 1995). The 950-cm^{-1} band in the Batho minus Rho spectrum could not be due to Lumi or Meta I because the photo-steady-state enriched with Iso did not show the absorbance band around 380 nm upon warming to room temperature as described under Materials and Methods.

Peptide Carbonyls. We examined the amide I regions in Figure 2 further. The positive bands at 1688, 1669, and 1656 cm^{-1} and the negative smaller bands at 1695, 1681, 1664, and 1642 cm^{-1} in the Batho minus Rho spectrum of the wild type (a) all persisted in D_2O (not shown). These bands are thus assigned to amide I (mainly composed of the stretching mode of peptide C═O). Similar bands at 1693 (−), 1688 (+), 1668 (+), 1658 (+), and 1642 (−) cm^{-1} are seen in the Batho minus Iso spectrum of the wild type (c). All of them are absent in the spectra of E113Q (b and d). The 1650-cm^{-1} band of E113Q (b) has a different mode because it disappears in D_2O (not shown). The intensities of these amide bands roughly correspond to single residues. The changes of the peptide backbone thus arise from changes in the strength of local H-bonding without gross conformational changes.

Water Structural Changes. Figure 3 shows the Batho minus Rho spectra in the 3700−3200-cm^{-1} region for the wild type (a) and E113Q (b) in H_2O (solid lines) and $H_2{}^{18}O$ (dashed lines). The spectra of the wild type in the 3700−3430-cm^{-1} region (a) are completely identical to those described previously (Kandori & Maeda, 1995). All the bands above 3500 cm^{-1} are shifted in $H_2{}^{18}O$, containing the O−H stretching vibration of water. In E113Q, a negative band appears at 3569 cm^{-1} (b) that is at a slightly higher frequency than the wild type at 3565 cm^{-1} (a). The positive band of E113Q at 3544 cm^{-1} (b) appears to be a band shifted upward from 3522 cm^{-1} of the wild type (a). However, this idea does not account for the shift on the small bilobe feature at 3545 (+) and 3537 (−) cm^{-1} of the wild type (a). More likely, the positive band at 3544 cm^{-1} in E113Q (b) originates

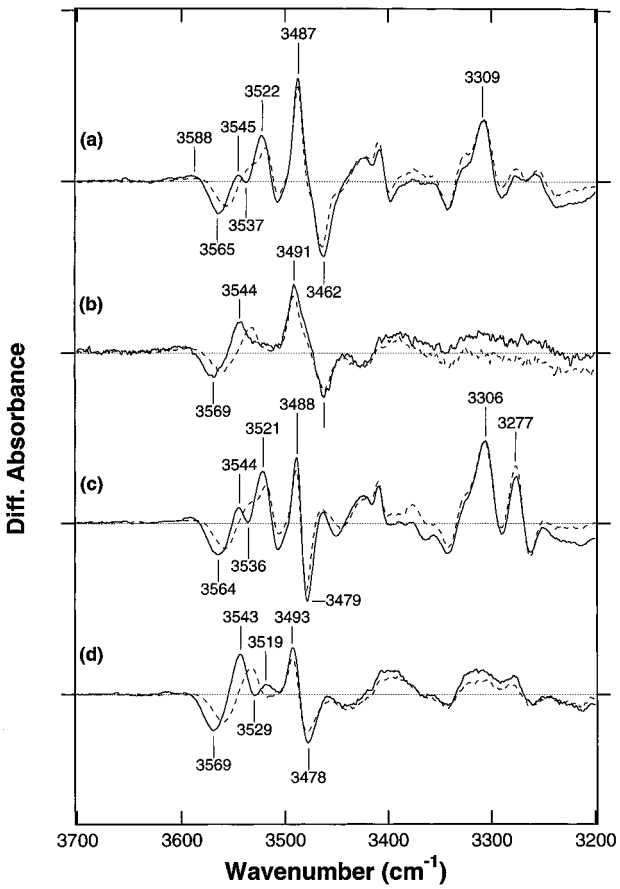

FIGURE 3: Batho minus Rho spectra of the wild type (a) and E113Q (b), and Batho minus Iso spectra of the wild type (c) and E113Q (d) in the 3700−3200-cm^{-1} region. The spectra in H_2O are shown by solid lines and those in $H_2{}^{18}O$ by dashed lines. The length of the ordinate from the top border to the bottom corresponds to 0.0095 (a), 0.0042 (b), 0.0102 (c), and 0.0073 (d) absorbance units for the spectra in H_2O (solid lines) and 0.0095 (a), 0.0051 (b), 0.0097 (c), and 0.0089 (d) for the spectra in $H_2{}^{18}O$.

from the absence of the cancelling negative band which appears as a small negative band at 3537 cm^{-1} in the wild type (a). According to this scheme, a bilobe in E113Q with the bands at 3569 (−) and 3544 (+) cm^{-1} (b) is present in the wild type at lower frequencies by ∼4 cm^{-1} (a), and another bilobe with the positive band at 3522 cm^{-1} and a negative band that cancels the positive band around 3540 cm^{-1} is present in the wild type (a), but absent in E113Q (b).

As described previously (Kandori & Maeda, 1995), the spectrum in this region contains smaller bands that do not shift in D_2O. Further analysis by curve fitting was therefore conducted on the spectra corrected for the D_2O-insensitive fraction. Figure 4 shows spectra in H_2O (solid lines in a and d) and in D_2O (dotted lines in a and d) for the wild type (a) and E113Q (d) in the 3620−3500-cm^{-1} region, respectively. The spectra after subtraction of the D_2O-insensitive fraction are shown by solid lines for the wild type (b) with bands at 3590 (+), 3566 (−), 3548 (+), 3537 (−), and 3523 (+) cm^{-1} and for E113Q (e) with bands at 3571 (−) and 3545 (+) cm^{-1}. According to the idea described above, the Batho minus Rho spectrum of the wild type (b) was resolved into six Gaussian curves (c), the sum of which (dotted line in b) reproduced the original spectrum (solid line in b). The bands at 3564 (−) and 3542 (+) were then combined with the smaller bands at 3591 (+) and 3573 (−) cm^{-1} in (c).

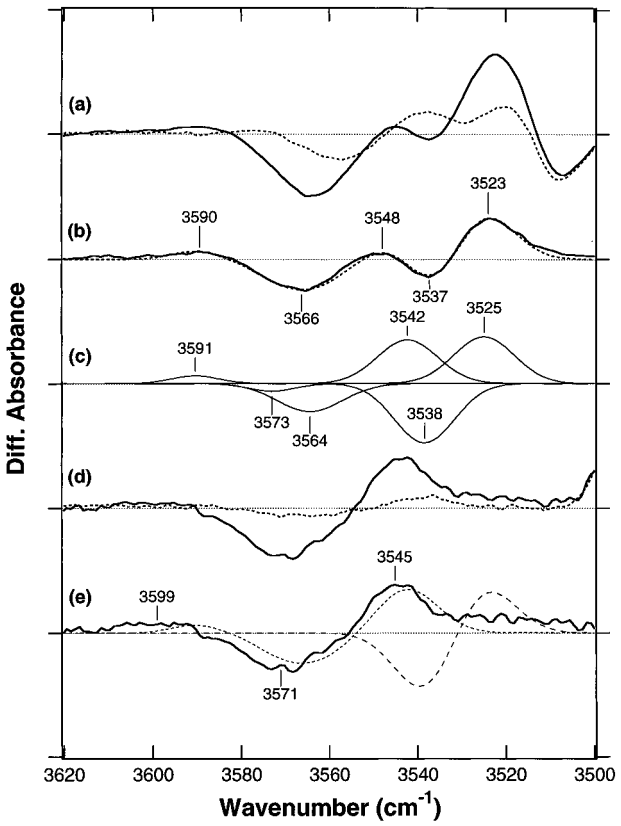

FIGURE 4: Batho minus Rho spectra in the 3620−3500-cm^{-1} region. Those in D_2O [dotted line in (a) for the wild type and (d) for E113Q] were subtracted from the corresponding spectra in H_2O [solid line in (a) for the wild type and (d) for E113Q]. The spectra thus obtained are shown as solid lines in (b) for the wild type and (e) for E113Q. The spectrum of the wild type was resolved into six Gaussian curves (c). The sum of all of them is shown by a dotted line in (b). The reconstituted spectrum of E113Q, as described in the text, is shown with a dotted line in (e). The dashed line in (e) is the sum of the abolished bilobe in E113Q. The length of the ordinate from the top border to the bottom corresponds to 0.0048 (a) and 0.0021 (d) absorbance units for the spectra in H_2O (solid lines) and 0.0039 (a) and 0.0022 (d) for the spectra in D_2O.

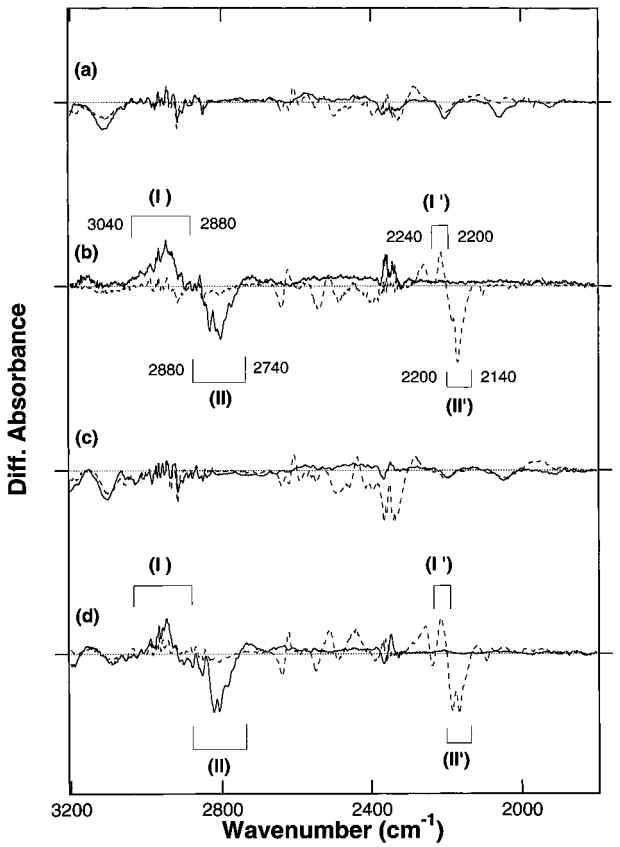

FIGURE 5: Batho minus Rho spectra of the wild type (a) and E113Q (b), and Batho minus Iso spectra of the wild type (c) and E113Q (d) in the 3200−1800-cm^{-1} region. The spectra in H_2O are shown with solid lines and those in D_2O with dashed lines. Features in the 2400−2300-cm^{-1} region are due to CO_2. The length of the ordinate from the top border to the bottom corresponds to 0.0085 (a), 0.0038 (b), 0.0091 (c), and 0.0065 (d) absorbance units for the spectra in H_2O (solid lines) and 0.0069 (a), 0.0038 (b), 0.0083 (c), and 0.0066 (d) for the spectra in D_2O.

Their sum (dotted line in e) nicely reproduced the spectrum of E113Q (solid line in e) if one shifted the 3545-cm^{-1} band by about 3 cm^{-1}, the 3571-cm^{-1} band by 5 cm^{-1}, and 3599-cm^{-1} band by 7 cm^{-1} toward the lower frequencies. These pictures explain why the bilobe that consists of the bands at 3525 (+) and 3538 (−) cm^{-1} (c) is absent in E113Q as shown by the dashed line in (e), and the bilobe with bands at 3542 (+) and 3564 (−) cm^{-1} (c) in the wild type appears with slightly higher frequency shifts in E113Q. These bands correspond to one or a few molecules of water involved in the reaction of rhodopsin for an observed absorbance change of 0.0002−0.0004 compared with the molar extinction of ∼100 in the same frequency region (Glew & Rath, 1971).

The same procedures were applied to the Batho minus Iso spectra (Figure 3c,d). The spectrum of the wild type was divided into two large bilobes at 3536 (−) and 3525 (+) cm^{-1} and 3569 (−) and 3542 (+) cm^{-1}, along with a small bilobe at 3555 (−) and 3590 (+) cm^{-1} (not shown). These are similar to those obtained with the Batho minus Rho spectra (Figure 2c) except for the small negative band at 3555 cm^{-1}. The Batho minus Iso spectrum of E113Q then can be reproduced by summing the bilobes at 3569 (−) and 3542 (+) cm^{-1} and at 3555 (−) and 3590 (+) cm^{-1} (not shown) and moving the summed spectrum by 4 cm^{-1} toward higher frequency (not shown). The bilobe at 3536 (−) and 3525 (+) cm^{-1} (not shown) is missing in E113Q as the Batho minus Rho spectrum.

N−H Stretching Vibrations. Figure 3 also shows bands that do not belong to water. A bilobe at 3487 (+) and 3462 (−) cm^{-1} in the Batho minus Rho spectrum of the wild type (a) could be attributed to the N−H stretching vibration of Batho and Rho, respectively. Iso exhibits it at 3479 cm^{-1} (c), distinct from 3462 cm^{-1} of Rho (Kandori & Maeda, 1995). These were conserved in E113Q (b and d) except for a slight shift of the 3487-cm^{-1} band of Batho to 3491 cm^{-1}. It should be noticed that the 3479-cm^{-1} band of Iso, which had been present in the raw Batho minus Rho spectrum before the corrections (not shown), was completely removed by the subtraction procedure described above (see Figure 2b). The positive bands at 3309 cm^{-1} in the Batho minus Rho (a in Figure 3) and at 3306 and 3277 cm^{-1} in the Batho minus Iso (c) spectra were insensitive to D_2O and are ascribed to amide A (stretching vibrational mode of peptide N−H) (Kandori & Maeda, 1995). Although some accompanying negative bands are present in this region, the intensities are much larger in the positive side of Batho. These bands are abolished in E113Q (b and d). This may be correlated with the abolition of amide I bands in E113Q (see above and Figure 2).

$N-H^+$ Stretching Vibrations. Figure 5 shows the spectra in the 3200−1800-cm^{-1} region for the Batho minus Rho

spectrum of the wild type (a) and E113Q (b) and the Batho minus Iso spectrum of the wild type (c) and E113Q (d) in H_2O (solid lines) and D_2O (dashed lines). Complex bands that are not present in the spectra of the wild type (a and c) are noted for E113Q in both the Batho minus Rho (b) and Batho minus Iso (d) spectra. The bilobe feature marked by I (+; 3040−2880 cm^{-1}) and II (−; 2880−2740 cm^{-1}) is shifted in D_2O to that marked by I′ (+; 2240−2200 cm^{-1}) and II′ (−; 2200−2140 cm^{-1}). These bands are located in the regions of the $N-H^+$ and $N-H_2^+$ stretching vibrations (Bellamy, 1954; Nakanishi et al., 1990). Although histidine $N-H^+$ could be a candidate (Lin-Vien et al., 1991), it would be absent in the intramembrane domain. The only likely candidate for it in rhodopsin is the protonated Schiff base, which would change the strength of H-bonding with chloride upon Batho formation in E113Q (b, d) and not in the wild type (a, c). These two can be distinguished definitely if ^{15}N labeling of either the lysine or the histidine side chains becomes possible in future studies. Arginine N−H is located above 3000 cm^{-1}, characteristic of free imine (Nakanishi et al., 1990).

DISCUSSION

The present studies aim at revealing the role and location of internal water molecules in relation to the structural features around the Schiff base in rhodopsin. For this purpose, FTIR spectral changes observed upon Batho formation (Kandori & Maeda, 1995) were analyzed for the mutant protein E113Q, in which the regular counterion of Glu113 was replaced by chloride. The water changes in the Batho minus Rho spectrum of the wild type can be reconstructed satisfactorily from three bilobe bands (Figure 4). In E113Q, a bilobe at 3564 (−) and 3542 (+) cm^{-1} is shifted by 3−5 cm^{-1} toward higher frequencies, and another at 3538 (−) and 3525 (+) cm^{-1} is absent. Besides these, a small bilobe at 3591 (+) and 3573 (−) cm^{-1} is shifted toward higher frequencies in E113Q. The bilobe at 3538 (−) and 3525 (+) cm^{-1} must originate from one or a few water molecules located close to Glu113, and the bilobe at 3564 (−) and 3542 (+) cm^{-1} may be due to other water molecules in the intramembrane region, which are influenced by the replacement of Glu113. These water molecules, with O−H stretching vibrations at 3564 and 3538 cm^{-1} in the unphotolyzed state, have a structural role in forming stronger H-bonding upon Batho formation.

Batho formation in the wild type is accompanied also by frequency shifts and intensity increases for both the peptide carbonyls (amide I) and amides (amide A). Most of them are absent in E113Q (Figures 2 and 3), suggesting connection of these residues to Glu113 and its associated water molecules. In this case, the amide hydrogen is protected from exchanging with D_2O by forming H-bonding, presumably with a peptide carbonyl (Earnest et al., 1990). Peptide carbonyls may participate through their polar character to interact with water molecules. One of these, associated with the 1688 (+) cm^{-1} band, has been detected in the Meta II minus Rho spectrum and has been attributed to the peptide carbonyl close to Gly90 (Zvyaga et al., 1996). This residue has been proposed to be located close to the Schiff base (Rao et al., 1994).

The C═N stretching vibrational mode in H_2O is a coupled mode of the intrinsic C═N stretching vibration and the C═N−H in-plane bending vibration (Rodman-Gilson et al., 1988). In D_2O, this coupling is eliminated because of the lower frequency of the C═N−D in-plane bending vibration mode. Thus, the C═N stretching vibrational frequency in D_2O is largely affected by the structure and electrostatic environment of the C═N bond. A previous resonance Raman spectral study (Lin et al., 1992) showed that the C═N stretching frequency is 1631 cm^{-1} in E113Q, whose Schiff base interacts with a chloride. This is almost identical with that of the free protonated Schiff base in solution at 1632 cm^{-1} (Bagley et al., 1985; Baasov et al., 1987). The lower value of 1624 cm^{-1} of the wild type suggests that the interaction of the Schiff base with Glu113 is different from a simple electrostatic interaction as observed with chloride. The present study indicates that the interaction of the Schiff base with Glu113 is constructed through H-bonding with water molecules, the peptide carbonyls, and amides. This seems to be valid for both the unphotolyzed states (Rho and Iso) and Batho. The water molecules and the peptide backbone participate with stronger H-bonding in Batho, while the H-bonding strength of the protonated Schiff base in Batho is identical with that in Rho or Iso [Figure 5; also see Deng and Callender (1987)]. It has also been shown that water molecules and peptide backbones are involved in the stabilization of the protonated Schiff base in octopus acid metarhodopsin (Nishimura et al., 1997), which is supposed to be responsible for the activation of Gq in analogy with another invertebrate rhodopsin (Terakita et al., 1993).

These results can be merged into a notion that the water molecule is the direct acceptor of H-bonding of the Schiff base and moves together upon isomerization to Batho. This idea has been first proposed by Deng et al. (1994) and explains also the invariable frequency upon going to Batho in octopus rhodopsin (Nishimura et al., 1997). The negative band at 3538 cm^{-1} due to water O−H close to Glu113 (Figure 4c) is hardly observable in the Lumi minus Rho spectrum (Maeda et al., 1993). This along with very weak H-bonding of the Schiff base (Ganter et al., 1989) suggests that this water molecule detaches from the Schiff base in Lumi.

In order to account for resonance Raman and NMR data (Palings et al., 1987; Han et al., 1993; Han & Smith, 1995), they have placed one of the carboxyl oxygens of Glu113 close to the C_{12} of the retinal to compensate for the positive electric charge and a water molecule as the acceptor of the H-bonding of the protonated Schiff base. The present FTIR study reveals the water molecule which forms H-bonding with Glu113 and the Schiff base. This water molecule also links to the peptide backbone. Another water molecule with the O−H stretching frequency at 3564 cm^{-1} (Figure 4c) located in the membrane may be connected to this structure. A chloride in place of Glu113 may be located also at a site close to the C_{12} in Batho and probably Rho of E113Q, because the C_{11}-HOOP is uncoupled from C_{12}-HOOP as the wild type. Nevertheless, distribution of the negative charge in chloride and the carboxylic oxygen might be different from each other and bring different interactions with the chromophore, resulting in different structures as reflected in the HOOP, and C═C and C═N stretching vibrations. The absence of the water molecule at the site for the direct acceptor of the protonated Schiff base in E113Q may account for weak H-bonding of the Schiff base (Lin et al., 1992), changes in H-bonding strength of the Schiff base upon the

formation of Batho (Figure 5), and the abolition of the structural changes of the peptide backbone (Figure 2).

ACKNOWLEDGMENT

We are grateful to Prof. Robert S. Molday (University of British Columbia) for providing us the hybridoma producing rho 1D4 antibody. Thanks are also due to Prof. Janos K. Lanyi for improving the style of the manuscript.

REFERENCES

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995−2999.

Baasov, T., Friedman, N., & Sheves, M. (1987) *Biochemistry* 26, 3210−3217.

Bagley, K. A., Balogh-Nair, V., Croteau, A. A., Dollinger, G., Ebrey, T. G., Eisenstein, L., Hong, M. K., Nakanishi, K., & Vittitow, J. (1985) *Biochemistry 24*, 6055−6071.

Bellamy, L. J. (1954) in *The Infrared Spectra of Complex Molecules*, pp 259−260, John Wiley & Sons, New York.

Beppu, Y., Kakitani, T., & Tokunaga, F. (1992) *Photochem. Photobiol. 56*, 1113−1117.

Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., & Lanyi, J. K. (1995) *J. Biol. Chem. 270*, 27122−27126.

DeGrip, W. J., Gray, D., Gillespie, J., Bovee, P. H. M., van den Berg, E. M. M., Lugtenburg, J., & Rothschild, K. J. (1988) *Photochem. Photobiol. 48*, 497−504.

Deng, H., & Callendar, R. H. (1987) *Biochemistry 26*, 7418−7426.

Deng, H., Huang, L., Callendar, R. H., & Ebrey, T. (1994) *Biophys. J. 66*, 1129−1136.

Earnest, T. N., Herzfeld, J., & Rothschild, K. J. (1990) *Biophys. J. 58*, 1539−1546.

Eyring, G., Curry, B., Mathies, R., Fransen, R., Palings, I., & Lugtenburg, J. (1980) *Biochemistry 19*, 2410−2418.

Fahmy, K., Jäger, F., Beck, M., Zvyaga, T. A., Sakmar, T. P., & Siebert, F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10206−10210.

Fahmy, K., Siebert, F., & Sakmar, T. P. (1994) *Biochemistry 33*, 13700−13705.

Fischer, W. B., Sonar, S., Marti, T., Khorana, H. G., & Rothschild, K. J. (1994) *Biochemistry 33*, 12757−12762.

Franke, R. R., König, B., Sakmar, T. P., Graham, R. M., Khorana, H. G., & Hofmann, K. P. (1990) *Science 250*, 123−125.

Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767−14774.

Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., & Siebert, F. (1989) *Biochemistry 28*, 5954−5962.

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.

Han, M., DeDecker, B. S., & Smith, S. O. (1993) *Biophys. J. 65*, 899−906.

Hárosi, F. I., & Sándorfy, C. (1995) *Photochem. Photobiol. 61*, 510−517.

Hatanaka, M., Sasaki, J., Kandori, H., Ebrey, T. G., Needleman, R., Lanyi, J. K., & Maeda, A. (1996) *Biochemistry 35*, 6308−6312.

Jäger, F., Fahmy, K., Sakmar, T. P., & Siebert, F. (1994) *Biochemistry 33*, 10878−10882.

Kandori, H., & Maeda, A. (1995) *Biochemistry 34*, 14220−14229.

Kandori, H., Yamazaki, Y., Sasaki, J., Needleman, R., Lanyi, J. K., & Maeda, A. (1995) *J. Am. Chem. Soc. 117*, 2118−2119.

Kayada, S., Hisatomi, O., & Tokunaga, F. (1995) *Comp. Biochem. Physiol. B 110*, 599−604.

Kojima, D., Oura, T., Hisatomi, O., Tokunaga, F., Fukada, Y., Yoshizawa, T., & Shichida, Y. (1996) *Biochemistry 35*, 2625−2629.

König, B., Arendt, A., MacDowell, J. H., Kahlert, M., Hargrave, P. A., & Hofmann, K. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6878−6882.

Lin, S. W., Sakmar, T. P., Franke, D. R., Khorana, H. G., & Mathies, R. A. (1992) *Biochemistry 31*, 5105−5111.

Lin-Vien, D., Colthus, N. B., Fateley, W. G., & Grassel, J. G. (1991) in *The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules*, p 165; Academis Press, Inc., Tokyo.

Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462−467.

Maeda, A., Ohkita, Y. J., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 12033−12038.

Maeda, A., Sasaki, J., Yamazaki, Y., Needleman, R., & Lanyi, J. K. (1994) *Biochemistry 33*, 1713−1717.

Molday, R. S., & MacKenzie, D. (1983) *Biochemistry 22*, 653−660.

Nakanishi, K., Solomon, P. H., & Furudate, N. (1990) in *Infrared Absorption spectrum* (in Japanese), p 38, Nankodo, Tokyo.

Nathans, J. (1990) *Biochemistry 29*, 937−942.

Nathans, J., Weitz, C. J., Arawal, N., Nir, I., & Papermaster, D. S. (1989) *Vision Res. 29*, 907−914.

Nishimura, S., Sasaki, J., Kandori, H., Lugtenburg, J., & Maeda, A. (1995) *Biochemistry 34*, 16758−16763.

Nishimura, S., Sasaki, J., Kandori, H., Matsuda, T., Fukada, Y., & Maeda, A. (1996) *Biochemistry 35*, 13267−13271.

Nishimura, S., Kandori, H., Nakagawa, M., Tsuda, M., & Maeda, A. (1997) *Biochemistry 36*, 864−870.

Ohkita, Y. J., Sasaki, J., Maeda, A., Yoshizawa, T., Groesbeek, M., Verdegem, P., & Lugtenburg, J. (1995) *Biophys. Chem. 56*, 71−78.

Oprian, D. D., Molday, R. S., Kaufmann, R. J., & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874−8878.

Palings, I., Pardoen, J. A., van den Berg, E. M. M., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987) *Biochemistry 26*, 2544−2556.

Palings, I., van den Berg, E. M. M., Lugtenburg, J., & Mathies, R. A. (1989) *Biochemistry 28*, 1498−1507.

Rao, V. R., Cohen, G. B., & Oprian, D. D. (1994) *Nature 367*, 639−642.

Resek, J. F., Farrens, D., & Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7643−7647.

Rodman-Gilson, H. S., Honig, B., Croteau, A., Zarrill, G., & Nakanishi, K. (1988) *Biophys. J. 53*, 261−269.

Sakmar, T. P., Franke, D. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.

Sakmar, T. P., Franke, D. R., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3079−3083.

Sasaki, J., Maeda, A., Shichida, Y., Groesbeek, M., Lugtenburg, J., & Yoshizawa, T. (1992) *Photochem. Photobiol. 54*, 1063−1071.

Scheiner, S., & Duan, X. (1991) *Biophys. J. 60*, 874−883.

Siebert, F., Mäntele, W., & Gerwert, K. (1983) *Eur. J. Biochem. 136*, 119−127.

Terakita, A., Hariyama, T., Tsukahara, Y., Katsukura, Y., & Tashiro, H. (1993) *FEBS Lett. 330*, 197−200.

Yamazaki, Y., Hatanaka, M., Kandori, H., Sasaki, J., Karstens, W. F. J., Raap, J., Lugtenburg, J., Bizounok, M., Herzfeld, J., Needleman, R., Lanyi, J. K., & Maeda, A., (1995) *Biochemistry 34*, 7088−7093.

Yamazaki, Y., Tuzi, S., Saito, H., Kandori, H., Needleman, R., Lanyi, J. K., & Maeda, A. (1996) *Biochemistry 35*, 4063−4068.

Zhukovsky, E. A., & Oprian, D. D. (1989) *Science 246*, 928−930.

Zvyaga, T. A., Fahmy, K., Siebert, F., & Sakmar, T. P. (1996) *Biochemistry 35*, 7536−7545.

BI962920T