Hydration of the Counterion of the Schiff Base in the Chloride-Transporting Mutant of Bacteriorhodopsin: FTIR and FT-Raman Studies of the Effects of Anion Binding When Asp85 Is Replaced with a Neutral Residue[†,‡]

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ABSTRACT: The chromophores of the D85T and D85N mutants of bacteriorhodopsin are blue but become purple like the wild type when chloride or bromide binds near the Schiff base. In D85T this occurs near neutral pH, but in D85N only at pH <4. The structures of the L and the unphotolyzed states of these proteins were examined with Fourier transform infrared spectroscopy. The difference spectra of the purple forms, but not the blue forms in the absence of these anions, resembled the spectrum of the wild-type protein. Shift of the ethylenic band toward lower frequency upon replacing chloride by bromide confirmed the contribution of the negative charge of the anions to the Schiff base counterion. These anions restored the change of water, which is bound near the protonated Schiff base but is absent in the blue form of the D85N mutant, though with stronger H-bonding than in the wild type. The C═N stretching vibration of the Schiff base in H_2O and ^2H_2O was detected by Fourier transform Raman spectroscopy. The H-bonding strength of the Schiff base in the unphotolyzed state was weaker when chloride or bromide was bound to the mutants than with Asp85 as the counterion in the wild type. Thus, although the geometry of the environment is different, there is at least one water molecule coordinated to the bound halide in these mutants, in a way similar to water bound to Asp85 in the wild type.

Bacteriorhodopsin is a retinal protein which spans the cellular membrane of *Halobacterium salinarium* and functions as a light-driven proton pump [see, for example, a review by Lanyi (1993)]. It consists of a single polypeptide chain folded into seven transmembrane α-helical segments (Henderson et al., 1990). The retinal forms a protonated Schiff base with Lys216 on the seventh helix near the center of the membrane, with its chain roughly parallel to the membrane plane. The counterion to the positive electric charge of the protonated Schiff base is a complex system composed of two negatively charged aspartates (Asp85 and Asp212) and a positively charged arginine (Arg82) (Marti et al., 1991). Replacement of Asp85 with a neutral residue, or its protonation at low pH, brings about a considerable red-shift of the absorption maximum in the visible, as recognized by change of the color of the chromophore from purple to blue (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Mogi et al., 1988; Brown et al., 1993; Turner et al., 1993).

The light-induced isomerization of the chromophore from the *all-trans* to 13-*cis* form triggers a sequential reaction of several spectroscopically and kinetically identified intermediates, named K, L, M, N, and O, followed by recovery of the initial state (Lozier et al., 1975). One of the important steps in determining the unidirectionality of the proton transport is the L-to-M conversion, in which the Schiff base proton is transferred to Asp85. This process is part of the mechanism that causes the switch of the access of the Schiff base from the extracellular side to the cytoplasmic side (Kataoka et al., 1994; Richter et al., 1996). Asp85 plays a key role, therefore, in the proton transfer reactions in the photocycle, in addition to stabilizing the protonated Schiff base in the unphotolyzed state.

The presence of bound water in the interior of the protein close to the Schiff base has been suggested from resonance Raman spectroscopy (Hildebrandt & Stockburger, 1984) and neutron diffraction (Papadopoulos et al., 1990). Molecular dynamics studies based on the Henderson structural model (Henderson et al., 1990) place six water molecules in the vicinity of the Schiff base (Zhou et al., 1993; Humphrey et al., 1994). Indeed, several water molecules that undergo structural changes in the photochemical process have been

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shown by Fourier transform infrared spectroscopy (FTIR)[1] (Maeda et al., 1992a, 1994; Fischer et al., 1994; Yamazaki et al., 1995b, 1996; Hatanaka et al., 1996). Among these, the water molecule that exhibits an O−H stretching band at 3643 cm^{-1} in the unphotolyzed state undergoes changes in the K, L, and M intermediates (Maeda et al., 1992a; Fischer et al., 1994). Replacement of Asp85 with Asn abolished this band (Maeda et al., 1994), suggesting that the water molecule is associated with Asp85. Resonance Raman measurements have revealed that H-bonding of the Schiff base with Asn in D85N is comparable to that in the wild-type protein that has a negative charge at the residue 85 (Rath et al., 1993). It is likely, however, that in the D85N mutant the water molecule is not accommodated in the same way as in the wild type.

The addition of an excess amount of chloride to the acid blue membrane (at pH <2) and the mutants of Asp85 replaced with Asn (at pH <4) or Thr (at pH as high as 6) restores the purple color with a maximum near 565 nm (for the wild type and D85N) or 556 nm (for D85T) (Dér et al., 1991; Rath et al., 1993; Sasaki et al., 1995; this report). These observations indicated that bound chloride can function as the counterion to the Schiff base, and interacts with it as it does in halorhodopsin where the residue that corresponds to Asp85 is a Thr (Walter & Braiman, 1994). Although their local structure might be different, an inorganic anion as the counterion of the Schiff base gives the same balance of electric charge near the Schiff base in these mutant proteins as the wild type. Interestingly, we recently found that the D85T mutant pumps chloride rather than protons (Sasaki et al., 1995).

In the work reported here, we studied H-bonding changes of the Schiff base and water molecules in the mutant proteins, D85N and D85T, with halides such as chloride and bromide added, by means of FTIR spectroscopy. FT-Raman spectroscopy was used in measurements of the C=N stretching vibrations of the Schiff base. The results indicate that, in spite of a different geometrical environment near the Schiff base, at least one water molecule is involved in the hydration of halide in these mutants, as Asp85 in the wild type.

MATERIALS AND METHODS

The D85T protein was expressed in *H. salinarium* Pho81 cells that lack all other bacterial opsins (Sundberg et al., 1990), and D85N was expressed in *H. salinarium* L33 cells as described previously (Ni et al., 1990). These proteins were purified as purple membranes by the method described by Oesterhelt and Stoeckenius (1974). For the synthesis of [15-^{2}H] derivatives, [15-^{2}H]retinal (provided by Kazuo Yoshihara) was mixed with apoprotein that was prepared by illumination with >500-nm light in 2 M hydroxylamine (pH 6.5) at 0 °C followed by five washings in 2% bovine serum albumin solution (Fraction V of Sigma) to remove the retinaloximes.

Wild-type or D85T membranes were suspended in 10 mM NaCl and 10 mM phosphate buffer (pH 6). Those of D85N were suspended in the same buffer except that the pH was 3. These samples are designated as D85T(Cl) and D85N-(Cl), respectively. The films were prepared by drying 50 μL aliquots of the suspensions on a BaF_2 window 18 mm in diameter in room air or under mild vacuum. These films were hydrated with 0.5 μL of water (or ^2H_2O, $H_2{}^{18}O$) and sealed with another window, with a silicon rubber O-ring between them. The sandwiched film was then placed in a sample holder. The film of D85T made from the suspensions in 10 mM NaBr in place of NaCl was denoted as D85T(Br).

The sample holder was mounted in an Oxford cryostat DN1704, equipped with an Oxford ITC-4 temperature controller. FTIR spectra were recorded in a Bio-Rad FTIR FTS60A/896 spectrometer at 2 cm^{-1} resolution by co-adding 128 interferograms for each measurement. FT-Raman spectra were recorded on a Bruker IFS66/S-FRA106/S spectrometer at 2 cm^{-1} resolution. Between 2 000 and 10 000 records were averaged. The measurements were at 22 °C, with the samples either as suspensions or encased in polyacrylamide gels equilibrated with the buffer specified, in a 4-mm pathlength quartz cuvette. Spectra measured for the buffer, or a gel without BR, were subtracted. The spectra in the visible region were recorded in a Shimadzu MPS-2000 or a Shimadzu UV-1601 spectrometer.

The light for illumination of the samples was provided from a 1 kW halogen−tungsten lamp through one of several Toshiba cutoff filters, Y52, R62, and R65, which pass >500-, >600-, and >630-nm light, respectively, and an interference filter, KL70, which passes 700-nm light.

Light adaptation of the samples was by illuminating with >500-nm light for 4 min at 274 K. The L intermediates of all samples, except for the blue form of D85N, were produced by the illumination with >600-nm light for 2 min at 170 K. The conditions used for blue D85N are given in the Results section. The difference FTIR spectrum was calculated from the spectra before and after the illumination. Spectra are depicted by normalizing to the intensity of the 1203 cm^{-1} band, except for D85N in which this band is slightly shifted. For the latter, the amplitude was adjusted instead by the intensity of the 1742 cm^{-1} band. The samples were warmed to about 273 K to revert them to the initial states, and the same process was repeated four to five times for averaging.

RESULTS

Purple Form of Mutants of Asp85. Chloride binding near the Schiff base will cause a blue-shift of the absorption maximum for mutants of Asp85. Figure 1 shows spectra in the visible region for D85N and D85T in 1 M Na_2SO_4 and 2 M NaCl, at two pH values. For D85N, the expected blue-shift with chloride is observed at pH 3 but not at pH 6 (Figure 1a). The skewed shape of the spectrum of this mutant with chloride indicates that chloride binding is not saturated even at 2 M NaCl. The calculated dissociation constant from titration experiments was 1.6 M (not shown). A minor pH dependent shift of the spectra is observed in sulfate, probably as a result of changing isomeric composition. With the membrane sheets we could not reproduce the earlier report on the formation of a purple form of D85N in detergent micelles in 4 M NaCl at pH 6 by Rath et al. (1993). Almost no chloride-dependent shift was observed by Marti et al. (1991) for D85N at the higher pH but in 0.5 M NaCl, consistent with Figure 1a. In contrast to D85N, the large blue-shifts in Figure 1b demonstrate that D85T binds chloride at both pH 4 and pH 6. At pH 4 the chloride binding in this mutant is stronger, with a dissociation constant of 0.6 M,

[1] Abbreviations: FTIR, Fourier transform infrared; BR, *all-trans* form of bacteriorhodopsin; HOOP, hydrogen-out-of plane.

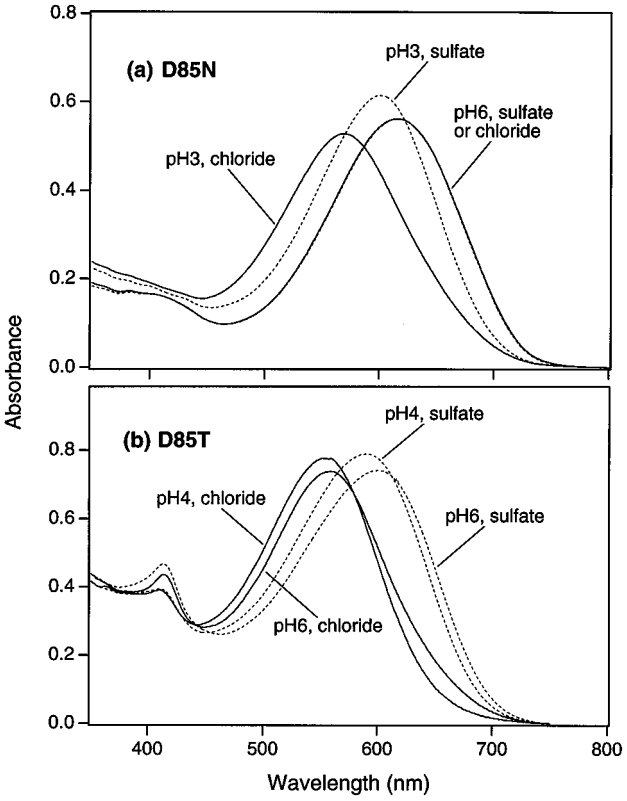

FIGURE 1: Absorption spectra of D85N (a) and D85T (b) bacteriorhodopsins in the presence and absence of chloride. Solid lines, 2.0 M NaCl; dashed lines, 1.0 M Na_2SO_4. The pH is as indicated. The membrane suspensions were encased in polyacrylamide gels; the buffer was 100 mM phosphate plus 100 mM succinate.

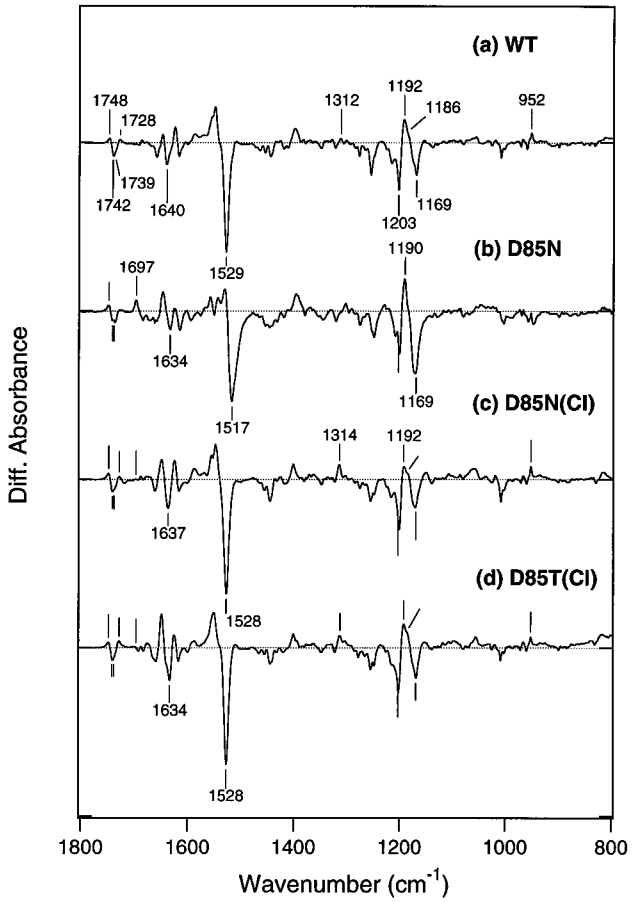

FIGURE 2: L *minus* BR spectra in the 1800-800-cm^{-1} region for the wild type (a), D85N (b), D85N(Cl) (c), and D85T(Cl) (d). The full length of the ordinate corresponds to 0.096 (a), 0.015 (b), 0.060 (c), and 0.033 (d) absorbance unit.

but at pH 6 it is 1.6 M. It was 0.6 M for bromide at pH 6 (not shown).

We ascertained by means of visible spectroscopy that the same purple form of the mutant is produced in the films as in 2 M NaCl in the suspensions (as in Figure 1). The spectral shapes of D85T at pH 6, and those of D85N at pH 3, were essentially the same in the films as in the suspensions, indicating that chloride binds to this protein under both conditions. The effective concentration of chloride in the films, once the salt became concentrated upon drying, was therefore not far from 2 M.

L Minus BR Spectra of the all-trans Species. Figure 2 shows the 1800−800 cm^{-1} region of the difference spectra recorded at 170 K upon illumination with >600-nm light of the wild type (a), D85N (b), D85N(Cl) (c), and D85T(Cl) (d). The spectra of D85T(Cl) were virtually identical at pH 4 and 6 (not shown). The spectrum of D85T without added salt was 30 times smaller in amplitude than the wild type (not shown). The earlier reported difference spectrum of the blue form of D85N obtained by illumination with >600-nm light exhibited two negative ethylenic bands, at 1519 and 1505 cm^{-1} (Maeda et al., 1994). These could arise from two different species. In the present study, they were separated by recording FTIR spectra after illumination first with 700-nm light and then >630-nm light. The difference spectrum with the ethylenic band at 1517 cm^{-1} in Figure 2b was calculated by subtracting the spectrum after the second illumination with >630-nm light from the spectrum after the first illumination with >700-nm light. The difference spectrum of the first photoreaction is different from the second one in exhibiting the negative ethylenic band only at 1509 cm^{-1} (not shown). The other bands were almost identical in the two spectra with each other. These results indicate that the blue form of D85N contains at least two different species with different absorption maxima but they convert to the same L-like species.

The spectra of D85N(Cl) (c) and D85T(Cl) (d) exhibited bands characteristic of the L *minus* BR spectrum of the wild type (a), with regard to the bands at 1748(+)/1742(−) and 1728(+)/1739(−) cm^{-1} due to perturbations of Asp96 and Asp115, respectively (Braiman et al., 1988; Maeda et al., 1992b), and to the 1192-cm^{-1} band with a shoulder at 1186 cm^{-1} due to the C−C stretching modes of the chromophore (Gerwert & Siebert, 1986).

A negative C−C stretching vibrational band around 1180 cm^{-1} and an N−H in-plane bending vibrational band at 1342 cm^{-1} in the light *minus* dark spectrum of the wild type are characteristic of the photoreaction of 13-*cis*,15-*syn* species of dark-adapted BR (Roepe et al., 1988). The same bands appear in the difference spectrum for the light-adaptation of the dark-adapted state of D85T(Cl) and D85T(Br) (not shown). The absence of these bands in the L *minus* BR spectra of these species indicates that the photoreaction arises only from the *all-trans* species. The rate for the dark-adaptation was too rapid for the D85N mutants to record the spectrum for the light-adaptation. We infer the absence of the photoreaction of the 13-*cis*,15-*syn* species from the assumption that the bands described above would appear as depletion bands if this species had undergone a photoreaction.

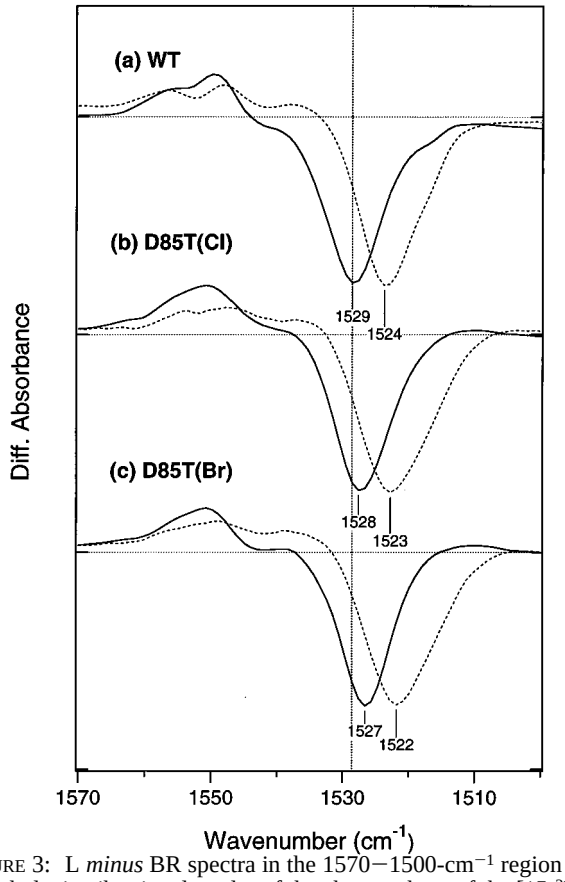

FIGURE 3: L *minus* BR spectra in the 1570−1500-cm^{-1} region for the ethylenic vibrational modes of the chromophore of the [15-^{2}H]-retinal-labeled BR (dashed lines) and unlabeled BR (solid lines) for the wild type (a), D85T(Cl) (b), and D85T(Br) (c) by adjusting peak intensities. A vertical dotted line is drawn at the peak frequency of the unlabeled wild type in order to show the shifts more clearly. The full length of the ordinate corresponds to 0.063 (a), 0.021 (b), and 0.035 (c) absorbance unit for the unlabeled BR (solid lines) and 0.063 (a), 0.021 (b), and 0.020 (c) absorbance unit for the labeled BR (dashed lines).

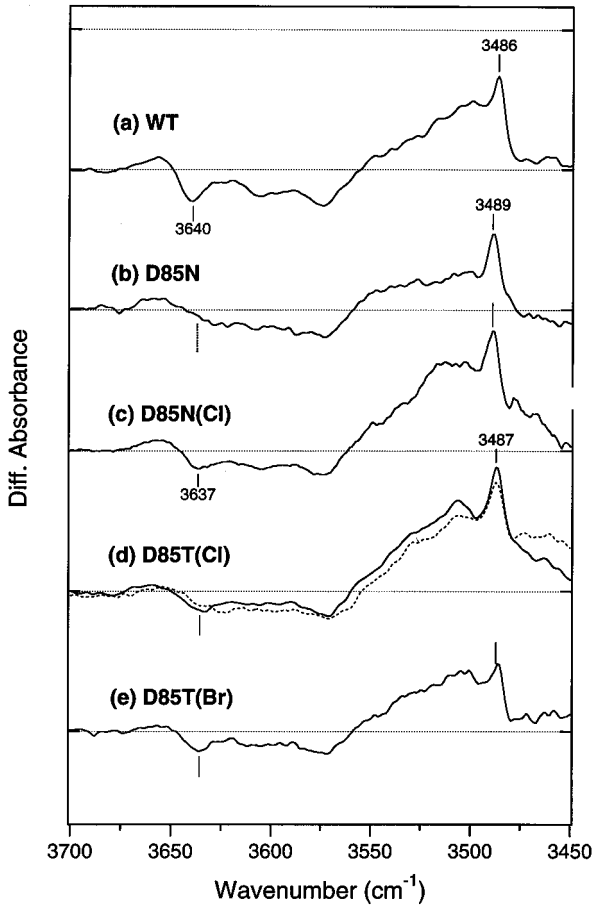

FIGURE 4: L *minus* BR spectra in the region for O−H stretching vibrational modes for the wild type (a), D85N (b), D85N(Cl) (c), D85T(Cl) (d), and D85T(Br) (e). A spectrum obtained for the film of D85T(Cl) hydrated with $H_2{}^{18}O$ is also shown in d by a dashed line. The vertical dotted line in b shows the absence of a corresponding band in a. The full length of the ordinate corresponds to 0.0116 (a), 0.0019 (b), 0.0073 (c), 0.0039 (d), and 0.0065 (e) absorbance unit in H_2O.

The spectrum of D85N in Figure 2b exhibits a more intense negative band at 1169 cm^{-1} than the chloride-binding purple form. The same tendency is observed for D85T (not shown). This is characteristic of blue pigments in general, such as the O photointermediate (Bousché et al., 1992) or the deionized blue membrane of the wild-type protein (Fahmy & Siebert, 1990). The positive band at 1697 cm^{-1} in D85N, presumably due to the C═O stretching vibration of Asn85, is not seen in the wild type (a) or in the Asp85 mutants with chloride (c and d). The 1728-cm^{-1} band due to the perturbation of Asp115 and the 952-cm^{-1} band due to a hydrogen-out-of-plane (HOOP) mode of the chromophore are absent in the blue form of D85N (b) but are restored in halides as the wild type.

The L *minus* BR spectra of Asp85 mutants in chloride are thus more similar to the wild type than to their blue forms, indicating that the negative charge of the chloride in place of Asp85 is sufficient to maintain wild-type-like structures for both the L and unphotolyzed states. The spectrum in bromide also had wild-type-like shapes (not shown). However, closer inspection revealed small but significant changes in the ethylenic modes of the chromophore, the C═N stretching mode of the Schiff base, and the O−H stretching mode of water molecules. These will be described below.

Effects of Anions on the Ethylenic Vibrations. The 1570−1500-cm^{-1} region (Figure 3) contains the ethylenic mode of the chromophore retinal. The spectra were shown for the wild type (a), D85T(Cl) (b), and D85T(Br) (c) (solid lines). These ethylenic bands are shifted parallel toward the lower frequency side in [15-^{2}H]retinal-labeled BR (dotted lines), indicating the absence of the contribution of the other bands such as amide II bands. The spectra of the BR regenerated with the unlabeled retinal were also shifted by about 1 cm^{-1} relative to the native BR (not shown) but in an extent not to affect the conclusion above. The negative band of D85T in the presence of these anions (b and c) is located clearly at a lower frequency than the wild-type band at 1529 cm^{-1} (a). The frequency shift for D85T(Br) (c) is slightly larger than for D85T(Cl) (b). Shift of the ethylenic mode to lower frequency reflects π-electron delocalization of the chromophore (Kakitani et al., 1983). Thus, larger shifts with increased size of the anion (Br vs Cl, Figure 3) confirm that the monovalent anions substitute for Asp85 as the counterion of the Schiff base in the unphotolyzed state of D85T, as proposed explicitly for halorhodopsin (Walter & Braiman, 1994).

Restoration of the H-Bonding Changes of Internal Water. Spectra in the 3700−3450-cm^{-1} region (Figure 4) include the O−H stretching vibrations of water which exhibit downshifts by about 10 cm^{-1} when H_2O is replaced with $H_2{}^{18}O$ (Maeda et al., 1992a; Yamazaki et al., 1995b). The band at 3486 cm^{-1}, which is attributed to the N−H stretching

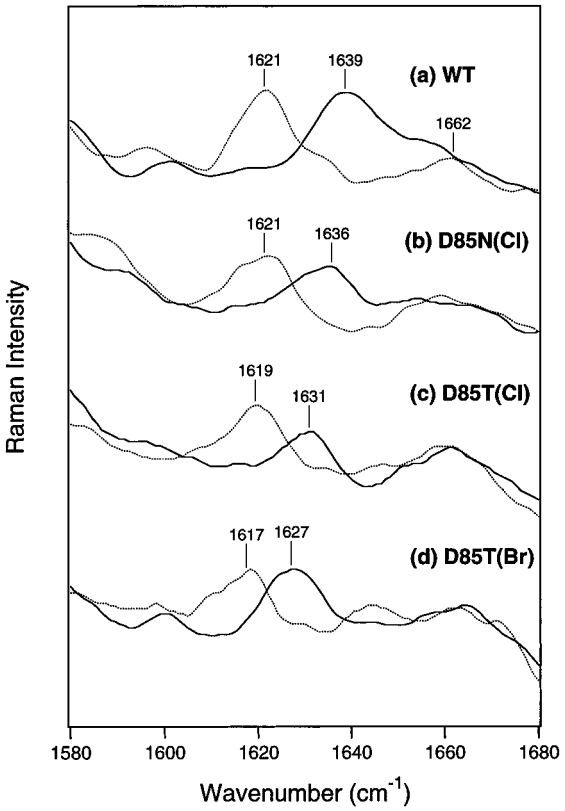

FIGURE 5: FT-Raman spectra in the 1680−1580-cm^{-1} region for the C═N stretching vibrational modes of the Schiff base for the wild type (a), D85N(Cl) (b), D85T(Cl) (c), and D85T(Br) (d) in H_2O (solid lines) and ^2H_2O (dotted lines). The amplitudes of the spectra were adjusted at the 1009- and 1455-cm^{-1} bands. Multiplication factors to a were 2.20 for b, 2.75 for c, and 1.83 for d.

vibration of the indole of Trp182 (Yamazaki et al., 1995a), is specific to the L intermediate. It shifted to 3489 cm^{-1} in D85N (b) and D85N(Cl) (c), and to 3487 cm^{-1} in D85T(Cl) (d). The water molecule of the wild type which exhibits a negative band at 3640 cm^{-1} (a) was suggested to be located in the immediate vicinity of Asp85, since in the blue form of D85N this band is completely absent (Maeda et al 1994; see also Figure 4b). D85T(Cl), which has chloride as the counterion of the Schiff base (d), shows a similar although less intense band. As in the wild type, it is attributable to water because it shifts to a lower frequency in $H_2{}^{18}O$ (dashed line). The corresponding band is located at similar frequencies for D85N(Cl) (c) and D85T(Br) (e) in H_2O.

H-Bonding of the Schiff Base. The C═N stretching vibrational bands can be identified as the bands sensitive to both [15-^{2}H] substitution of the retinal and deuteration of the Schiff base. However, all the bands of the regenerated mutant proteins in this region became much less intense than native mutant proteins, and that made the band assignments difficult. For this reason, we used FT-Raman spectroscopy with unphotolyzed samples (Sawatzki et al., 1990). These spectra are free from any photoproducts, and the amide I bands are so weak that they do not interfere. Recordings were done for the dark-adapted samples because the long duration of the recordings (2 h with the wild type and 10 h with the mutants) did not allow measurements with light-adapted states in any case. The spectra in the 1800−700-cm^{-1} region (not shown) are similar to that of the dark-adapted BR reported by Sawatzki et al. (1990). Figure 5 shows the spectra in the 1700−1560-cm^{-1} region (solid lines) together with those in ^2H_2O (dotted lines). The ^2H_2O-insensitive bands around 1662 cm^{-1} may be due to amide I, since it exhibits the maximum intensity at 1665 and 1658 cm^{-1} in the absolute FTIR spectrum (Cladera et al., 1992). Hence, the contribution of amide I in the C═N stretching region is negligible. The band at 1639 cm^{-1} of the wild type (a) is completely shifted to 1621 cm^{-1} in ^2H_2O. Hence, it can be assigned to the C═N stretching vibrational mode. Its frequency at 1639 cm^{-1} is very close to that of both light-adapted and dark-adapted BR at 1639 cm^{-1} in previous FT-Raman spectra (Sawatzki et al., 1990). The centers of the C═N stretching vibrational bands of D85N(Cl) (b) are located at 1636 cm^{-1} in H_2O and 1621 cm^{-1} in ^2H_2O. They are coincident with those obtained from resonance Raman spectra (Rath et al., 1993). D85T(Cl) exhibits relatively sharp bands at 1631 cm^{-1} in H_2O and 1619 cm^{-1} in ^2H_2O (c). These bands for D85T(Br) show the centers at 1627 and 1617 cm^{-1}, respectively (d).

The C═N−H stretching modes of the protonated Schiff base in the mutants with bound chloride are located at frequencies by about 3−8 cm^{-1} lower (b and c) than the wild type. A further slight down-shift upon replacing chloride with bromide (c vs d) is consistent with that observed earlier for halorhodopsin (Walter & Braiman, 1994).

Since the C═N stretching mode of the protonated Schiff base is coupled with the C═N−H in-plane bending mode, whose frequency is raised with increasing strength of H-bonding of N−H with the counterion, the extent of the upshift of the C═N stretch mode reflects the strength of H-bonding of the Schiff base proton. When the proton on the Schiff base is replaced by deuteron, the coupling is eliminated. The H-bonding strength of the Schiff base proton can be estimated therefore from the down-shift of the C═N stretching frequency upon replacing the proton with deuteron (H−D shift) (Kakitani et al., 1983; Baasov et al., 1987; Rodman-Gilson et al., 1988). The H−D shifts of D85T(Cl) (c, 12 cm^{-1}) and D85T(Br) (d, 10 cm^{-1}) are much smaller than the wild type (a, 18 cm^{-1}). The value for D85N(Cl) (b, 15 cm^{-1}) is only slightly smaller than the wild type. The H-bonding of the Schiff base with halides is thus weaker in D85T than with the aspartate in the wild type but not so much in D85N, as reported previously for Asp85 mutant proteins in micelles by Rath et al. (1993).

DISCUSSION

Bacteriorhodopsin mutants whose Asp85 is replaced with a neutral residue such as Asn, Ala, or Thr are known to bind monovalent anions, and the bound anions function as counterions of the Schiff base (Marti et al., 1991, 1992; Rath et al., 1993; Sasaki et al., 1995). The observation of the anion-dependent shift of the ethylenic frequency (Figure 3) confirms this. Of these mutant proteins only D85T has been demonstrated so far to transport chloride instead of protons (Sasaki et al., 1995). Inability of D85N to transport chloride is suggested also by the observation (Brown and Lanyi, unpublished result) that the photocycle of D85N does not contain a red-shifted intermediate with chloride-dependent decay even at pH 3, such as described for D85T both at pH 6 (Sasaki et al., 1995) and at pH 4. It is not clear why D85N is inactive in anion transport. One possibility is that the H-bonding geometry of Thr at position 85 is unique and

required for the binding of the anion appropriate for translocation. Stronger H-bonding of the Schiff base in D85N than D85T is another possibility (the present results). The other possibility is that the critical difference resides in the pH dependence of the anion binding. For reasons that are not yet clear, D85N shows halide binding, as revealed by blue-shift of its absorption maximum, only at and below pH 4, while D85T binds the anions near neutral pH (Figure 1). At such a low pH, and in both the presence and absence of chloride, it appears that neither the blue nor the acid purple forms of the wild-type bacteriorhodopsin nor D85N has any transport activity (Moltke & Heyn, 1995; Moltke et al., 1995).

The negative charge of Asp85 in the wild-type protein is implicated not only in the formation of an electrostatic interaction with the positively charged protonated Schiff base, but also in the complex H-bonding network that includes several important residues in the extracellular region, Asp212, Arg82, Tyr57, Glu204, water, etc. (Marti et al., 1991; Needleman et al., 1991; Balashov et al., 1993; Brown et al., 1993; Maeda et al., 1994; Kandori et al., 1995; Govindjee et al., 1995; Richter et al., 1996; Hatanaka et al., 1996). The local conformation in the absence of the negative charge of Asp85 (in the blue form) must therefore be different from that of the wild type. The chloride-free, blue form of D85N with the *all-trans* chromophore contains two different species distinguishable by their different ethylenic frequencies. Both of these L *minus* BR spectra of D85N are distinct from the wild type, indicating that the absence of the negative charge at position 85 brings about perturbations of the structure in both the L and unphotolyzed states. The analogous blue form of D85T shows an L *minus* BR spectrum similar to the spectrum of D85N but with an amplitude about 4 times smaller. D85T may have a generally similar structure to D85N but with a somewhat different arrangement between residue 85 and the Schiff base. The L *minus* BR spectra of the mutants thus restored a nearly wild-type-like shape upon anion binding. The effects of the halides on the ethylenic frequencies indicate that their location is close to the Schiff base. This suggests that the negative charge of the anion in place of Asp85 maintains the structure not only in the unphotolyzed state but also in the L intermediate.

Information about H-bonding of the Schiff base in the D85N and D85A proteins in the presence and absence of chloride was reported earlier from resonance Raman spectroscopy (Rath et al., 1993). In terms of the H−D shift, the interaction is particularly weak in D85A. The H-bonding of the protonated Schiff base in D85N is as strong as that in the wild type, in spite of the fact that Asn does not have a negative charge. It was proposed that one or a few water molecules intervene between the protonated Schiff base and Ala, whereas the carbonyl of Asn forms a direct H-bonding with the protonated Schiff base. However, they did not propose the model for chloride binding.

The weak H-bonding of the Schiff base in BR, as revealed by ^{15}N solid state NMR, has been attributed, however, to intervening water molecules (de Groot et al., 1989). The presence of water molecules between the protonated Schiff base and the counterion was suggested by the energy calculations for a simple model with the protonated Schiff base and the carboxylate (Beppu et al., 1992; Scheiner & Duan, 1991) and for the entire bacteriorhodopsin (Humphrey et al., 1994) on the basis of the structure of Henderson et al. (1990).

The frequency region higher than 3600 cm^{-1} reveals structural changes of weakly H-bonding water molecules (Maeda et al., 1992a). The disappearance of the negative band at 3640 cm^{-1} due to the O−H stretch mode of a water molecule upon substitution of Asn for Asp85 indicates that in the wild type the water molecule associates with the negatively charged Asp85 (Maeda et al., 1994). Upon restoration of the negative charge at this location in D85N-(Cl), D85T(Cl), and D85T(Br) (Figure 4), the corresponding water band reappeared, although at a slightly lower frequency. These results indicate that water molecule will associate with either the negative charge of Asp85 or the chloride bound close to residue 85.

The water band at 3640 cm^{-1} is affected by the replacement of any of the residues in the H-bonding network that forms complex counterion in the extracellular domain, Asp212 (Kandori et al., 1995), Glu204 (Brown et al. 1995a), Arg82 (Hatanaka et al., 1996), and Tyr57 (Fischer et al., 1994). However, the location of the water molecule is most likely between the Schiff base and Asp85, because the replacements of residues other than Asp85 cause only a shift of the O−H stretching mode rather than its abolition as in the uncharged Asp85 mutants.

The lower frequency of the O−H stretch mode in the Asp85 mutants in the presence of halides appears to be related to the lesser H−D shift of the C═N stretching mode of the protonated Schiff base. Thus, stronger H-bonding of water O−H is accompanied by weaker H-bonding of the Schiff base. These can be ascribed to the slightly different location of the binding site of halide relative to position of 85. The positive charge of Arg82 is not essential for chloride binding, since R82Q and D85N/D212N/R82Q (Marti et al., 1991, 1992) both bind anions (Brown et al., 1995b), in contrast to the corresponding residue Arg108 in halorhodopsin which is a site for the binding of chloride (Rüdiger et al., 1995). The distance between the water molecule and the protonated Schiff base will be thus greater when halide is the counterion, causing the H-bonding of the Schiff base proton with the water molecule to be weaker than in the wild type with Asp85. Conversely, the H-bonding of the water molecule with the halides will be stronger in the mutants.

A hydrated negative charge close to position 85 seems to be crucial for the normal structure in both the unphotolyzed state and the L intermediate. In the L state this water plays a critical role in determining the pathway for the proton conduction from the Schiff base to Asp85, and not to Asp212, by binding strongly to the Schiff base and Asp85 (Kandori et al., 1995; Humphrey et al., 1995). The water molecule that hydrates the halide also forms strong H-bonding in the L intermediate, as the water of the wild type, and may have the role of keeping the halide tightly bound to position 85. Connection of the Schiff base N−H with the counterion at position 85 with an intervening water molecule in the L intermediate (Maeda et al., 1994) can be accomplished only by chain distortion of the 13-*cis* chromophore of the wild type (Schertler et al., 1991; Humphrey et al., 1995). A similar structure of the L intermediate may be necessary before transfer of halide to the cytoplasmic side. In D85T these interactions occur at neutral pH and result in transport, but in D85N they can take place only at much lower pH and do not appear to be followed by translocation.

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