Interaction of Internal Water Molecules with the Schiff Base in the L Intermediate of the Bacteriorhodopsin Photocycle†

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Received October 12, 2001; Revised Manuscript Received January 18, 2002

ABSTRACT: In the photocycle of bacteriorhodopsin (BR), the first proton movement, from the Schiff base to Asp85, occurs after the formation of the L intermediate. In L, the C═N bond of the Schiff base is strained, and the nitrogen interacts strongly with its counterion. The present study seeks to detect the interaction of internal water molecules with the Schiff base in L using difference FTIR spectroscopy at 170 K. The coupled modes of the hydrogen-out-of plane bending vibrations (HOOPs) of the N−H and C_{15}−H of the protonated Schiff base are detected as a broad band centered at 911 cm^{-1} for BR. A set of bands at 1073, 1064, and 1056 cm^{-1} for L is shown to arise from the coupling of the HOOP with the overtones of interacting water O−H vibrations. Interaction with water was shown by the decreased intensity of the HOOPs of L in $H_2{}^{18}O$ and by the influence of mutants that have been shown to perturb specific internal water molecules in BR. In contrast, the HOOP band of initial BR was not affected by these mutations. In D85N, the coupled HOOP of BR is depleted, while the coupled HOOPs of L are shifted. The results indicate that the Schiff base interacts with water in the L state but in a different manner than in the BR state. Moreover, the effects of mutations suggest that cytoplasmic water close to Thr46 (Wat46) either interacts stronger with the Schiff base in L or that it is important in stabilizing another water that does.

Bacteriorhodopsin is a transmembrane, seven-helix protein. It utilizes light energy, absorbed by its retinylidene chromophore, to cause the unidirectional transport of protons across the cell membrane, from the cytoplasm to the extracellular surface (*1*). The polyene chain of the retinal lies in the middle of the membrane and is linked to Lys216 by forming a protonated Schiff base (−C═NH^+−). The chromophore roughly divides the membrane into the extracellular domain, which is relatively rich in polar residues, and the cytoplasmic domain, with few polar residues (*2*). In the dark, the chromophore of bacteriorhodopsin is present as a mixture of all-trans and 13-cis, 15-syn forms. Both forms of the chromophore absorb light energy and isomerize the C_{13}═C_{14} bond, but only the species with the all-trans chromophore (BR)[1] pumps protons by being driven through a series of intermediates called K, L, M, N, and O (*3*). The inactive 13-cis, 15-syn species does not form a blueshifted L-type intermediate (*4*), and it does not pump protons. The formation and the decay of L are crucial steps in the operation of the light driven proton pump. FTIR studies have shown many perturbations of amino acid residues and internal water molecules in going from BR to L (*5*). Nevertheless, no gross conformational changes were detected by relatively low-resolution electron diffraction studies (*6*, *7*). The structure of the chromophore is strained in L (*8*, *9*). This distinguishes L from the later intermediate N, which has a relaxed, 13-cis chromophore.

The interaction of the Schiff base with a water molecule in BR was previously shown by resonance Raman (*10*), solid-state NMR (*11*), and neutron diffraction (*12*) studies. Recent X-ray studies have shown that, in BR, the N−H of the protonated Schiff base H bonds with a water molecule on the extracellular side (*13*−*17*). Light-induced isomerization of the chromophore leads to the formation of K, in which the H-bonding interaction of the Schiff base nitrogen is quite weak (*18*). This could result from the change of orientation of the Schiff base N−H upon isomerization. X-ray crystallographic studies indicate that the lone pair of electrons of the unprotonated nitrogen in late M is oriented toward the cytoplasm, roughly opposite to the orientation of the N−H in BR (*17*, *19*). The orientation of the Schiff base in L is uncertain. The cis form for both the C_{13}═C_{14} and C_{14}−C_{15} bonds of the retinal chromophore, which would orient the Schiff base N−H toward the extracellular domain, was not

† This work was supported by NIH Grants GM 52023 (to T.G.E.), HL 16101 (to R.B.G.), and GM 36810 (to J.H.).

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[1] Abbreviations: BR, all-trans bacteriorhodopsin; HOOP, hydrogen-out-of plane vibration; Wat85, water molecule responsible for the O−H stretching frequencies which are affected by the mutation of Asp85; Wat46, water molecule responsible for the O−H stretching frequencies which are affected by the mutation of Thr46.

10.1021/bi011923p CCC: $22.00
Published on Web 02/20/2002

detected by resonance Raman (*20*) and solid-state NMR (*9*) studies. However, the protonated Schiff base of the L intermediate exhibits moderately strong H bonding and distortion around the C=N bond (*9*, *18*). In the L-to-M transition, the proton of the Schiff base is transferred to Asp85. What is the structure of the protonated Schiff base of the L intermediate that makes its H bonding stronger than that in K, while positioning the Schiff base proton to be transferred to Asp85 in the L-to-M transition?

Previous studies, using the photoreaction of L to its photoproduct, L′, at 80 K (*21*, *22*) suggested that the internal water molecules in the cytoplasmic domain in BR relocate close to the Schiff base in L (*23*). Water is the most likely H-bonding partner of the Schiff base in L, and its interaction can be detected by examining the vibrations of the Schiff base that are affected by water molecules. The C=N stretching vibration is difficult to detect in L because it overlaps with amide I bands in the difference FTIR spectrum (*24*). The in-plane bending vibrations of the Schiff base are informative but are too weak in L to distinguish clearly from other overlapping bands (*18*). A more satisfactory analysis can be achieved from another Schiff base vibration, the hydrogen-out-of plane vibration (HOOP). In low-temperature FTIR spectroscopy, the K intermediate shows a very intense band at 957 cm^{-1} due to the C_{15}−HOOP coupled weakly with the N−HOOP (*18*). However, the corresponding bands in BR, L, and N have not yet been identified. In D_2O, the C_{15}−HOOP band is uncoupled and appears with large intensity, at 976 cm^{-1} in BR and 985 cm^{-1} in L (*25*). In the present study, we identified the HOOP bands of the Schiff base in BR and in L using isotope-labeled bacteriorhodopsin.

Difference FTIR spectra show internal water O−H stretching bands that shift upon formation of L (*26*). Among the water O−H bands of BR, a 3642 cm^{-1} band disappeared in D85N (*27*). This band is probably due to water molecules around Asp85. We call this Wat85. Figure 1 shows the locations of amino acid residues and internal water molecules that are discussed in the text. A water O−H band of BR at 3575 cm^{-1} and a large part of a wide water band for L in the region between 3560 and 3500 cm^{-1} disappeared in T46V. These bands are partially restored with an additional mutation of D96N in T46V/D96N (*28*). We call the water(s) responsible for these bands Wat46. FTIR studies have shown that Wat46 strengthens its H bonding and becomes more polarized upon formation of L (*24*). Such changes of Wat46 are characteristic of L and were not detected in K and M. These results are consistent with the locations of water molecules found in X-ray diffraction studies (*14*, *17*). As noted earlier, the water molecules surrounding Asp85 are candidates for the 3642 cm^{-1} Wat85 band. Among them, W402 is located between the Schiff base and Asp85, while W401 and W406 water molecules are located between Asp85 and Asp212. Thr46 is a residue in the cytoplasmic domain, far from the Schiff base (∼10 Å). A water molecule (W502), which forms H bonds to the peptide carbonyl groups of Thr46 and Lys216 in BR, is close to the methyl groups of both Thr46 and Val49 (∼3.5 Å). Val49 is also close to the side chain of Lys216. In V49A, the L minus BR spectrum exhibits a larger water O−H band at 3565 cm^{-1} in BR (*29*), suggesting the perturbation of Wat46 or the appearance of additional water in the region between Thr46 and Ala49 (in place of Val49).

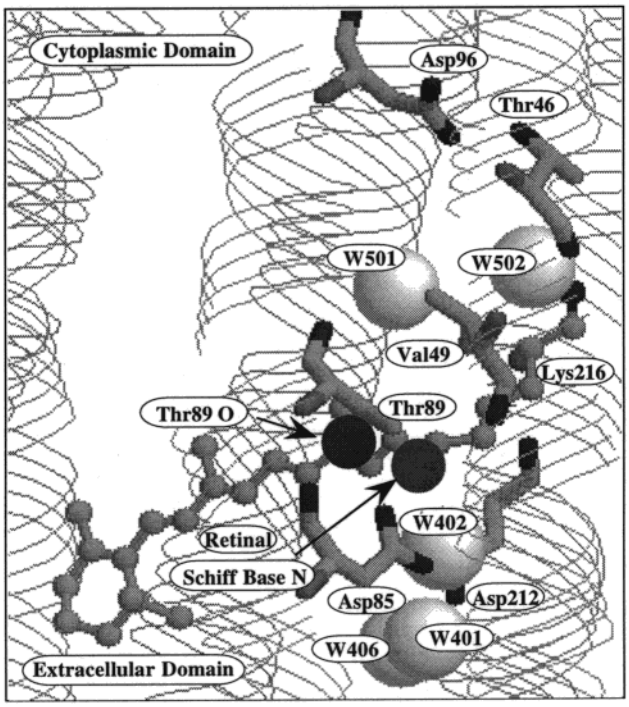

FIGURE 1: Locations of amino acid residues and internal water molecules of bacteriorhodopsin that are discussed in this text. These are based on the X-ray structure of BR at 1.55 Å resolution by Luecke et al. (*14*) (Protein Data Bank entry 1C3W). For emphasis, water molecules are shown by large space filling spheres. The N of the Schiff base and γ-O of Thr89 are also emphasized by larger circles.

The frequency of vibration is affected by its coupling with a second vibration having a similar frequency. The effect depends on the distance and relative orientation of the dipoles (*30*, *31*). In the present paper, the interaction between the Schiff base HOOP and water vibrations was investigated using two approaches, isotope substitution and mutation.

MATERIALS AND METHODS

All-trans retinal chromophores, with the C_{14} or C_{15} hydrogen atom replaced with deuterium, were synthesized according to the literature (*32*).

Bacteriorhodopsin and its mutant pigments in purple membranes were prepared by standard methods (*33*). The spectroscopic properties of the mutant pigments of T46V, T46V/D96N, V49A, T89A (donated by J. K. Lanyi and L. S. Brown), and D85N (donated by R. Needleman and E. P. Lukashev) were described previously (*23*, *27*−*29*, *34*, *35*). Bacteriorhodopsin, in which all of the ϵ-positions of the lysine residues were labeled with ^{15}N, was prepared as described previously (*36*). This sample will be denoted as ^{15}N bacteriorhodopsin in this text. Comparison of the C=N stretching bands in the difference FTIR spectrum with that of native bacteriorhodopsin shows that the Schiff base nitrogen is almost completely labeled.

Bacterio-opsin was prepared by illuminating native bacteriorhodopsin (donated by M. Lu) in 1.75 M hydroxylamine (pH 6.5) at 0 °C for 6 h with yellow light from a 500 W slide projector. After being washed with water three times by centrifugation at 40 000 rpm in a Beckman L7 Ultracentrifuge with a Titan O60 rotor, the remaining retinal oxime was removed by suspending the membrane in a pH

6.9 phosphate buffer containing 2% fatty acid-free bovine serum albumin (Sigma). Centrifugation and resuspension were repeated four times in the same buffer, and then again in water to wash out free and complexed albumin. Bacterioopsin thus obtained was suspended in 0.2 mM phosphate buffer (pH 7.0) and all-trans retinal (unlabeled, C_{14}-deuterated, or C_{15}-deuterated) in ethanol was added in stoichiometric amounts. The absorbance changes were measured in a Shimadzu spectrophotometer UV2101PC. The final amount of the added ethanol was less than 2% in volume. The pigments thus reconstituted are denoted as unlabeled bacteriorhodopsin, C_{14}-D bacteriorhodopsin, and C_{15}-D bacteriorhodopsin, respectively. These samples exhibited the same visible spectra as native bacteriorhodopsin. Unlabeled bacteriorhodopsin showed the same L minus BR FTIR spectrum as native bacteriorhodopsin. Also, C_{15}-D bacteriorhodopsin exhibited the same spectral shape as that described previously (*18*). Both C_{14}-D bacteriorhodopsin and C_{15}-D bacteriorhodopsin exhibited differences in the chromophore bands but had the same C═O stretching vibration frequencies for Asp96 and Asp115 as native bacteriorhodopsin.

Before the films were made, the samples were washed three times in water by centrifugation in an Eppendorf centrifuge 5415C. Air-dried films on the BaF_2 windows were hydrated by placing 0.2 μL of either H_2O or D_2O at the edge of the film. Bacteriorhodopsin hydrated with D_2O contains more than 90% D_2O, as judged from the water O−H band in the difference spectra.

The procedures for installing the hydrated film into the cryostat and the FTIR measurements were described previously (*23*). The hydrated film in the cryostat was light-adapted by illuminating with yellow light (Corning 3-72 filter, >450 nm) at 273 K and then cooling to 170 K. The L minus BR spectra were obtained as the difference between the averages of the eight spectra, each of which contained the sum of 256 scans taken before and after illumination of the initial BR with red light (2−62 filter, >590 nm) for 2 min at 170 K. The same procedure was repeated after the sample was warmed to 273 K to allow all of the intermediates to relax into the initial BR state and recooled to 170 K. At least three cycles were repeated, and the spectra were averaged. The M minus BR spectra were obtained at 230 K by illuminating with orange light (3−69 filter, >520 nm) for 1 min. Four repeated measurements were done after warming to 273 K. A small contamination by M which was produced along with L was subtracted using the M minus BR spectrum scaled to the amplitude of the small band at 1761 cm^{-1} which is due to protonated Asp85 in M.

RESULTS

Effects of Isotope Substitution. Figure 2a−d shows the effects of the isotope labeling around the Schiff base on the L minus BR spectra in the 1100−850 cm^{-1} region. These spectra (solid lines) were superimposed on the L minus BR spectrum of native bacteriorhodopsin (dotted lines). A wide negative band with the center at 911 cm^{-1} for BR was depleted in bacteriorhodopsin in D_2O (Figure 2a) and in C_{15}-D bacteriorhodopsin (Figure 2c) and showed a slight shift in ^{15}N bacteriorhodopsin (Figure 2d) but was preserved in C_{14}-D bacteriorhodopsin (Figure 2b). On the positive side for L, a set of three bands at 1073, 1064, and 1056 cm^{-1}

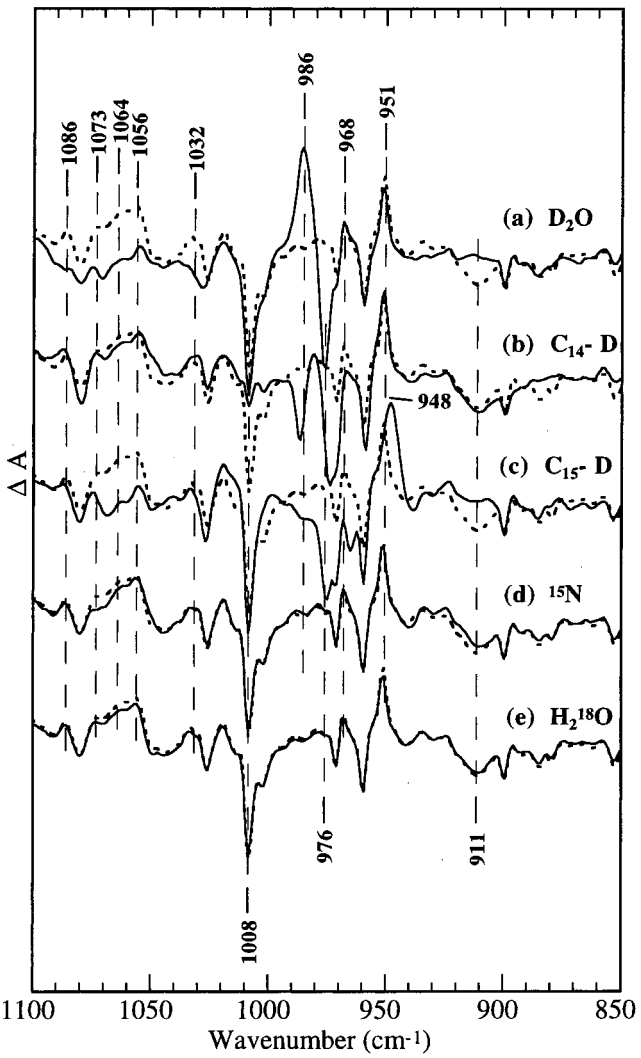

FIGURE 2: L minus BR spectra of isotope-labeled bacteriorhodopsins in the 1100−850 cm^{-1} region: (a) bacteriorhodopsin in D_2O, (b) C_{14}-D bacteriorhodopsin, (c) C_{15}-D bacteriorhodopsin, (d) ^{15}N bacteriorhodopsin, and (e) bacteriorhodopsin in $H_2{}^{18}O$. The spectrum of native bacteriorhodopsin in H_2O (dotted lines) is superimposed on each. The amplitude of the spectrum of bacteriorhodopsin in D_2O (a) was adjusted to that of native bacteriorhodopsin by normalizing to the chromophore bands at 1201 and 959 cm^{-1}. The amplitudes of the spectra of C_{14}-D bacteriorhodopsin (b) and C_{15}-D bacteriorhodopsin (c) were adjusted by the 1740 cm^{-1} band due to C═O stretching vibrations of Asp96 and Asp115 of BR. For ^{15}N bacteriorhodopsin (d) and bacteriorhodopsin in $H_2{}^{18}O$ (e), all of the bands, except for the HOOP bands of the Schiff base, coincided completely. The full length of the ordinate corresponds to 0.076 absorbance units for native bacteriorhodopsin.

along with separate bands at 1086 and 1032 cm^{-1} disappeared in D_2O (Figure 2a). Among these, the two bands at 1073 and 1064 cm^{-1} had lower intensity with the heavier isotope in ^{15}N bacteriorhodopsin (Figure 2d). The 1056 cm^{-1} band underwent a lower frequency shift in ^{15}N bacteriorhodopsin. These bands disappeared in C_{15}-D bacteriorhodopsin (Figure 2c). The two bands at 1064 and 1056 cm^{-1} underwent slight frequency shifts in C_{14}-D bacteriorhodopsin (Figure 2b). These bands of L exhibit similar responses to isotope substitutions as the broad band of BR at 911 cm^{-1}. From the frequencies and the responses to isotope substitutions, the three bands of L at 1073, 1064, and 1056 cm^{-1} and the 911 cm^{-1} band of BR can be assigned to a coupled mode of the N−HOOP and the C_{15}−HOOP of the retinal

Schiff base. The C_{14}−HOOP further couples with them for at least the two bands at 1064 and 1056 cm^{-1}. These bands were neither detected in the resonance Raman spectra of L and BR (*20*, *37*, *38*) nor identified in previous difference FTIR spectra.

The 986 cm^{-1} band of L and the 976 cm^{-1} band of BR, both of which appeared in the spectrum of bacteriorhodopsin in D_2O (Figure 2a), disappeared in the spectrum for the C_{15}-D bacteriorhodopsin in D_2O (*18*). Hence, these bands were assigned to the C_{15}−HOOP (*25*). The uncoupled 986 cm^{-1} band might arise from the 1073, 1064, and 1056 cm^{-1} bands by eliminating the contribution of N−HOOP. These uncoupled C_{15}−HOOP bands of L and BR have been detected in the resonance Raman spectra (*20*, *37*). The 948 cm^{-1} band in the spectrum of C_{15}-D bacteriorhodopsin (Figure 2c), which partially overlaps the band at 951 cm^{-1}, is not due to the N−HOOP because it was not affected in D_2O (*18*). It is probably due to a C_{15}-D in-plane bending vibration in L. Thus, the uncoupled N−HOOP has a quite low intensity and can be detected only if it couples with the C_{15}−HOOP. It was not detected in the resonance Raman spectrum of C_{15}-D bacteriorhodopsin (*20*) in the >800 cm^{-1} region where this mode is expected to be located. An apparent depletion of the positive band at 968 cm^{-1} in C_{14}-D bacteriorhodopsin (Figure 2b) and in C_{15}-D bacteriorhodopsin (Figure 2c) could be due to the appearance of the negative bands of C_{14}-D and C_{15}-D in-plane bending vibrations at similar frequencies (*20*). The negative band at 1008 cm^{-1} that is depleted in C_{14}-D bacteriorhodopsin (Figure 2b) was assigned to the in-plane bending vibrations of the 9- and 13-methyl groups of the chromophore. They are further coupled with C_{10}−H and C_{14}−H in-plane bending vibrations, respectively (*39*).

The set of HOOP bands with a similar mode most likely acquires intensity from Fermi resonance because of the coupling of a fundamental C_{15},N−HOOP mode of the Schiff base with double-frequency modes (overtones) of the HOOPs of interacting O−H groups (*40*). These bands are expected to be located in the 300−700 cm^{-1} region, which is out of range of our FTIR system. The O−H of Thr89, which is located close to the Schiff base (*13*, *15*, *41*), is a candidate for this interaction. However, Figure 2e shows that ^{18}O-substitution of water decreases the intensity of the 1073, 1064 and 1056 cm^{-1} HOOP bands. The lower intensity of these HOOP bands could result because the ^{18}O−H vibrations of expected partners of the Fermi resonance have lower intensities and more separated frequencies than the ^{16}O−H counterparts. A lower intensity of a vibration band by heavier isotope substitution was observed for the Wat85 band of BR (*26*). In this study, the HOOP bands of L appeared with lower intensity in ^{15}N bacteriorhodopsin (Figure 2d). These results suggest that the C_{15},N−HOOP bands of L arise from the interaction of the Schiff base with a water molecule. The negative HOOP band of BR at 911 cm^{-1} did not show such an effect. The effect in L is consistent with previous results suggesting that water molecules in the cytoplasmic domain relocate closer to the Schiff base in L (*23*). The absence of the effect in BR is consistent with the very weak H bonding of the Schiff base in BR (*42*).

Effects of Mutations. As described in the introduction, we have identified several mutant pigments that specifically affect the water O−H bands in the L minus BR spectra and the corresponding water molecules can be tentatively identified in the X-ray crystallographic structures of BR (*14*, *15*, *17*). Figure 3 shows the L minus BR spectra of these mutants in the 1800−800 cm^{-1} region. These spectra exhibit bands at 1400, 1300, and 1191 cm^{-1} typical for the chromophore of the L intermediate (*24*). The 1527, 1201, and 1168 cm^{-1} bands of BR are nearly preserved in these mutants except for D85N (see below). The C═O stretching vibration frequency of Asp96 in L is at 1756 cm^{-1} for T46V (Figure 3b) and at 1728 cm^{-1} for V49A (Figure 3d) (*28*, *29*).

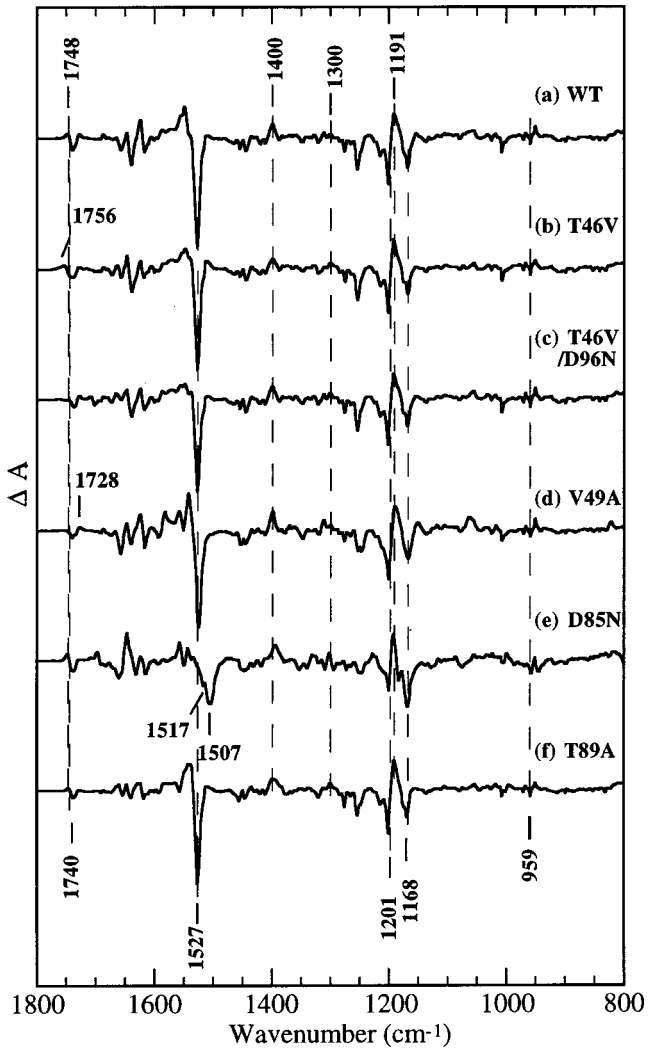

FIGURE 3: L minus BR spectra in the 1800−800 cm^{-1} region of the mutants that are used in the present study: (a) the wild type, (b) T46V, (c) T46V/D96N, (d) V49A, (e) D85N, and (f) T89A. All of the spectra were normalized by the 1201 and 959 cm^{-1} bands of BR. The full length of the ordinate corresponds to 0.326 absorbance unit for the wild type.

We investigated the effects of these mutations on the HOOP bands (see the expanded spectra in the 1100−850 cm^{-1} region of the L minus BR spectra in Figure 4). The corresponding spectra for the films hydrated with D_2O (dotted lines) are shown to help identify the C_{15},N−HOOP coupled bands which only appear in H_2O. The spectra of the wild type are repeated in Figure 4a for comparison with the mutants. The T46V mutation depleted the 1064 cm^{-1} band and decreased the intensities of the 1073 and 1056 cm^{-1} bands of L (Figure 4b). The additional mutation of D96N in T46V/D96N partially restored the intensity of the band at 1064 cm^{-1} and increased the intensities of the band at 1056

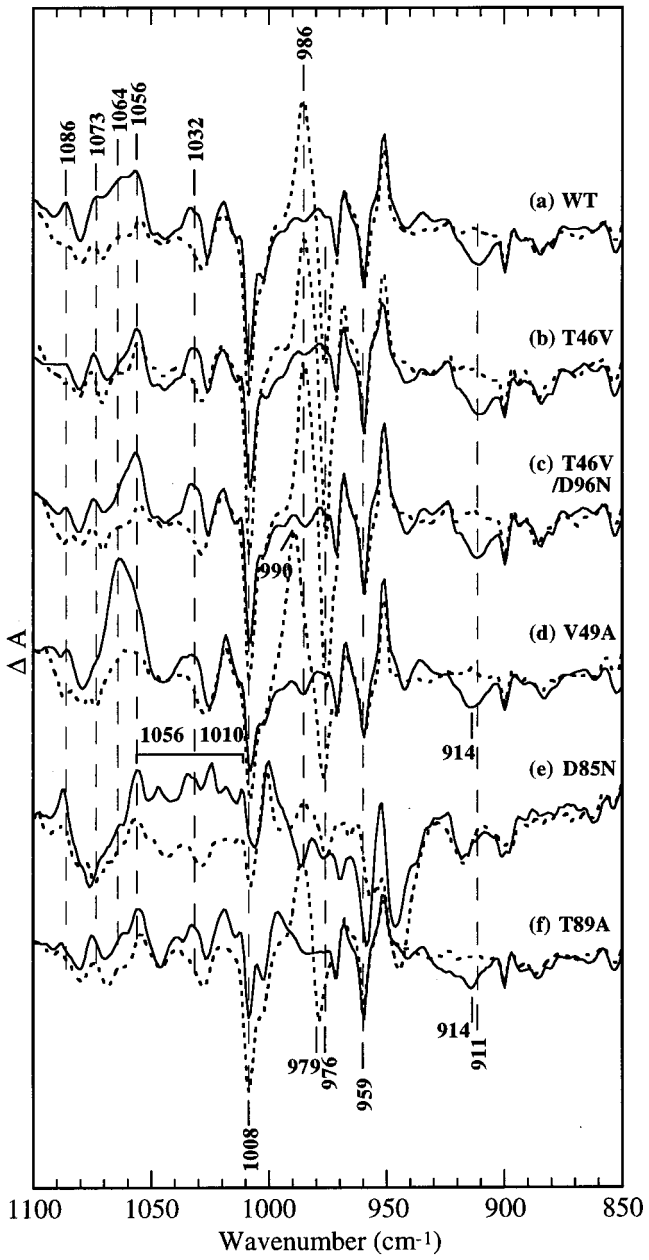

FIGURE 4: Effects of mutations on the HOOP bands in the L minus BR spectra: (a) the wild type, (b) T46V, (c) T46V/D96N, (d) V49A, (e) D85N, and (f) T89A. The corresponding spectra in D_2O are superposed by dotted lines. All of the spectra in H_2O were depicted by expanding the spectra in the 1100−850 cm^{-1} region from Figure 3. The spectra of scan a were duplicated from Figure 2a. All of the spectra were normalized by the 1201 and 959 cm^{-1} bands of L. The full length of the ordinate corresponds to 0.098 absorbance unit for the wild type.

cm^{-1} (Figure 4c). An intense 1064 cm^{-1} band emerged in V49A (Figure 4d). These changes of the C_{15},N−HOOP bands might be related to the disappearance of water bands because of Wat46 in T46V, its partial restoration in T46V/D96N, and a large increase in V49A (*28*, *29*; see, also, the introduction).

The effects of the mutants are not due to the different structures of the chromophore because the fingerprint bands of the chromophore of these mutant pigments are same as those of the wild type (Figure 3). More importantly, the C_{15}−HOOP band at 986 cm^{-1} in D_2O (where it is uncoupled from the N−HOOP) is not affected by T46V (Figure 3b) and T46V/D96N (Figure 3c). A small frequency shift toward 990 cm^{-1} in V49A (Figure 3d) may be related to the proximity of the Val49 side chain to the side chain of Lys216 and the perturbation of Wat46 or the appearance of an additional water upon replacement by a smaller side chain in V49A (*29*). This change is different from the intensity increase due to the coupled HOOP bands of L in H_2O. The results in D_2O suggest that the T46V mutation does not affect C_{15}−H of the chromophore, and intensity changes due to the interacting water are manifest only for the C_{15}−HOOP coupled with N−HOOP in H_2O. In other words, the N−HOOP bands appear because of coupling not only with C_{15}−HOOP but also with the overtones of the O−H vibrations of water. On the other hand, the 911 cm^{-1} band of the coupled C_{15},N−HOOP in BR was not affected at all by these mutations (Figure 3a−c) except for a slight shift toward 914 cm^{-1} in V49A (Figure 3d) and T89A (Figure 3f). This may reflect the configuration of the chromophore in BR or the H-bonding interaction of the Schiff base with water in initial BR.

Asp85 is close to the Schiff base and is the acceptor for the Schiff base proton in the L-to-M transition. In BR, Asp85 interacts directly with the Schiff base via electrostatic attraction and indirectly via an H-bonding chain through W402 (*14*, *16*, *17*; see Figure 1). In D85N, this interaction is perturbed, redshifting the visible spectrum (*43*) and distorting the chromophore (*44*) similar to that seen in BR at low pH (*42*). The photoreaction for the formation of L is 4-fold less extensive than in the wild type, rendering the L minus BR spectrum noisier. This redshifted pigment gave two C═C stretching vibrations for BR at 1517 and 1507 cm^{-1} (Figure 3e) because of two species. Each produced an L intermediate, which was similar to the L of the wild type (*45*) with respect to a characteristic chromophore band at 1191 cm^{-1} (*24*), the indole N−H stretching vibration band at 3486 cm^{-1} (*46*), and the C═O stretching vibration band of Asp96 at 1748 cm^{-1} (*47*, *48*) (see, also, Figure 3e). On the other hand, the L minus BR spectrum of D85N is devoid of the 3642 cm^{-1} band because of water O−H stretching vibration (*27*). Furthermore, the structure of the chromophore around the Schiff base is different from the wild type, as shown by the much smaller intensities of the uncoupled C_{15}−HOOP bands in D_2O at 986 cm^{-1} for L and 976 cm^{-1} for BR and by the in-plane bending vibration of the retinal methyl groups at 1008 cm^{-1} (Figure 4e). In the present study, the L intermediate of D85N exhibited widely distributed subbands in the 1056−1010 cm^{-1} region (Figure 4e) in place of the three HOOP bands of L seen for the wild type (Figure 4a). Thus, the D85N mutation preserves the coupled HOOP bands of L with some perturbations. On the other hand, the D85N mutation depletes the 911 cm^{-1} band of BR. Because this band was not affected by $H_2{}^{18}O$ (Figure 2e) and appeared as a broad structureless band, rather than as a set of bands that is typical for Fermi resonance, its depletion probably reflects the absence of the negative charge of Asp85.

Among the mutants used, T89A (Figure 4f) is the only mutant in which the 1086 cm^{-1} band of L is depleted. This suggests that the band is due to the O−H in-plane bending vibration of Thr89 and that its perturbation is consistent with the earlier result that the O−H stretching vibration of Thr89 is perturbed in L (*49*). The T89A mutation retains the HOOP bands at 1073, 1064, and 1056 cm^{-1} in the original band shape of the wild type (Figure 4a) but with some decreases

in intensity. In this mutant, the water O−H stretching bands of L at around 3490 cm^{-1} were also largely retained, except for a shift in frequency to around 3550 cm^{-1} (not shown in the figure). Thus, the O−H group of Thr89 affects the Schiff base only slightly and indirectly. This is consistent with previous evidence showing the absence of a strong interaction between Thr89 and the Schiff base in L (*41*). The 1086 cm^{-1} band is not affected by the other mutants used in the present study (Figure 4a−e). Thus, the effect of T46V on the HOOP bands is not due to perturbation of Thr89. T89A shifts the 911 cm^{-1} HOOP band of BR toward 914 cm^{-1} and lowers its intensity. This might be due to perturbation of Asp85, which H bonds with Thr89 in BR (*13*−*17*).

DISCUSSION

Identification of the HOOP Bands of L Caused by Coupling of C_{15},N−HOOP Vibrations with Interacting Water O−H Groups. The present results suggest that the coupled C_{15},N−HOOP bands of the Schiff base for L at 1073, 1064, and 1056 cm^{-1} arise from the interaction of the Schiff base with water molecules. (1) The response of these HOOP bands to isotopic substitutions (Figure 2a−d) are typical for Fermi resonance bands. This resonance may be derived from the coupling of the HOOP vibrations of the Schiff base with the frequency-doubled HOOP bands (overtones) of interacting O−H groups (*40*). (2) These HOOP bands for L are affected by $H_2{}^{18}O$ substitution, indicating an interaction between the dipoles of water O−H and the Schiff base (Figure 2e).

The results further suggest that the water molecules involved originate from the cytoplasmic domain or are stabilized by water originating in the cytoplasmic domain. (1) The mutations of the residues in the cytoplasmic domain (T46V, T46V/D96N, and V49A) affect these HOOP bands (Figure 4a−d) and the water molecules in the cytoplasmic domain (Wat46) in parallel, implying interaction of the Schiff base with Wat46. (2) The effects of these on the HOOP bands also parallel the effect on the L-to-M equilibrium. The L-to-M equilibrium is shifted in favor of M in T46V, and it is reversed in T46V/D96N (*28*). The equilibrium is shifted toward L in V49A (*34*). These shifts can be explained by the interaction of water with the Schiff base which contributes to the stabilization of the protonated state. It is an open question, however, whether this water directly participates in the proton transfer from the Schiff base to Asp85.

One could argue that Wat85 interacts differently with the Schiff base in L and undergoes the perturbation by T46V. However, this possibility is less likely. The D85N mutation, which depleted both the HOOP band and the Wat85 band of BR (*27*), preserved the HOOP bands of L with lower frequency shift. This is in contrast to the T46V mutation, which depleted at least one of the HOOP bands as well as Wat46 bands (*28*).

Mode of Interaction of the Schiff Base with a Water Molecule in L. The HOOP bands of L appeared as three similar bands affected by $H_2{}^{18}O$. The corresponding vibration of BR was a single broad band at 911 cm^{-1} insensitive to $H_2{}^{18}O$. These results imply a different interaction of water in L from that in BR, in which the H bonding of the Schiff base with the neighboring water oxygen is very weak (*11*).

Theoretical calculations have shown that either the N−H or C_{15}−H could be an H-bonding donor to water (*50*). H bonding of C_{15}−H in L is suggested by the effect of V49A on the uncoupled C_{15}−HOOP in D_2O (Figure 4). However, the uncoupled C_{15}−HOOP in D_2O was not affected by T46V. Therefore, the effect of Thr46 on the coupled C_{15}−HOOP in H_2O must be through some other mode of interaction, possibly the H bonding of water with the N−H group. The reason the uncoupled C_{15}−HOOP exhibits different responses to water must be pursued in future studies.

Possibility of Two Different Structures around the Schiff Base in L. In L, we find that the interaction of the Schiff base with a water molecule results in the multiple bands at higher frequencies for the coupled mode of the C_{15}−HOOP and the N−HOOP. On the other hand, a time-resolved FTIR study of BR in H_2O (*51*) showed a narrow C_{15}−HOOP band at 983 cm^{-1} in L at room temperature. Thus, the structure around the Schiff base in L at room temperature may be different from that at low temperature. The 983 cm^{-1} band of L is similar to the sharp band at 984 cm^{-1} in KL or late K (*52*−*54*). The two structures of L may arise from different couplings between the N−HOOP and C_{15}−HOOP due to different structures of the chromophore around the Schiff base or to different interactions of the protonated Schiff base with water molecule(s). An example of the presence of two sites of water has been found in another protein. At the active site of the $p21^{ras}$ protein, a water molecule required for the hydrolysis of GTP is present in either of two proximal locations at room temperature, but at 100 K, the two sites merge into a single site (*55*). A similar change might occur in bacteriorhodopsin by the dynamic transition from harmonic thermal motion about an equilibrium position at low temperature to anharmonic vibrations jumping between positions above a critical temperature at ∼200 K (*56*). A structure that allows the presence of two alternative sites for the water molecule might be present around the Schiff base in L. These ideas must be explored in future studies.

In conclusion, by using isotope-labeled bacteriorhodopsin, a set of bands at 1073, 1064, and 1056 cm^{-1} was identified as arising from water coupling with C_{15},N−HOOP vibrations of the L chromophore. These bands are sensitive to mutations of Thr46, Asp96, Val49, Thr89, and Asp85. The effects for the three residues far from the Schiff base (Thr46, Asp96, and Val49) parallel their effects on water O−H bands and the L-to-M equilibrium. This reflects stronger H bonding of the Schiff base and possible relocation of water in the cytoplasmic domain in the BR-to-L transition.

ACKNOWLEDGMENT

We thank J. K. Lanyi, L. S. Brown, R. Needleman, and E. P. Lukashev for their gift of the mutant proteins and M. Lu for his gift of native bacteriorhodopsin.

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BI011923P