Relocation of Internal Bound Water in Bacteriorhodopsin during the Photoreaction of M at Low Temperatures: An FTIR Study†

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ABSTRACT: Changes in the FTIR difference spectra upon photoconversion of the M intermediate to its photoproduct(s) M′ were studied in wild-type bacteriorhodopsin and several mutants at low temperatures. The studies aimed at examining whether internally bound water molecules interact with the chromophore and the key residues Asp85 and Asp96 in M, and whether these water molecules participate in the reprotonation of the Schiff base. We have found that three water molecules are perturbed by the isomerization of the chromophore in the M → M′ transition at 80 K. The perturbation of one water molecule, detected as a bilobe at 3567(+)/3550(−) cm^{-1}, relaxed in parallel with the relaxation of an Asp85 perturbation upon increasing temperature from 80 to 100 and 133 K (before the reprotonation of the Schiff base). Two water bands of M at 3588 and 3570 cm^{-1} shift to 3640 cm^{-1} upon photoconversion at 173 K. These bands were attributed to water molecules which are located in the vicinity of the Schiff base and Asp85 (Wat85). In the M to M′ transition at 80 and 100 K, where the Schiff base remained unprotonated, the Wat85 pair stayed in similar states to those in M. The reprotonation of the Schiff base at 133 K occurred without the restoration of the Wat85 band around 3640 cm^{-1}. This band was restored at higher temperatures. Two water molecules in the region surrounded by Thr46, Asp96, and Phe219 (Wat219) were perturbed in the M to M′ transition at 80 K and relaxed in parallel with the relaxation of the perturbation of Asp96 upon increasing the temperature. Mutant studies show that upon photoisomerization of the chromophore at 80 K one of the Wat219 water molecules moves closer to Val49 (located near the lysine side chain attached to retinal, and close to the Schiff base). These data along with our previous results indicate that the water molecules in the cytoplasmic domain participate in the connection of Asp96 with the Schiff base and undergo displacement during photoconversions, presumably shuttling between the Schiff base and a site close to Asp96 in the L to M to N transitions.

Bacteriorhodopsin is a heptahelical transmembrane protein from the highly halophilic bacterium *Halobacterium salinarum*. Bacteriorhodopsin uses light energy to transport protons unidirectionally from the cytoplasm to the extracellular medium [see reviews (*1*−*3*)]. The light absorbed by the pigment's chromophore, *all-trans*-retinal attached to Lys216 through a protonated Schiff base linkage, causes the isomerization from all-trans to 13-cis, which then induces the formation and decay of a series of intermediates: K, L, M, N, and O. The bacteriorhodopsin with *all-trans*-retinal will be called BR[1] in this text. The retinal's polyene chain lies roughly in parallel to the membrane plane and near its center, conceptually dividing the protein into extracellular and cytoplasmic domains (*4*). The L → M transition is associated with proton transfer from the Schiff base to Asp85, which is located at the extracellular side of the Schiff base. The Schiff base is then reprotonated in the M → N transition by a proton from initially protonated Asp96, located on the cytoplasmic side of the Schiff base. Internal water molecules participate in stabilization of the protonated Schiff base in L and N (*5*, *6*) and in proton transport from and to the Schiff base (*5*−*7*). They are also likely to play a crucial role in light-induced changes of the pK_a of key residues (*8*) and the formation of a pathway for proton transport (*7*, *9*).

In the M to N intramolecular transition, the proton affinity of Asp96 decreases (*10*, *11*), and that of the Schiff base increases. This causes transfer of a proton from Asp96 to the Schiff base. The mechanism of this long-range proton transfer is unknown. One possibility is that it is mediated

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[1] Abbreviations: BR, *all-trans*-bacteriorhodopsin; M′, photoproducts of M; M′80, M′100, M′133, and M′173, photoproducts produced by illumination of the M intermediate at 80, 100, 133, and 173 K, respectively; λ_{max}, wavelength for maximum absorption; Wat85, water molecules responsible for the O−H stretching frequencies which are affected by the mutation of Asp85; Wat219, water molecules responsible for the O−H stretching frequencies which are affected by the mutation of Phe219 (see text).

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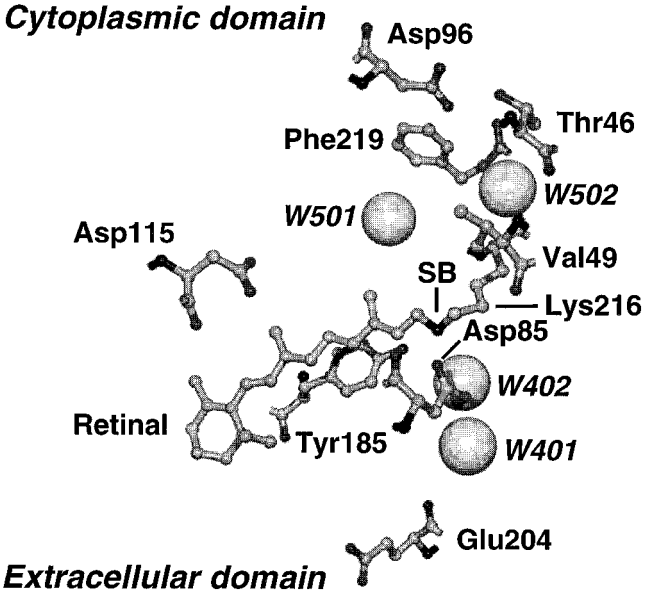

FIGURE 1: Protein residues and internal water molecules of BR discussed in this paper, drawn on the basis of the X-ray crystallographic structure by Luecke et al. (*18*). Water oxygens are depicted by larger spheres. SB stands for the Schiff base.

by the migration of internal water molecules from the region close to Asp96 in M to the Schiff base in N, as suggested by the previous FTIR studies (*6*). Recent X-ray structural data showed changes in Phe219 and a water molecule close to it in the transition from BR to the M-like state (*7*).

Low-temperature difference FTIR spectroscopy has provided important information on the changes of internal water molecules of bacteriorhodopsin during the photocycle (*12*−*14*). Changes of several water O−H stretching bands were detected, and their possible locations were identified using different mutants [reviewed by Maeda, (*2*)] before the first X-ray structural data on water molecules became available (*7*, *15*−*18*). Residues and water molecules that are discussed in the present paper are shown in Figure 1 using coordinates of the 1.55 Å structure of the wild-type bacteriorhodopsin (*18*).

In the BR → L transition, changes of some amino acid residues and internal water molecules in the extracellular and cytoplasmic domains have been detected by low-temperature FTIR studies (*19*, *20*). Studies of the back-photoreaction of L at 80 K, a temperature at which the restoration to BR is blocked, revealed amino acid residues and internal water molecules that interact with the chromophore in L. We proposed that these water molecules are relocated from the cytoplasmic domain to a site near the Schiff base and participate in stabilizing the protonated form of the Schiff base (*5*).

In this study, we investigated FTIR difference spectra accompanying the photoconversion of the M intermediate in order to obtain information on the interaction of water molecules and key residues with the chromophore in M and the relocation of water molecules during the L → M transition. The photoproducts obtained by illuminating M were previously characterized by visible absorption (*21*−*27*), resonance Raman (*28*), and FTIR spectroscopy (*29*), as reviewed by Balashov (*30*). The restoration of the initial BR state was achieved after several intermediate thermal steps. Initial photoproducts with unprotonated Schiff bases (P421 and P433), which are stable below 100 K, were changed to species with protonated Schiff bases (P565 → P575 → P585) upon warming before finally returning to the initial BR state at 230 K.

An earlier FTIR study (*29*) reported changes in Asp85, Asp96, and some other amino acid residues produced by the photoreaction of M. We studied changes of the internal water molecules in parallel with the changes in carboxyl and chromophore bands, to reveal the role of internal water molecules and the carboxylic acid residues in determining the structure of the M intermediate and in effecting proton transfer between the Schiff base and donor/acceptor groups. To identify the origin of carboxyl bands and probable location of water molecules, a number of mutants were utilized in our studies: D115N, D96N, F219L, V49A, and T46V. The questions we addressed were as follows: *(i) Are there any water molecules that interact with the chromophore in M, directly or indirectly?* These water molecules might be perturbed by the photoisomerization of the chromophore during the primary M → M′ photoreaction at 80 K and exhibit a shift of their vibrational bands. Their possible location can be tested with the mutant studies. *(ii) Is there some connection (or interaction) of the chromophore with the proton donor (Asp96) in M?* Photoisomerization of the chromophore in L did not cause any noticeable perturbation of Asp96 (*5*). Is this also true for the M state? A previous FTIR study (*29*) suggested that some perturbation of Asp96 might occur. This is tested in studies of the D96N and D115N mutants. *(iii) How does light-induced reprotonation of the Schiff base correlate with changes in water structure?* Previous studies (*31*, *32*) suggest that the protonated state of the Schiff base is stabilized by a water molecule between the positively charged Schiff base and negatively charged Asp85. Photoconversion of M provides an opportunity to follow the process of reprotonation of the Schiff base and re-formation of the initial BR state gradually (*30*), and check whether this process correlates with changes in the water bands.

MATERIALS AND METHODS

Wild-type bacteriorhodopsin was prepared by the method of Oesterhelt and Stoeckenius (*33*). The mutant proteins were expressed in *Halobacterium salinarum* (*34*). The D96N, D115N, T46V, T46V/D96N, V49A, and F219L pigments were described previously (*6*, *35*). T46V and T46V/D96N were generously provided by J. K. Lanyi and L. S. Brown (University of California, Irvine), and the other mutants were provided by Y. Yamazaki of Kyoto University. The dried films were prepared by placing an aliquot of a suspension of purified purple membranes in 0.01 M borate buffer (pH 10) on a BaF_2 window under room air. After hydrating with 0.2 μL of H_2O, D_2O, or $H_2{}^{18}O$, the film was installed in an Oxford cryostat Optistat, whose temperature was controlled by an Oxford Intelligent Temperature Controller. The spectra were measured using a BioRad FTS6000 FTIR Spectrometer as described previously (*5*).

The sample was light-adapted at 273 K by illumination with yellow light (Corning 3-73 filter, wavelengths >400 nm) for 2 min from a slide projector with a 500 W halogen−tungsten lamp. The sample was then cooled to 230 K, and illuminated with deep yellow light (3−69 filter, wavelengths

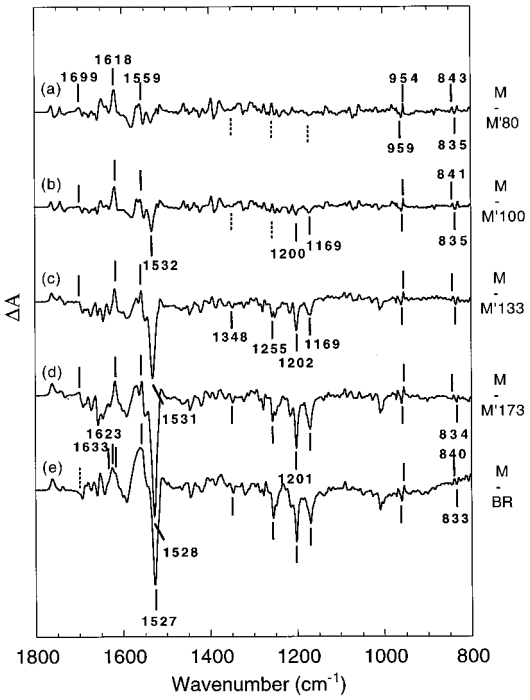

FIGURE 2: Comparison of the spectra of M′ in the 1800−800 cm^{-1} region at various temperatures: (a) M minus M′80, (b) M minus M′100, (c) M minus M′133, (d) M minus M′173, and (e) M minus BR spectra. The bands that were not observed but were observed in other spectra are represented by dotted lines at the corresponding frequencies. Labels were omitted if the frequency of the corresponding bands is identical with that in the first spectrum in which it appeared. The amplitudes of the M minus M′100 (b), M minus M′133 (c), and M minus M′173 (d) difference spectra were normalized so that the bands at 1618 and 954 cm^{-1} would be identical because the temperature shift study (see text) shows that these bands appear with the same intensity. The same was applied to the similar spectra at 80 K (a) and 100 K (b). The M minus M′173 (d) and M minus BR spectra (e) were adjusted by the 1762 cm^{-1} band of Asp85 and the C═C and C−C stretching vibration bands of BR. All five spectra were depicted in these ways to allow comparison of the intensities with each other. Full length of the ordinate corresponds to 0.081, 0.095, 0.126, 0.113, and 0.176 absorbance units for spectra a−e, respectively.

>520 nm) to produce M. The M minus BR spectrum was recorded. The film was then cooled to the temperature indicated in the text, and M was transformed by illumination with blue light (7-59 filter, wavelengths 350−500 nm) followed by red light (2-64 filter, >650 nm) illumination to convert K formed from residual BR. The M minus M′ spectrum at each temperature was obtained as a difference between the average of 8 absolute spectra, each obtained by adding 256 interferograms, taken before and after the illumination.

RESULTS

Photoreaction of M at Various Temperatures. The photoreaction of M was studied at four temperatures: 80, 100, 133, and 173 K. At the first two temperatures, illumination of M results in formation of its primary photoproducts (50% of P421 and ca. 5% of P433) which have unprotonated Schiff bases (*21*−*25*). At 133 K, part of these photoproducts converts into a species with a protonated Schiff base (P565), and at 173 K a larger fraction of the photoproducts has a protonated Schiff base and the maximum shifts to 575 nm (P575) (*23*−*25*). Figure 2 shows the FTIR difference spectra in the 1800−800 cm^{-1} region for the photoreactions of M at 80 K (a), 100 K (b), 133 K (c), and 173 K (d). The spectra are arranged so that the contributions of M are in the positive direction in all the spectra and those of the photoproduct of M, M′, are in the negative direction. Positive bands at 1618 and 954 cm^{-1} are present in all the spectra, indicating that their frequencies reversed to those they had in BR upon the photoisomerization of the chromophore. The spectra at different temperatures were normalized so that the amplitudes of M are identical at 1618 and 954 cm^{-1} (see the legend of Figure 2). The photoproducts of M formed at 80, 100, 133, and 173 K were named M′80, M′100, M′133, and M′173, respectively. Note that some of the M′s will have protonated Schiff bases (see below). The M minus BR spectrum, recorded at 230 K (e), is shown for comparison. The corresponding spectra for the film hydrated with D_2O did not exhibit the hydrogen out-of plane bands at 951 and 986 cm^{-1} (data not shown), characteristic of the K and L intermediates in D_2O, respectively (*36*), excluding the possibility that these spectra were contaminated by the K and L intermediates.

The difference spectra were also obtained by warming the samples to 133 and 173 K after illumination at 100 and 133 K, respectively (data not shown). These spectra exhibited nearly identical spectral shapes to those produced by the photoreaction at 133 K (c) and 173 K (d), respectively. These results show that the spectra obtained by illumination of M at various temperatures can be regarded as the spectra of the intermediate states formed in the processes that follow the photoreaction of M at 80 K

Protonation State of the Schiff Base of M′. Previous visible (*21*, *24*, *25*) and FTIR (*29*) spectroscopic studies have shown that the Schiff bases of M′80 and M′100 are unprotonated, but about half of the photoproduct becomes protonated in M′133. This protonated species exhibited a λ_{max} of 565 nm (P565). In M′173, the Schiff base is completely protonated, and the λ_{max} shifted to 575 nm (P575). These conclusions were confirmed in our spectra. The N−H bending vibrations of the protonated Schiff base of BR (*36*) at 1348 and 1255 cm^{-1} (Figure 2, a through d) seen in the M minus BR spectrum (e) are absent in M′80 and M′100 (a and b), indicating that these species have an unprotonated Schiff base. These bands appear in M′133 with an intensity half of that in BR, indicating that only 50% of the photoproducts have protonated Schiff bases (c). The band intensities of the chromophore vibrations are in general much lower in a species with an unprotonated Schiff base [see review (*14*)]. Intensities of the C═C and C−C stretching bands at 1531, 1200, and 1169 cm^{-1} in M′133 (c) are about half of the corresponding bands in BR (e). Their bands are absent in M′80 (a). Bands at 1532, 1200, and 1169 cm^{-1} in M′100 (b) are likely due to amide II and amide III (mainly due to the peptide amide) because these bands shift in D_2O (data not shown). FTIR results do not show the isomeric state of the unprotonated photoproducts of M, but previous resonance Raman experiments (*28*) indicated that the chromophore is *all-trans*-retinal.

The C═C stretching band due to M′173 (P575) at 1528 cm^{-1} is almost the same as the BR band at 1527 cm^{-1} (d and e), but different from that of M′133 at 1531 cm^{-1}. Nevertheless, some protein structure still has not completely recovered in M′173, because the M minus M′173 spectrum

does not show the amide I bands at 1633 and 1623 cm^{-1}, which are characteristic of the M minus BR spectrum (e). The C═C stretching frequency of M′133 (P565) at 1531 cm^{-1} is as expected for its λ_{max}, judging from the linear relation between the λ_{max} and the C═C stretching frequency (*37*).

Structural Changes around Glu204 Do Not Revert in Any M′ Species. A small positive band at 1699 cm^{-1} appears as a common feature of all the M minus M′ spectra (Figure 2, a−d), but is absent in the M minus BR spectrum (e) probably because a negative band at the same frequency cancels it. The 1699 cm^{-1} band was detected in the M minus BR spectrum of E204Q at 230 K (*38*) and at room temperature (*11, 39*). Thus, the 1699 cm^{-1} band of the initial state is sensitive to the E204Q mutation and most likely belongs to Glu204 or a peptide carbonyl close to it. The fact that the 1699 cm^{-1} band is present in the M minus M′ spectra indicates that it is not canceled by the Glu204 band, implying that the environment around Glu204 was changed upon M formation and the change persists in all the M′ species.

Isomerization of the Chromophore Perturbs Tyr185. The vibration at 840 cm^{-1}, seen as a positive band in the M minus BR spectrum (Figure 2, e), was assigned to the benzene ring out-of-plane vibration of Tyr185 (*40*). The corresponding vibration in BR is located at 833 cm^{-1}. The Tyr185 band undergoes a slight shift in frequency from 843 cm^{-1} toward 835 cm^{-1} in M′80 (a). A reasonable explanation is that this shift is caused by the isomerization of the chromophore. Upon increasing temperature from 100 to 173 K, this band gradually moves toward 834 cm^{-1} (b through d). The benzene ring of Tyr185 is located relatively close to the C_{13}═C_{14} bond of the retinal (about 4 Å) (*17, 18*). The slightly different frequencies in M′ might be caused by the different environments around the chromophore in M′ compared to BR.

Perturbation and Deprotonation of Asp85 in the M → M′ Transitions. The spectra in the 1780−1720 cm^{-1} region contain the C═O stretching vibrations of the protonated carboxylic acids (Figure 3). Previous studies have shown that the positive band at 1761 cm^{-1} in the M minus BR spectrum (e) is due to the protonation of Asp85 in M (*41, 42*). The M → M′133 and the M → M′173 conversions to form protonated Schiff base species are accompanied by the deprotonation of Asp85, giving rise to a positive band at 1761−2 cm^{-1} (c and d). The M → M′ transitions at 80 K (a) and 100 K (b), which are not accompanied by the reprotonation of the Schiff base, still exhibit the positive band at 1762 cm^{-1}. These bands are accompanied by negative bands at 1754 cm^{-1} due to the perturbation of protonated Asp85 in both M′80 and M′100, as observed by Takei et al. at 100 K (*29*). The perturbation at 100 K is smaller than at 80 K, as indicated by the decrease in the intensity of the 1762 and 1754 cm^{-1} bilobe at 100 K relative to that at 80 K (a and b).

Perturbations of Asp96 and Asp115 in the M → M′ Transitions. The M minus M′80 spectrum (solid line in Figure 3, a) shows a positive band at 1742 cm^{-1}. Takei et al. (*29*) suggested that this band might be due to a perturbation of Asp96. According to Sasaki et al. (*35*), the carboxyl group of protonated Asp115 also has a band at a similar frequency in M. To determine the contributions of Asp96 and Asp115 to the 1742 cm^{-1} band, we studied the

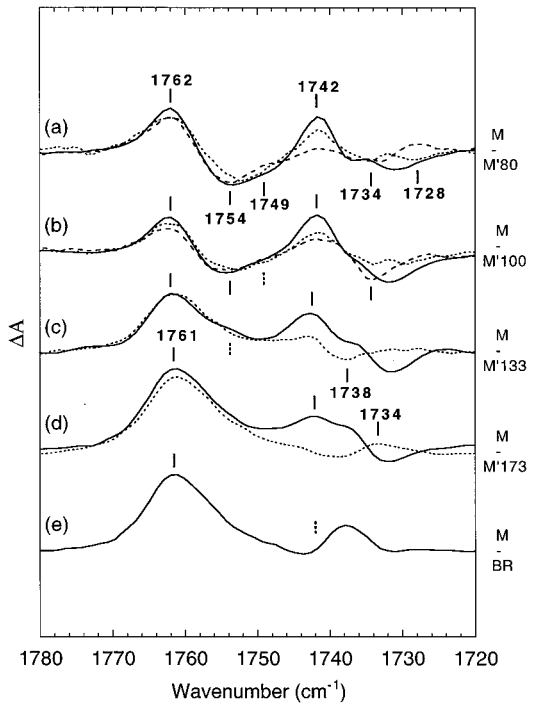

FIGURE 3: Comparison of the perturbations of the C═O stretching vibrations of carboxylic acid residues in various M′ states in the 1780−1720 cm^{-1} spectral region for (a) M minus M′80, (b) M minus M′100, (c) M minus M′133, (d) M minus M′173, and (e) M minus BR spectra. Solid lines are for the wild-type protein, dotted lines (a−d) for D115N protein, and a broken line (a and b) for D96N protein. The bands that were not observed but were observed in other spectra at the corresponding frequencies are represented by dotted lines. Labels were omitted if the frequency of the corresponding bands is identical with that in the first spectrum in which it appeared. Intensities of the spectra of mutant pigments were adjusted by normalization at the 1618 and 954 cm^{-1} bands. Full length of the ordinate corresponds to 0.0069, 0.0069, 0.0067, and 0.0055 absorbance units for the spectra of D115N (dotted lines in a−d), and 0.0055 and 0.0069 for the spectra of D96N (broken lines in a−b), respectively. For the wild type, see Figure 2.

D96N and D115N mutants. In the D96N mutant, the 1742 cm^{-1} band is greatly reduced (broken line in a). In the D115N mutant (dotted line in a), the positive band at 1742 cm^{-1} in the M minus M′80 spectrum is only slightly smaller than in the wild type. These results indicate that the 1742 cm^{-1} band at 80 K is composed of two parts: the larger part is associated with a perturbation of Asp96, and the minor part is due to a perturbation of Asp115. Takei et al. (*29*) showed the presence of the negative band at 1749 cm^{-1} and tentatively assigned it for Asp96. The negative band around this frequency is smaller in D96N (broken line) but not in D115N (dotted line) than the wild type (solid line), indicating that the negative band at 1749 cm^{-1} is due to Asp96. The 1736 cm^{-1} band of Asp96 in M, whose amplitude is much smaller than that of the 1742 cm^{-1} band (*35*), arose either from a small shift of the 1742 cm^{-1} band that takes place in all the molecules or from a large shift (from 1742 to 1736 cm^{-1}) that takes place in a small fraction. The latter possibility suggests heterogeneity of M in which part of Asp96 absorbs 1742 cm^{-1} and a minor part at 1736 cm^{-1}. Further study is necessary in order to distinguish these two possibilities. Changes in the 1734 cm^{-1} band, which appeared as a bilobe at 1734 (+)/1739-(−) cm^{-1} in the M minus M′173 spectrum of the D115N mutant pigment (dotted line in d), might be due to reversal

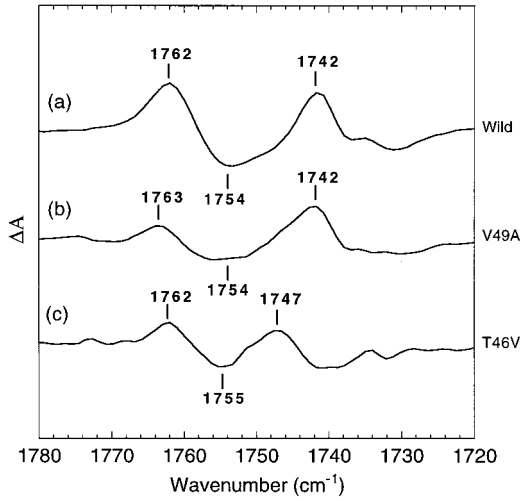

FIGURE 4: Comparison of the perturbations of the C═O stretching vibrations of carboxylic acid residues in the 1780−1720 cm^{-1} spectral region in the M to M′80 transition in various mutants. (a) Wild type, (b) V49A, and (c) T46V. (a) is from Figure 3, a. Intensities of the spectra of mutant pigments were adjusted to that of the wild type by normalization of their 1618 and 954 cm^{-1} bands. The full length of the ordinate corresponds to 0.0037, 0.0054, and 0.0063 absorbance units for (a), (b), and (c), respectively.

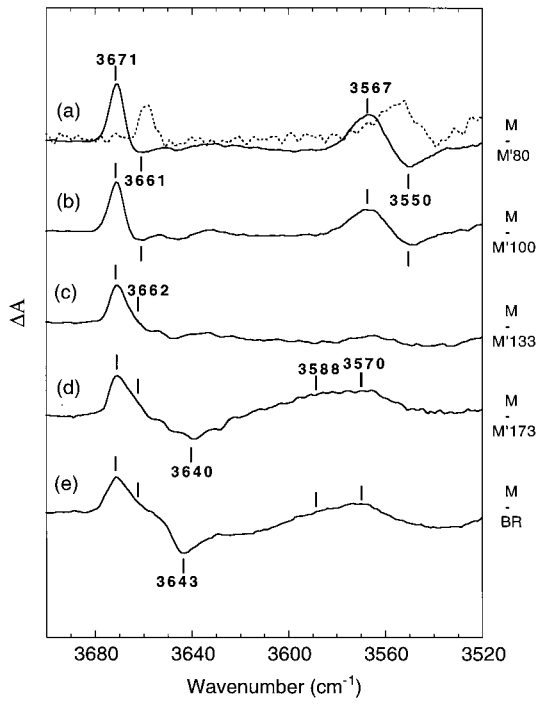

FIGURE 5: Comparison of the perturbations of the O−H stretching vibrations of water molecules in various M′ states of the wild type in the 3700−3520 cm^{-1} spectral region for (a) M minus M′80, (b) M minus M′100, (c) M minus M′133, (d) M minus M′173, and (e) M minus BR spectra. Solid lines are for the film hydrated with H_2O, a broken line (in a) for the film with $H_2{}^{18}O$. Labels were omitted if the frequency of the corresponding bands is identical with that in the first spectrum in which it appeared. Full length of the ordinate corresponds to 0.027 absorbance unit for the wild type.

of the shift of the Asp96 band seen in the M minus BR spectrum (*35*).

The amplitude of the 1742 cm^{-1} band of Asp96 in the M minus M′ spectrum in the D115N mutant decreases upon warming to 100 K, decreases further upon warming to 133 K (dotted line in b and c), and almost disappears at 173 K (dotted line in d). The negative band at 1749 cm^{-1} also decreases at 100 K. These results indicate that Asp96 undergoes a perturbation at 80 K and then returns to the initial BR state at higher temperatures.

As noted above, in the spectrum of the M → M′80 transition, the contribution from the perturbation of Asp115 to the 1742 cm^{-1} band is smaller than the contribution from Asp96, but it increases at higher temperatures (Figure 3, b−d). The 1742 cm^{-1} band of Asp115 in M shifts to 1734 cm^{-1} in M′80 and M′100 (broken lines in a and b). At 173 K, most of changes around 1742 cm^{-1} are due to Asp115 (compare solid line and broken lines in d). The latter observation suggests that Asp115 undergoes a small perturbation upon isomerization of the chromophore, but experiences larger additional changes upon reprotonation of the Schiff base.

Inhibition of the Perturbation of Asp85 in the M → M′ Transition by Mutations of the Cytoplasmic Residues, Val49 and Thr46. Some mutants in the cytoplasmic domain are known to affect the formation and decay of the M intermediate. T46V accelerates both the formation and the decay of M (*19, 41*). V49A enormously decelerates the formation of M, so that the steady-state amplitude of M is very low (*41*). To establish whether the mutation of these residues affects the environment of Asp85, we examined the FTIR difference spectra that accompany the photoconversion of M in these mutants.

Figure 4 shows the effects in the M to M′ transition of V49A (b) and T46V (c) on the 1762 cm^{-1} band of Asp85, and on the 1742 cm^{-1} band of Asp96 seen in the wild-type pigment (a). These mutations did not affect the 1761 cm^{-1} band of Asp85 in the M minus BR spectra (*6*). However, it is worth examining the 1762 cm^{-1} band in the M to M′80 transition (Figure 3, a) because here the 1762 cm^{-1} band is due to the perturbation rather than the deprotonation of Asp85. The T46V mutation markedly decreases the 1762 cm^{-1} band of Asp85 (Figure 4, c), suggesting an indirect interaction from Thr46 to Asp85 in M. The 1762 cm^{-1} band decreases even more in the V49A mutation (b). This suggests that Val49 is at or near the locus that perturbs Asp85 in the M to M′ transition.

Evidence for the Interaction of Thr46 and Asp96 in M. The 1742 cm^{-1} band of Asp96 of M is not seen in the M minus BR spectrum (see Figure 3, e), but as described above, it is present in the M minus M′80 spectrum (Figure 3, a). This band shifts from 1742 cm^{-1} in the M of wild type (Figure 4, a) to 1747 cm^{-1} in the M of T46V (c). This shifted band is due to Asp96 because it disappears in T46V/D96N (not shown). This shift of the C═O stretching vibration of Asp96 to a different frequency in the M of T46V indicates an interaction of Thr46 with Asp96 in M. A similar interaction between Thr46 and Asp96 is present in L (*19, 44*).

Changes in Internal Water Bands Produced by the Photoconversion of M. The M to M′ transition at 80 K causes two prominent spectral changes in the 3700−3520 cm^{-1} region which contains the O−H stretching vibrations of water (Figure 5): one is a sharp band of M at 3671 cm^{-1} which is accompanied by a small negative band at 3661 cm^{-1}, and the other is a bilobe with 3567(+) and 3550(−) cm^{-1} bands. These bands shift in $H_2{}^{18}O$ (a, dotted line) and can be assigned to water molecules undergoing a perturbation upon

the photoisomerization of the chromophore during the M → M′ transition at 80 K. These changes of water bands produced by the M → M′ photoreaction are entirely different from what we observed for the L → L′ photoreaction at 80 K (*5*) or those described for the BR → K photoreaction (*13*, *45*).

Upon reprotonation of the Schiff base at 133 and 173 K, other changes occur. At 133 K, the bilobe of 3567 cm^{-1}(+) and 3550 cm^{-1}(−) bands almost disappears (c). At 173 K, a broad positive band of M in the 3600−3550 cm^{-1} region appears simultaneously with a negative band at 3640 cm^{-1}, and also a shoulder at 3662 cm^{-1} (c) becomes visible.

To elucidate the origin of these bands and their probable location, it is necessary to consider the water bands identified earlier in the M minus BR spectrum (*6*). Generally, water exhibits two O−H stretching vibrations, asymmetric and symmetric vibrations, that are separated by ∼100−150 cm^{-1} from each other in the region above 3000 cm^{-1}. Different hydrogen-bonding strength in a heterogeneous environment of a protein would also contribute to the two bands. In this case, the higher frequency band is due to weaker hydrogen bonding. The bands we observed were probably only the higher frequency bands of different water molecules. The lower frequency bands (below 3520 cm^{-1}) are difficult to observe because of band widening, larger noise, and baseline distortions. Nevertheless, some speculation on the hydrogen bonding strength can be made regarding the higher frequency bands (see *12*, *14*, and references cited therein).

Perturbation of Wat85 in the M → M′ Transitions. The 3643 cm^{-1} band of BR is absent in the D85N mutant (*46*). The water molecules that comprise this band in BR must be close to Asp85, and are collectively named Wat85 in the present paper. The 3643 cm^{-1} band of BR seen in the M minus BR spectrum (Figure 5, e) is nearly missing in M′80 (a), M′100 (b), and M′133 (c). It appears as a broad band at 3640 cm^{-1} in the M minus M′173 spectrum (d). A broad positive band in the 3600−3550 cm^{-1} region in the M minus BR spectrum (e) shifts in $H_2{}^{18}O$ (*6*) and was previously assigned to the O−H stretching vibration of water in M. A similar feature is present in the M minus M′173 spectrum, but absent in the M minus M′ spectrum at 80 K (c), 100 K (b), and 133 K (a). Thus, this feature corresponds to the negative band at 3643 cm^{-1} and can be assigned to Wat85 in M.

This broad positive band seems to be composed of two bands at 3588 and 3570 cm^{-1}. Since the bandwidth of a water O−H stretching vibration tends to be narrower with a slight lower frequency shift at lower temperature, the M minus BR spectrum was recorded at 80 K. Though the baseline was greatly distorted, it is clear that the broad water band in the spectrum of M is resolved into two bands at 80 K. The frequencies of the maximum intensities of these bands were determined in the second derivative of this spectrum at 3584 and 3561 cm^{-1} (not shown in figures). The corresponding 3643 cm^{-1} band of BR may arise from two water O−H bonds as suggested by its intensity decreasing by half in the L minus BR spectrum of V49A (*20*). The positive band of the bilobe at 3567 cm^{-1} observed in the M minus M′80 spectrum might correspond to the 3570 cm^{-1} band of M at 230 K, though other water molecules cannot be excluded.

In M′133, half of the Schiff bases are protonated. However, no substantial changes in water bands take place except for the 3671 cm^{-1} band. The previous visible spectroscopic experiments (*24*, *25*) show that P565 (the species with the protonated Schiff base in M′133) reverts to M by the photoreaction at 100 K. The absence of these water bands in the difference spectrum between M′133 and M at 100 K (data not shown) further confirms that these water molecules are not perturbed in the M ⇔ P565 transitions.

Perturbations of Wat219 in the M to M′ Transitions. A water O−H band at 3671 cm^{-1} and its shoulder band at 3662 cm^{-1} seen in the M spectrum (*44*) are completely depleted in F219L and are affected in T46V and D96N[2] (*6*). These water molecules are called Wat219 in the present paper. The frequencies at 3671 and 3662 cm^{-1} indicate that the O−H bonds of Wat219 in M must be engaged in a very weak hydrogen bond. The corresponding bands in BR have not been detected (*6*) and so must be below 3520 cm^{-1} or present even in the 3620−3520 cm^{-1} region with a very low intensity or overlapping with other bands. Other water molecules in the cytoplasmic domain, which show the 3607 and 3577 cm^{-1} bands in the BR state (*19*), did not appear in the M → M′ transitions.

The band around 3671 cm^{-1} in the M minus BR spectrum (Figure 5, e) exhibits an asymmetrical shape due to the presence of a shoulder at 3662 cm^{-1} (*47*). The 3671 cm^{-1} band continues to be asymmetrical in the M minus M′173 spectrum (d), and in the M minus M′133 spectrum (c), but appears symmetrical in the M minus M′80 and M minus M′100 spectra (a and b). A small negative band at 3661 cm^{-1} is observed in these spectra. The symmetrical shape of the 3671 cm^{-1} band did not arise from a sharpening of the band by lowering the temperature, because the 3671 cm^{-1} band of M produced by illumination at 230 K (e) has the same asymmetrical shape, when recorded at 80 and 100 K (not shown). Thus, the symmetrical shape must arise from the depletion of the shoulder at 3662 cm^{-1}. This shoulder belonging to M must be present in all the spectra, and the lack of the shoulder near 3662 cm^{-1} in the M minus M′80 (a) and M minus M′100 (b) spectra must be due to its cancellation by a negative band at the same frequency, which either could arise by a slight shift of the 3671 cm^{-1} band to 3662 cm^{-1}, or be due to an unchanged position of the 3662 cm^{-1} band in the M → M′ transitions at 80 K, and 100 K.

The presence of the small negative band at 3661 cm^{-1} in the M minus M′80 spectrum of the wild type (Figure 5, a) and the results on the V49A mutant (see below) can be better explained by assuming that the shoulder at 3662 cm^{-1} undergoes a shift to a frequency below 3620 cm^{-1} whereas the 3671 cm^{-1} band undergoes only a slight shift of the O−H stretching vibration to 3662 cm^{-1}. Superposition of these two shifts (the large one for the 3662 cm^{-1} band and a small one for the 3671 cm^{-1} band) would correspond to the changes seen in Figure 5, a. The presence of both the 3671 cm^{-1} band and the 3662 cm^{-1} shoulder in the M minus M′ spectra at temperatures above 133 K and the absence of a sharp negative band at 3662 cm^{-1} (c, d) indicate that both bands shift toward lower frequencies in the region below 3620 cm^{-1}, or are transformed into a very broad band in the 3640−3560 cm^{-1} region seen in the M minus M′133

[2] The 3671 cm^{-1} band in the M minus BR spectrum appeared at 3673 cm^{-1} in the corresponding spectrum of D96N, suggesting that Wat219 is also close to Asp96 (Yamazaki, Kandori, Needleman, Lanyi, and Maeda, unpublished results).

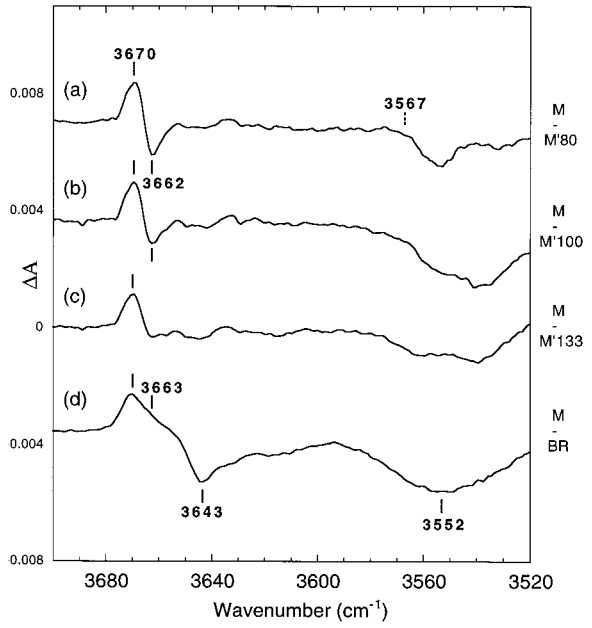

FIGURE 6: The O−H stretching vibrations of internal water molecules of V49A in the 3700−3520 cm^{-1} spectral region observed as a difference spectrum between various species: (a) the M minus M′80, (b) the M minus M′100, and (c) the M minus M′133 spectra, and (d) the M minus BR spectrum. Labels were omitted if the frequency of the corresponding bands is identical with that in the first spectrum in which it appeared. The dotted line in (a) shows the absence of the 3567 cm^{-1} band. Intensities of the spectra were adjusted by normalization at the 1618 and 954 cm^{-1} bands. Full length of the ordinate corresponds to 0.0094, 0.0123, 0.0123, and 0.0182 absorbance units.

spectrum (c). We call the water O−H bonds responsible for the 3671 and 3662 cm^{-1} bands Wat219a and Wat219b, respectively, because these bands are absent in the F219L mutant.

Effect of the V49A Mutation on Wat219. An interesting feature of the V49A mutant is that instead of the 3671 cm^{-1} band, a bilobe with 3670(+) cm^{-1} and 3662(−) cm^{-1} bands is present in the M minus M′80 spectrum (Figure 6, a). The 3662 cm^{-1} negative band becomes smaller at 100 K (b) and disappears at 133 K (c). The shoulder band at 3663 cm^{-1} is, however, present in the M minus BR spectrum (d). The appearance of the negative band at 3662 cm^{-1} in the M → M′80 transition of the V49A mutant (a) can be explained by assuming that the V49A mutation abolishes the large shift seen in the wild type of the Wat219b band from 3662 cm^{-1} to <3620 cm^{-1}, which usually occurs in the M → M′ transition at 80 K (Figure 5, a). Under these conditions, a slight shift of the 3671 cm^{-1} band of Wat219a (3670 cm^{-1} in the mutant) is clearly seen. In the wild type, this shift is barely detectable because of its overlap with the 3662 cm^{-1} positive band of M which undergoes a large shift. This inhibition of the shift of the 3662 cm^{-1} band by the V49A mutation diminishes with increasing temperature; there remains a smaller negative band in the M′100 spectra (Figure 6, b) but none in M′133 (c).

It is interesting to note that the T46V mutation affects the frequency of both the 3671 and 3662 cm^{-1} bands in the M state, causing them to downshift, indicating that the water molecules responsible for these bands are close to Thr46 in M (*6*). The V49A mutation does not affect these frequencies in M, as was shown previously (*6*), but precludes changes of the 3662 cm^{-1} band in the M → M′ transition at 80 K as we found in this study. From these results, we conclude that in the wild type the M → M′ transition involves a change of interaction of Wat219b and Val49. Presumably this water moves closer to Val49 in the M → M′ transition at 80 K. The inhibition in V49A is readily overcome by increasing the temperature to 133 K.

The M minus M′ spectra for the V49A mutant did not exhibit the positive band at 3567 cm^{-1} seen in the wild type (compare Figure 5, a, with Figure 6, a). This band was not detected in the M minus BR spectrum of V49A either (Figure 6, d). However, this could be a result of the cancellation by a large negative band at 3566 cm^{-1}, which is present in the M minus BR spectrum as in the L minus BR spectrum (*20*). It would cancel the 3567 cm^{-1} band in the M minus M′80 spectrum and would leave the negative band at 3552 cm^{-1}.

DISCUSSION

In this study we found that photoisomerization of the chromophore in the M intermediate causes perturbation of water molecules and several other identified residues (Tyr185, Asp115) along with earlier described perturbations of Asp85 and Asp96 (*29*) at 80 K. Subsequent reprotonation of the Schiff base and re-formation of initial pigment are also accompanied by further changes in water bands. Below we discuss the possible origin of these changes and the probable location of participating water molecules.

Relation of Wat85 to the Schiff Base−Asp85 Ion Pair. The present results strongly suggest that the 3643 cm^{-1} band of Wat85 water molecules of BR changes to two bands at 3588 and 3570 cm^{-1} in M. X-ray crystallographic structures show that two water molecules (W402 and W401) are hydrogen-bonded to Asp85 in BR (*17, 18*), and that W402, between Asp85 and the Schiff base, is displaced in K (*48*) and becomes invisible in M_N (*7*). The reason for the latter could be due to the water moved out of the original site, or to the waters becoming more mobile and so not giving rise to a well-defined diffracting site. The frequency at 3570 cm^{-1} of Wat85 in M, which reflects a relatively strong hydrogen bond, may be due to W401, which forms a hydrogen bond with the protonated Asp85 in M_N (*7*), though the possibility that the band is due to Wat402 cannot be excluded completely. FTIR spectroscopy also shows that Wat85 water molecules change to different states in the BR to K transition (*13, 45*).

In the M → M′ transition, the Schiff base does not reprotonate at 80 K even though it should be oriented toward Asp85 after the 13-cis to trans photoisomerization. Meanwhile, Wat85 stayed almost in the same state as in M except for a possible slight shift of the 3567 cm^{-1} band to 3550 cm^{-1}. Reprotonation of the Schiff base occurs in M′133 even without the restoration of the O−H stretching vibration band to around 3640 cm^{-1}. However, the restoration of the same C═C stretching frequency was attained only with restoration of the O−H stretching frequency in M′173 to that of BR. Thus, Wat85 is involved in determining the extent of electron delocalization of the retinal chromophore, as reflected in the C═C stretching frequency, by providing a polar environment around the Schiff base and Asp85 ion pair.

Environmental Changes around Asp85 and Asp96 in the M to M′ Transitions and Their Relationship with the

Perturbations of Wat219 and Wat85. While the Schiff base remains unprotonated in going from M to M′80, the C=O stretching vibration of the protonated Asp85 changes from 1762 cm^{-1} in M to 1754 cm^{-1} in M′ (Figure 3, a). Some of the perturbed Asp85 in M′ at 80 K returns to the 1762 cm^{-1} state at 100 K (Figure 3, b) before its deprotonation. The perturbation and relaxation of Asp85 occurs in parallel with a slight shift of a water band from 3567 to 3550 cm^{-1} in the M → M′80 transition and its relaxation in the M to M′133 transition. In this regard, W401, which is hydrogen-bonded to Asp85 in the M-like structure of D96N (*7*), is the best candidate for the water O−H stretching vibration at 3567 cm^{-1}. As described under Results, the 3567 cm^{-1} band of M is most likely derived from the 3570 cm^{-1} band of Wat85 in the M minus BR spectrum.

In M′80 the band of Asp96 at 1742 cm^{-1} undergoes a perturbation (Figure 3, a). The fraction that exhibits the perturbation decreases with increasing temperature. The relaxation of these perturbations of Asp96 occurs in parallel with the relaxation of the perturbation of Asp85. The 3671 cm^{-1} band of Wat219 undergoes a slight perturbation in the M → M′80 and M → M′100 transitions (Figure 5, a), and nearly returns to the initial BR state along with the protonation of the Schiff base, probably at 133 K. In M, this water molecule is present in the region surrounded by Thr46, Asp96, and Phe219 [see above and (*6*)]. Asp96 is perturbed at 80 K and relaxed by increasing temperature. Thus, perturbation of Asp96 is coupled with the perturbation of nearby water molecules that are also close to Phe219 and Thr46.

How are Asp85 and Asp96 affected by the isomerization of the chromophore in the M to M′80 transition? One possibility is that water molecules interacting with Asp85 and Asp96 are perturbed in the M to M′ transition at 80 K in response to structural changes of the amino acid residues around the chromophore that occur upon isomerization. The present study detected changes of environments of Val49 (Figure 6), Asp115 (Figure 3), and Tyr185 (Figure 2), of the interaction between Thr46 and Asp96 (Figure 6), and of the effects on Asp85 by V49A (Figure 6), T46V (Figure 6), and F219L mutations (not shown). The γ-methyl group of Val49, which is separated by about 3.5 Å from the side chain of Lys216 in BR (see Figure 1) (*17, 18*) but upon isomerization (in M_N) moves closer to the γ-methylene of Lys216 (3.3 Å), is a possible site that is affected by the isomerization. Several effects of this contact were discussed previously (*43*). An alternative possibility is that changes of the dipole moment of the chromophore during the photoreaction perturb water molecules and residues interacting with them (*30*). Finally, one cannot exclude a possibility that one of the water molecules (Wat85) is bound to the Schiff base and is directly perturbed by photoisomerization. Such a water molecule bound to the Schiff base in M was proposed earlier in molecular dynamics calculation (*9*) but has not been observed in the X-ray study of the M-like intermediate of D96N mutant pigment (*7*).

Relocation of Wat219b to Val49 in the M → M′ Transition. In the M → M′ transition at 80 K, the shoulder band at 3662 cm^{-1} due to Wat219b is perturbed. This perturbation is inhibited in V49A at 80 K (Figure 6, a), suggesting the relocation of Wat219b closer to Val49 in M′. Thus, the relocation of Wat219 to the site close to Val49 in the M to M′80 transition may be one of the reasons causing the perturbations of both Asp96 and Asp85 by the isomerization of the chromophore (see below). In agreement with this, the perturbation of Asp85 in the M to M′80 transition is markedly suppressed in the V49A pigment (Figure 4, b).

Water Relocation in the L to M to N Transitions. Our previous studies on the photoreaction of L at 80 K (*5*) revealed that at least two internal water molecules have similar O−H stretching vibration frequency. This was taken as evidence for a water cluster around the Schiff base in L and possibly in its photoproduct, L′ (*5*). The size of the water cluster became smaller in T46V and was restored in T46V/D96N, in parallel with the speeding up and restoration of the L to M transition (*19*). We suggested that this cluster is formed by the water molecules that are present in the Thr46−Asp96 region (Wat46) and another internal water molecule in the cytoplasmic domain. In contrast to the effect of the T46V mutation, enlargement of the water cluster by additional water molecules in the V49A pigment (*5*) is accompanied by a retardation of the L → M transition (*43*). These results suggest that L is favored in the L ⇔ M equilibrium when the size of the cluster of water molecules is large. The water molecules in the cluster are in a strong hydrogen bonding state and may be involved in the stabilization of the protonated form of the Schiff base. The hydrogen bonding state as reflected in the O−H stretching frequency of the water cluster is affected in the V49A mutant protein (*5*). Hence, this cluster must be located close to Val49 in L.

Water molecules act as a mobile mediator in the functioning of BR, as suggested in this and in an earlier study (*5*). The same might occur in other proteins also. Relocation of internal water molecules was suggested by a similar FTIR study in the functioning of bovine rhodopsin (*49*). Other examples based on X-ray crystallographic results have been described for serine proteases (*50*), cytochrome *c* (*51, 52*), and cytochrome P450 (*53*).

One important difference between the L → L′ and M → M′ photoreactions is that Asp96 is perturbed only in the latter transition. It is reasonable to suppose that water molecules are absent around Asp96 in L and L′ (*5*). On the other hand, in M, Wat219 is present close to the Asp96−Thr46−Phe219 region and undergoes a perturbation. Thus, the water environment of Asp96 changes in the M → M′ transition, but not in the L → L′ transition. The perturbation of Asp96 is not observed in the BR → K transition at 80 K either. In this photoreaction, relatively small changes of O−H bands presumably due to water molecules in the vicinity of Asp85 and the Schiff base (Wat85) were detected (*13, 45*).

The cluster of water molecules in L forms strong hydrogen bonds presumably with the protonated Schiff base. Our present results indicate the presence of at least two water molecules in the cytoplasmic domain that undergo perturbation along with Asp96 upon 13-cis → trans photoisomerization of the chromophore in the M → M′ transition. However, a hydrogen-bonded chain connecting Asp96 to the Schiff base by only water molecules does not seem to exist in the M state. Such a pathway was also not detected in the X-ray structure of M_N of D96N (*7*). A network of hydrogen bonds might be formed in a transition state between M and N. An alternative possibility is that such a complete network does not form and the proton is conveyed by a water molecule which shuttles between a site close to Asp96 and

the Schiff base in the M to N transition. This suggests that the relocation of Wat219 closer to Asp96 in M (*6*) may initiate the transfer of a proton from Asp96 to the Schiff base. In N, Wat219 is closer to the Schiff base and may be involved in stabilizing its protonated form (*6*).

It is worthwhile to point out some similar features of the M to M′ photoreaction and the M to N transition despite different configuration of the chromophore: both reactions are accompanied by the lower frequency shifts of the 1761 cm^{-1} band of Asp85 to 1754 cm^{-1} (*54*), and the 3671 cm^{-1} band of Wat219 to 3654 cm^{-1} (*6*). These similar perturbations could be brought about by the relocation of Wat219 toward Val49 in M′ as in N (*6*) from the site surrounded by Thr46, Asp96, and Phe219 in M.

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