Interaction of Tryptophan-182 with the Retinal 9-Methyl Group in the L Intermediate of Bacteriorhodopsin†

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ABSTRACT: An intense indole N−H stretching vibrational band at 3486 cm^{-1} in the difference Fourier transform infrared spectrum is one of the characteristic features of the L intermediate of bacteriorhodopsin [Maeda, Sasaki, Ohkita, Simpson, & Herzfeld (1992) *Biochemistry 31*, 12543]. This band is now assigned to tryptophan-182. The Trp182→Phe (W182F) protein shows specific features in the difference spectrum in the visible region upon L formation, and exhibits great delay in the L−M conversion. Fourier transform infrared difference spectra further indicate that while the intensity of the C-methyl in-plane bending vibration at 1009 cm^{-1} is lost in the L intermediate of the wild type, its intensity remains high in the W182F protein. The intensity of the N−H stretching vibration upon L formation is diminished considerably in an artificial bacteriorhodopsin containing 9-desmethylretinal. It also exhibits delayed M formation. These results suggest that Trp182 interacts with the retinal side chain through the 9-methyl group, and thereby affects the L-to-M conversion.

Bacteriorhodopsin is a light-dependent proton pump in the purple membrane of *Halobacterium salinarium*. The function of bacteriorhodopsin is carried our mainly by three residues: Asp85 located in the extracellular domain, Asp96 in the cytoplasmic domain, and the intervening Schiff base of *all-trans*-retinal linked to Lys216 (Mathies et al., 1991; Lanyi, 1993). In the photocycle, proton transfer from the Schiff base to Asp85 (the L-to-M conversion that occurs in the microsecond time range) triggers proton release to the extracellular aqueous phase, and then the proton of Asp96 restores the protonated state of the Schiff base (the M-to-N conversion that occurs in the millisecond time range). Proton transfer from the Schiff base to Asp85 is thus an obligatory step in the pumping process.

The structural features that induce this proton transfer would be detected in the L intermediate. Previous Fourier transform infrared (FTIR)[1] analysis in the O−H and N−H stretching regions revealed L-specific features; stronger H-bonding of a specific water molecule (Maeda et al., 1992b) is one of them. This water was localized in the proximity of Asp85 (Maeda et al., 1994). L has a distorted 13-cis structure different from the relaxed 13-cis form of N (Maeda et al., 1991; Pfefferlé et al., 1991). Thus, water coordinated to Asp85 was proposed to participate in the formation of the distorted 13-cis retinal conformation of L, which may be prerequisite for proton transfer from the Schiff base to Asp85 (Fahmy et al., 1989). Elsewhere, we discuss changes in the geometry of the Schiff base relative to Asp85 in L (Brown et al., 1994); in this report, we focus on the configuration of the retinal chain and its interaction with residues in the binding pocket.

A sharp positive N−H stretching band at 3486 cm^{-1} is another characteristic of L, and was identified as the indole of a tryptophan residue by Maeda et al. (1992a). However, its assignment to a particular tryptophan residue remained unsolved. Since this N−H is insensitive to ^2H_2O substitution, the most likely candidates are buried Trp182 and Trp189. The present study on these mutants reveals that the indole can be assigned to Trp182. Interaction of the indole of Trp182 with the 9-methyl group of the retinal is revealed by FTIR and visible absorption spectroscopic methods for the mutant protein of Trp182 and 9-desmethylretinal-containing bacteriorhodopsin (9-dmr bacteriorhodopsin).[1] It appears that this interaction plays a role in the L-to-M conversion.

MATERIALS AND METHODS

Trp189→Phe and Trp182→Phe genes were expressed in *Halobacterium salinarium* L33 strain, transformed with a vector described previously (Ni et al., 1990). Bacteriorhodopsin in the purple membrane of W189F and W182F, and the wild type was purified by the standard method

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[1] Abbreviations: FTIR, Fourier transform infrared; 9-dmr bacteriorhodopsin, 9-desmethylretinal-containing bacteriorhodopsin; rec bacteriorhodopsin, reconstituted bacteriorhodopsin containing regular *all-trans*-retinal; BR, light-adapted form of bacteriorhodopsin.

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(Oesterhelt & Stoeckenius, 1974). The wavelength of the maximum absorbance is located at 548 and 570 nm for W182F abd W189F, respectively. Apoprotein of bacteriorhodopsin was prepared by irradiation of bacteriorhodopsin in the presence of 2 M hydroxylamine (pH 7). The retinal oximes were removed by five washings in a 2% solution of bovine serum albumin, fraction V of Sigma (Katre et al., 1981). It was removed by three washings in water. 9-Desmethylretinal was prepared by the reported method (Blatz et al., 1969), and the structure was identified by comparison of the NMR data with those reported by Broek et al. (1983). The artificial bacteriorhodopsin containing 9-desmethylretinal was produced by reconstituting the apoprotein with the retinal analogue. The excess retinal was removed by five washings in 2% bovine serum albumin, which was then removed by three washings in water. The reconstituted bacteriorhodopsin containing regular *all-trans*-retinal (rec bacteriorhodopsin)[1] was prepared from the apoprotein in the same way.

The film of the sample was prepared by drying aqueous suspensions on a BaF_2 window, and then humidified by placing 1 μL of water or ^2H_2O. The light-adapted form of bacteriorhodopsin (BR)[1] was obtained by irradiation at 294 K. The sample was cooled to 170 K in an Oxford cryostat DN1754. Difference FTIR and visible spectra were recorded as described previously (Maeda et al., 1994). The data are presented as difference spectra between after and before the irradiation of BR with >600 nm light for 2 min at 170 K. They present averages of five recordings.

Flash photolytic experiments at a single wavelength at room temperature were carried out for the mutant proteins as described by Brown et al. (1994), and for 9-dmr BR as described by Okada et al. (1991). The BR samples for the kinetic experiments were in 0.1 M NaCl containing 5 mM phosphate buffer (pH 7 or 5).

RESULTS

Assignment of the N−H Stretching Vibration of L to Trp182. Figure 1 shows difference FTIR spectra in the 3750−3450 cm^{-1} region upon irradiation with >600 nm light at 170 K. For the wild type (a), a sharp positive band at 3486 cm^{-1} superimposed on a broad band between 3560 and 3490 cm^{-1} was observed only for the L intermediate but not for the M and N intermediates (Maeda et al., 1992b). The 3486 cm^{-1} band, which persists in ^2H_2O, was previously assigned to the N−H stretching vibration with [^{15}N]indole-labeled bacteriorhodopsin (Maeda et al., 1992a). We attempted to assign this mode to a specific tryptophan residue by using W189F and W182F. The negative water band at 3642 cm^{-1} and a positive broad O−H stretching vibrational band between 3560 and 3490 cm^{-1} (Maeda et al., 1992b) persist in both mutants (b and c) as in the wild type (a). The negative band located in the lower frequency region than the 3642 cm^{-1} band probably arose by the shift of the O−H stretching vibrations of the wild-type protein in the same region. However, the sharp positive band at 3486 cm^{-1} is completely missing only in W182F (c). Thus, this indole N−H bond is assigned to that of Trp182.

Visible L/BR Spectra of W182F. The corresponding spectral changes in the visible region were recorded upon irradiation with >600 nm light at 170 K (Figure 2). The spectrum of W182F (c) is similar to those of the wild type (a) and W189F (b) with respect to the minimum at 580 nm

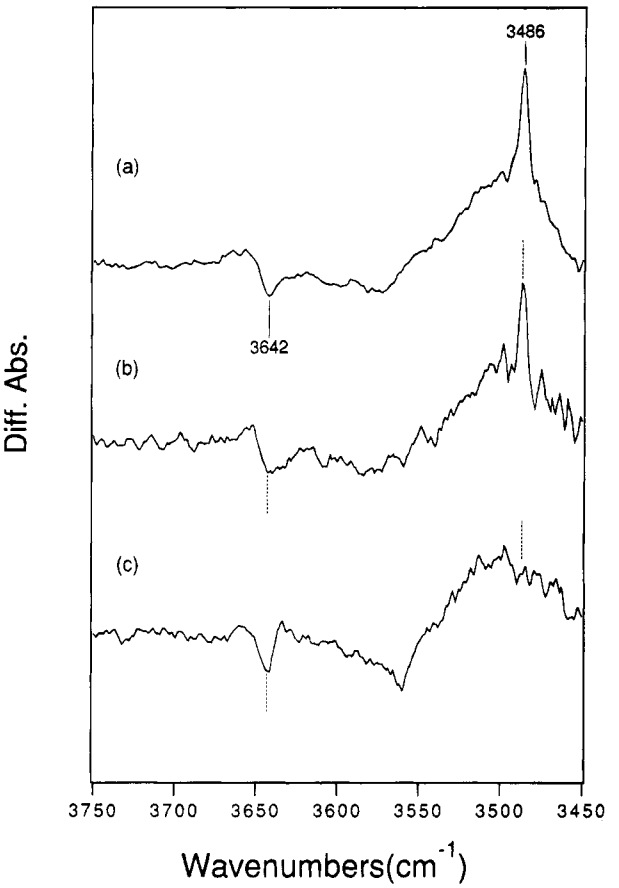

FIGURE 1: Comparison of the FTIR difference spectra in the 3750−3450 cm^{-1} region of the wild type (a), W189F (b), and W182F (c) at 170 K. The amplitudes were scaled to the negative band at 1201 cm^{-1} (see Figure 4). The full length of the vertical axis is 0.025, 0.010, and 0.009 absorbance unit for (a), (b), and (c), respectively. The spectrum of the wild type is the duplicate of that presented by Maeda et al. (1992b).

and a broad positive feature between 440 and 500 nm. However, the spectrum of W182F (c) shows different features from other (a, b): an additional broad positive band around 330 nm, which is similar to the N intermediate of the wild type (Kouyama et al., 1988), a broader negative 580 nm band with a shift of the zero-crossing point by about 10 nm from 520 to 510 nm, and more intense positive and negative bands below 300 nm. This may arise from the perturbation of the π-electron system due to a change in interaction of the retinal polyene chain with Trp182.

Effect of Trp182 on Schiff Base Deprotonation. The effect of the mutation of Trp182 on the L-to-M conversion was examined by measuring the absorbance increase at 410 nm after flash photoexcitation at room temperature (Figure 3). The M rise for W182F was greatly delayed as compared with the wild type or W189F. This observation is similar to an earlier one with the protein expressed in *Escherichia coli* and reconstituted in vesicles with the polar lipid of *Halobacterium* (Wu et al., 1991). Close inspection of the time courses, however, reveals the presence of a small fraction of M even in the earlier time domain where M of the wild type rises. These results show a shift of the L-to-M equilibrium toward L, as discussed for the V49A mutant protein (Brown et al., 1994). A lowering of amplitude of the M signal at pH 5 is compatible with this notion (Zimanyi et al., 1992). No appreciable differences were observed for W182F from the wild type on the rate of proton release

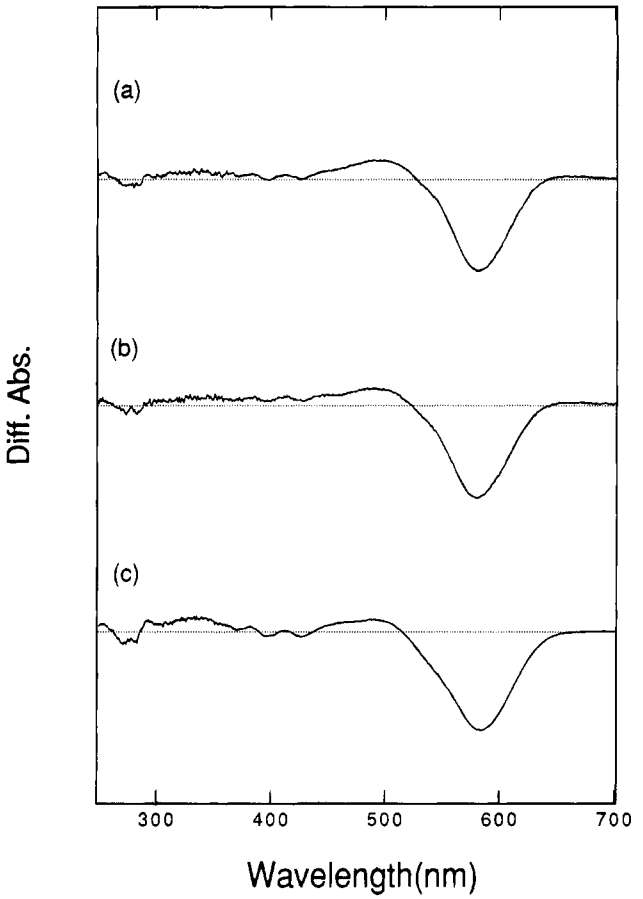

FIGURE 2: Difference spectra in the visible region of the hydrated films of the wild type (a), W189F (b), and W182F (c) before and after irradiation with >600 nm light for 2 min at 170 K. The maximal intensities of the negative peak at 580 nm in the difference spectra were read as 0.08, 0.18, and 0.08 absorbance unit, respectively, and those in the absolute spectra of the unphotolyzed state (not shown in figures) were 0.40, 0.72, and 0.43 absorbance unit, respectively.

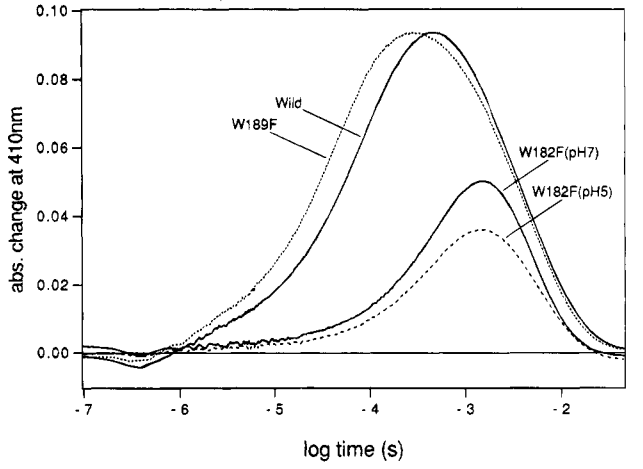

FIGURE 3: Single-wavelength kinetics at 410 nm for the wild type (solid line labeled as wild), W189F (dashed line), W182F [solid line labeled as W182F (pH 7)] at pH 7.0, and W182F (dotted line) at pH 5.0 in 0.1 M NaCl and in 50 mM phosphate buffer. The maximum absorbance of the samples used was 0.8 absorbance unit for the 1 cm path length.

detected by the absorbance changes of the pH indicator dye pyranine at 457 nm (not shown in figures). Thus, interaction between the indole of Trp182 with the retinal plays a role in accelerating M formation, but not proton release.

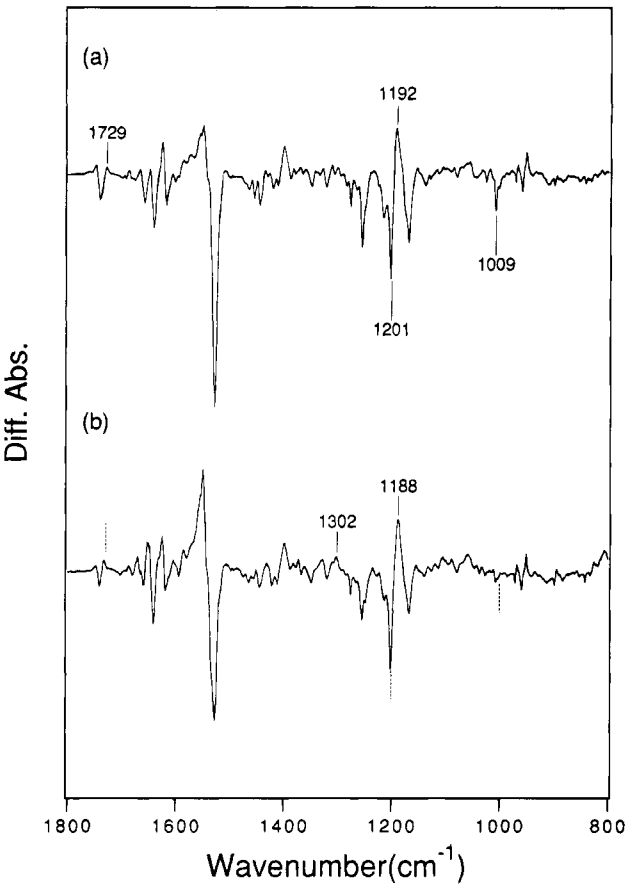

FIGURE 4: FTIR difference spectrum in the 1800−800 cm^{-1} region of the L/BR spectrum of the wild type (a) and W182F at 170 K (b). The corresponding spectra were presented in Figure 1.

Effect of Trp182 on the Methyl Group in L. Changes in the chromophore structure were studied for W182F on the FTIR spectrum in the 1800−800 cm^{-1} region (Figure 4). The photoproduct of W182F at 170 K (b) showed some differences from the L intermediate of the wild type (a). The C−C stretching vibration at 1188 cm^{-1} for W182F (b) is different from the corresponding band at 1192 cm^{-1} in L (a) but rather close to that in N. We focused attention on a negative band at 1009 cm^{-1}. It is observed in the L/BR spectrum of the wild type but absent in the corresponding spectrum of W182F (b).

The 1009 cm^{-1} band is assigned to the in-phase combination of the two C-methyl in-plane bending vibrations at C_9 and C_{13} with the contribution of the C−C stretching vibrations (Curry et al., 1985). Its frequency is almost invariable for the unphotolyzed bacteriorhodopsin and various intermediates as seen by resonance Raman spectroscopy (Ames et al., 1990). The same negative band was also observed in the M/BR spectrum (Maeda et al., 1991). Thus, its absence in the N/BR spectrum (Pfefferlé et al., 1991) must be due to the cancellation with a positive band at the same frequency, and the appearance of the negative band at 1009 cm^{-1} in the L/BR and M/BR spectra is a result of the loss of the intensity in these intermediates.

For W182F protein, the negative band at 1009 cm^{-1} is seen in the M/BR spectrum (not shown in figures). Therefore, its absence in the L/BR spectrum of W182F is a result of the cancellation of the positive band at the same frequency as was observed for the N/BR spectrum of the wild type. In other words, the replacement of the indole of Trp182 restores

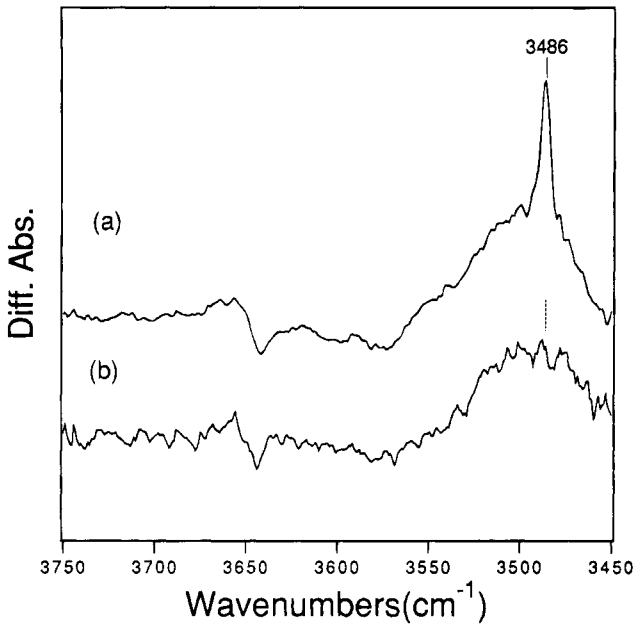

FIGURE 5: Comparison of the FTIR difference spectra in the 3750–3450 cm^{-1} region of (a) the wild type and (b) 9-dmr BR at 170 K. The spectrum of the wild type is the same as in Figure 1a. The full length of the vertical axis is 0.016 and 0.008 absorbance unit for (a) and (b), respectively.

the intensity of the C-methyl bond in L. On the other hand, the C-methyl bond of the unphotolyzed W182F is in the same state as the corresponding wild type. These results suggest that upon L formation the C_9 or C_{13} methyl group moves into close porximity to the indole of Trp182. Additionally, the C=O stretching vibration of Asp115 in the L intermediate at 1729 cm^{-1} gained more intensity in W182F.

Effect of the 9-Methyl Group on Trp182. From the foregoing, it is evident that reciprocal effects on the N–H stretching vibration are expected by the removal of either C_9 or C_{13} methyl groups. Testing 13-desmethylretinal-containing bacteriorhodopsin is not practical because it is mainly in the 13-cis form even after light adaptation (Gärtner et al., 1983). Importantly, we find that the removal of the 9-methyl group depletes the 3486 cm^{-1} band of the N–H stretching vibration of Trp182 in the L intermediate (Figure 5), though the negative water band at 3642 cm^{-1} and the positive broad band between 3560 and 3490 cm^{-1} remain unchanged.

The 3486 cm^{-1} band of Trp182 is more clearly discernible with a film hydrated with ^2H_2O, a treatment which depletes all other bands in the 3750–3450 cm^{-1} region. In Figure 6, the spectrum of 9-desmethyl-BR (c) was compared with the wild type (a) and W182F in ^2H_2O (b). Intensity adjustment could be accomplished by comparing the whole of features in the 1800–800 cm^{-1} region because there are many changes in the vibrational bands upon deuteration (b) or desmethylation (c). The 3486 cm^{-1} band is completely extinguished in W182F (b). On the other hand, the intensity of 9-dmr BR (c) reduced to about one-third in comparison with the wild type. This also indictes that a small part of the 3486 cm^{-1} band remains within the broad O–H stretching bands of H_2O (Figure 5b), though it is hardly discernible.

L-to-M Conversion in 9-Desmethylretinal-Containing BR. M formation at pH 7 was followed at 412 nm after the flash at 540 nm (Figure 7) for the native BR (a), rec BR as control (b), and 9-dmr BR (c). The rate of rise in rec BR (b), with

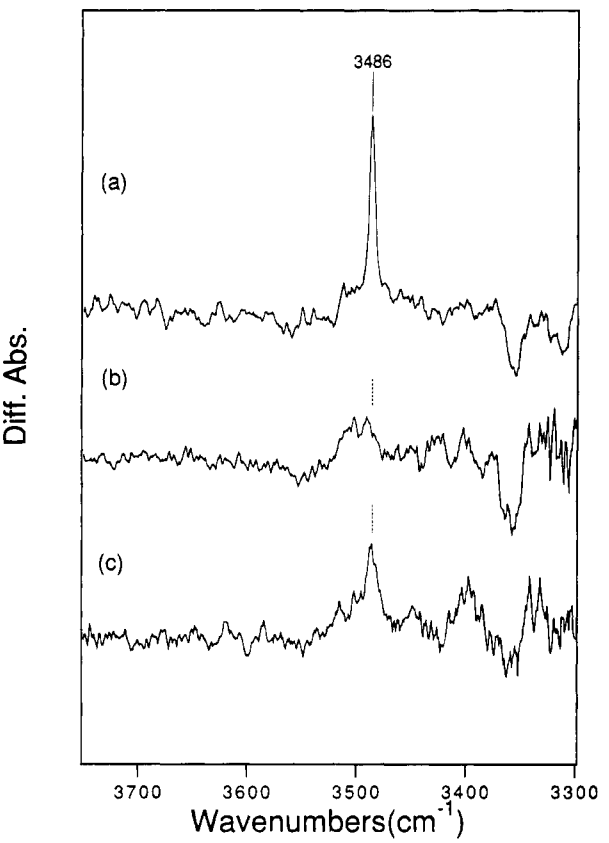

FIGURE 6: Comparison of the FTIR difference spectra in the 3750–3300 cm^{-1} region for the wild type (a), W182F (b), and 9-dmr BR (c) in ^2H_2O at 170 K. The spectrum of the wild type is the duplicate of an earlier presented spectrum in Maeda et al. (1992b). The full length of the vertical axis is 0.018, 0.008, and 0.005 for (a), (b), and (c), respectively.

a maximum concentration of M at 0.8 ms, was slightly behind that of the wild type (a) with the maximum at 0.5 ms. In comparison, with these, M formation of 9-dmr BR (c) showed great delay, attaining the maximum concentration around 3 ms, similarly to that of W182F (Figure 3). Also like in W182F, the maximum amplitude of M is extremely small for 9-dmr BR (c). The efficiency of the photoreaction was compared by monitoring the absorbance change at the wavelengths for the maximum difference between BR and L: at 580 nm for the wild type and 560 nm for 9-dmr BR (not shown in figures). An increase of the absorbance at zero time, which was assumed to correlate with the amount of L formed, was 32% for 9-dmr BR as compared with the wild type. The maximum amplitude of 12% in the 412 nm trace for 9-dmr BR (c) relative to wild type (a) and rec BR (b) thus cannot simply be explained by lowered quantum yield. The additional diminuation in the amplitude of the 412 nm signal could be brought about by delayed formation and more rapid decay of M. We suppose that a delay in M formation by the removal of the 9-methyl group is due to a change in the L–M equilibrium similar to the replacement of Trp182 by phenylalanine.

DISCUSSION

The N–H stretching vibrational band at 3486 cm^{-1} in the L intermediate shifted in [^{15}N]indole-labeled bacteriorhodopsin (Maeda et al., 1992a) and disappeared in W182F (Figure 1c). Thus, it is assigned to the N–H stretching vibration of Trp182. This perturbation differs from that previously

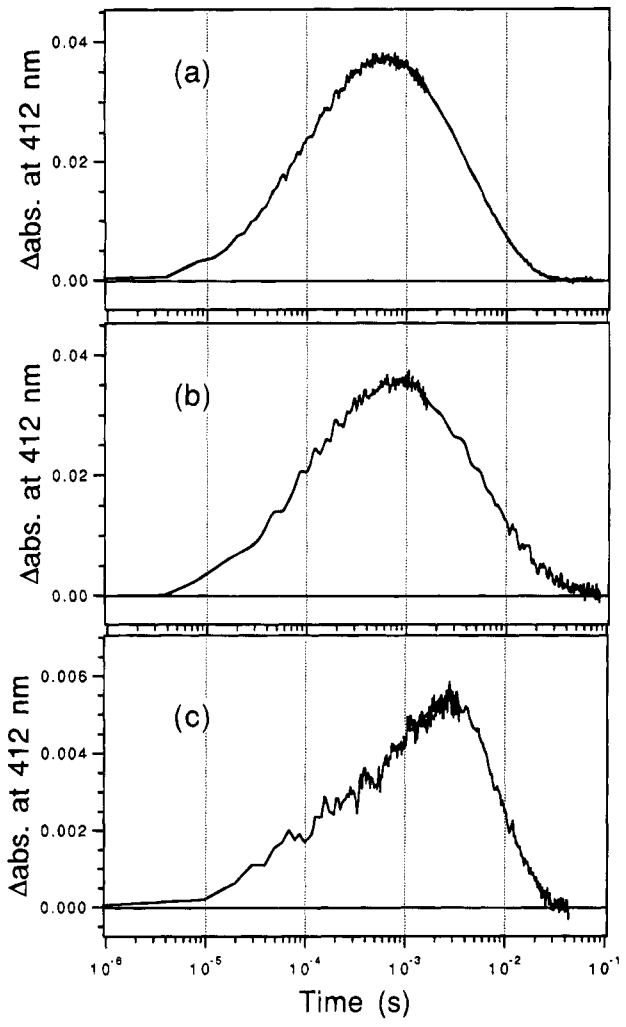

FIGURE 7: Single-wavelength kinetics at 412 nm for the native BR (a), rec BR (reconstituted with authentic retinal) (b), and 9-dmr BR (c) at pH 7 after excitation at 540 nm, where the absorbance was adjusted to the same value for three samples (0.5 absorbance unit in a cell with 2 mm path length).

envisaged in a 742 cm^{-1} band (Roepe et al., 1988), which was assigned to the C−H out-of-plane bending mode of Trp86 (Rothschild et al., 1989b).

The relatively high frequency of the N−H stretching vibration of Trp182 at 3486 cm^{-1} (Figure 1a) indicates that this indole N−H is a very weak H-bonding donor. The observed extinction of 400 for this band (Maeda et al., 1992a) is much larger than the extinction of 100 for indole in an apolar solvent, such as carbon tetrachloride (Fuson et al., 1952). The reason for this may be that the N−H bond is in a more polarized environment, a result of the interaction of the indole ring with the 9-methyl group of the retinal. This is supported by the much decreased extinction of this band in L of 9-dmr BR (Figures 5b and 6b).

The 1009 cm^{-1} band due to the C-methyl vibration loses its intensity upon L formation in the wild type but keeps it in W182F (Figure 4), indicating the interaction of either the 9- or the 13-methyl group with Trp182. That the 9-methyl group is responsible for interaction is shown by a reduction in intensity of the N−H stretching vibration of Trp182 in the L intermediate of 9-dmr BR (Figures 5b and 6b). This is in accordance with models that Trp182 is located close to the 9-methyl group of the retinal (Rothschild et al., 1989a; Henderson et al., 1990). The upward movement of the retinal side chain toward the cytoplasmic side, as suggested for the M intermediate by Heyn and Otto (1992), may bring the 9-methyl group of the retinal and the indole of Trp182 closer to one another in the L intermediate.

M formation is delayed in 9-dmr BR (Figure 7) as well as in W182F (Figure 3), in correlation with the perturbation or depletion of the N−H bond which normally exhibits an intense N−H stretching vibration at 3486 cm^{-1} (Figures 1c and 5b). This indicates a role for the interaction of Trp182 with the 9-methyl group in the proton transfer reaction from the Schiff base to Asp85.

The delay in the L-to-M conversion must result from a decrease in the difference between pK_a values of the Schiff base and Asp85 (Lanyi, 1993). One of the possible causes is the relaxation of the distorted chromophore characteristic of the normal L intermediate. The intensity increase of the 1302 cm^{-1} band due to the C_{15}−H in-plane bending vibration in the N intermediate was ascribed to the relaxation of the distorted 13-*cis*-retinal in the L intermediate (Pfefferlé et al., 1991). A similar increase in the intensity of the same band in the L intermediate of D85N thus accounted for the relaxed structure of the chromophore in its L intermediate (Maeda et al., 1994). Since the increase of the 1302 cm^{-1} band is much smaller for W182F (Figure 4) and 9-dmr BR (not shown in figures), the chromophore of these L intermediates persists in a distorted state as the wild type.

A proper geometry between the Schiff base and Asp85 is necessary for proton transfer between them (Scheiner & Duan, 1991). A perturbation of this geometry by replacement of Val49 with alanine or Ala53 with valine in the site near the side chain of Lys216 results in the shift of the L−M equilibrium to L (Brown et al., 1994) as in W182F. The frequency shift of the water band at 3642 cm^{-1} associated with L formation persists with W182F (Figure 1c) and 9-dmr BR (Figure 5b), in contrast to the Val49 and Ala53 mutants that abolish a normal change. However, an intensification of the Asp115 band of L at 1729 cm^{-1} (Figure 4) and a different feature in the O−H stretching vibration (Figure 1) indicate some steric perturbations in the protein.

Thus, the delayed M formation from the L intermediate of the W182F protein is not likely to be due to relaxation of the specific distortion nor the geometrical disturbance of the Schiff base N−H bond. The indole of Trp182 works instead to decrease the proton affinity of the Schiff base by interacting with the retinal skeleton at the site of the 9-methyl group. Changes in electronic structures are seen in both Trp182, as exemplified in the increased intensity of its N−H stretching vibration (see above in Discussion), and the retinal, as shown by a different visible spectral feature (Figure 2) upon L formation. Besides the interaction at the 9-methyl group, other changes as seen in the perturbation of Asp115 located close to Trp182 (Henderson et al., 1990) and the water or O−H-bearing residues can be envisaged as an additional mechanism to affect the environment of the Schiff base and Asp85. Future studies are required to solve this mechanism.

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