Hydrogen Bonds of Water and C═O Groups Coordinate Long-Range Structural Changes in the L Photointermediate of Bacteriorhodopsin†

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ABSTRACT: Fourier transform infrared spectra of light-adapted bacteriorhodopsin exhibit a band at 1618 cm^{-1} that shifts to 1625 cm^{-1} upon formation of the L intermediate. It is assigned to the peptide C═O of Val49 from the fact that it shifts in [1-^{13}C]valine-labeled bacteriorhodopsin and appears perturbed in the Val49→Met mutant. The intensity of the BR→L difference band is reduced in the Thr46→Val mutant but restored by the additional mutation of Asp96→Asn. These intensity changes are closely correlated with the H-bonding change of water molecules, suggesting that the peptide C═O of Val49 is hydrated. This could arise in the Thr46→Val mutant because of perturbation of the C═O of Val46, which points toward Val49. The Val49→Ala mutation influences a peptide N−H, presumably of Val49, and the carboxylic C═O of Asp96, as well as water molecules proximal to Asp85. Conversely, the water molecule assumed to be in the cavity that arises from the missing two methyl groups in V49A could be affected in the mutant of Asp96→Asn. We propose that the perturbation exerted on Asp85 by the Schiff base in the L intermediate is transmitted to Asp96 through H-bonding of water molecules in the Asp85−Val49 region, the C═O of Val49, H-bonding between Val49 and Thr46, and H-bonding between Thr46 and Asp96.

Bacteriorhodopsin is a transmembrane protein of *Halobacterium salinarium* that contains an all-trans retinal chromophore linked to Lys216, forming a protonated Schiff base. Transport of proton is triggered by light dependent 13-trans to cis isomerization of the retinal, followed by partial reactions consisting of proton transfer from the Schiff base to Asp85 in the L-to-M transition, and reprotonation of the Schiff base by the proton of Asp96 in the subsequent M-to-N reaction [reviewed by Mathies et al. (1991) and Lanyi (1993)]. The former reaction is induced by strong interaction of the protonated Schiff base and Asp85 with intervening water molecules in the L intermediate (Maeda, 1996).

By applying Fourier transform infrared (FTIR)[1] spectroscopy, we have described several structural motifs in the L intermediate. Strong H-bonding of the Schiff base (Maeda et al., 1991) with intervening water molecules causes distortion of the retinal close to the Schiff base (Maeda et al., 1994). The anionic Asp85 (Maeda et al., 1994) and Asp212 (Kandori et al., 1995) are necessary coordinators for these water molecules. At the same time, Trp182 interacts with the retinal skeleton via the 9-methyl group (Yamazaki et al., 1995a). These changes induce the deprotonation of the Schiff base.

The structural changes in the L intermediate include also water molecuels that are affected by mutations of Thr46 and Asp96, located in the cytoplasmic proton uptake domain (Yamazaki et al., 1995b). Such changes in water molecules have been correlated also with the protonation equilibrium in the L-to-M process in Val49 mutants (Brown et al., 1994a), as well as in Thr46 mutants (Yamazaki et al., 1995b). Thus, structural alterations in both extracellular and cytoplasmic regions are induced as early in the photocycle as the L intermediate. This is compatible with the fact that structure of the N intermediate can be produced even with the unprotonated Schiff base (Sasaki et al., 1992).

Infrared spectroscopy reveals structural changes in the peptide backbone as changes of the amide I and amide II bands [see reviews by Krimm and Bandekar (1988) and Maeda (1996)]. From the functional standpoint, peptide carbonyls are supposed to be sites for interaction with the ions to be translocated and also for hydration in channels (Eisenman & Dani, 1987). A recent X-ray crystallographic study on cytochrome *c* oxidase (Iwata et al., 1995) suggests the participation of peptide carbonyls along the proton channel. Changes in these bands are indeed revealed in bacteriorhodopsin as early as in the K intermediate (Diller et al., 1992) and become prominent in the N and M_N intermediates (Pfefferlé et al., 1991; Sasaki et al., 1992; Rothschild et al., 1993). To understand the role of these

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

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peptide functions it is important to assign these amide I and amide II changes to specific residues. Recent studies by Ludlam et al. (1995) used site-directed isotope labeling to identify one of the peptide C═O with Tyr185. This technique is the ultimate tool for such identification but requires chemical modification of tRNA in the case of amino acids other than tyrosine (Ellman et al., 1992). In some cases, we may obtain clues without such a technically difficult approach. For example, the carbonyl C═O of Lys216 was proposed as a possible candidate for the change of an amide I band at 1620 cm^{-1} upon M formation (Takei et al., 1994).

Previously, we had described new amide I bands that arise in the BR→L spectrum of a mutant with valine substituted for Thr46 (Yamazaki et al., 1995b). In the present study, we attempted to confirm these as changes of the peptide C═O of Val46 by labeling this mutant protein with [1-^{13}C]-valine. A necessary parallel study by labeling the wild type was carried out also, in order to examine the presence of amide I bands of valine residues present in the wild type. We expected a specific role of the peptide C═O of Val49 in the photocycle, because this residue is located close to the site of the interaction between Asp85 and the Schiff base (Brown et al., 1994a). Val49 could facilitate the transmission of structural changes in the L intermediate from the Asp85 region to the Asp96 region. Effects of the mutation of Val49 on water molecules and Asp96 were therefore also examined.

MATERIALS AND METHODS

The double mutants V49A/D96N, V49A/D115N, and V49M/D96N, expressed in *H. salinarium*, were prepared as described previously (Needleman et al., 1991). For isotope labeling, the bacteria were grown in the TS medium of Ohnishi et al. (1965) in which unlabeled D,L-valine was replaced with half of the amount of L-[1-^{13}C]valine purchased from CIL, Cambridge, MA (Tuzi et al., 1993). Purple membranes were isolated by the standard method of Oesterhelt and Stoeckenius (1974).

Difference FTIR spectra upon formation of the L intermediate were recorded as differences before and after the irradiation of light-adapted bacteriorhodopsin (BR)[1] with >600 nm light at 170 K, as described previously (Yamazaki et al., 1995b). All of the spectra were scaled by normalizing to the amplitudes of the negative bands at 1202 cm^{-1}.

RESULTS

Effects of Val49 on Valine-Specific Peptide C═O. Figure 1 shows changes in the amide I and amide II regions of the BR→L spectrum in [1-^{13}C]valine-labeled wild type (b). The spectra below 1540 cm^{-1} were practically identical with each other (not shown). [1-^{13}C]Valine-labeled wild type (b) lost much of the intensities of the positive and negative bands at 1625 and 1618 cm^{-1} of the unlabeled wild type (a) and instead exhibits positive and negative bands at 1587 and 1581 cm^{-1}, respectively. These shifts toward lower frequency, by 38−37 cm^{-1}, are similar to those in previous studies with synthetic peptides (Tadesse et al., 1991), and identify the bands as originating from the peptide C═O of valine(s). Incorporation of isotope into the C═O of valine was more than two-thirds, judging from the ratio of intensities at 1618

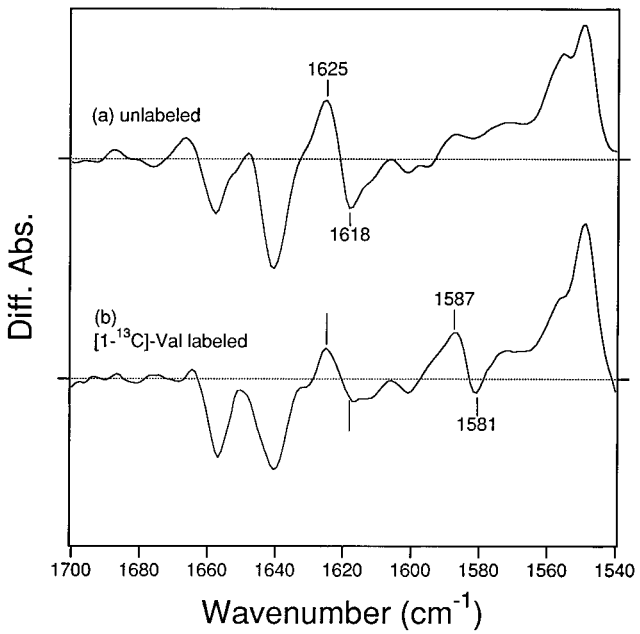

FIGURE 1: BR→L spectra in the 1700−1540 cm^{-1} region of the unlabeled (a) and [1-^{13}C]valine-labeled (b) wild types. One division in the ordinate represents 0.02 absorbance unit for a. The spectrum of the wild type (a) was taken from that described in Yamazaki et al. (1995b).

cm^{-1} (−) of the unlabeled (a) and labeled (b) samples,[2] and evidently sufficient to detect the shift. The smaller changes at >1635 cm^{-1} are not as clear. Scrambling to other amino acids from [U-^{14}C]valine is reported to be less than 2% (Kinsey et al., 1981). NMR spectra of the same sample showed only a small amount of scrambling to the lipids (Tuzi et al., 1993). Thus, the intensity changes for the bands in the frequency region higher than 1635 cm^{-1} could be due to the C═O of other valines or to transition-dipole coupling of the same valine with other residues (Krimm & Abe, 1971). It has been reported (Ludlam et al., 1995) that even the single C═O of Tyr185 is scattered in various bands.

Similar shifts were observed in the BR→K spectrum also, with less intensity changes (not shown), indicating that the C═O changes arise already in the isomerization process of the retinal. No corresponding changes were noted in the BR→M spectrum, however (not shown). The BR→K spectrum of [1-^{13}C]Tyr185-labeled BR exhibited a smaller decrease in intensity of these bands (Sonar et al., 1995).

The L-to-M transition is affected in two mutants of Val49, V49A, and V49M (Brown et al., 1994a). The spectra of these mutant proteins, recorded upon irradiation with >600 nm light at 170 K, along with that of the wild type are shown in Figure 2 for the 1800−800 cm^{-1} region. First of all, the C−C stretching vibrational bands at 1202 (−), 1169 (−), and 1192 (+) cm^{-1}, which are characteristic of the BR→L spectrum of the wild type (a), were all present in V49A (b) and V49M (c). The different shape of the C═C stretching vibrational region of the mutant proteins (b and c) could be ascribed partly to slight differences of the frequencies in the unphotolyzed state, 1525 cm^{-1} for V49A (b) and 1532 cm^{-1} for V49M (c) from 1528 cm^{-1} of the wild type. The negative

[2] The incorporation of [1-^{13}C]valine in T46V almost completely abolished the 1673 cm^{-1} band (see below in Figure 3a,d). This demonstrates that the incorporation of the isotope is nearly complete, as the consequence of the fact that valine is essential for growth (Ohnishi et al., 1965). Thus, the remaining amplitudes of the 1625 and 1618 cm^{-1} bands cannot originate from valine C═O groups.

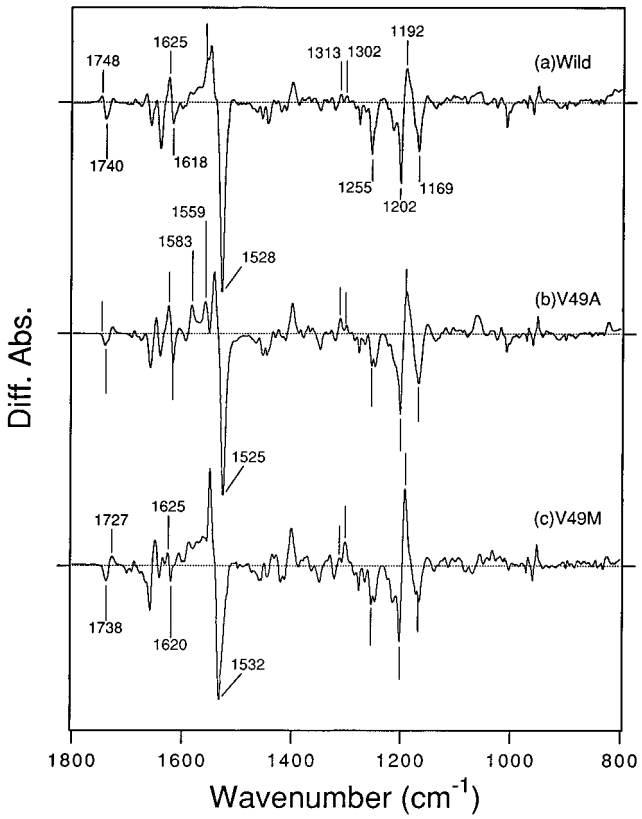

FIGURE 2: BR→L spectra in the 1800−800 cm^{-1} region of the wild type (a), V49A (b), and V49M (c). One division in the ordinate represents 0.05 absorbance unit for (a).

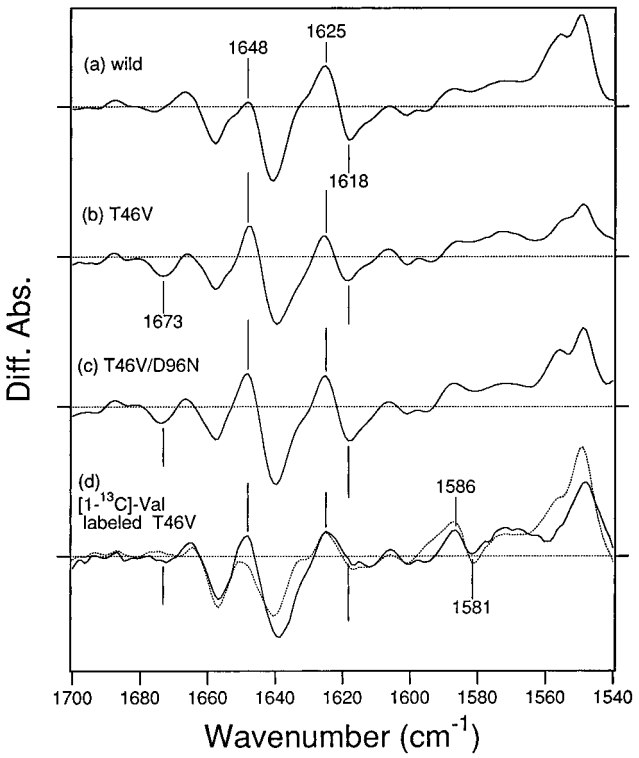

FIGURE 3: BR→L spectra in the 1700−1540 cm^{-1} region of the wild type (a), unlabeld T46V (b), unlabeled T46V/D96N (c), [1-^{13}C]-valine-labeled T46V (solid line in d), and the [1-^{13}C]valine-labeled wild type (dotted line in d). The spectrum of a and that with the dotted line in d are duplicates of Figure 1a and b, respectively, for the sake of comparison. One division in the ordinate represents 0.02 absorbance unit for a.

band at 1254 cm^{-1}, though smaller in intensity in the mutant proteins (b and c), shifted to 1263 cm^{-1} in ^2H_2O (not shown) as the wild type, in which it was assigned to a mode containing the N−H in-plane bending vibration of the Schiff base (Maeda et al., 1991). As shown below (Figure 5) like the wild type, the spectra of mutant proteins contain the characteristic N−H stretching vibrational band around 3486 cm^{-1} for the L intermediate. On the other hand, different from the L intermediate of the wild type (a) are the larger intensities of the vibrational bands at 1313 cm^{-1} for V49A (b) and 1302 cm^{-1} for V49M (c), both of which are presumably due to the C_{15}−H in-plane bending vibration (Pfefferlé et al. 1991). Changes in these bands are most likely associated with the structural perturbations around the Schiff base in the L intermediate of these mutant proteins, as expected from the shift of the L-to-M equilibrium (Brown et al., 1994a).

The bands at 1618 (−) and 1625 (+) cm^{-1} are conserved completely in the spectrum of V49A (b), although there are other alterations in the amide I region. The BR→L spectrum of V49M (c) is, however, devoid of a large part of the positive band at 1625 cm^{-1} and shows a shift of the negative band to 1620 cm^{-1}. These results strongly suggest that both these bands arise from the peptide C═O of Val49. Methionine has a longer chain without a branch, which can fold back to the proximity of the C═O of Met49, and perturbs more at this position than alanine.

Positive bands appeared at 1583 and 1559 cm^{-1} in V49A (b). These are probably due to amide II, mainly the in-plane bending vibration of the peptide N−H. Though the 1559 cm^{-1} band seems to be present also in the wild type, the other band at 1583 cm^{-1} is unique for V49A and absent in V49M. It is possible that a water molecule that is brought into the cavity after the removal of the two methyl groups at the β-position (see below), and affects the proximal peptide N−H of Val49.

Effects of Thr46 Mutation on the Valine Specific Peptide C═O. Intensity changes in those amide I bands in the BR→L spectra of the mutants of Thr46 have appeared previously (Yamazaki et al., 1995b). These spectra in the 1700−1540 cm^{-1} region were duplicated in Figure 3. The intensity of the positive band at 1625 cm^{-1} of T46V (b) is about half of that of the wild type (a). The intensity was partially restored in T46V/D96N (c). The corresponding band in the negative side at 1618 cm^{-1} also lost intensity in T46V (b) and restored in T46V/D96N (c). In agreement with this, the positive and negative ^{13}C-shifted bands at 1586 and 1581 cm^{-1} in [1-^{13}C]valine-labeled T46V, respectively, displayed less intensities (solid line in d) than [1-^{13}C]valine-labeled wild type (dotted line in d), as the 1625 and 1618 cm^{-1} bands in T46V vs the wild type (a and b). Such reduction and restoration occurred in parallel with the loss and regain of H-bonding change of the water molecules in the Thr46−Asp96 segment (Yamazaki et al., 1995b).

A negative band at 1673 cm^{-1} in T46V (b) and T46V/D96N (c) is unique to these mutants, as was observed by Yamazaki et al. (1995b). It lost almost all of its intensity in [1-^{13}C]valine-labeled T46V (d). The positive band at 1648 cm^{-1} of T46V (b) also seems to have lost some intensity in [1-^{13}C]valine-labeled T46V (d). The corresponding band upon ^{13}C-shift may be obscured by the overlapping larger band around 1635 cm^{-1}, where the ^{13}C-shifted band is expected to be. It is likely that the 1648 cm^{-1} band is shifted to about 1625 cm^{-1}, because the positive band at this frequency is not diminished even though it should have

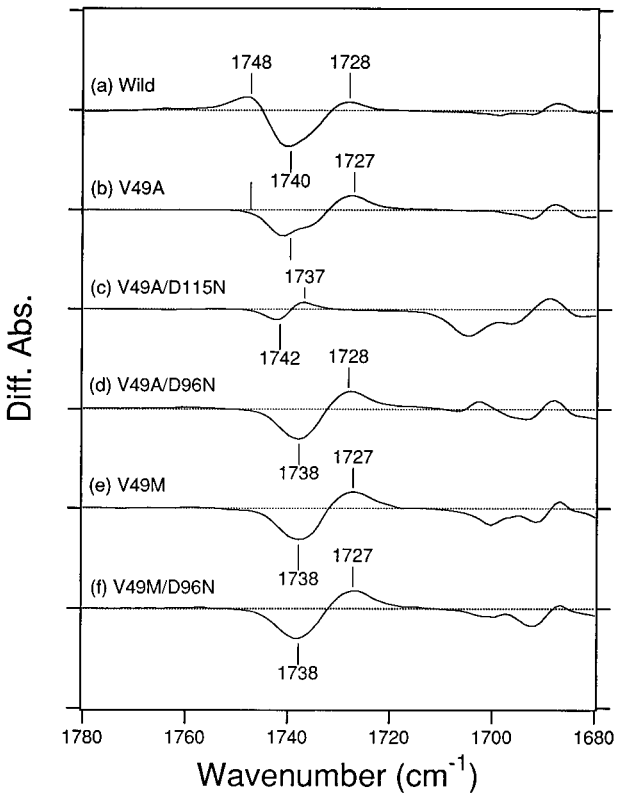

FIGURE 4: BR→L spectra in the 1780−1680 cm^{-1} region of the wild type (a), V49A (b), V49A/D115N (c), V49A/D96N (d), V49M (e), and V49M/D96N (f). One division in the ordinate represents 0.01 absorbance unit for a. The spectrum of the wild type a is a duplicate of Figure 2a.

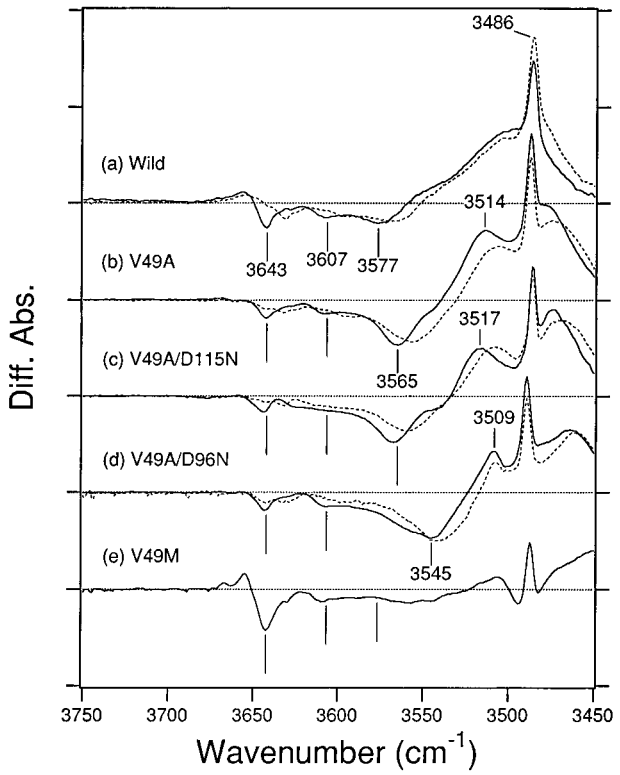

FIGURE 5: BR→L spectra in the 3750−3450 cm^{-1} region of the wild type (a), V49A (b), V49A/D115N (c), V49A/D96N (d), and V49M (e). The solid and dashed lines are with H_2O and $H_2{}^{18}O$, respectively. One division in the ordinate represents 0.005 absorbance unit for a. Spectrum a is presented by duplicating Figure 4a of Yamazaki et al. (1995b) for the sake of comparison.

shifted away as in the [1-^{13}C]valine-labeled wild type (Figure 1). Thus, the bands at 1673 and 1648 cm^{-1} are susceptible to ^{13}C-substitution, and assigned to the peptide C═O of a valine residue. It is most likely Val46, the valine introduced in the T46V mutation.

Interaction of Val49 with Asp96. Figure 4 compares the C═O stretching vibrations of the protonated carboxylic acids of Val49 mutants in the 1800−1700 cm^{-1} region. The absence of the C═O stretching band at 1762 cm^{-1} due to Asp85 indicates that the spectrum does not contain any contributions of the M intermediate. The striking difference in V49A is the loss of the shift of the C═O stretching vibration of Asp96 (b), which appears as a shift from 1740 to 1748 cm^{-1} due to a weaker H-bonding state in the L intermediate of the wild type (a) (Braiman et al., 1988; Maeda et al., 1992a). In V49A/D115N (c), the positive band appeared with low amplitude at 1737 cm^{-1} with a negative band at 1742 cm^{-1}, indicating that the C═O stretching vibration of Asp96 shifted only slightly, and to a lower rather than a higher frequency as would be in the wild type. Thus, the V49A mutation renders the C═O of Asp96 to a somewhat stronger H-bonding states in the L intermediate than the unphotolyzed state. The small intensities may reflect overlapping between the only slightly shifted bands. The spectrum of V49A/D96N (d) shows that Asp115 shifts its C═O from 1738 to 1728 cm^{-1} as in the wild type. The spectrum of V49A (b) is exactly the sum (not shown) of these of the double mutants (c and d), indicating independent perturbations of Asp96 and Asp115 in V49A. The spectral shape of V49A (b) is largely contributed from Asp115 in (d) and the positive band of Asp96 at 1737 cm^{-1} (c) produced a small concave in the negative band of V49A (b). The shape of the spectrum of V49M (e) is similar to V49A/D96N (d) and V49M/D96N (f), suggesting that Asp96 undergoes only a very slight shift as in V49A. These results indicate that Val49 is located to be in position to alter the H-bonding state of Asp96 in the L intermediate.

Water Structural Changes in V49A Mutants. The corresponding spectra in the 3750−3450 cm^{-1} region of Val49 mutants were determined previously for both V49A and V49M (Brown et al., 1994a). These were reexamined (Figure 5) in view of new assignments of the O−H stretch vibrations of the water molecules in the Thr46−Asp96 region (Yamazaki et al., 1995b). The 3486 cm^{-1} band is characteristic of the L intermediate (Maeda et al., 1992b; Yamazaki et al., 1995a). The negative band at 3643 cm^{-1}, which is due to depletion of a water molecule coordinated with Asp85 in the wild type (a) (Maeda et al., 1994), has reduced intensity in V49A mutants (b−d) but increased intensity in V49M (e), as shown previously (Brown et al., 1994a). V49A shows a large negative band at 3565 cm^{-1} (b). It is not affected in V49A/D115N (c) but moved to 3545 cm^{-1} in V49A/D96N (d). All these are shifted in $H_2{}^{18}O$ (dashed lines), indicating that they originate from water.

These negative bands could arise by shift of the 3577 cm^{-1} band, which was assigned to the O−H stretching vibration of one of the water molecules in the Thr46−Asp96 domain (Yamazaki et al., 1995b), but more likely as additional bands that obscure the 3577 cm^{-1} band, because of the presence of corresponding positive bands at 3514 (b), 3517 (c), and 3509 (d) cm^{-1}, respectively. Thus, these water O−Hs form stronger H-bonding upon L formation. The negative band at 3607 cm^{-1} was preserved. As shown by Yamazaki et al. (1995b), this band is lost in D96N and restored in T46V/D96N. The same band is also restored in V49A/D96N (d).

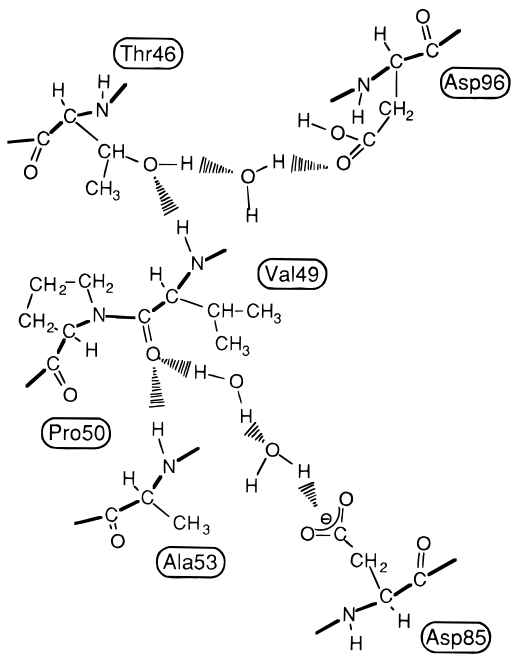

FIGURE 6: Possible structures in the Asp85−Val49−Thr46−Asp96 region in the wild type. The structure is drawn on the basis of the proposed model by Humphrey (1994) except for Thr46, which is written to form bifunctional H-bonding with the N−H of Val49 and the oxygen of water. Bonds in the backbone are stressed by thick lines. Shaded lines represent H-bonding.

DISCUSSION

The many and varied changes in the peptide C═O stretching vibrational frequencies in the photocycle and in different mutants indicate that the backbone has considerable local structural flexibility. The correlation of some of these changes with the O−H stretching vibrational frequencies of bound water indicates that the structural changes can arise through changing interaction with water. In the structure between Asp85 and Asp96 depicted in Figure 6, we emphasize H-bonding networks including water and peptide bonds. Changes in this network appear typically in the BR→L spectrum.

Assignment of the Peptide C═O Bonds of Val49 and Val46 (in T46V). The H-bonding change of the peptide C═O of a valine residue, as indicated by a shift of an amide I band from 1618 to 1625 cm^{-1}, occurs in the K intermediate, persists in the L intermediate, and disappears in the M intermediate. It is affected by the V49M mutation but not by the V49A mutation (Figure 1). Thus, we tentatively assign this peptide C═O to Val49. The BR→K spectrum of P50G had been reported to show intensity decrease for the 1618 cm^{-1} band (Rothschild et al., 1990), consistent with our assignment of this band, since the Val49 C═O forms a peptide bond with the imino-nitrogen of Pro50. The new negative and positive bands at 1673 and 1648 cm^{-1}, respectively, that appear in the BR→L spectrum of T46V (Figure 2b) should be then due to the peptide C═O of Val46 that replaces Thr46. Although these results are suggestive, the definite assignments of the peptide C═O bands must be done in the future by site-directed isotope labeling, as was first done by use of *Escherichia coli* suppressor $tRNA^{Tyr}$ for the peptide C═O of Tyr185 (Ludlam et al., 1995).

Water Changes at the C═O of Val49. The intensities of the amide I bands of Val49 are reduced in the BR→L spectrum of T46V (Figure 2a,b) and restored in T46V/D96N (Figure 2c). It is plausible that at least one of the water molecules that exhibit the O−H stretching vibrational bands at 3607 and 3577 cm^{-1} should interact with the peptide C═O of Val49. Indeed, the 3577 cm^{-1} band is really absent in V49M (Figure 4e), where the C═O of Val49 is perturbed.

The peptide backbone segment that includes the Thr46−Ala53 region is generally assumed to be in an α-helix (Henderson et al., 1990), but the frequencies of the peptide C═O of Val49 are located outside the 1660−1650 cm^{-1} region assigned to α-helices. This could arise by transition-dipole coupling (Torii & Tasumi, 1992), but it is more likely a result of strong H-bonding of the peptide C═O group of Val49, to both an externally bound water molecule and the regular peptide N−H of Ala53 in the α-helical segment (Sundaralingam & Sekhardu, 1989). A similar argument was made previously for the 1620 cm^{-1} band in the BR→M spectrum (Takei et al., 1994).

H-Bonding between Val49 and the Bifunctional O−H of Thr46. The unpaired peptide nitrogen of Pro50, to which Thr46 would point its C═O in a regular α-helix, and the consecutive β-branched amino acid residues, of Thr46, Thr47, and Val49 may disrupt the α-helix in the Thr46−Pro50 segment (Padmanabhan et al., 1990; Horovitz et al., 1992). In such a local, nonhelical state the oxygen of the O−H of Thr46 would be in an appropriate location to enter into H-bonding with the N−H of Val49 (Figure 6), which is three residues away, similarly to H-bonding in calmodulin (Satyshur et al., 1988), or more generally in helix caps (Harper & Rose, 1993). Its side chain O−H would, in turn, donate H-bonding to Asp96, as evidenced by weaker H-bonding of the carboxyl C═O of Asp96 in T46V (Rothschild et al., 1992; Yamazaki et al., 1995b). This could also account for the stronger H-bonding of the Asp96 carboxyl C═O in the L intermediate of Val49 mutants (Figure 4).

The frequencies at 1673 and 1648 cm^{-1} of amide I in T46V (Figure 3b) are also out of the range for α-helix. The former value could be due to the C═O with weakly H-bonding, and the latter value was not far from the observed values for a type III β-turn, the building unit of 3_{10}-helix (Kennedy et al., 1991). The 3_{10}-helix is also observed in the synthetic peptide consisting of α-aminoisobutylic acid (α-branched residue) and valine (Karle et al., 1989). These changes in amide I bands could arise from the changes in a single C═O of Val46. Thus, the perturbation of the amide I in T46V might be the result of displacement of the peptide C═O of Val46 toward the peptide N−H of Val49 but not of a conformation change spanning several residues. Such a change as large as 25 cm^{-1} could be produced. The peptide C═O of Val49 with its hydrated water might be affected in the L intermediate of T46V through the change of H-bonding between the oxygen of Thr46 with either the amide N−H of Val49 or water outside the side chain of Val49.

Interaction between Val49 and Asp96. Structural and functional interaction between Thr46 and Asp96 has been demonstrated before (Rothschild et al., 1992, 1993; Brown et al., 1994b; Humphrey et al., 1994; Yamazaki et al., 1995b). The results in the present paper indicate that there is interaction between Val49 and Asp96 also. As discussed above, the carboxyl C═O of Asp96 is affected by the mutants of Val49, as well as Thr46. Thus, we propose an extended H-bonding system that connects the C═O of Asp96 to the O−H of Thr46, the oxygen of the O−H of Thr46 to the peptide N−H of Val49, and the peptide C═O of Val49 to a water molecule, as depicted in Figure 6. Interaction between Thr46 and Asp96 could be through an intervening water

molecule with its O−H stretching vibration at 3607 cm^{-1} because this band diminishes in D96N and is restored in both T46V/D96N (Yamazaki et al., 1995b) and V49A/D96N (Figure 5d).

The newly appearing O−H stretching vibrational band of water in the Val49→Ala mutant (Figure 5b) is probably due to water that occupies the cavity produced by the replacement of the valine with a smaller residue. As expected from its position close to Thr46, the frequency of the O−H stretching vibration was affected by the additional mutation of Asp96 (Figure 5d).

Interaction between Asp85 and Asp96. On the one hand, the mutants of Val49 (Brown et al., 1994a) and Thr46 (Yamazaki et al., 1995b) have *local* effects in perturbing the L-to-M conversion. The Val49 mutations also affect the water molecule coordinated with Asp85 (Brown et al., 1994a). On the other hand, there is *long-range* interaction between the Schiff base and Asp96 in the L intermediate (Yamazaki et al., 1995b). This is now suggested to occur through H-bonding between the peptide C═O of Val49 and hydrated water molecules in the Asp85−Val49 region toward one side, and H-bonding between the peptide N−H of Val49 to carboxylic acid C═O of Asp96 through the O−H of Thr46 toward the other side (Figure 6).

The effect of the mutation of Val49 on the water molecules proximal to Asp85 was ascribed previously to steric interaction between the side chain of Val49 and Lys216 (Brown et al., 1994a). The present study suggests an H-bonded string of water from Asp85 to the C═O of Val49 as an additional possible mediator (Figure 6). According to the structural model of Humphrey et al. (1994), which is based on complete α-helical structure in this region, the water molecule that we propose to be coordinated with C═O of Val49 is close to the C═O of Val49 but is not at an H-bonding distance (∼0.4 nm) and is in the neighborhood of another water molecule proximal to Asp85. The observed O−H stretching vibrations of water in the L intermediate are always at lower frequencies, suggesting that their structural role is to form stronger H-bonds in the L intermediate.

This is a mechanism proposed for long-range interaction between Asp85 in the extracellular region, with Asp96 in the cytoplasmic region. It is not an uninterrupted H-bonded chain for conducting protons. Rather, it could be the mechanism by which structural changes are induced in the cytoplasmic region, that leads ultimately to the deprotonation of Asp96 in the M-to-N reaction.

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