Interaction of the Protonated Schiff Base with the Peptide Backbone of Valine 49 and the Intervening Water Molecule in the N Photointermediate of Bacteriorhodopsin†

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ABSTRACT: The effects of replacing Val49, Thr46, Asp96, and Phe219 in the cytoplasmic domain of bacteriorhodopsin on water O−H stretching vibrational bands and the amide I and imide II bands of the peptide backbone were examined in the M, N, and M_N intermediates. This study is an extension of previous work on the L photointermediate [Yamazaki, Y., Tuzi, S., Saitô, H., Kandori, H., Needleman, R., Lanyi, J. K., and Maeda, A. (1996) *Biochemistry 35*, 4063−4068]. The O−H stretching bands at 3671 cm^{-1} in the M intermediate and at 3654 cm^{-1} in the N intermediate are shown to originate from the same water molecule. It is located in the region surrounded by the Schiff base, Val49, Thr46, and Phe219 in the M intermediate, and moves closer to Val49 in the M to N reaction. The peptide C−N bond between Val49 and Pro50 and the C═O bond of Val49 undergo perturbations upon formation of the N intermediate but not the M and N-like M_N states in which the Schiff base is unprotonated. The carbonyl oxygen of Val49 is proposed to be the acceptor in H-bonding with the protonated Schiff base in the N intermediate. The results suggest that water molecules may be involved in this interaction in the cytoplasmic region, and may play a role in the accessibility change of the Schiff base in the L to M to N photocycle steps.

Bacteriorhodopsin is a protein present in the purple membrane of *Halobacterium salinarium*. Using light energy absorbed in the retinylidene chromophore attached to Lys216 through the protonated Schiff base, it carries out unidirectional transport of protons across the membrane. Bacteriorhodopsin, containing the *all-trans*-retinal chromophore (BR),[1] undergoes a cyclic photochemical reaction with a series of intermediates called J, K, L, M, N, and O, and finally returning to the initial BR state (*1*). The proton pumping function of bacteriorhodopsin is coupled to this photocycle. Proton release is coupled to proton transfer from the Schiff base to Asp85 in the L to M process, and the subsequent uptake from the opposite side is induced by the reprotonation of the Schiff base by the protonated Asp96 in the M to N process. How access is switched in these two directions is the most important problem in studying this protein. This event can be revealed by analyzing the structure of the L, M, and N intermediates, which are distinguished by steady-state spectra at low temperatures or time-resolved spectra. Fourier transform infrared spectroscopy is one of the most useful methods (*2*).

The O−H stretching vibrational band of water at 3643 cm^{-1} in the unphotolyzed state is absent in the L minus BR spectrum of the D85N mutant protein. Hence, it was ascribed to a water molecule present close to Asp85 (*3*). The same band is also affected by mutations of various extracellular regions, Asp212 (*4*), Arg82 (*5*), Trp86 (*6*), Tyr57 (*7*), and Glu204 (*8*). The structural features of this water molecule have been elucidated on the basis of the orientation angle of the O−H bond of this water molecule (*9*) and of the atomic coordinates by Grigorieff et al. (*10*) for the residues that surround it: the Schiff base, Asp85, Asp212, and Trp86. A recent X-ray diffraction study (*11*) has shown that among eight water molecules one is located close to the Schiff base but clearly not to Asp85. It also shows a pocket including water molecules in the space surrounded by Asp85, Asp212, Tyr57, and Arg82. The water molecule that is affected by the mutation of Asp85 could be one of these water molecules.

The Schiff base, together with this water molecule, forms strong H-bonding with Asp85 upon formation of L (*3*). Molecular dynamics calculations have proposed its possible structure, indicating distortion around the Schiff base in the chromophore (*12*). This structure further extends to the region of Asp96 in the cytoplasmic domain through the peptide carbonyl of Val49 with a proposed interacting water molecule and Thr46 (*13, 14*). The L to M conversion is attained by deprotonation of the Schiff base with accompanied protonation of Asp85. The reverse flow of the proton to the Schiff base is blocked by the increased proton affinity of Asp85, resulting from (a) the interaction with the

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[1] Abbreviations: BR, *all-trans*-bacteriorhodopsin; FTIR, Fourier transform infrared.

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unprotonated Glu204 upon proton release to the extracellular surface (*15*) and (b) the low dielectric environment around Asp85 established in the M state (*16*). The former might be mediated by water molecules present between Asp85 and Glu204 (*5, 8*). The latter might be brought about by loss of the water molecule which had been complexed with the unprotonated Asp85 in the L intermediate. The reprotonation of the Schiff base should be accomplished after its accessibility to the protons changes at the cytoplasmic side (*17, 18*). Nevertheless, the H-bonding groups with the Schiff base in the N intermediate have never been examined. Some insight is provided by the fact that the decay of the M intermediate becomes slower with increasing dehydration (*19, 20*). This suggests the necessity of internal water molecules for the stabilization of the protonated state of the Schiff base in the N intermediate and for the stabilization of the anionic state of Asp96 (*21, 22*). In a previous FTIR study for the L intermediate, we had observed water molecules which are present between the peptide carbonyl of Val49 and the protonated Schiff base (*14*). The present study deals with the effects of the mutations, which change the residues in the cytoplasmic domain, on the internal water molecules and the peptide backbone during the formation of the M and N intermediates. A possible structure around the Schiff base in the N intermediate is proposed.

MATERIALS AND METHODS

The mutants of T46V, T46V/D96N, D96N, V49A, and V49M are identical with those used in earlier studies (*13, 14*). F219L was constructed by the method previously described (*23, 24*). [1-^{13}C]-Val-labeled bacteriorhodopsin was prepared as described previously (*14*).

Bacteriorhodopsin in the purple membrane of these mutants and the wild-type was prepared by the standard method (*25*). Films of the sample were prepared by drying in a vacuum a 40 μL aliquot of the purple membrane suspension ($A_{568\,nm} = 3-4$) in 0.005 M borate buffer (pH 10) on a BaF_2 window with a diameter of 18 mm, and then hydrating with $\sim$1 μL of water. The films at neutral pH were prepared from suspensions in water. The hydrated films were mounted in an Oxford DN1704 cryostat. Difference FTIR spectra were recorded in a BioRad FTS-60A/896 spectrometer as the difference before and after the illumination from a 1 kW halogen−tungsten lamp. The M minus BR spectrum was obtained from films at pH 10 and 230 K by four alternate illuminations with >500 and 420 nm light for 60 s. These conditions are not sufficient for establishing the photosteady state. Illumination of the film at 273 K with >500 nm light for 30 s yielded predominantly N intermediate with small amount of the M intermediate. These decayed in less than 5 min. The illuminations and the recordings at intervals of 10 min were repeated, and 10 difference spectra were averaged. From it the M minus BR spectrum was subtracted so as to delete the 1761 cm^{-1} band due to the protonated Asp85 in the M minus BR spectrum. The shape of the N minus BR spectrum in the 1800−800 cm^{-1} region was almost identical with that previously recorded for highly hydrated films (*26, 27*). The N minus BR spectrum at neutral pH was obtained in the same way from the spectrum recorded at 265 K. The intensities of the negative bands at 1201 cm^{-1} have been adjusted in all the spectra presented in the figures.

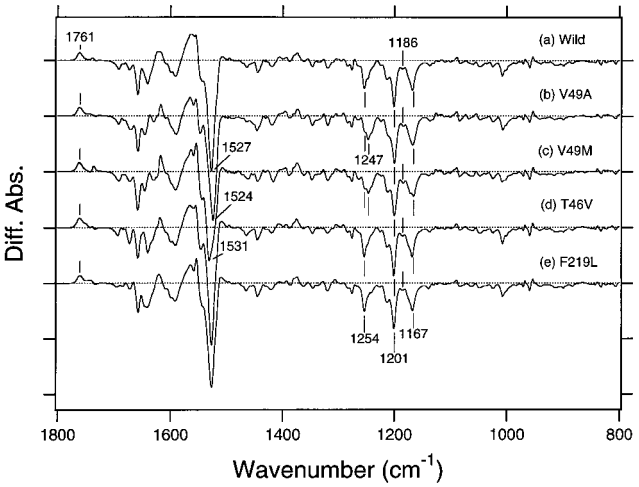

FIGURE 1: M minus BR spectra in the 1800−800 cm^{-1} region for the wild-type (a), V49A (b), V49M (c), T46V (d), and F219L (e). One division of the ordinate for (a) corresponds to 0.02 absorbance unit. The other spectra are scaled to (a) for comparison.

RESULTS

FTIR Spectra of the Mutant Proteins. Figure 1 shows the M minus BR spectra of the wild-type (a), V49A (b), V49M (c), T46V (d), and F219L (e). The negative bands at 1201 and 1167 cm^{-1}, together with the absence of the negative band around 1186 cm^{-1}, indicate that the photoreaction occurred only from the all-trans species. All spectra exhibit the positive C═O stretch band of Asp85 at 1761 cm^{-1} which is typical of the M intermediate. Both V49A (b) and V49M (c) exhibit lowered intensity for the 1254 cm^{-1} band. It is composed of two bands, one of which is sensitive to ^2H_2O substitution and assigned to the combined mode of the N−H and C_{15}−H in-plane bending vibrations (*28*). The V49A (b) and V49M (c) mutations shifted it to 1247 cm^{-1}. This could arise from the perturbation of the N−H bond of the Schiff base from steric interaction between the side chains of Lys216 and Val49 (*29*). The assignment of this band to methylene vibration of lysine (*30*) is applicable to the other, ^2H_2O-insensitive band. The negative C═C stretching bands of V49A (b) and V49M (c) are located at 1524 and 1531 cm^{-1}, respectively, in contrast to that of the wild-type at 1527 cm^{-1}. They reflect slight shifts in the visible spectra of these mutant proteins, in accordance with the well-known linear relationship of the ethylenic stretch frequency and the absorption maximum in the visible (*31*).

Figure 2 shows the N minus BR spectra of the wild-type (a), V49A (b), V49M (c), T46V (d), and F219L (e). The C═O stretching band of Asp85 at 1754 cm^{-1}, the amide I bands of 1669 (−) and 1648 (+) cm^{-1}, the amide II band at 1556 (+) cm^{-1}, the C_{15}−H in-plane bending band at 1400 and 1302 cm^{-1}, and the C−C stretching vibration at 1186 cm^{-1} are typical for the N intermediate (*26, 27*). The negative C═C stretching band at 1525 cm^{-1} of the wild-type (a) appears at a slightly lower frequency than the corresponding band in the M minus BR spectrum at 1527 cm^{-1}, due to cancellation with a positive band, presumably located at 1530 cm^{-1} as deduced by the resonance Raman spectrum (*32*). The C═C stretching band of the N intermediate of V49M may cancel the corresponding negative band at 1531 cm^{-1}, which was detected in the M minus BR spectrum of V49M (see Figure 1c). The lower intensity of

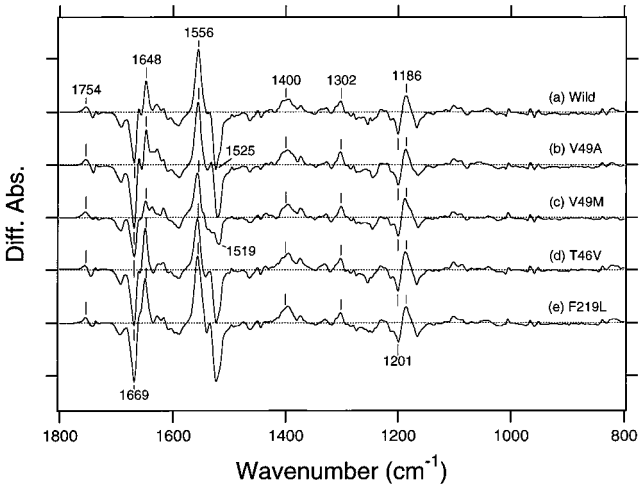

FIGURE 2: N minus BR spectra in the 1800−800 cm^{-1} region for the wild-type (a), V49A (b), V49M (c), T46V (d), and F219L (e). One division of the ordinate for (a) corresponds to 0.04 absorbance unit.

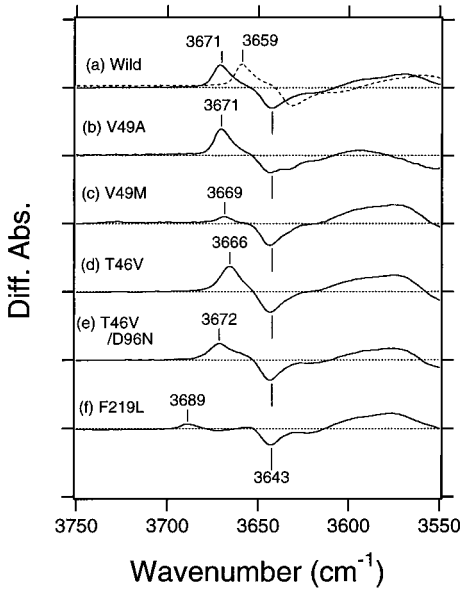

FIGURE 3: M minus BR spectra in the 3750−3550 cm^{-1} region for the wild-type (a), V49A (b), V49M (c), T46V (d), T46V/D96N (e), and F219L (f). The dashed line in (a) shows the spectrum recorded in $H_2{}^{18}O$. One division of the ordinate for (a) corresponds to 0.005 absorbance unit.

this negative band could be accounted for also by the presence of a positive band at the same frequency which is suggested by the emerging negative band at 1531 cm^{-1} in ^2H_2O (not shown). Another negative band of V49M at 1519 cm^{-1} (c) seems to be present in the wild-type (a), which cannot be assigned at present. No significant differences were detected in the spectra of T46V (d) and F219L (e).

Water Structural Changes in the M and N Intermediates. The M minus BR spectra in the 3750−3550 cm^{-1} region (Figure 3) contain the O−H stretching bands of water. The band of the wild-type at 3671 cm^{-1} (a), which is shifted to 3659 cm^{-1} in $H_2{}^{18}O$, does not change in the spectrum of V49A (b) but almost disappears in V49M (c). In the spectrum of T46V (d), the corresponding band appears at 3666 cm^{-1}. This change is abolished in T46V/D96N (e), which nearly restores the original frequency at 3672 cm^{-1}. Similar responses to the single replacement of Thr46 and

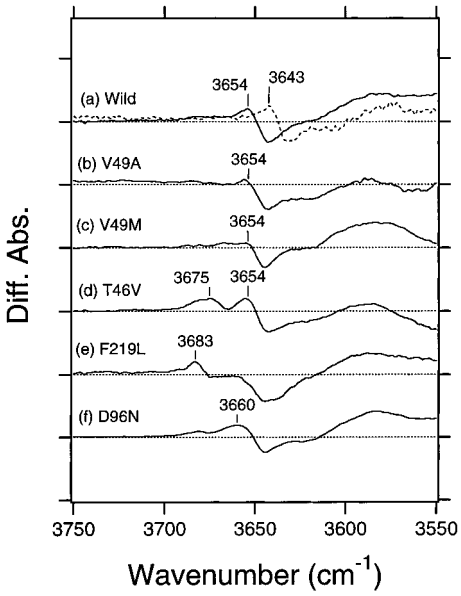

FIGURE 4: N minus BR spectra in the 3750−3550 cm^{-1} region for the wild-type (a), V49A (b), V49M (c), T46V (d), F219L (e), and D96N (f). The dashed line in (a) shows the spectrum recorded in $H_2{}^{18}O$. One division of the ordinate for the solid line in (a) corresponds to 0.005 absorbance unit.

the additional replacement of Asp96 were observed for the rate of the Schiff base deprotonation in the L to M process (*13*) and for the structural changes of the hydrated peptide carbonyl of Val49 (*14*). The effects of T46V might be due to local structural alterations by the perturbation of the peptide carbonyl of position 46, Val46 (*13*). In F219L, the 3671 cm^{-1} band completely disappears, and a substitute band appears at 3689 cm^{-1} with smaller intensity (f). These results strongly suggest that the water molecule with the O−H stretching vibration at 3671 cm^{-1} is present in the domain surrounded by the Schiff base, Val49, Thr46, and Phe219.

The same region in the N minus BR spectra is shown in Figure 4. The 3654 cm^{-1} band in the wild-type (a), which shifts to 3643 cm^{-1} in $H_2{}^{18}O$, is due to the water O−H stretching vibration. Shifts are, however, unclear for the broad band below 3620 cm^{-1} because of distortion of the base line. The 3654 cm^{-1} water O−H band almost disappears in V49M (c). F219L (e) shows a higher frequency band at 3683 cm^{-1} in place of the 3654 cm^{-1} band of the wild-type (a). These effects of the mutations to the water O−H stretching mode are therefore common to both the M and N intermediates (see Figure 3c and 3f vs 3a), indicating that the water O−H bands at 3671 cm^{-1} of the M intermediate and at 3654 cm^{-1} of the N intermediate arise from the same water molecule. In contrast to the M intermediate, however, the band of T46V (d) remains unchanged at 3654 cm^{-1}, though with an accompanied band at 3675 cm^{-1}. A decrease in intensity is to be noted for V49A (b) in contrast to the M minus BR spectrum, indicating that this water molecule is more affected in V49A but less in T46V. Thus, this water may be located more closely to Val49 in N than in the M intermediate. D96N at alkaline pH produces the M_N state, in which changes in the protein backbone occur as the N intermediate forms but the Schiff base remains unprotonated like the M intermediate (*27*). The formation of the M_N state also causes a downshift of the O−H stretching band toward 3660 cm^{-1} (Figure 4f), although to

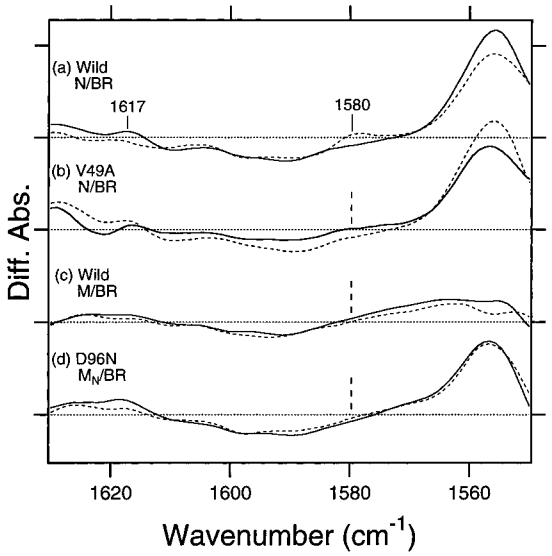

FIGURE 5: N minus BR spectrum of the unlabeled (solid lines) and [1-^{13}C]-Val-labeled (dashed lines) samples for the wild-type (a), the N minus BR spectrum for V49A (b), the M minus BR spectrum for the wild-type (c), and the M_N minus BR spectrum for D96N (d) in the 1630−1550 cm^{-1} region. One division of the ordinate for (a) corresponds to 0.04 absorbance unit.

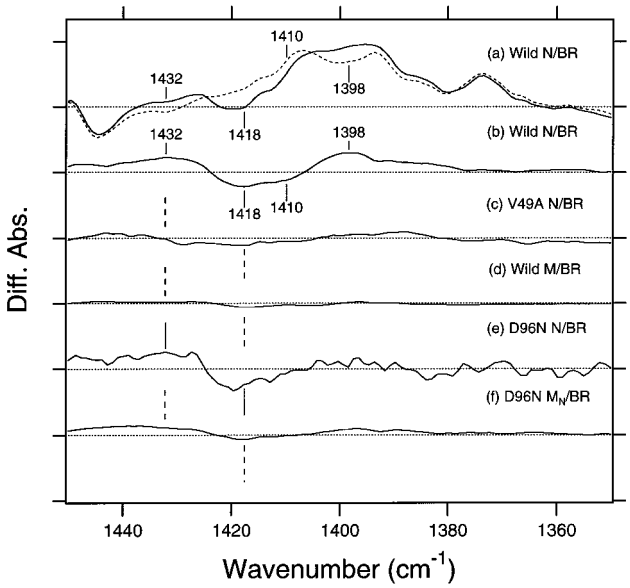

FIGURE 6: N minus BR (a) spectra in the 1450−1350 cm^{-1} region for the unlabeled (solid line) and [1-^{13}C]-Val-labeled (dashed line) wild-type. Differences by subtracting the spectrum of the [1-^{13}C]-Val-labeled sample from the spectrum of the unlabeled sample for the wild-type (b) and V49A (c). The corresponding differences of the M minus BR spectrum (d) of the wild-type, the M minus BR spectrum of D96N at neutral pH (e), and the M_N minus BR spectrum of D96N (f). One division of the ordinate for (a) corresponds to 0.010 absorbance unit.

a slightly different extent than in the N intermediate of the wild-type. Hence, the downshift in the N intermediate is not the result of the protonation of the Schiff base, but of structural changes characteristic of the N intermediate in the protein in the cytoplasmic domain.

Peptide Backbone of Val49. The previous study of the L intermediate (*14*) had shown that the frequency of the C═O stretching mode of the peptide carbonyl of Val49 at 1618 cm^{-1} in the unphotolyzed state changed to 1625 cm^{-1} in L. Changes in the carbonyls were also detected, due to the presence of amide I bands for [1-^{13}C]-Val-labeled BR at 1587 (+) and 1582 (−) cm^{-1}. The spectra in the 1630−1550 cm^{-1} region are shown in Figure 5, for unlabeled (solid lines) and [1-^{13}C]-Val-labeled (dashed lines) samples. The N minus BR spectrum (a) of the unlabeled wild-type (solid line) exhibits a positive band at 1617 cm^{-1} and the corresponding band in the [1-^{13}C]-Val-labeled sample (dotted line) at 1580 cm^{-1}. These are ascribable to Val49, as confirmed by their absence in V49A (b). The absence of corresponding negative bands is the result of an intensity increase which does not affect the frequency. These amide I bands are observed in neither the M (c) nor the M_N state of D96N (d), indicating that these changes correlate with the protonation state of the Schiff base.

Figure 6 shows the region between 1450 and 1350 cm^{-1}. The N minus BR (a) spectrum of the [1-^{13}C]-Val-labeled wild-type (dashed lines) exhibits differences from that of the unlabeled wild-type (solid lines). The shifts by isotope labeling are shown below (Figure 6b−f) in spectra obtained by subtracting the [1-^{13}C]-Val-labeled spectra from the unlabeled spectra. A bilobic feature at 1432 (+) and 1418 (−) cm^{-1} for the N minus BR spectrum (b) is ascribable only to imide II, which is composed of the peptide C−N stretching mode between valine carbonyl and proline imide. The corresponding change of the labeled sample occurs from 1398 to 1410 cm^{-1}. These isotope shifts of about 20−25 cm^{-1} are expected for a peptide bond. Previous studies of [1-^{15}N]-Pro-labeled BR showed similar frequency changes in the imide II bands of the K minus BR spectrum (*33*, *34*). In the amino acid sequence of bacteriorhodopsin, there are three Val-Pro sequences, Val49-Pro50, Val69-Pro70, and Val199-Pro200. Only Val49-Pro50 is in the intramembrane domain (*10*). Thus, the bands that are shifted in [1-^{13}C]-Val-labeled BR might be ascribed to the imide II of Val49-Pro50. This can be confirmed from the absence of the 1432 (+) and 1418 (−) cm^{-1} bands in the spectrum of V49A (c). The M minus BR spectrum (d) does not show these bands. While the N intermediate of D96N (e) at neutral pH retains the shifts, the M_N state of D96N (f) at pH 10 does not show these bands. Thus, the changes in the imide II bands are dependent on the protonated Schiff base.

DISCUSSION

The dynamic properties of the purple membrane in the normal photocycle are dependent on the temperature and hydration (*35*), and the conditions have to be chosen with this in mind. Our hydrated membranes satisfy this criterion above 230 K, where the photoreactions to the M and N intermediates were carried out in the present study. The spectra under physiological relevant conditions (*36*) are quite similar to those we obtained in the present study, at least in the 1800−800 cm^{-1} region. Time-resolved FTIR difference spectra by use of an attenuated total reflection cell are believed to largely conserve the biological state. The spectra thus obtained are quite similar to those in the present study except for differences of intensity inherent to the oriented membrane in the film sample (*37*).

Water Molecule in the Schiff Base, Val49, Thr46, and Phe219 Regions of the M and N Intermediates. In the unphotolyzed state of bacteriorhodopsin, one of the internal

water molecules is located in the extracellular domain because it is affected by mutations of Asp85, Asp212, Arg82, Glu204, Tyr57, and Trp86 (*9*). In the L intermediate, one of its O−H bonds forms strong H-bonding with Asp85, and the other responsible for the O−H stretching vibration band at 3643 cm^{-1} is directed to Asp212, though with weaker H-bonding (*4*). The L minus BR spectrum exhibits a large positive feature in the range between 3560 and 3450 cm^{-1}, as a result of the lower frequency shifts of the 3643 cm^{-1} band (*3*) and of the others at 3607 and 3577 cm^{-1} (*13*, *14*). The latter two are the O−H stretching modes of the water molecules that were suggested to be located at Asp96 and the peptide carbonyl of Val49, respectively. The X-ray diffraction study showed a water molecule close to Asp96 but not to Val49 (*11*). The relation between these residues and internal water molecules will have to be carefully examined after the coordinates by the recent X-ray and electron diffraction studies become available (*11*, *38*). These water molecules comprise an H-bonding network between Asp85 and Asp96 in the L intermediate. This structure was proposed to be a prerequisite for the deprotonation of Asp96 in the subsequent M to N process (*14*), as well as the deprotonation of the Schiff base in the L to M process (*3*).

In the M intermediate, the O−H stretching band of water appears at 3671 cm^{-1} (*5*, *39*). This is not affected by replacement of the residues in the extracellular domain such as Arg82 (*5*), Glu204 (*8*), and Trp86 (*6*), although it forms a distinct bilobe with the 3643 cm^{-1} band that is affected in these mutants. The present study suggests that this water molecule in the M intermediate is present in the region surrounded by the Schiff base, Val49, Thr46, and Phe219. The O−H stretching frequency of 3671 cm^{-1} changes to 3654 cm^{-1} in the M to N conversion in response to the mutations of residues in its environment. In this regard, V49A is more influential while T46V is less so. Most distinct protein changes common for both the N and M_N intermediates appear for the amide I bands at 1669 (−) and 1648 (+) cm^{-1} and the C═O stretching vibration band of the protonated Asp85 at 1754 cm^{-1} (*27*). The present study shows further that shifts of the O−H stretching vibrations at 3671 cm^{-1} to lower frequency occur in the M to either N or M_N conversion, as a reflection of the protein environment characteristic of these states. However, a small difference in the frequency of 3654 cm^{-1} for N from 3660 cm^{-1} for M_N is attributable to the protonated Schiff base.

Perturbation of the Peptide Bond of Val49 in the N Intermediate. The perturbations of the C−N bond between Val49 and Pro50 and the C═O stretching vibration of Val49, as indicated by the frequency changes in imide II and the intensity increase in amide I, respectively, are characteristics that distinguish the N intermediate from the M_N state. The frequencies of the imide II band at 1418 (−) and 1432 (+) cm^{-1} are much lower than the 1465 cm^{-1} band for the trans peptide bond of polyproline II but close to 1435 cm^{-1} of the cis peptide bond of polyproline I (*40*). According to the model of the tertiary structure of bacteriorhodopsin (*10*), however, a cis configuration is quite unlikely for this bond. The imide II frequency of the oligoproline peptide varies from 1445 to 1485 cm^{-1} depending on solvent (*41*). The frequencies of 1432 and 1418 cm^{-1} are much lower than these values. Such low frequencies could be brought about by the movement of π electrons from the C−N bond to the carbonyl moiety, which would confer more single bond character to the C−N bond. It has been shown that the binding of the retinal to the apoprotein of the P50A mutant is extremely slow, while the same as the wild-type in P50G (*42*). Only a glycine residue which has a wider allowed region of dihedral angles in the Ramachandran plot can substitute for proline. This suggests a distorted conformation around the Val49−Pro50 bond. The structure is relaxed slightly in the N intermediate by interaction with the protonated Schiff base, but not in the M and M_N intermediates with the unprotonated Schiff base. Imide II bands of Tyr185−Pro186, which shows a shift from 1429 to 1434 cm^{-1} in the K minus BR spectrum (*43*), could behave similarly, but no corresponding changes have been reported for the N intermediate.

The frequency of the amide I band in the N intermediate at 1617 cm^{-1} is also quite low, as expected for the amide I band of Val49 which is supposed to be in an α helix. Such a low frequency had been explained as arising from the peptide bond forming two H-bonds, one with a water molecule and the other with peptide N−H, presumably Ala53 (*14*). The absence of the corresponding negative band indicates that the band arises from an increase in intensity without affecting the frequency. Intensity increase is expected if the carbonyl band perpendicular to the membrane plane changes its orientation in the photoreaction. Such a band in the BR state should be detected by changing the angle of the membrane plane to an incident polarized light (*44*). This is, however, not the case.[2] Another possible source for the increase in intensity of the infrared band may be electrical interactions, possibly with the positive electric charge of the protonated Schiff base. This idea is supported by the fact that an increase in intensity is attained in the N intermediate with the protonated Schiff base but not at all in the M_N state with the unprotonated Schiff base, although otherwise both show the same protein environment.

Possible Interaction of the Schiff Base with the C═O of Val49 in the N Intermediate. Asp85 is the persistent counterion to the positive electric charge of the chromophore and also the acceptor of H-bonding of the protonated Schiff base in the L intermediate in the photolyzed state (*3*). The protonated Schiff base retains its strong H-bonding in the N intermediate (*26*). The protonated Asp85 in the N intermediate could be neither a counterion nor a H-bonding acceptor of the protonated Schiff base. The negative charge of Asp96 does not work as the counterion because the chromophore of the N intermediate of D96N is nearly identical with that of the wild-type (*26*). In the N intermediate, the protonated Schiff base must be stabilized by the negative charge of Asp212 (*45*, *46*). The red shift for the N intermediate in T89N (*47*) may be the result of changes in the interaction of Thr89 with Asp85 and Asp212 (*11*). These Asp residues, however, could not be the acceptor for H-bonding of the Schiff base. The acceptor must be present in the cytoplasmic domain.

The present FTIR study strongly suggests that the H-bonding acceptor could be the peptide carbonyl of Val49 or the water molecule coordinated to Val49. The Schiff base and this C═O might be connected by water molecules. In

[2] Kandori et al., unpublished experiments.

the L minus BR spectrum, we had proposed that two water molecules exist between the Schiff base in the extracellular domain and the C═O of Val49 (*13*, *14*). These are detected as the water molecule with the O−H stretching vibration in the unphotolyzed state at 3643 and 3577 cm^{-1}. In the N intermediate, these water molecules might form H-bonding between the Schiff base in the cytoplasmic domain and the C═O of Val49. Thus, a string of the water molecules may be involved in directing the accessibility change of the Schiff base to the cytoplasmic side.

The M to N conversion due to the proton transfer from Asp96 to the Schiff base follows a protein backbone change, which can occur even in the absence of protonation of the Schiff base (*27*). Depletion of internal water, which also inhibits the reprotonation of the Schiff base (*20*, *48*), stabilizes an N-like protein change (*49*). Such an M_N-like state is accumulated even with the deprotonation of Asp96 in the wild-type in guanidine hydrochloride (*50*). Water molecules may be involved in the stabilization of the protonated Schiff base besides deprotonated Asp96 as proposed earlier (*48*). The requirement for the protonation of the Schiff base is more rigorous than the deprotonation of Asp96.

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