Trp86 → Phe Replacement in Bacteriorhodopsin Affects a Water Molecule near Asp85 and Light Adaptation†

Minoru Hatanaka,‡ Ryuji Kashima,‡ Hideki Kandori,‡ Noga Friedman,§ Mordechai Sheves,§ Richard Needleman,|| Janos K. Lanyi,⊥ and Akio Maeda*,‡

Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-01, Japan, Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201, and Department of Physiology and Biophysics, University of California, Irvine, California 92717

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ABSTRACT: Illumination of the Trp86 → Phe mutant of bacteriorhodopsin causes anomalous light adaptation, i.e., isomerization of the retinal from *all-trans* to 13-*cis*, 15-*syn*. FTIR spectral analysis shows that illumination at 250 K yields two 13-*cis* photoproducts, the conventional 13-*cis*, 15-*syn* state, BR_C, and another termed BR_X. BR_X is different from BR_C because it has a lower N−H in-plane bending frequency and a higher $C_{14}-C_{15}$ stretching frequency, as well as an absence of coupling between these modes. BR_X, which is stable at 275 K, is more abundant in the photosteady state produced by longer wavelength light and detected as the only photoproduct at 170 K. Its different structural features result from distortion of the $C_{14}-C_{15}$ bond of the chromophore. In the W86F mutant protein, the small structural changes of a water molecule in the conversion between the *all-trans* and 13-*cis*, 15-*syn* forms and in the formation of the K photointermediate are absent, but the larger changes of water molecule(s) that normally occur in the L and M intermediates are present. We propose that Trp86, together with Asp85, is involved in binding the water molecule and in preventing the formation of the 13-*cis*, 15-*syn* photoproducts, BR_C and BR_X, when the wild type protein is illuminated.

Bacteriorhodopsin is the transmembrane protein in the purple membrane patches of *Halobacterium salinarium* that functions as a light-dependent proton pump. The chromophore retinal, linked to Lys216 through a protonated Schiff base, lies in the center of the membrane. The extracellular region of the protein contains the residues that participate in proton release and is more abundant in polar amino acid residues than the opposite cytoplasmic region (Henderson et al., 1990). In the dark, the chromophore is a thermally equilibrated mixture of the *all-trans* and 13-*cis*, 15-*syn* forms (Harbison et al., 1984). The latter is converted to the *all-trans* form upon sustained illumination, the "light adaptation". Only the *all-trans* form is active in proton pumping, and its photochemical cycle contains the successive intermediates called K, L, M, N, and O (Lozier et al., 1975).

In the unphotolyzed state, the protonated Schiff base forms a weak H bond with Asp85 (de Groot et al., 1989), which accepts a proton from the Schiff base in the L-to-M process. Asp85 is located on the extracellular side of the retinal as part of a complex counterion system with Arg82 and Asp212. Asp212 makes further H bonds with the aromatic amino acid residues, Trp86, Tyr57, and Tyr185. Trp86 on the extracellular side, and Trp182 on the opposite side, together immobilize the proximal end of the retinal. Of the eight tryptophan residues, Trp86 and Trp182 are the only ones that exhibit perturbations of the indole ring in the photocycle, indicating steric interaction with the retinal (Roepe et al., 1988; Rothschild et al., 1989; Weidlich et al., 1996). Trp182 is located close to the 9-methyl group of the retinal and takes part in the L-to-M conversion by accelerating the deprotonation of the Schiff base (Yamazaki et al., 1995; Weidlich et al., 1996). Mutations of these residues cause blue shifts and reduced proton pumping activity (Mogi et al., 1989).

Previous FTIR studies with the mutant protein of Trp86 expressed in *Escherichia coli* (Rothschild et al., 1989) showed incomplete light adaptation, as revealed by the observation of the K intermediate of the 13-*cis*, 15-*syn* species at 77 K. Upon reexamining its light adaptation, we found a new photoproduct with altered structure in the retinal moiety close to the Schiff base. The indole N−H of Trp86 is near the carboxylate of Asp85 also (Grigorieff et al., 1996). One of the internal water molecules detected by FTIR spectra (Maeda et al., 1992) is located close to Asp85, Asp212 (Maeda et al., 1994; Kandori et al., 1995), and Tyr57 (Fischer et al., 1994). Thus, we suspected that Trp86 is also involved in binding this water molecule. The present studies on the effects of the W86F mutation on changes of the O−H stretching vibration of water indeed reveal such an additional role of Trp86.

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* To whom correspondence should be addressed: Department of Biophysics, Graduate School of Science, Kyoto University, Kita-shirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-01, Japan. Telephone and Fax: 81 75 753 4210. E-mail: maeda@photo2-biophys.kyoto-u.ac.jp.

‡ Kyoto University.

§ Weizmann Institute of Science.

|| Wayne State University School of Medicine.

⊥ University of California.

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MATERIALS AND METHODS

Sample Manipulation for FTIR Studies. The W86F protein was expressed in *H. salinarium* as described previously for other mutants of bacteriorhodopsin (Needleman et al., 1991). The protein was prepared in the form of purple membranes, by the method of Oesterhelt and Stoeckenius (1974). Reconstitution with ^{13}C-labeled bacteriorhodopsin was done by adding the equimolar amount of $^{13}C_{14}-^{13}C_{15}$ retinal to bacterioopsin, which was prepared by photobleaching in the presence of 2 M hydroxylamine and subsequent washings with a 2% solution of bovine serum albumin. The labeled retinal was synthesized by the method described previously (Lugtenburg, 1985; Friedman et al., 1989). A 40 μL aliquot of the protein in water or 5 mM borate buffer (pH 9) (for the M intermediate) was put on a BaF_2 window with a diameter of 10 mm and dried in room air. Hydration with H_2O or D_2O and subsequent insertion of the sample film in the cryostat were described previously (Hatanaka et al., 1996). Light adaptation was with >500 nm light for 2 min at 275 K. Although this does not convert completely to the *all-trans* form, as described below, no further spectral changes were observed upon repeated illuminations under the same conditions. Photoreactions for the formation of the K, L, and M intermediates were produced by illuminating with 500 nm light for 3 min at 77 K, >600 nm light for 2 min at 170 K, and >500 nm light for 1 min at 230 K, respectively.

Isomer Analysis of the Retinal Chromophore. The sample of either illuminated or unilluminated films was rinsed off the window and subjected to brief sonication. The protein in the solution was denatured by adding 2.5 volumes of ethanol, and the retinals were extracted immediately into the same volume of hexane (Scherrer et al., 1989). The composition of the retinal isomers was determined from the chromatographic pattern monitored at 365 nm from a Zorbax-Sil column in a Shimadzu HPLC LC-7 system. The solvent was 12% diethyl ether in hexane, and the flow rate was 1.5 mL/min.

RESULTS

Spectra of the K, L, and M Intermediates. Figure 1 compares spectra in the 1800−800 cm^{-1} region for the photoreactions of the light-adapted state (BR)[1] of the W86F protein that produce the K (a), L (b), and M (c) intermediates (solid lines) with the corresponding states of the wild type (dotted lines). The spectral shapes of the mutant protein are not very different from those of the wild type. The only significant changes are that the negative band at 1215 cm^{-1} of the wild type is replaced by a more intense band at 1219 cm^{-1} and that the W86F protein shows a negative C═C stretching vibrational band at 1532 cm^{-1} instead of the 1527 cm^{-1} band of the wild type. The latter must arise from the blue shift of the absorption spectrum (the maximum is at 534 nm for the dark-adapted state and 547 nm for the light-adapted state)[2] on the basis of an inverse linear relation between the absorption maximum and the frequency of the

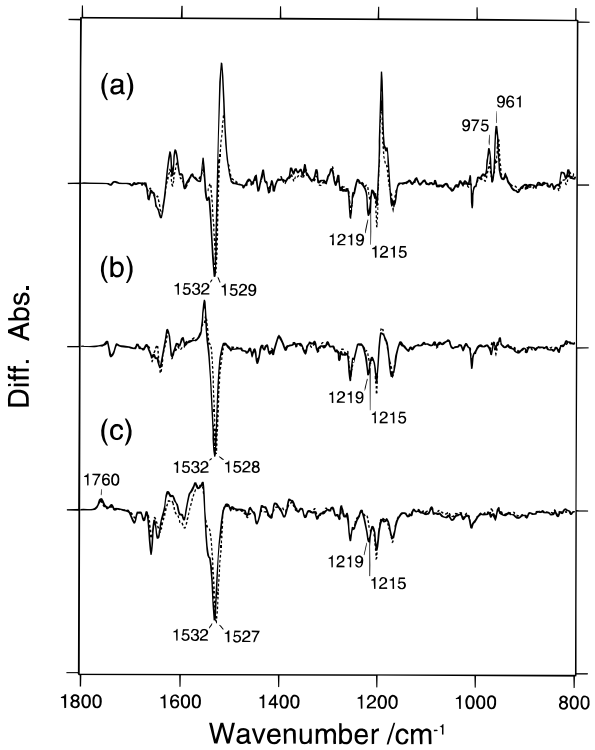

FIGURE 1: K minus BR (a), L minus BR (b), and M minus BR (c) spectra in the 1800−800 cm^{-1} region. Solid lines are for W86F, and the dotted lines are for the wild type. One division in the ordinate in part a corresponds to 0.09 and 0.03 absorbance unit for the wild type and W86F, respectively. All spectra were normalized at the negative band around 1530 cm^{-1}.

C═C stretching vibration (Aton et al., 1977). Slight shifts of the hydrogen out-of-plane (HOOP) bands at 974 and 957 cm^{-1} of the wild type to 975 and 961 cm^{-1}, respectively, in the K minus BR spectrum are also characteristics of W86F. These bands are due to the modes containing C_{15}, N−HOOP (Maeda et al., 1991). In contrast to the previous results (Rothschild et al., 1989), a nearly normal K minus BR spectrum was obtained by cooling the sample to 77 K after the normal procedure for light adaptation. The difference could arise from the fact that our sample is in the purple membrane in contrast to the *E. coli*-expressed protein in the pervious studies.

Photoreversibility of the Light Adaptation. The difference spectrum for light adaptation of W86F by >500 nm light at 275 K is shown in Figure 2a. No further changes were observed in an additional 2 min of illumination (not shown). HPLC analysis of the extracted retinals showed, however, that, in addition to *all-trans*-retinal, the light-adapted state of W86F contains 13-*cis* (26%), along with small fractions of 11-*cis* (2%) and 9-*cis* (1%). Such incomplete light adaptation suggests the occurrence of reverse light adaptation, i.e., photoreaction of the *all-trans* to produce the 13-*cis*, 15-*syn* form, or very rapid dark adaptation. In order to test these possibilities, the light-adapted state formed with >500 nm light was illuminated with >600 nm light for 30 min, and the procedure for the light adaptation was repeated. The spectrum for the light adaptation after >600 nm illumination (b) shows the same shape with 40% in amplitude as that of the first light adaptation (a), indicating that this 40% was reversed from the *all-trans* to the 13-*cis* form. No photoreaction occurred in the absence of the prior illumination with >600 nm light (c). These results indicate the existence

[1] Abbreviations: BR, bacteriorhodopsin in the light-adapted state; HOOP, hydrogen out-of-plane.

[2] Our data are different from those at 529 and 539 nm previously reported for the protein expressed in *E. coli*, respectively (Mogi et al., 1989).

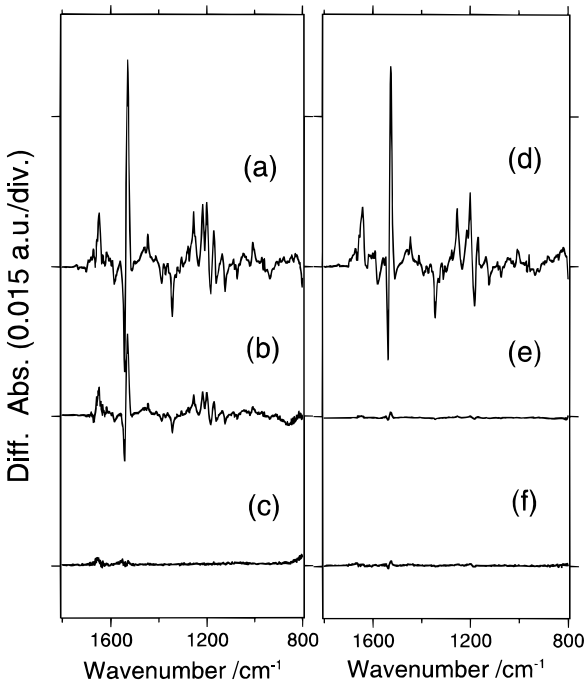

FIGURE 2: Spectra for the light adaptation at 275 K of W86F (a) and the wild type (d). Spectra b and e are for light adaptation after preillumination with >600 or >620 nm light, for W86F and the wild type, respectively. Spectra c and f are light adaptation spectra upon reillumination after 30 min of incubation in the dark, for W86F and the wild type, respectively.

of a forward and a backward photoreaction in the W86F protein that will produce a photostationary mixture of *all-trans*- and 13-*cis*-15-*syn*-retinal. The spectrum for the light adaptation of W86F (a) is nearly identical with the corresponding spectrum of the wild type (d) except for the differences in the C−C and C═C stretching vibrations as discussed above (see Figure 1). A second illumination with >500 nm light of the previously light-adapted state of the wild type with >620 nm light did not yield any photoreaction (e). The very small amount of the 13-*cis*, 15-*syn* species formed was to the same extent as in a previously unilluminated sample (f).

Two Species upon Photoreversal. In order to obtain a completely light-adapted state, Rothschild et al. (1989) illuminated the W86F protein with yellow light at 250 K. We find that illumination of the light-adapted W86F at 250 K with >500 nm light yields an unstable photoproduct, presumably the N photointermediate that decays completely in 30 min (not shown). However, a stable photoproduct remains. This stable photoproduct was warmed to 275 K and then illuminated with >500 nm light in the same procedure as the light adaptation. The spectral shape upon the second light adaptation (Figure 3b) is similar to that obtained after the reversal of the light adaptation at 275 K (Figure 3a, reproduced from Figure 2a), except for the emergence of a negative band at 1333 cm^{-1} besides the 1344 cm^{-1} band and a less intense positive band at 1217 cm^{-1}. Figure 3 further demonstrates that illumination at different wavelengths and temperatures results in a mixture of at least two photoproducts, normal 13-*cis*-bacteriorhodopsin and another one, BR_X. A pure BR minus BR_X spectrum was obtained at 170 K. The stable photoproducts produced by illumination at 250 K under various illumination conditions (c−f) were examined in the same way. Illumination under various conditions was continued until photosteady states

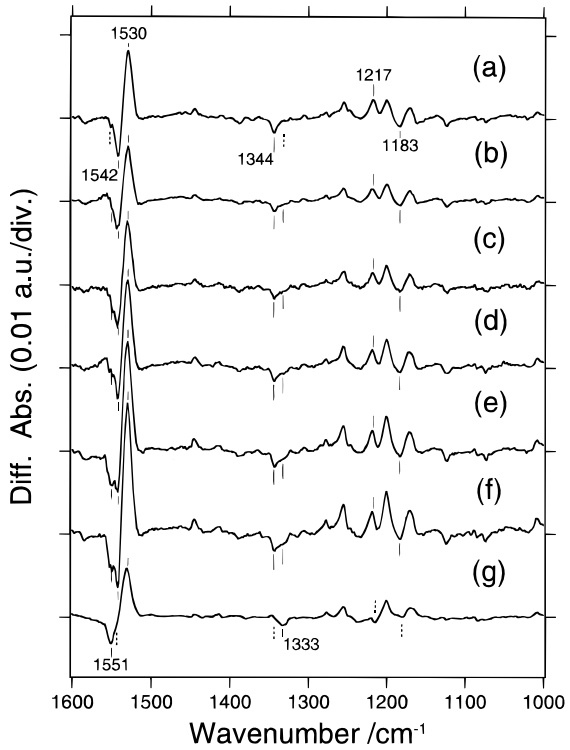

FIGURE 3: Spectra from light adaptation of W86F, which was previously illuminated at 275 K with >620 nm light for 30 min (a), at 250 K with >500 nm light for 6 min (b), at 250 K with >520 nm light for 9 min (c), at 250 K with >540 nm light for 12 min (d), at 250 K with >560 nm light for 15 min (e), at 250 K with >580 nm light for 20 min (f), and at 170 K with >580 nm light for 2 h (g).

were reached. With longer wavelength light for the illumination, the amplitude of the positive band at 1530 cm^{-1} and a negative band at 1551 cm^{-1} increased, in addition to the 1542 cm^{-1} band in the C═C stretching vibrational region. This suggests the formation of two stable photoproducts with different absorption maxima at 250 K. A single species with the C═C stretching vibration at 1542 cm^{-1} was produced by illumination at 275 K with >620 nm light for 2 h (a). Illumination with >580 nm light at 170 K yielded virtually only the other species, with a C═C stretching vibration at 1551 cm^{-1} (g). Both were stable at 275 K and produced *all-trans* species upon light adaptation with the normal procedure. The positive C═C stretching vibration is located at 1530 cm^{-1} for both, but the C−C stretching vibration at 1217 cm^{-1} of the former (a) is absent in the latter (g). The 1344 cm^{-1} band in the former (a) is replaced by the 1333 cm^{-1} band in the latter (g). The species with the 1344 cm^{-1} band should have a normal 13-*cis*, 15-*syn* structure because it arises under conventional conditions for the light adaptation. This 13-*cis*, 15-*syn* species, formed at 275 K (a), will be referred to as BR_C. The other, with the 1333 cm^{-1} band, is a new photoproduct that we name BR_X. Thus, the spectrum at 170 K (g) can be regarded as the pure BR minus BR_X spectrum for W86F. The absence of BR_X in the photoreaction at 275 K is assured by the absence of the 1333 cm^{-1} band. The absence of the positive band at 1217 cm^{-1} and the negative band at 1183 cm^{-1} are also characteristic of BR_X. Illumination by >500 nm light at 250 K produces substantially more BR_C compared to illumination at 275 K (b). On the contrary, the accumulation of BR_C does not take place at 170 K under 580 nm illumination (f vs g). These results suggest that BR_C and BR_X are produced from the

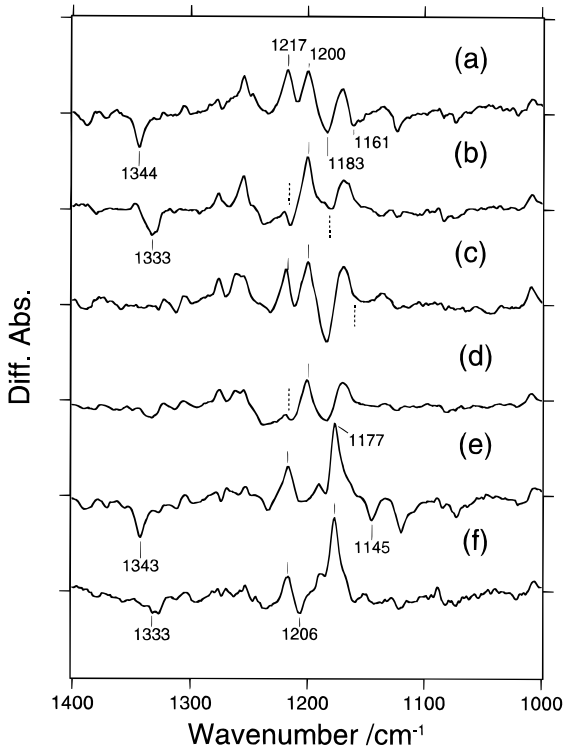

FIGURE 4: BR minus BR_C (a, c, and e) and BR minus BR_X (b, d, and f) spectra in H_2O (a and b) and D_2O (c and d) and for $^{13}C_{14}-{}^{13}C_{15}$-reconstituted BR (e and f).

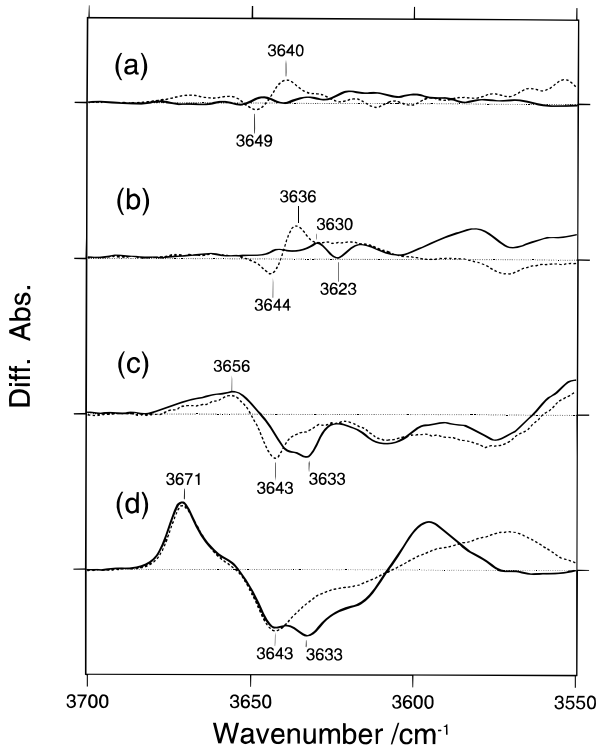

FIGURE 5: BR_C minus BR (a), K minus BR (b), L minus BR (c), and M minus BR (d) spectra in the 3700−3550 cm^{-1} region of W86F (solid lines) and the wild type (dotted lines). The spectra presented were scaled for the 1200 cm^{-1} band. One division in the ordinate in part a corresponds to 0.0067 and 0.0021 absorbance unit for the wild type and W86F, respectively.

photointermediates characteristic of these low temperatures. The BR_X-like photoproduct was detected also in the wild type under the same conditions, but its amount is only about 2% in the light-adapted products, estimated from the amplitudes of the difference spectra. In order to determine the isomeric state of BR_X, the extracted retinal was analyzed with HPLC. The dark-adapted state of W86F is composed mainly of 13-*cis*-retinal (90%). In the photoreaction which forms only BR_X at 170 K, the content of 11-*cis*-retinal increased from 2 to 5%, but more significantly, the amount of 13-*cis*-retinal increased from 26 to 38%. No increase was observed for 9-*cis*-retinal. The sum of the increased amount of the 13-*cis*- and 11-*cis*-retinal is almost comparable with the fraction of BR_X produced at 170 K.

Chromophore Structure of BR_X Different from BR_C. We have shown that 13-*cis*-retinal is the main constituent of the chromophore in both stable photoproducts, BR_C and BR_X. Their vibrational modes were examined also after D_2O substitution and ^{13}C labeling of the $C_{14}-C_{15}$ bond by recording difference spectra for light adaptation (Figure 4). One of the differences detected between BR_C and BR_X is in the negative band at 1344 cm^{-1} for BR_C (a) that is shifted to 1333 cm^{-1} for BR_X (b). These bands disappear in D_2O (c and d), being ascribable to the N−H in-plane bending vibrations of the Schiff base. The BR minus BR_X spectrum of W86F (b) exhibits neither the positive band at 1217 cm^{-1}, which may be the mode of the C_8-C_9 stretching vibration similar to the corresponding band at 1214 cm^{-1} of the wild type (Smith et al., 1987), nor the negative band at 1161 cm^{-1} present in the BR minus BR_C spectrum (a). However, when the $C_{14}-C_{15}$ bond was doubly labeled by ^{13}C, a new band appeared at 1217 cm^{-1} in the BR minus BR_X spectrum (f). The negative band at 1206 cm^{-1} of ^{13}C-labeled BR_X (f) is due to the $C_{14}-C_{15}$ stretching vibration shifted from 1217 cm^{-1} (b). On the other hand, the 1217 cm^{-1} band in the BR minus BR_C spectrum (a) does not undergo any changes upon the labeling (e). Most likely, the negative band at 1161 cm^{-1} of BR_C (a) that contains the $C_{14}-C_{15}$ stretching mode, as revealed by its shift upon ^{13}C labeling to 1145 cm^{-1} (e), is located at around 1217 cm^{-1} in BR_X (f) and cancels the positive band at a similar frequency that does not contain this mode. The positive band at 1217 cm^{-1} of the BR minus BR_X spectrum (f) thus arises from the abolition of the canceling negative band. The 1161 cm^{-1} band in the BR minus BR_C spectrum (a) due to the $C_{14}-C_{15}$ stretching vibration is upshifted in D_2O to around 1183 cm^{-1} as shown by the increase in the intensity around this frequency (c). A similar upshift toward higher frequencies was observed for the 13-*cis*, 15-*syn* form of the wild type (Smith et al., 1984; Roepe et al., 1984). On the other hand, no shifts occur in the BR minus BR_X spectra with D_2O substitution (b vs d). Smith et al. (1984) had proposed that the coupling between the N−H in-plane bending and $C_{14}-C_{15}$ stretching vibrations lowers the frequency of the latter. If coupled, the increase in the frequency of the $C_{14}-C_{15}$ stretching mode in D_2O results from the downshift of the N−H in-plane bending vibration to below 1000 cm^{-1}. Thus, the absence of a D_2O effect for BR_X indicates that the 1217 cm^{-1} band of $C_{14}-C_{15}$ stretching vibration is not coupled with the N−H in-plane bending vibration. Since there are no other differences in the positive sides between the spectra of BR_X and BR_C (a and b), both produce the same *all-trans* species. Both BR_C and BR_X have the 13-*cis*-retinal and are stable at 275 K. The greater amount of BR_X produced upon illumination with longer wavelength light, and the higher frequencies of the C═C stretching vibrations, indicate that the absorption spectrum of BR_X is at shorter wavelengths than that of BR_C.

No BR_X appears upon the illumination at 275 K, but it is stable at this temperature once formed at a lower temperature.

Water Molecule Located Close to Trp86. Figure 5 shows the BR_C minus BR (a), K minus BR (b), L minus BR (c), and M minus BR (d) spectra of W86F (solid lines) in the 3700−3550 cm^{-1} region, as compared with the corresponding spectra of the wild type (dotted lines). A small bilobe at 3649 (−) and 3640 (+) cm^{-1} in the BR_C minus BR spectrum of the wild type disappears in W86F (a). These bands are also not observed in the BR_X minus BR spectrum (not shown). The bilobe with the 3644 (−) and 3636 (+) cm^{-1} bands in the K minus BR spectrum (Fischer et al., 1994) is also completely absent in W86F (b). The L minus BR (c) and M minus BR (d) spectra of W86F show a negative band of a water molecule at 3633 cm^{-1}, in addition to the normal band at 3643 cm^{-1}.

DISCUSSION

We have detected the reverse photoreaction of the *all-trans* to the 13-*cis*, 15-*syn* state, in addition to the more usual forward reaction, in the light adaptation process of W86F. In the wild type protein, such a reaction was found only in dry films (Kouyama et al., 1985) and after detergent solubilization (Casadio & Stoeckenius, 1980; Dencher et al., 1983). Photoisomerization in both directions was suggested to occur for Y57N (Soppa et al., 1989; Govindjee et al., 1995), R82K below pH 9 (Balashov et al., 1995), and artificial pigments with retinal analogs (Steinberg et al., 1991). Apparently, incomplete light adaptation as is the case for D85E (Soppa et al., 1989) and the wild type with the 13-desmethylretinal (Gärtner et al., 1983) is due largely to rapid dark adaptation, but there may be some contribution also from reversible photoisomerization. The residues whose replacements cause the reverse photoisomerization, Trp86 (this study), Tyr57 (Soppa et al., 1989; Govindjee et al., 1995), and Arg82 (Balashov et al., 1993, 1995; for R82K but not for R82A), are involved in an H bonding network including water molecules in the extracellular domain around the Schiff base (Fischer et al., 1994; Hatanaka et al., 1996).

A greater tendency to the 13-*cis* form was observed for 13-desmethylbacteriorhodopsin (Gärtner et al., 1983), and the ionone ring-modified bacteriorhodopsin (Towner et al., 1980; Steinberg et al., 1991), as well as for the dry films (Kouyama et al., 1985) and detergent-solubilized samples (Casadio & Stoeckenius, 1980; Dencher et al., 1983) of the wild type. Thus, altered interactions between the protein and retinal seem to stimulate the formation of the 13-*cis* isomer. Trp86 is located beneath the polyene chain of the retinal in the region connecting the ring and the Schiff base (Grigorieff et al., 1996). The replacement of Trp86 by phenylalanine specifically affects the 1215 cm^{-1} band (Figure 1) due to the C_8-C_9 stretching vibration (Smith et al., 1987) and leads to the formation of the additional 13-*cis* photoproduct, which can be distinguished from BR_C by the absence of the coupling between the N−H in-plane bending vibration at 1333 cm^{-1} and the $C_{14}-C_{15}$ stretching vibration at 1217 cm^{-1}. According to Livnah and Sheves (1993), the coupling occurs for the 13-*cis* chromophore irrespective of the configuration of the C=N bond, but not for the *all-trans* species. The absence of the coupling in the K, L, and N photointermediates (Smith et al., 1984; Fodor et al., 1988) is ascribed to the distorted structure around the Schiff base (Livnah & Sheves, 1993). The structure around the Schiff base of BR_X would be also distorted. The 15-*syn* structure of BR_C is required for interaction with Asp85, which stabilizes otherwise short-living 13-*cis* species. The BR_X, which is stable at 275 K, also must have the 15-*syn* structure. BR_X must be produced from transient structure stabilized at low temperatures much more favorably in W86F.

Another characteristic feature of the W86F protein is the absence of structural changes of a water molecule with the O−H stretching vibration at 3644 cm^{-1} upon K formation. A similar change for the light adaptation is also absent in W86F. Thus, the conversion to BR_X occurs independently of water changes. A structural change of a water molecule at a similar frequency (Maeda et al., 1992), however, remains in the process for the formation of the L and M intermediates of W86F. It is uncertain whether these two kinds of change in the water molecule in the wild type are ascribable to the same water molecule. The changes upon formation of the K intermediate in the wild type appear with smaller intensity than those observed in the L minus BR (c) and M minus BR (d) spectra of the wild type (Figure 5), and half of the K population in the K minus BR spectrum does not shift in $H_2{}^{18}O$ (Fischer et al., 1994). The recent structural model of Grigorieff et al. (1996), however, exhibits space to accommodate at most a single water molecule in the region surrounded by Trp86 and Asp85. If this is the case, the changes in water must originate from the single water molecule, which can undergo small structural changes in the process for the formation of the K intermediate or the 13-*cis*, 15-*syn* form. These reactions may not be accompanied by gross positional alteration of the N−H bond of the Schiff base. The formation of the K intermediate is accompanied by the perturbation of the indole ring of Trp86 (Rothschild et al., 1989; Weidlich et al., 1996), and the unusual photoreactions to the 13-*cis* forms, BR_C and BR_X, occur in the absence of the indole N−H at position 86. The structural model by Grigorieff et al. (1996) has shown that the N_ϵ of Trp86 is located at a distance of 0.48 nm from the $O_{\delta 2}$ of Asp85. These results suggest that the water molecule coordinated with Asp85 (Maeda et al., 1994) is present also in the close proximity to the indole N−H of Trp86 and is affected upon isomerization to the 13-*cis* form in the K intermediate or to the 13-*cis*, 15-*syn* form in the dark adaptation. Slight shifts in the C_{15}, N−HOOP vibrational bands in the K minus BR spectrum of W86F may reflect the interaction between the Schiff base and Trp86. The mutation may eventually cause a different response in the water molecule through the disruption of the H bond with Asp212. A change in Tyr57, which is also an H bonding partner of Asp212 as is Trp86 but located farther from the Schiff base (Grigorieff et al, 1996), loses the change of the same water molecule upon formation of the K intermediate (Fischer et al., 1994) and causes the isomerization from the *all-trans* to the 13-*cis*, 15-*syn* form (Soppa et al., 1989; Govindjee et al., 1995). In this case, however, the corresponding O−H stretching vibrations in the negative side of the L minus BR and M minus BR spectra appear at a lower frequency, 3635 cm^{-1} (Fischer et al., 1994) as in the D212N protein (Kandori et al., 1995). This is not a case for the

[3] The 3625 cm^{-1} band in the previous study (Hatanaka et al., 1996) corresponds to the 3633 cm^{-1} band characterized by deconvolution (M. Hatanaka, H. Kandori, and A. Maeda, unpublished experiments).

W86F protein. In the case of W86F, therefore, a more direct effect on the water molecule might also be involved. Larger structural changes around the Schiff base in the L and M intermediates may not be affected in W86F. Another water O−H stretching vibration appears in the spectra of the L and M intermediates of this mutant protein. Its O−H stretching vibration at 3633 cm^{-1} is similar to those observed at 3625 cm^{-1} with smaller intensity in the wild type,[3] which disappears in the mutants of R82A and R82K (Hatanaka et al., 1996) and E204Q and intensifies in E204D (Brown et al., 1995). These were supposedly due to the water molecules in the Arg82−Glu204 region which are connected to the Asp212−Trp86 region (Humphrey et al., 1994). R82K also shows the photoisomerization from the *all-trans* to the 13-*cis*, 15-*syn* form below pH 9 (Balashov et al., 1995). Arg82 is located one turn away from the Asp85−Trp86 region. However, the cavity formed by replacing the indole with a phenyl ring may accommodate such an additional water molecule. Very rapid reaction with hydroxylamine in W86F (not shown) is also compatible with the presence of the cavity for a hydroxylamine molecule in place of the indole N−H. The Asp85 to Asn mutation removes the water molecule which undergoes structural changes upon formation of the L and M intermediates (Maeda et al., 1994). This structure may allow the light-dependent formation of unusual 9-*cis*- and 11-*cis*-retinals (Tittor et al., 1994), in analogy with the acid form of bacteriorhodopsin, in which Asp85 is protonated (Maeda et al., 1980; Chang et al., 1987). In the presence of the indole at position 86, the water molecule is perturbed together with the indole ring when the Schiff base moves upon such isomerization to the K state or from the 13-*cis*, 15-*syn* to the *all-trans* form. This indicates that the N−H of Trp86 places the water molecule at the right position close to Asp85 (Maeda et al., 1994). The structure composed of the indole ring of Trp86, together with surrounding residues, including possibly the water molecule, then prevents an unusual reaction which forms BR_C as well as distorted BR_X either thermally or by absorbing a second photon.

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