Interaction of Aspartate-85 with a Water Molecule and the Protonated Schiff Base in the L Intermediate of Bacteriorhodopsin: A Fourier-Transform Infrared Spectroscopic Study†

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ABSTRACT: Fourier-transform infrared spectra were recorded at 170 K before and after irradiating the $Asp^{85} \rightarrow Asn$ mutant of bacteriorhodopsin. The difference spectrum exhibits protein bands such as those due to the perturbations of Asp^{96} and Asp^{115} and the N–H stretching vibration of tryptophan, characteristic of the L minus *all-trans*-bacteriorhodopsin spectrum of the wild-type protein. However, some vibrational bands of the peptide backbone and the chromophore are different from L and more characteristic of N of the wild-type protein. Remarkably, the shift observed for the vibrational band due to an internal water molecule upon L formation [Maeda, Sasaki, Shichida, and Yoshizawa (1992) *Biochemistry 31*, 462–467] is absent. These changes in the spectrum of the mutant could originate from the destruction of a hydrogen-bonding system consisting of Asp^{85}, the water molecule, and the Schiff base, upon replacement of Asp^{85} with asparagine. These observations constitute direct evidence for the interaction of water with Asp^{85} at the time when it is protonated by the Schiff base.

Bacteriorhodopsin (bR[1]) is a protein which transports protons across the membrane by using light energy absorbed in the all-*trans* retinal linked to Lys^{216} through the protonated Schiff base. This is the simplest system in which elucidation of the mechanism of the light energy conversion process in proteins can be attempted, and many studies, especially by use of spectroscopic methods, have been devoted to this problem (Kitagawa and Maeda, 1989; Mathies et al., 1991).

The N–H bond of the Schiff base in unphotolyzed *all-trans*-bacteriorhodopsin points toward Asp^{85} and Asp^{212} (Lin and Mathies, 1989; Lin et al., 1991) which comprise a hydrogen-bonding network including other residues and water (Hildebrandt and Stockburger, 1984; de Groot et al., 1989, 1990; Mathies, et al. 1991) in the extracellular domain of the membrane (Henderson et al., 1990). In this state, Asp^{85} is in the unprotonated form (Braiman et al., 1988; Metz et al., 1992) and works as an effective countercharge to increase the pK_a value of the Schiff base (Marti et al., 1991; Needleman et al. 1991; Brown et al., 1993).

The first step in the light-induced reaction of the all-*trans* form of bacteriorhodopsin is the isomerization of the retinylidene chromophore to yield the highly twisted 13-*cis* form of the J intermediate (Doig et al., 1991). After passing the K and KL intermediates, strong interaction of the Schiff base with the protein is made in the L intermediate (Maeda et al., 1991). Proton transfer from the Schiff base to Asp^{85} in the L-to-M conversion (Braiman et al., 1988; Metz et al., 1992; Fahmy et al., 1992) would suggest an orientation of the N–H bond toward Asp^{85} in L. In the relaxation steps that follow, the N–H bond is likely to change its orientation. In the M-to-N conversion, the unprotonated Schiff base accepts a proton from Asp^{96} in the cytoplasmic side (Otto et al., 1989; Pfefferlé et al., 1991), with accompanying structural changes in the protein (Fodor et al., 1988; Pfefferlé et al., 1991; Braiman et al., 1991; Sasaki et al., 1992; Souvignier & Gerwert, 1992).

Kinetic analysis of the photocycle revealed the presence of two M subspecies, M_1 and M_2 (Váró & Lanyi, 1991a), with a large difference in free energy between them (Váró & Lanyi, 1991b). The large decrease in entropy at M formation proposed by Ort and Parson (1979) was then attributed to this conversion. The switch of the Schiff base connectivity from the side of Asp^{85} to Asp^{96} was thus placed at this step (Váró & Lanyi, 1991b). This would suggest that the Schiff base is connected to Asp^{85} in the L state.

Characteristic for L is the formation of strong hydrogen bonding of an internally bound water molecule (Maeda et al., 1992a), which in contrast forms a weak hydrogen bond in the unphotolyzed state and in the M intermediate as well. These results suggest a specific interaction of the protonated Schiff base with the water molecule in the L intermediate. Replacement of Asp^{96} or Asp^{115} by asparagine does not affect this water molecule (Maeda et al., 1992c), indicating that this water molecule is not one of those located between Asp^{96} and the Schiff base (Cao et al., 1991).

In the present approach, we studied the Fourier-transform infrared (FTIR[1]) spectrum of the L intermediate formed from

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[1] Abbreviations: bR, bacteriorhodopsin; BR, the light-adapted sample of bacteriorhodopsin; FTIR, Fourier-transform infrared.

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the mutant protein in which Asp^{85} is replaced by asparagine (D85N[2]) in order to elucidate whether the interaction of the water involves Asp^{85} and discussed the orientation of the N–H bond of the Schiff base in the L intermediate.

MATERIALS AND METHODS

A *Halobacterium halobium* strain with the $Asp^{85} \rightarrow$ Asn *bop* gene was obtained by transforming strain L33 with a vector similar to those described previously for D96N (Ni et al., 1990). Purple membranes containing this protein were isolated by a standard method (Oesterhelt and Stoeckenius, 1974). For the synthesis of the [15-^{2}H]D85N, [15-^{2}H]retinal (provided by Dr. Kazuo Yoshihara of Suntory Institute for Bioorganic Research) was mixed with apoprotein of D85N that was prepared by irradiation with >400-nm light for 40 h in 2 M hydroxylamine (pH 6) at 20 °C because of slow reaction of D85N with hydroxylamine as reported by Subramaniam et al. (1991). Its binding of exogenous retinal was also very slow, taking about 10 h at 20 °C to completion.

The film for FTIR spectroscopy was prepared by drying aqueous purple membrane suspensions, and then humidifying by H_2O, or ^2H_2O, to a water content of 50% by weight as described by Maeda et al. (1992a). The sample mounted in a copper block was installed in an Oxford cryostat DN-1754 and cooled to 170 K. The FTIR spectra were measured in a Nicolet SX-60 by summing 512 interferograms before and after irradiation with >600 nm light for 2 min. The sample was warmed to 220 K, where the photoproducts decayed completely, and then cooled again to 170 K. The same process for the measurements was repeated 10 times with the same sample and the results were averaged. The data presented are the averages of measurements for three independently prepared films.

RESULTS

The irradiation of the D85N film with >470-nm light for 1 min at 274 K did not cause detectable FTIR spectral changes due to light adaptation as would be observed for wild-type bR (Roepe et al., 1988). This is expected from the properties of the blue membrane of wild-type bR at acid pH which undergoes rapid dark adaptation (Ohno et al., 1977; Maeda et al., 1980). The pH-dependent spectral change of D85N revealed (Turner et al. 1993) an equilibrium between an O-like species containing predominantly the all-*trans* form and an N-like species containing predominantly the 13-*cis* form. The fraction of the N-like species is negligibly small below pH 7 where the sample film in this experiment was prepared. The isomeric composition of the chromophore after the light adaptation of D85N was analyzed by the method of Scherrer et al. (1989) and the ratio of the all-*trans* to 13-*cis* retinal was estimated to be 68:32, which was in a close agreement with the results obtained for the O-like species of D85N by Turner et al. (1993).

The light-adapted sample (BR[1]) was cooled to 170 K and irradiated with >600-nm light for 2 min. Under these conditions, the wild-type bR yields an L intermediate (Maeda et al., 1991). The subtraction between the spectra after and before the irradiation of D85N yields a difference spectrum

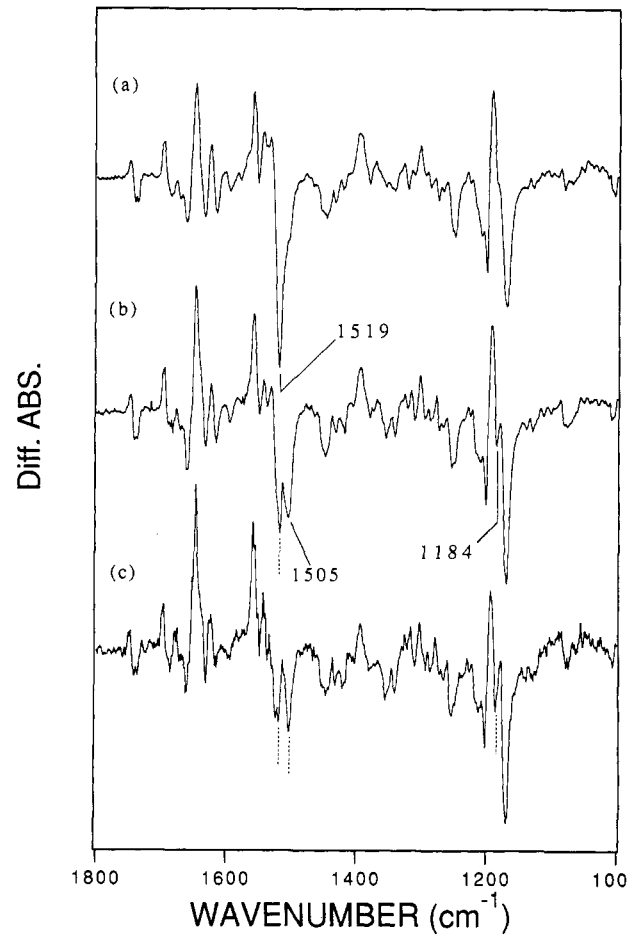

FIGURE 1: The L/BR spectra of the D85N (a), another preparation of D85N (b) and the same sample used for b but added with 10 mM phosphate buffer (pH 5.0) before drying (c). The amplitude was adjusted with a by the negative band at 1742 cm^{-1} for b and c. The vertical dashed lines show the same wavenumbers as those depicted in a and b. The full scale of the vertical axis is 0.011, 0.022, and 0.012 absorbance unit for a, b, and c, respectively.

of the photoproducts versus the unphotolyzed state (Figure 1a). From the two facts described below, it is inferred that this spectrum arises mainly by the photoreaction of the all-*trans* species and is almost free from the contribution due to the photoreaction of the 13-*cis*,15-*syn* bR.[3]

The film prepared from another sample of D85N in water (Figure 1b) showed an additional negative band at 1184 cm^{-1}, presumably due to 13-*cis*,15-*syn* bR (Roepe et al., 1988) along with a shift of the 1519-cm^{-1} band to 1505 cm^{-1}. This change became larger for a film prepared from this sample in 10 mM phosphate buffer (pH 5.0) (Figure 1c), whereas it almost disappeared in the spectrum of the other sample of D85N (Figure 1a).

Although the reason why two different preparations of D85N exhibit different features has not been solved, an increasing intensity of the 1184-cm^{-1} band at low pH (Figure 1b vs 1c) suggests that possible different surface states of the membrane are influential in the different preparations. The

[2] bR mutants were designated by the wild-type amino acid residue with the standard one-letter code and its position number followed by the substituted amino acid residue. D85N, D96N, D85E, and D212N mean the mutant proteins in which aspartic acid at position 85, 96, 85, and 212 are replaced by asparagine, asparagine, glutamic acid, and asparagine, respectively. Y185F is a mutant protein in which tyrosine-185 is replaced by phenylalanine.

[3] Fahmy et al. (1992) attributed intensity decrease in the 1169-cm^{-1} band upon ^2H_2O substitution for the 610-nm species of D85E to the photoreaction of the 13-*cis*,15-*syn* bR. In the present experiment for D85N, however, the same band retained intensity even upon 15-^{2}H substitution of the retinal (Figure 2d). This excludes a possibility of the 1169-cm^{-1} band of D85N to be C_{14}–C_{15} stretching mode, and ^2H_2O sensitivity of this band is not an indication for the photoreaction of 13-*cis*,15-*syn* bR.

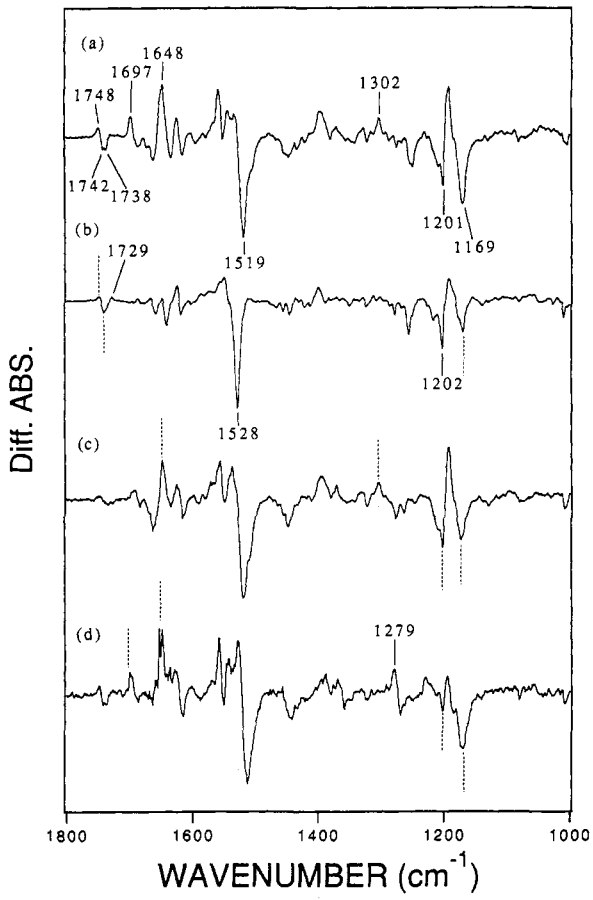

FIGURE 2: The L/BR spectra of D85N (a), wild-type bR (b), D85N in ^2H_2O (c), and D85N with C_{15}–^2H retinal chromophore (d) in the 1800–1000-cm^{-1} region. The sample for a was used for the experiments of c and d. The amplitude was adjusted with a by the negative band at 1742 cm^{-1} for b and d. For c it was adjusted by the negative band at 1201 cm^{-1} with a. The spectrum d was smoothed on five points. The vertical dashed lines show the same wavenumbers as those depicted in a. The full scale of the vertical axis is 0.041, 0.16, 0.025, and 0.0079 absorbance unit for a, b, c, and d, respectively.

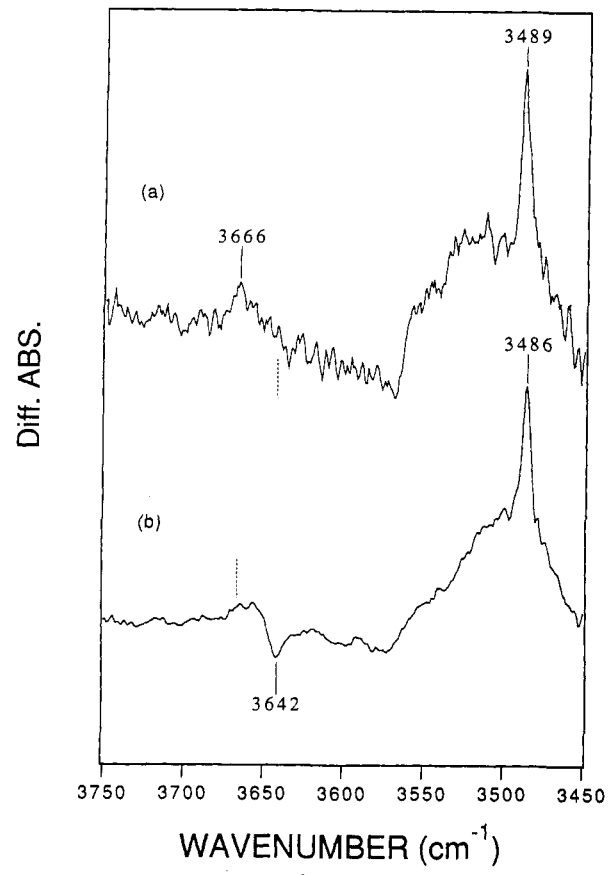

FIGURE 3: The L/BR spectra of D85N (a) in the 3750–3450-cm^{-1} region. The spectrum of wild-type bR (b) presented previously (Maeda et al., 1992a) was shown for a sake of a comparison. The vertical broken line in a shows the location of the 3642-cm^{-1} band in b, and that in b shows the location of the 3666-cm^{-1} band in a. The full scale of the vertical axis is 0.0038 and 0.00095 absorbance unit for a and b, respectively.

decay of the photoproduct, which exhibits the same spectral shape as that at 170 K, proceeds as a single component at 210 K (not shown in figures), further supporting the notion for the absence of the contribution of the 13-*cis*,15-*syn* species.

The spectrum of D85N shown in Figure 1a was duplicated in Figure 2a, and compared with that of the L/BR spectrum of the wild-type (Figure 2b). In spite of many distinct features from those of the wild-type, negative bands at 1742 and 1738 cm^{-1} and a positive band at 1748 cm^{-1} of D85N (Figure 2a) can be attributed to the C=O stretching vibration of the Asp^{96} and Asp^{115}, characteristic of the L/BR spectrum of the wild-type bR (Braiman et al., 1988; Maeda et al., 1992b) shown in Figure 2b. Hence, the corresponding spectrum of D85N was shown by scaling the intensity of the negative 1742-cm^{-1} band of the C=O stretching vibration (Figure 2a). The amount of the intermediate produced in the photoreaction was about one-fourth of the wild-type bR. This normalization also made the amplitude of a positive band at 3489 cm^{-1} of D85N (solid line in Figure 3a) comparable to the 3486 cm^{-1} band of the L/BR spectrum of wild-type bR (Figure 3b), which was assigned to the indole N–H stretching vibration of tryptophan (Maeda et al., 1992b). Broad positive and negative features of D85N below about 3550 cm^{-1} (Figure 3a) were also similar to the corresponding ones of wild-type BR (Figure 3b), which were assigned to the O–H stretching vibrations of Asp^{96} and Asp^{115} (Maeda et al., 1992c). On the basis of these facts, the spectrum obtained can be regarded as the L/BR spectrum of D85N.

A negative band at 1519 cm^{-1} of D85N, which may be due to the C=C stretching vibration of the chromophore (Figure 2a), is commonly observed for the O intermediate of Y185F (Bousché et al., 1992). A greater intensity of the 1169-cm^{-1} band than the 1201-cm^{-1} band (Figure 2a) in the negative side for D85N is distinct from wild-type bR (Figure 2b). The same relation was also observed for the O-like species of Y185F (Bousché et al., 1992; He et al. 1993). These results suggest that the difference spectrum obtained with D85N is due to the photoreaction of the O-like species.

Prominent among the vibrational bands of the photoproduct of D85N (Figure 2a) are the presence of intense bands at 1648 and 1302 cm^{-1}, which are not seen in the L/BR spectrum of wild-type bR at 170 K (Figure 2b). The intense 1648-cm^{-1} band for unlabeled D85N (Figure 2a) remains at the same frequency even after ^2H_2O exchange (Figure 2c) and 15-^2H substitution (Figure 2d), indicating that it is the amide I band. On the other hand, the 1302-cm^{-1} band of unlabeled D85N (Figure 2a) shifts to 1279 cm^{-1} by 15-^2H substitution (Figure 2d), showing that it is a chromophore band.

The positive band of D85N at 1697 cm^{-1} (Figure 2a), which is not present in wild-type bR (Figure 2b), is sensitive to ^2H_2O (Figure 2c) but not to 15-^2H exchange (Figure 2d). It is likely due to the perturbation of Asn^{85}, although this conclusion is tentative without isotope-substitution experiments. The C=O stretching bands of the protonated aspartic acid of D85N in the positive side exhibit increased intensity for the

1748-cm^{-1} band and decreased intensity for the 1729-cm^{-1} band (Figure 2a), as compared with corresponding bands in the L/BR spectrum of wild-type (Figure 2b).

The spectrum of D85N in the 3750-3450-cm^{-1} region is shown in Figure 3a. The 3642-cm^{-1} negative band of a water molecule in BR (Maeda et al., 1992a; the spectrum was duplicated in Figure 3b) is clearly absent for D85N (Figure 3a). Thus, the weakly hydrogen-bonding water which undergoes structural changes upon L formation is missing in this mutant. A positive band of L of D85N at 3666 cm^{-1} is more intense and located at higher frequency than similar positive bands of L of the wild-type (Figure 3b). The 3486-cm^{-1} band of tryptophan residue of the wild-type (Figure 3b) shifts slightly to 3489 cm^{-1} for D85N (Figure 3a).

DISCUSSION

Irradiation of the all-*trans* blue species of D85N with >600-nm light at 170 K yields an L-like photoproduct. The difference FTIR spectrum for D85N at 170 K shares the characteristic bands of the L/BR spectrum of wild-type bR for Asp^{96} and Asp^{115} and the indole N–H stretching vibrational band of tryptophan. These indicate similar environments of L in D85N to wild-type bR around Asp^{96}, Asp^{115}, and some tryptophan residues.

The involvement of the structural change of a water molecule in the BR-to-L conversion of wild-type bR was derived from the analysis of O–H stretching vibrational bands above 3450 cm^{-1} (Maeda et al., 1992a). Strikingly, the 3642-cm^{-1} band due to the weakly hydrogen bonding water molecule of the wild-type is missing for D85N. This is due either to the disappearance of the weakly bonding water molecule from the water binding site, its formation of strong hydrogen bonding, or the absence of a structural change in the photoreaction. For the O–H stretching vibration of water, increase in hydrogen-bonding strength is accompanied by increased intensity and bandwidth along with decreased frequency (Mohr et al., 1965; Glew & Rath, 1971). In the L/BR spectrum of D85N, however, such bands would be buried under the intense bands of O–H stretching vibrations of carboxylic acids (Maeda et al., 1992c). In any event, Asp^{85} is essential to keep the specific water molecule which exhibits the 3642-cm^{-1} band, and its replacement brings the water into a different state. The protonation of Asp^{85} in M and N causes the appearance of the water band at 3665 cm^{-1} (Maeda et al., 1992a). A similar band is seen at 3666 cm^{-1} for L of D85N (Figure 3a).

Other distinct features upon its replacement of Asp^{85} by asparagine are the intense positive bands at 1302 cm^{-1} due to the chromophore and at 1648 cm^{-1} due to the backbone peptide bond. These bands are characteristic of N in wild-type in both their frequencies and the responses to ^2H_2O and 15-^2H substitutions (Pfefferlé et al., 1991; Sasaki et al., 1992). The L/BR spectrum of D85N also exhibits a more increased intensity at 1748 cm^{-1} for the protonated Asp^{96} than the wild-type. These changes in L relative to wild-type bR are also observed for the blue form of D212N (Cao et al. 1993) and Y185F (Bousché et al., 1992), whereas the mutants of either other possible partners of the Schiff base, Asp^{96} and Asp^{115}, show similar L/BR spectra to the wild type (Maeda et al., 1992c). The fact that strong hydrogen bonding of the water is lost upon L-to-M conversion (Maeda et al., 1992a) suggests the influence of the protonated Schiff base on strong hydrogen bonding of the water molecules. Thus, a system containing Asp^{85}, Asp^{212}, and Tyr^{185} is more likely to be the partner of hydrogen bonding of the Schiff base (Braiman et al., 1988; Mathies et al., 1991) in L than the hydrogen-bonding system containing Asp^{96}.

The spectral shape of the negative side for the unphotolyzed state of D85N resembles that of deionized bR (Fahmy & Siebert, 1990) and the 610-nm component of D85E (Fahmy et al. 1992), suggesting that the purple-to-blue transition is the specific consequence of the protonation of Asp^{85} or Glu^{85}, respectively, rather than a general effect of lipid surface charge due to increases in the proton concentration as argued by Szundi and Stoeckenius (1987). The same conclusion was drawn from a solid state ^{13}C-NMR study (Metz et al., 1992). The L/BR FTIR spectrum obtained with D212N at pH 8 at room temperature (Cao et al., 1993), where the unphotolyzed state exhibits a red-shifted visible spectrum, is also similar to the difference spectrum of D85N at 170 K, suggesting that the blue shift by replacement of Asp^{212} is caused by a similar environment change in both the unphotolyzed state and L, even though Asp^{85} is unprotonated. An FTIR difference spectrum with the same shape was reported upon photolysis of the O-like species of Y185F under the same conditions, consistent with a notion that Asp^{85} remains protonated in O (Bousché et al., 1992).

Although a possibility that the replacement of Asp^{85} by asparagine indirectly affects the L/BR spectrum could not be rigorously excluded, we propose that the Schiff base becomes more directly connected to Asp^{85} once L is formed. Since the Schiff base of the all-*trans* form of BR points to the system containing Asp^{85} (Henderson et al. 1989, Mathies et al., 1991; Zhou et al., 1993), the orientation change of the Schiff base upon L formation will be small, and its 13-*cis* chromophore will be distorted as suggested by many authors (Zhou et al., 1993; Fahmy et al., 1989; Pfefferlé et al., 1991). As suggested above, the absence of the negative charge at residue 85 in D85N may render the structure of L to a more relaxed 13-*cis* form like in N (Pfefferlé et al. 1991; Sasaki et al., 1992).

The present results indicate that a water molecule weakly interacting in the interior of the protein of wild-type bR and entering into strong hydrogen-bonding interaction in L (Maeda et al., 1992a) is lost upon removal of the negative charge of aspartate-85. This water is most likely included among the water molecules previously suggested to be present close to the Schiff base by means of resonance Raman spectroscopy (Hildebrandt and Stockburger, 1984), neutron diffraction (Papadopoulos et al., 1990), solid-state ^{15}N-NMR (de Groot et al., 1989, 1990), and calculations on energetics (Beppu et al., 1992; Zhou et al., 1993). Our results provide direct evidence for the hydration of Asp^{85}.

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