Water Structural Changes at the Proton Uptake Site (the Thr46-Asp96 Domain) in the L Intermediate of Bacteriorhodopsin†

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ABSTRACT: Fourier transform infrared spectra of the L intermediate of light-adapted bacteriorhodopsin were examined for recombinant proteins with amino acid substitutions at Thr46 and Asp96. Two O−H stretching vibrational bands of water, at 3607 and 3577 cm^{-1}, change into stronger H-bonding states in L of the wild type. Thr46→Val substitution abolished these bands in spite of the fact that [3-^{18}O]threonine-labeling did not shift them, indicating that they correspond to coordination of the water with Thr46. The two water bands were restored, although with changed frequencies, by an additional Asp96→Asn substitution in Thr46→Val/Asp96→Asn. A single Asp96→Asn substitution abolished the 3607 cm^{-1} band. Thus, Asp96 also takes part in structural changes in water. The perturbations of these water molecules in the L intermediate displayed a weak correlation with the ratio of intensity change in the two vibrational bands of the Schiff base mode at 1312 and 1301 cm^{-1} and the rate for the deprotonation of the Schiff base at the L-to-M reaction of the photocycle. We find, therefore, that the water molecules in the cytoplasmic Asp96-Thr46 domain, which comprises the site of proton uptake after formation of the M intermediate, undergo structural changes in the L intermediate already. These changes are transmitted to the extracellular domain and affect interaction of the Schiff base with Asp85, that is far removed from this region.

Bacteriorhodopsin is the protein in the purple membrane of *Halobacterium salinarium* that transports a proton across the membrane upon absorption of a photon. The chromophore is retinal bound to the ϵ-amino group of Lys216 via a protonated Schiff base. In the absence of light, the retinal is in an equilibrium mixture of all-trans, 15-anti and 13-cis, 15-syn forms. Proton transport is mediated by the photocycle triggered by light-induced isomerization of the all-trans species to the 13-cis, 15-anti form. Several states called J, K, L, M, and N appear successively, and then the chromophore returns to the all-trans state [see reviews of Mathies et al. (1991) and Lanyi (1993)].

Among these states, the L intermediate is the first one in which the Schiff base N−H forms strong interaction with a system consisting of Asp85, Asp212, and water (Maeda et al., 1991, 1992a, 1994; Kandori et al., 1995). This interaction is lost in the M intermediate with the unprotonated Schiff base that follows. The chromophore of the subsequent N intermediate with a reprotonated Schiff base is in a more relaxed conformation than in L (Pfefferlé et al., 1991). Changed amide bands in N indicate a changed protein structure, but these changes will occur even without reprotonation of the Schiff base (Sasaki et al., 1992). Thus, at least some transmission of conformational energy from the chromophore to the protein will have taken place in the L-to-M conversion already.

It is well-known that L formation is accompanied by H-bonding changes in Asp96 (Braiman et al., 1988; Maeda et al., 1992b), a residue separated very considerably from the region where the Schiff base interacts with Asp85 in L (Henderson et al., 1990). Recent investigations have shown, however, that the C=O of Asp96 increases its H-bonding strength between L and M (Sasaki et al., 1994). Previously, interaction of Asp96 with Thr46 was suggested from the perturbation of the Fourier transform infrared (FTIR)[1] spectrum in L and M of T46V and T46D (Rothschild et al., 1992, 1993). Acceleration of M decay and deceleration of N decay in T46V (Marti et al., 1991; Brown et al., 1993) result from the effect of Thr46 on the deprotonation and reprotonation of Asp96. Thr46 is thus supposed to be located close to Asp96, and Asp96 is connected to the Schiff base via several intervening water molecules (Cao et al., 1991).

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

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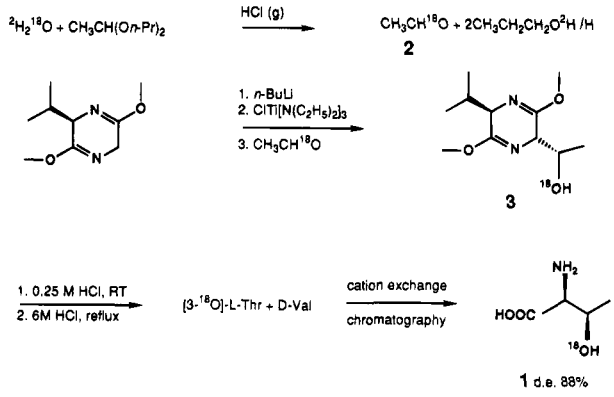
Scheme 1



These residues form a H-bonding cluster and constitute the proton uptake machinery on the cytoplasmic side (Brown et al., 1994b).

Our FTIR analysis of the L intermediate revealed several O−H stretching vibrational bands in the 3750−3450 cm^{-1} region (Maeda et al., 1992a,b; Kandori et al., 1995). Among them, the prominent negative band at 3642 cm^{-1} was assigned to the O−H stretching vibration of a water molecule coordinated with Asp85 (Maeda et al., 1994; Kandori et al., 1995), and small contributions of O−H stretching vibrations were assigned tentatively to Asp96 (Maeda et al., 1992b). However, almost all the complex bands in this region remained unidentified. In the present FTIR studies, the other O−H stretching vibrational bands in the 3750−3450 cm^{-1} region are assigned to the water molecules in the Thr46-Asp96 domain. We suggest that the L intermediate forms an interaction between the protonated Schiff base and the Thr46-Asp96 domain with intervention of water molecules.

MATERIALS AND METHODS

Synthetic Methods. The synthesis of L-[3-^{18}O]threonine (**1**) was performed according to Scheme 1, starting from commercially available (2*R*)-2,5-dihydro-2-isopropyl-3,6-dimethoxypyrazine (Merck) and [^{18}O]acetaldehyde (**2**), which was obtained by acid hydrolysis of 1,1-dipropoxyethane with $^2H_2{}^{18}O$ (98% ^{18}O; ICON, New York). (2*R*)-2,5-Dihydro-2-isopropyl-3,6-dimethoxypyrazine was converted to [5′-^{18}O]-(2*R*,5*S*,5*R*′)-2,5-dihydro-5-(5′-hydroxy)ethyl-2-isopropyl-3,6-dimethoxypyrazine (**3**) on treatment with *n*-butyllithium, chlorotris(diethylamino)titanium, and **2** following a modified procedure, which was described by Schöllkopf et al. (1985). The acid-catalyzed hydrolysis of **3** is performed in two steps to prevent epimerization at the carbon bearing the hydroxyl group. Finally, the product mixture is separated by cation-exchange chromatography, and subsequent treatment of the remaining HCl salt of the product with 1,2-epoxybutane affords a mixture of L-*allo*-threonine and L-threonine in a 45% yield based on the starting material $^2H_2{}^{18}O$. The diastereomeric excess was determined by HPLC using precolumn derivatization with *o*-phthalicdicarboxaldehyde (*o*-phthalaldehyde) and *N*-acetyl-L-cysteine, giving a L-Thr:L-*allo*-Thr ratio of 15:1. No D-isomers could be detected. GC-MS analysis of the TBDMS derivative (Mawhinnery et al., 1986) of **1** indicated an isotope enrichment of 93 ± 3%. The loss of label incorporation can be ascribed to the ease by which acetaldehyde exchanges oxygen with water: ^{1}H-NMR (300 MHz, ^2H_2O, pH 5) d (ppm) 4.36 (m, 1 H, $^3J_{HH}$ = 3.9 Hz, $^3J_{HH}$ = 6.6 Hz, H3), 3.90 (d, 1 H, $^3J_{HH}$ = 3.9 Hz, H2), 1.31 (d, 3 H, $^3J_{HH}$ = 6.6 Hz, H4); ^{13}C-NMR (75 MHz, ^2H_2O, pH 5) d (ppm) 172.2 (C1), 65.3 (C2), 59.8 (C3), 18.9 (C4).

Bacteriorhodopsin. The mutated structural genes for T46V, D96N, T46V/D96N, T46V/D115N, and T46V/D96N/D115N were constructed and introduced into *Halobacterium salinarium* as described previously (Needleman et al., 1991). [3-^{18}O]Threonine-labeled bacteriorhodopsin was prepared by growing *H. salinarium* (JW-3) in a defined medium similar to that of Gochnauer and Kushner (1969), except that the D-amino acids and NH_4Cl were omitted and the L-threonine was replaced by 0.25 g/L L-[3-^{18}O]threonine. Under these conditions, lipid extraction and amino acid analysis with radiotracers typically show that approximately two-thirds of the threonine residues are labeled, with no scrambling. The purple membrane was isolated by the method of Oesterhelt and Stoeckenius (1974).

FTIR Spectroscopy. The sample in water was air-dried on a BaF_2 window, humidified by H_2O or $H_2{}^{18}O$ before being mounted into an Oxford cryostat DN1704, and irradiated at 274 K for 2 min with >500 nm light to obtain the light-adapted state of bacteriorhodopsin which will be denoted as BR.[1] It was cooled after 10 min, once the relatively long-lived N of T46V had decayed. The L/BR spectra were measured at 170 K as the differences between spectra with 256 interferograms before and after irradiation with ≥600 nm light for 2 min in a BioRad FTIR FTS60A/896 spectrometer with 2 cm^{-1} resolution. The spectra on the O−H stretching region are scaled by normalizing to the amplitudes of the bands in the 1800−800 cm^{-1} region.

Nanosecond Flash Photolysis. Nanosecond flash photolytic experiments were performed by using the apparatus described previously (Okada et al., 1991; Yamazaki et al., 1995). The sample was excited with a 17 ns (FWHM) pulse from an excimer-pumped dye laser (Lambda Physik EMG101 MSC, FL3002). The wavelength was selected at 540 nm. A xenon continuous lamp (L2274, Hamamatsu) coupled with a shutter system was used as a light source of a probe light, which was focused onto the sample through a blue glass filter (Toshiba, VY-42). The angle between the planes of polarization of the excitation and probe lights was 54.7°. The intensities of the probe light passing through the sample and a monochromator were monitored by a photomultiplier (R666S, Hamamatsu). Its output was recorded in a Stragescope (TS-8123, Iwatsu).

The purple membranes of A_{568nm} = 1.0 in 50 mM phosphate buffer (pH 7) were light-adapted at 20 °C with >500 nm light for 5 min. The sample was left for 30 s before the measurements to ensure the decay of the photointermediates. The laser excitation frequency was 0.1 Hz. It sufficed for the complete decay of the intermediates, even for the mutants. The time courses of absorbance change measured 500 times at 412 nm were averaged. No changes of the absorption spectra were detected after the experiments. The formation and decay of M were depicted by combining two kinetic measurements with 0.98 or 3.9 μs/point. The data were analyzed by a multiexponential fitting for the contribution of the decay components, except for D96N and D96N/T46V with very slow decays in M.

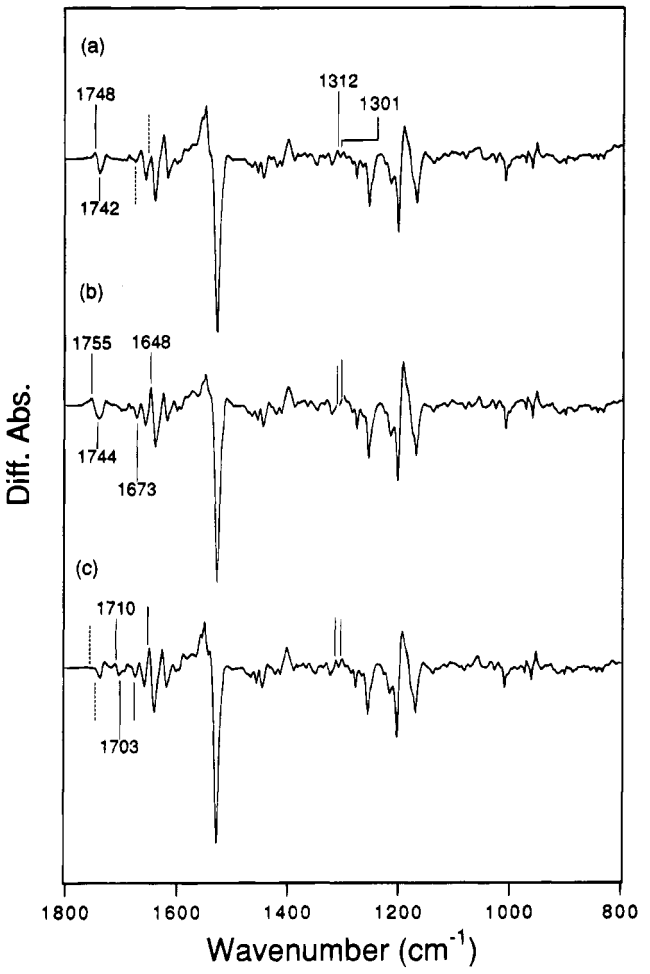


FIGURE 1: L/BR spectra in the 1800−800 cm^{-1} region for T46V (b) and T46V/D96N (c) are compared with that of the wild-type protein (a). The vertical solid lines in (b) and (c) indicate the same wavenumbers as shown in (a) or (b). The vertical dotted lines in (a) and (c) indicate the absence of the corresponding bands in (b). The entire length of the vertical axis is 0.10, 0.22, and 0.10 absorbance units for (a), (b), and (c), respectively.

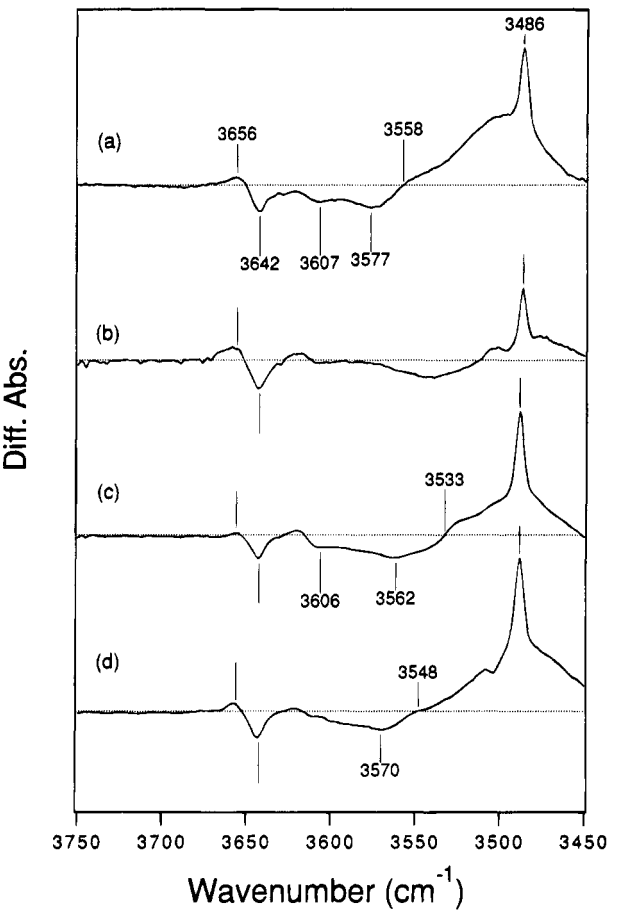


FIGURE 2: L/BR spectra in the 3750−3450 cm^{-1} region for T46V (b), T46V/D96N (c), and D96N (d) are compared with that of the wild-type protein (a). The vertical solid lines in (b), (c), and (d) indicate the same wavenumbers as shown in (a). Horizontal dotted lines represent the zero line of the spectra. The entire length of the vertical axis is 0.014, 0.030, 0.014, and 0.008 absorbance units for (a), (b), (c), and (d), respectively.

RESULTS

Differences in T46V and T46V/D96N. FTIR difference spectra of T46V, and the double mutant, T46V/D96N, upon irradiation at 170 K with >600 nm light were examined. The spectra in the 1800−800 cm^{-1} region (Figure 1b,c) were quite similar to the L/BR difference spectrum of the wild-type protein (Figure 1a) under the same conditions. However, some differences are to be noted. The positive band at 1755 cm^{-1} and the negative shoulder band at 1744 cm^{-1} in T46V (Figure 1b) disappeared in T46V/D96N (Figure 1c). This confirms the previous suggestion (Rothschild et al., 1992, 1993) that the C=O stretching vibrations in T46V and T46D, that are located at higher frequencies than the wild-type protein at 1748 and 1742 cm^{-1}, are due to Asp96. The negative C=O stretching at 1703 cm^{-1} in the unphotolyzed state of T46V/D96N shifted to 1710 cm^{-1} upon L formation (Figure 1c), in contrast to the shift to 1699 cm^{-1} in D96N (Gerwert et al., 1989; Maeda et al., 1992b). The positive band at 1648 cm^{-1} and the negative band at 1673 cm^{-1} in the amide I region in T46V (Figure 1b) and T46V/D96N (Figure 1c) are not present in the wild-type protein (Figure 1a). Of the two coupled bands of the N−H and C_{15}−H in-plane bending vibrations of the Schiff base at 1312 and 1301 cm^{-1} (Maeda et al., 1991), the former is more intense in the wild type (Figure 1a) but the latter in T46V (Figure 1b). In T46V/D96N (Figure 1c), this relation was partly reversed, in spite of the fact that D96N exhibited a similar ratio to the wild type (not shown in figures). These changes were unaffected with an additional substitution for Asp115 to asparagine, in T46V vs T46V/D115N and T46V/D96N vs T46V/D96N/D115N (not shown).

Figure 2 shows the spectra in the 3750−3450 cm^{-1} region. The wild type exhibits an essentially identical shape with that previously reported (Maeda et al., 1992a), but the use of an improved FTIR system with decreased noise level allowed more clear identification of two negative bands at 3607 and 3577 cm^{-1}. These disappeared in T46V (Figure 2b). However, additional substitution of D96N (T46V/D96N, Figure 2c) afforded two negative bands at 3606 and 3562 cm^{-1}, which are similar to the bands of the wild-type protein at 3607 and 3577 cm^{-1} (Figure 2a). On the other hand, a single D96N substitution itself abolished the band at 3607 cm^{-1} and retained the band at 3577 cm^{-1} (Figure 2d).

The abolition and reappearance of these negative bands were accompanied by the disappearance of a considerable part of the broad positive feature in the 3558−3450 cm^{-1} region (Figure 2a) in T46V (Figure 2b), and a restoration of a new feature in the 3533−3450 cm^{-1} region in T46V/D96N (Figure 2c). The D96N mutation itself decreased some of

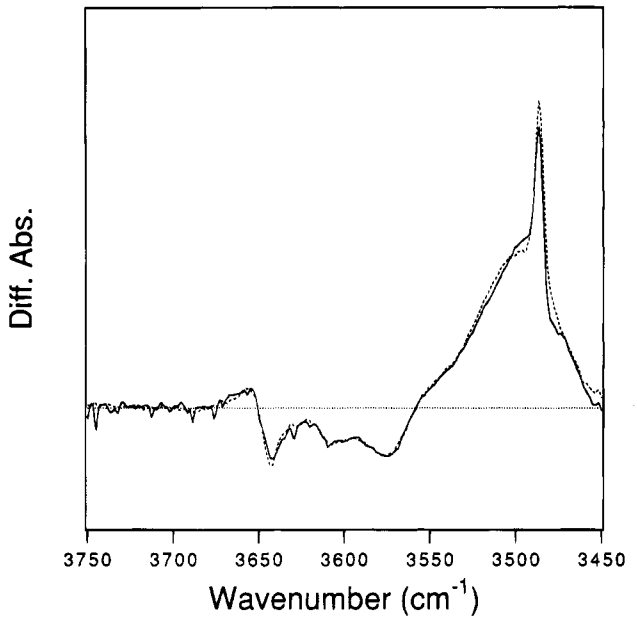


FIGURE 3: L/BR spectrum in the 3750−3450 cm^{-1} region for [3-^{18}O]threonine-labeled BR (solid line) is compared with that of the unlabeled BR (dashed line), the duplicate of Figure 2a. The horizontal dotted line represents the zero line of the spectrum. The entire length of the vertical axis is 0.006 and 0.013 absorbance units for labeled and unlabeled BR, respectively.

the intensity of this positive feature (Figure 2d). A 3656 cm^{-1} positive band is present in both T46V (Figure 2b) and D96N (Figure 2d) but has lost considerable intensity in T46V/D96N (Figure 2c). No striking changes of these features due to an additional Asp115 substitution were observed upon comparing T46V/D115N with T46V and T46V/D96N/D115N with T46V/D96N (not shown in figures).

Effects of ^{18}O Substitution. The L/BR spectra of [3-^{18}O]-threonine-labeled BR were compared with unlabeled BR, in order to distinguish whether the depletion of the O−H stretching bands is ascribable to that of Thr−O−H or others, for example, water. In Figure 3, the L/BR spectrum of [3-^{18}O]threonine-labeled BR (solid line) is overlaid on that of unlabeled BR (dashed line). The spectrum of [3-^{18}O]-threonine-labeled BR completely coincided with that of unlabeled BR. Thus, the L/BR spectrum in this region does not contain any of the O−H stretching mode of threonine, even though the whole O−H stretching vibrational feature is considerably less intense in T46V (Figure 2b).

The effect of $H_2{}^{18}O$ hydration on the L/BR spectrum of the wild type is shown in Figure 4a. The broad positive band in the 3558−3450 cm^{-1} region and the negative bands at 3607 and 3577 cm^{-1} shifted in $H_2{}^{18}O$. These O−H stretching vibrations are now identified as due to water. In comparison with the intensity of the 3642 cm^{-1} band that was assigned to the water coordinated with Asp85 (Maeda et al., 1994), those bands at 3607 and 3577 cm^{-1} are attributed to two O−H's of one or two water molecules. These are susceptible to substitution of Thr46 (see Figure 2b) probably due to the coordination of these water molecules to Thr46. Among these two O−H stretching bands, the one at 3607 cm^{-1} is susceptible to substitution of Asp96 too. Thus, the water molecule with the O−H stretching vibration at 3607 cm^{-1} is associated with both Asp96 and Thr46. The decrease in intensity of the broad feature reported earlier for

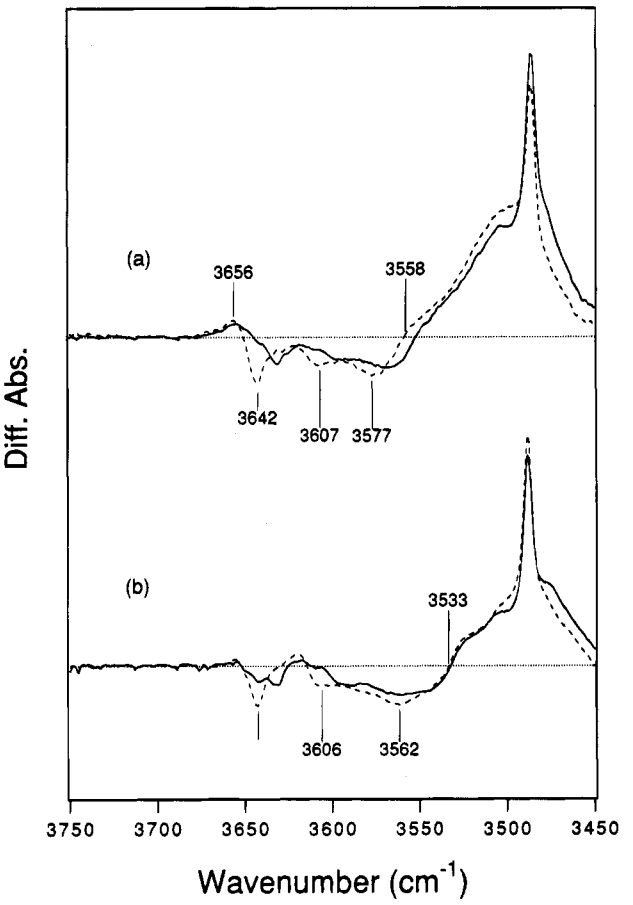


FIGURE 4: L/BR spectra in the 3750−3450 cm^{-1} region for the wild-type protein (a) and T46V/D96N (b) hydrated with [^{18}O]water (solid line) are overlaid on those hydrated with [^{16}O]water (dotted line), respectively. The vertical solid lines in (b) indicate the same wavenumbers as shown in (a). The dotted lines in (a) and (b) are the duplicates of Figures 2a and 2c, respectively. Horizontal dotted lines represent the zero lines. The entire length of the vertical axis is 0.011 and 0.024 absorbance units for (a) and (b), respectively.

D96N (Maeda et al., 1992b) is thus attributed to the depletion of the O−H stretching vibration of water located closely to Asp96 and not to the O−H stretching band of the O−H of the carboxylic acid in Asp96. The observed shift of the positive band at 3656 cm^{-1} (Figure 4a) was too small to regard this band as entirely a water O−H stretching vibration.

In T46V/D96N (Figure 4b), $H_2{}^{18}O$ shifts were observed in the positive broad feature at 3533−3450 cm^{-1} and the two negative bands at 3606 and 3562 cm^{-1}. These bands were thus assigned to the water O−H stretching vibration. These are similar to the water change in the wild-type protein. The negative band at 3642 cm^{-1} is due to water which resembles the corresponding water band in the wild-type protein (Maeda et al., 1992a), but its half is insensitive to $H_2{}^{18}O$. The corresponding shifts of the bending vibrations of water below 1700 cm^{-1} were not detected, probably owing to the fact that small $H_2{}^{18}O$ shifts are obscured by other intense bands.

Effects on the Rate of L-to-M Conversion. In order to assess the effects of the substitutions of Asp96 and Thr46 on the properties of the Schiff base of the L state, the M rise kinetics of T46V, T46V/D96N and D96N (solid lines in panels a, b, and c, respectively, of Figure 5) were compared with those of the wild-type protein (dashed lines). The M rise of T46V was more rapid than the wild-type

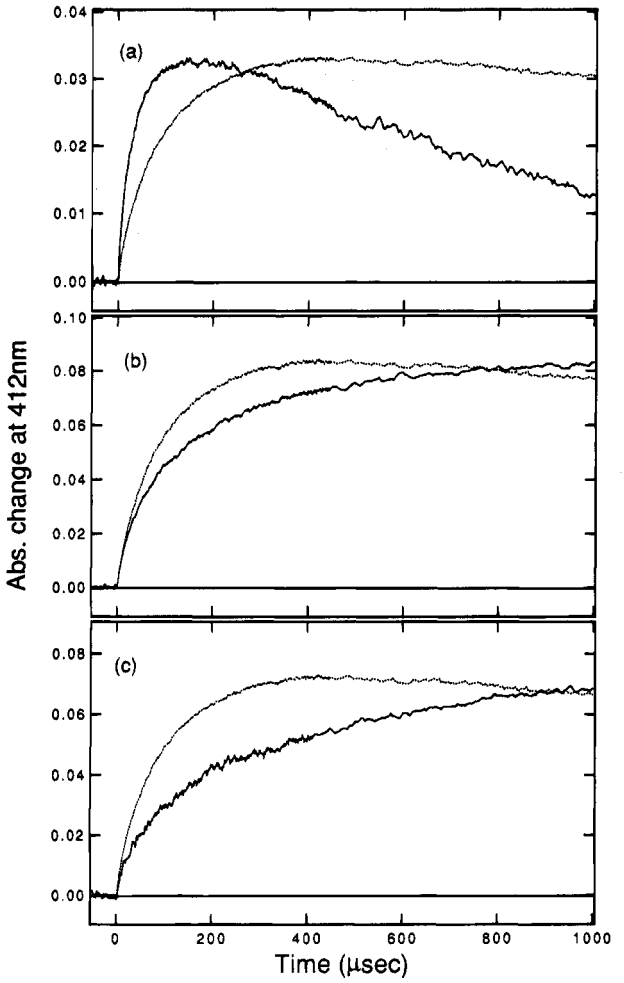


FIGURE 5: Single-wavelength kinetics at 412 nm (solid lines) for T46V (a), D96N (b), and T46V/D96N (c) after flash excitation at 540 nm. Each was compared with that of the wild-type protein (dotted lines) multiplied by adjusting the maximum amplitude to the same. The maximum absorbance attained with the wild type was 0.086. All the rising phases were well fitted by two exponentials. The two rise time constants, τ_1 and τ_2, were 31 μs (24%) and 140 μs (76%) for wild type, 20 μs (47%) and 92 μs (53%) for T46V, 39 μs (33%) and 260 μs (67%) for D96N, and 55 μs (30%) and 450 μs (70%) for T46V/D96N, respectively. The maximum absorbances obtained with two increasing exponentials for M formation were 0.096 for the wild type and 0.045 for T46V.

protein (Figure 5a). The maximum amplitude for T46V was about one-third, and the M decay is very rapid as reported previously (Marti et al., 1991; Brown et al., 1993). M of a single substitution of D96N rose behind the wild type (Figure 5b), and the M rise of T46V/D96N was much slower in T46V/D96N (Figure 5c). Cao et al. described the rising phase of M with three kinetic components. The present time resolution allowed, however, two constants τ_1 and τ_2. These values of 31 and 140 μs of the wild-type protein are consistent with the corresponding values of 35 and 144 μs by Cao et al. (1995), if the fastest phase with a time constant of 1.7 μs was neglected in view of a low amplitude (11%). In the model $L \leftrightarrow M_1 \rightarrow M_2$ (Váró & Lanyi 1991), τ_1 is the equilibration of L and M_1, and τ_2 is related to the unidirectional formation of M_2 (Brown et al., 1994a; Cao et al., 1995). Both values became smaller (20 and 92 μs, respectively) for T46V and larger for D96N (39 and 260 μs, respectively). These values further increased to 55 and 450 μs for T46V/D96N. Thus, the proton transfer from the Schiff base is rendered more rapid in T46V, while the additional mutation of D96N retards it. Both τ_1 and τ_2 values of T46V/D96N are higher than in the single D96N mutant. Thus, the water structural change upon L formation is roughly correlated with the rate of proton transfer from the Schiff base to Asp85, in spite of an observed tendency of D96N to slow M rise. It may be responsible for the increased retardation in T46V/D96N.

DISCUSSION

All the O−H stretching vibration bands in the 3750−3450 cm^{-1} region in the L/BR spectrum are now assigned. In addition to the water signal associated with Asp85, the L/BR spectrum of the wild-type protein exhibits structural changes of water molecules, one coordinated with both Thr46 and Asp96 and the other with Thr46 only. The unresolved positive band in the 3558−3450 cm^{-1} region is also associated with these two residues. These frequency shifts upon L formation are correlated with the strength of H-bonding (Mohr et al., 1965; Maeda et al., 1992a). Thus, the O-H's of these water molecules form relatively weak H-bonding in BR, though stronger than the water coordinated with Asp85 (Maeda et al., 1994), and change into a state with strong H-bonding in L. These water molecules remain in similar places in T46V but do not undergo structural changes upon L formation. The restored water structural changes in T46V/D96N could be due to the same water molecules as in the wild type but present in different sites as suggested by the different shifts, though structural changes of water molecules in another site cannot be completely excluded.

These facts provide some insights to the structure in the Thr46-Asp96 domain. Upon formation of L, the water molecules associated with Thr46 and Asp96 form stronger H-bonding. Its acceptor must be the hydroxyl oxygen of Thr46, because of the observed loss of H-bonding of the water hydrogen in T46V. The most straightforward interpretation of the results of the additional D96N substitution (T46V/D96N) is that the water acquires new binding sites (peptide carbonyl, for example) upon being released from H-bonding with both Asp96 and Thr46. The frequency shift of the C=O stretchng vibration from 1703 cm^{-1} to 1710 cm^{-1} for Asn96 in T46V/D96N is the opposite direction from the frequency shift to 1699 cm^{-1} in D96N (Gerwert et al., 1989; Maeda et al., 1992b). Thus, H-bonding of the C=O of Asn96 in the L intermediate of T46V/D96N becomes weaker than D96N. This suggests that the C=O of Asn96, which may support H-bonding of the water in D96N, does not function as an acceptor in T46V/D96N. The frequency shifts of the amide I bands in T46V and T46V/D96N proteins suggest additional H-bonding formation of the peptide carbonyls in this domain.

The disappearance of stronger H-bonding formation of the water molecules in T46V is accompanied by a shift of the in-plane bending vibration band related to the Schiff base, and the acceleration of the deprotonation of the Schiff base. The reappearance of the water structural change in T46V/D96N is also correlated with the restoration of the Schiff base vibrational bands and the rate of the deprotonation of the Schiff base. Thus, the water molecules in the Thr46-Asp96 domain appear to interact with the Schiff base of the chromophore in L. A similar influence of bound waters was observed for the N state upon addition of osmotically active

solutes or perturbants (Cao et al., 1991) that retarded its formation.

The presence of a water molecule near Thr46 is consistent with a model based on molecular dynamic calculations (Humphrey et al., 1994). However, H-bonding of water with Thr46 must be weaker in the BR state than in L. This model also exhibits a string of H-bonding of water molecules from the carbonyl of Lys216 to the oxygens of Thr46 and Asp96. According to it, structural changes of the water molecules might be brought simply by the isomerization of the chromophore. However, no such structural changes in water were noticed in the K/BR spectrum (Fischer et al., 1994; our unpublished data). The structural changes of the water molecules are only attained in the particular structure in L.

It should be noted that H-bonding changes in these water molecules have been observed in the L/BR spectra of other mutant proteins. The L/BR spectrum of V49M completely extinguished the O−H stretching vibrations of the water in the positive broad band, and to some extent the negative broad band (Brown et al., 1994). Since Val49 is located only one turn away from Thr46, substitution of a longer side chain, or the presence of the lone-pair electron of methionine sulfur, may affect the water system connected to Thr46. The negative water band at slightly lower frequencies than the wild type in the L/BR spectrum of W182F (Yamazaki et al., 1995) is similar to that of T46V/D96N. This suggests the connection of Trp182 with Asp96, as was envisaged from the perturbed Asp96 signal of the L intermediate of W182F. Similar perturbation was also observed for V49M and V49A (our unpublished data).

The present results show that the Thr46-Asp96 domain with the intervening water molecules, which is responsible for the M-to-N conversion leading to proton uptake, already undergoes structural changes in the L intermediate, and they also have an interaction with the Schiff base.

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