Relocation of Water Molecules between the Schiff Base and the Thr46−Asp96 Region during Light-Driven Unidirectional Proton Transport by Bacteriorhodopsin: An FTIR Study of the N Intermediate†

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ABSTRACT: A key event in light-driven proton pumping by bacteriorhodopsin is the formation of the L intermediate, whose transition to M is accompanied by the first proton transfer step, from the Schiff base to Asp85 on the extracellular side. Subsequent reprotonation of the Schiff base from the other side of the membrane to form the N intermediate is crucial for unidirectional proton transport. Previous FTIR studies have suggested that the intense water O−D stretching vibration bands which appear in L at 2589, 2605, and 2621 cm^{-1} are due to a cluster of polarized water molecules connecting the Schiff base to the Thr46−Asp96 region closer to the cytoplasmic surface. In the present study the difference spectrum was obtained of the N intermediate with its photoproduct N′, formed after irradiating N at 80 K. The water O−D stretching vibrations of N appear as a broad feature in a similar frequency region with a similar intensity to those of L. This feature is also affected by T46V like in L. However, the intensities of these water vibrations of N nearly returned to the initial unphotolyzed state upon formation of N′, unlike those of L which are preserved in L′. An exception was V49A, which preserved the intense water vibrations of N in N′. The results suggest that both L and N have a water cluster extending from the Schiff base to Thr46. The surrounding protein moiety stabilizes the water cluster in L, but in N it is stabilized mostly by interaction with the Schiff base.

Bacteriorhodopsin carries out unidirectional proton transport across the purple membrane of *Halobacterium salinarum* utilizing light energy absorbed by its all-trans retinal chromophore. The retinal is linked to Lys216 of the apoprotein through a protonated Schiff base linkage. This site is directly involved in proton transport which occurs as a cyclic multistep process. After the all-trans-to-13-cis photoisomerization of the chromophore during the primary light reaction (BR-to-K transition),[1] the Schiff base undergoes deprotonation in the L-to-M transition and subsequent reprotonation in the M-to-N transition (*1*). The protonation of Asp85 on the extracellular side in M (*2*) and the deprotonation of Asp96 on the cytoplasmic side in N (*3*) are crucial steps in proton transport from and to the Schiff base, respectively.

The L-to-M transition is driven by changes in the chromophore and its environment which cause the lowering of the pK_a value of the Schiff base (*4*) and an increase of the pK_a value of Asp85, to >11 (*5*). L may be like a transition state, which creates conditions for proton transfer to Asp85 while keeping the Schiff base in a protonated state. The free energy level of L is not far from that of M (*6, 7*). The protonated state of the Schiff base in L is stabilized by its interaction with water molecules (*8, 9*). Stabilization of the protonated state of Asp85 in M also involves rearrangement of waters (*10, 11*).

Previous FTIR studies have found that L formation is accompanied by the rise of intense water bands (*8, 12*). These probably are the result of the polarization of water O−H bonds in L due to their interactions with polar bonds of the apoprotein and the chromophore. Mutant studies have suggested that L formation induces a polarized water cluster extending from the Schiff base to the region consisting of the backbone amide of Gly220 and the side chain of Thr46 (*9, 13*). Upon formation of L′[2] by the irradiation of L at 80 K, which is presumably accompanied by a change in the orientation of the Schiff base, the intense water band of L remains almost unchanged, showing only a slight frequency shift (*14*). The vibration bands of Asp96 and Val49 are also unchanged. The vibration bands of perturbed Asp96 and Val49 are also unchanged. The intense water band is detected even in the initial unphotolyzed states of the L93M and W182F, two residues located close to the chromophore, in which the formation of L occurs at much lower temperatures

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[1] Abbreviations: BR, initial, unphotolyzed state of light-adapted bacteriorhodopsin; HOOP, hydrogen out-of-plane vibration.

[2] L′, M′, and N′ are photoproducts of the L, M, and N intermediates at 80 K. Note that N′ in this paper is different from the N′ used by Schobert et al. (*22*).

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than for wild type (*15*). These observations suggest that a specific structure of L on the cytoplasmic side of the Schiff base accommodates a string of polarized water molecules which stabilizes L relative to M and helps to retain the proton on the Schiff base in L. A similar string of water molecules was recently detected in an X-ray crystallographic structure of L (*16*).

The isomeric form of the chromophore of N is 13-cis, 15-anti (*17, 18*). The Schiff base, deprotonated in forming M, is reprotonated by the proton of Asp96 (*3*), which has a pK_a of ~7 in late N (*19, 20*). The pK_a value of the Schiff base in N is 8.3 for D96N (*21*). The changes in proton affinity of the key groups in going from L through M to N ensure unidirectional proton transfer in the photocycle. The recent X-ray structure of the N intermediate of the V49A mutant by Schobert et al. (*22*) shows a string of H-bonding water molecules from the Schiff base to Asp96. This water chain is similar to that in the L structure mentioned above (*16*).

Earlier FTIR studies on the water vibration bands in M and N have revealed water O−H bands at 3671 cm^{-1} for M and 3654 cm^{-1} band for N (*23*). However, the frequency region corresponding to the intense water bands in L was not examined due to low signal/noise and baseline drift. In order to circumvent these difficulties, N produced by irradiation at 260 K was rapidly cooled to 80 K and the difference spectrum between N and its photoproduct at this temperature, called here N′, was measured to obtain the N-minus-N′ spectrum (*24, 25*). This spectrum is expected to reveal the water bands of N, in a manner similar to the main water bands of L and M which were previously observed in the L-minus-L′ (*14, 15*) and M-minus-M′ (*10*) spectra at 80 K, respectively. This approach is also useful in order to see whether the orientation change of the Schiff base in going from N to N′ affects water molecules in N.

In the present study FTIR spectra in the region where water vibrations are observed were examined for the N-minus-N′ spectra of wild type and of the T46V, T46V/D96N, and V49A mutants. These spectra were compared with those of L minus L′ for these same species. Our results indicate that an intense set of vibrations, including water bands similar to the intense water bands in L (*13, 15*), are observed in wild-type N. This intense feature disappears in the N-to-N′ photoreaction. The results suggest that the water cluster in N is mainly associated with the Schiff base and is returned to its initial unphotolyzed state upon the change in orientation of the Schiff base after photoisomerization of the chromophore by the irradiation of the pigment at 80 K to form N′. In contrast, the water cluster in L is surrounded by a more rigid protein structure and so is largely unchanged in the L-to-L′ photoreaction. These findings have several implications for the mechanism of proton transport from and to the Schiff base and are discussed below.

MATERIALS AND METHODS

The mutant pigment proteins of bacteriorhodopsin, expressed in *H. salinarum*, and then purified in the form of purple membranes, T46V, T46V/D96N, and V49A, were described previously (*8, 23, 26, 27*). These pigment proteins were kindly provided by Janos K. Lanyi of the University of California, Irvine.

The films of bacteriorhodopsin were made from purple membrane suspensions in water, which were obtained after three cycles of centrifugation in 1.4 mL of water starting from 0.2 mL of initial suspensions of the purple membranes in 1 mM phosphate buffer (pH 7.0). A 70-μL aliquot was placed on a BaF_2 window (13 mm in diameter) and dried in a vacuum. The films were hydrated by placing 1 μL of D_2O or $D_2{}^{18}O$ (obtained from ICON, D 99%, ^{18}O 95%) at the edge of the film before sealing it in a brass cell-holder with a 2-mm-thick Teflon spacer and a BaF_2 blank window. Rehydration with D_2O or $D_2{}^{18}O$ shifts the water vibrations to a frequency region of the spectrum where there is little interference from other vibration modes.

The film was installed into an Oxford cryostat Optistat with an Oxford Intelligent Temperature Controller, and was illuminated with >440-nm light (a Corning 3-73 filter transmitting light of wavelengths longer than 440 nm) from a slide projector for 3 min at 283 K for light-adaptation before cooling to the desired temperature. Photoconversion of light-adapted bacteriorhodopsin (BR) to the N intermediate was carried out by irradiation with >520-nm light (a Corning 3-69 filter transmitting light of wavelengths longer than 520 nm) for 1 min. The irradiation of wild-type bacteriorhodopsin at 260 K produced N and M, both of which decayed in a few minutes (*28*). The photoproducts were cooled rapidly by fully opening the screw for liquid nitrogen flow in the cryostat (~10 K/min), followed by shutting off the irradiation when the temperature decreased to 257 K. It reached 80 K in ~20 min. L, with a small amount of M, is produced by irradiation at 170 K with >590-nm light (a Corning 2-62 filter transmitting light of wavelengths longer than 588 nm). L was stable at this temperature, and was cooled to 80 K after the recording of the L-minus-BR spectrum at 170 K. No transformation of L to K while lowering the temperature as described by Kouyama et al. (*16*) for their crystals was observed in the hydrated films (not shown). In order to induce the photoconversions of the L and N intermediates at 80 K and obtain the L-minus-L′ and N-minus-N′ difference spectra, the photoproducts formed at 170 K and at 260 K were illuminated at 80 K with 520-nm light (a Corning interference filter transmitting light at 520 nm) for 2 min, and subsequently at 80 K with >640-nm light (a Corning 2-64 red filter) for 1 min to photoconvert any K formed back to BR.

FTIR spectra were measured with a BioRad (Varian) FTS6000 FTIR spectrometer as described previously (*13*). Absolute spectra were obtained as an average of 8 spectra, each of which was constructed from 256 interferograms (2-cm^{-1} resolution). The difference spectra at 80 K were calculated by subtracting the absolute spectra after irradiation from the absolute spectra before irradiation. Finally, all the photoproducts were returned to the initial unphotolyzed state by warming the samples above 283 K. All the data presented are the averages of eight independent measurements.

RESULTS

Water O−D Vibration Bands in N and N′. The difference spectrum for the water O−D vibrations in N and N′ can be obtained by optimizing conditions for N and N′ formation and subtracting out contributions from other species. It was previously shown, by conventional rapid scan spectroscopic methods, that formation of N together with a comparable amount of M can be observed after irradiation of bacteriorhodopsin with orange light at 260 K (*28, 29*). M and N

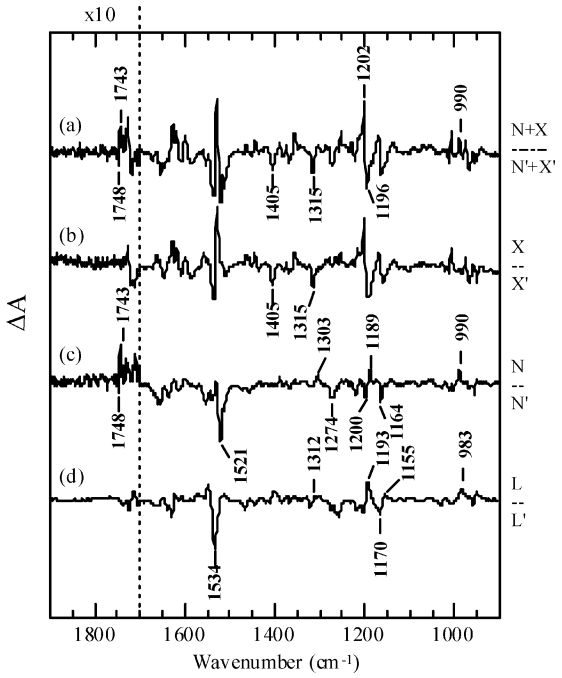

FIGURE 1: The FTIR difference spectra for the region showing the bands characteristic of N. The amplitudes of the spectra were expanded 10-fold in the 1900−1700 cm^{-1} region. (a) The spectrum upon irradiation at 80 K with 520-nm light and then >640-nm light of a sample with prior irradiation with orange light at 260 K to form N. (b) The corresponding spectrum without prior irradiation at 260 K; and so in the absence of N. Spectrum b was normalized by adjusting the intensities of the negative bands at 1405 and 1315 cm^{-1} to those of spectrum a. (c) The N-minus-N′ spectrum obtained by subtraction of spectrum b from spectrum a. (d) The L-minus-L′-spectrum. The sum of the absolute intensities of the bilobe of 1189 (+) and 1164 (−) cm^{-1} for the N-minus-N′ spectrum (spectrum c) was adjusted to those of the bilobe of 1193 (+) and 1170 (−) cm^{-1} for the L-minus- L′ spectrum (spectrum d). This adjustment of the amplitude of the spectrum for the intensity change in the C−C vibration bands upon isomerization gave an appropriate scaling of the two spectra. The full length of the ordinate is 0.0110 absorbance unit for spectra a and c, 0.0139 absorbance unit for spectrum b, and 0.266 absorbance unit for spectrum d in the 1700−900 cm^{-1} region. These numbers should be multiplied 10-fold in the 1900−1700 cm^{-1} region.

decayed in a few minutes. However, both M and N can be trapped by rapid cooling to 80 K. The spectral features of N were examined at 80 K by irradiation with 520-nm light, followed by a second irradiation with >640 nm light. The 520 nm light does not induce any changes in M, whereas the irradiation with >640-nm light was used to return any K, which is present (produced from BR at this temperature), back to the initial unphotolyzed state. The difference spectrum obtained by subtracting the spectrum after these two irradiations at 80K from that before these irradiations should contain the N-minus-N′ spectrum.

Before analyzing the water bands we examined the difference spectrum in the 1900−900 cm^{-1} region for the presence of N-specific bands (Figure 1a). The positive band at 1743 cm^{-1} is due to protonated Asp85 and is specific to N (*3*) as is the chromophore band at 990 cm^{-1}, due to the C_{15}-hydrogen out-of plane vibration (HOOP) in D_2O (*30*). However, this difference spectrum also exhibits positive and negative bands at 1202 and 1196 cm^{-1}, respectively, due to the conversion of the all-trans chromophore to the 13-cis form. These unexpected bands suggest the presence of photoproducts other than N. Figure 1b shows a control, in which the sample had not been irradiated at 260 K and so contained no N, was cooled to 80 K, and was irradiated. A similar bilobe band shape, due to the photoconversion of the all-trans chromophore to the 13-cis form, appears in the difference spectrum. This bilobed band must arise from a species other than K because it remains after irradiation with >640-nm light (which eliminates all the K). We call this unknown species X. Judging from the intensities it may comprise about 1−2% of the whole sample. The spectrum without N (Figure 1b) was subtracted from the one with N (Figure 1a) by scaling the common bands at 1405 and 1315 cm^{-1} in both spectra. The resulting spectrum is shown in Figure 1c.

The spectrum in Figure 1c can be regarded as the N-minus-N′ spectrum of wild type. Its positive side exhibits bands characteristic of N. The 1743-cm^{-1} band and the negative band at 1748 cm^{-1} are due to a slight perturbation of protonated Asp85, whose vibration appears at 1745 cm^{-1} in the N-minus-BR spectrum recorded in D_2O (*3*). The M-specific positive band of Asp85 at 1749 cm^{-1} in D_2O is clearly absent. The intense 1303-cm^{-1} band due to the C_{15}−H in-plane bending vibration of the chromophore (*28*), the 1189-cm^{-1} band due to the 13-cis chromophore, and the 990-cm^{-1} HOOP band are all characteristic of N. These bands are distinct from the corresponding bands of L, as shown by comparison to the L-minus-L′ spectrum (Figure 1d; see also ref *31*). The C−C stretching bands of N′ at 1200 and 1164 cm^{-1} are similar to those of BR (reviewed in ref *31*), indicating that the N′ chromophore is all-trans. The negative C=C band at 1521 cm^{-1} suggests that the absorption spectrum of N′ is red shifted compared to N.

Figure 2 shows the spectral region where the water O−D stretching vibrations occur. The spectrum obtained by irradiation at 80 K of the photoproduct formed at 260 K (solid line in Figure 2a) exhibits a broad feature in the 2640−2570 cm^{-1} region. This feature is shifted by ∼16 cm^{-1} toward lower frequencies in $D_2{}^{18}O$ (dotted line in Figure 2a) showing that it is due to O−D vibrations of water. The corresponding spectrum without prior irradiation at 260 K, and so without N (solid line in Figure 2b), is nearly flat in this region. The broad noisy feature of N in the N-minus-N′ spectrum (solid line in Figure 2c) roughly coincides with the set of water O−D bands of L at 2621, 2605, and 2589 cm^{-1} (superimposed dotted line in Figures 2c and 2d) in frequency and relative amplitude. This suggests that N has similar water O−D vibration bands to those of L, which are due to a water cluster. For the L-minus-L′ spectrum, the intensity of the water O−D band at 2589 cm^{-1} and possibly of the 2605-cm^{-1} band was preserved in L′ with a slight frequency shift to 2629 cm^{-1} (solid line in Figure 2d). This band is quite unlike the original water band in BR which only has weak intensity at 2666 cm^{-1}, as seen in the L-minus-BR spectrum (dotted line in Figure 2c,d). This suggests that besides the Schiff base the apoprotein is involved in the interaction with the water molecules in L and L′ and its conformation largely determines the high intensity of these water bands. In contrast to L, when N is converted to N′, its water band is changed to a much less intense water band at 2657 cm^{-1} (solid line in Figure 2c). This suggests that waters in N interact with only the Schiff base and the movement of the Schiff base upon the isomerization from N-to-N′ transition disrupts its interaction with these waters.

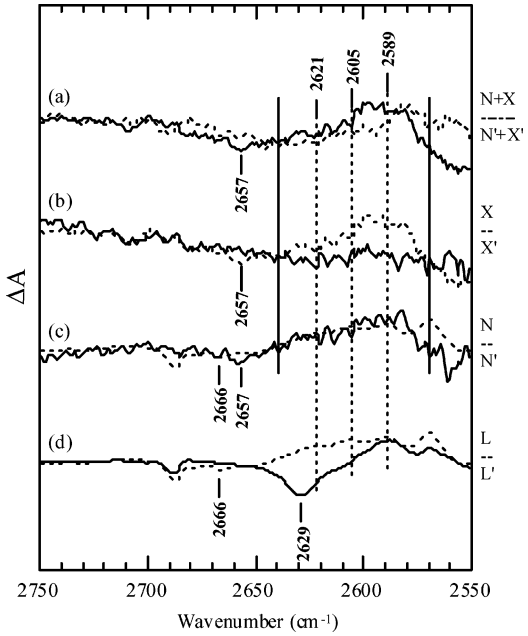

FIGURE 2: The water O−D stretching vibration bands of wild-type BR for the difference spectra accompanying the photoconversion of N and of L. (a) Spectra obtained as for Figure 1a. Solid and dotted lines are for the film hydrated with D_2O (the other part of Figure 1a) and with $D_2{}^{18}O$, respectively. A broad feature due to a cluster of waters is delineated by the two vertical solid lines at 2640 and 2570 cm^{-1}. (b) Solid line is the spectrum obtained as for Figure 1b; and so in the absence of N (the X-minus-X′ spectrum). The spectrum in the presence of N is duplicated from part a by a dotted line for comparison. (c) Solid line is the N-minus-N′ spectrum (obtained as described in Figure 1c), compared with the L-minus-BR spectrum (dotted line). (d) Solid and dotted lines are the L-minus-L′ and L-minus-BR spectra, respectively. These two spectra are scaled by the HOOP band at 983 cm^{-1} specific to L in D_2O (Figure 1d). The full length of the ordinate is 0.00125 absorbance unit for both the spectra in part a, the dotted line in part b, and the solid line in part c and 0.00167, 0.0305, and 0.139 absorbance unit for the solid line in part b, the solid line in part d, and both the dotted lines in parts c and d, respectively. The legend on the outside of the right ordinate gives the components of the difference spectra shown with solid lines.

Effects of T46V and T46V/D96N Mutations on the Water Bands in N and N′. It is known that the intensities of the water O−D bands of L at 2621, 2605, 2589 cm^{-1} are decreased by the T46V mutation with only a small band remaining at 2587 cm^{-1} (*13*). These water bands are partially restored with the additional mutation of D96N in T46V/D96N. Asp96 is close to Thr46, and relatively far from the Schiff base (∼1 nm). Hence these water bands are assigned to the water molecules present in the region between the Schiff base and the Thr46-Asp96. In the N-minus-BR spectrum of the hydrated film at pH 7 a large fraction of Asp96 appears protonated in contrast with fully unprotonated Asp96 at pH 10 (*28*). This is in agreement with time-resolved spectra at room temperature and with estimates of the pK_a of Asp96 in N of ca. 7.5 (*19, 20*). In contrast, Asp96 is unprotonated between pH 4 and 8 in the N of T46V (*32*). Under our conditions in T46V, Asp96 should be completely unprotonated in N, while in wild type it should be largely protonated. The effect of the T46V mutation without the negative charge at position 96 can be studied in the double T46V/D96N mutant. The N-minus-N′ spectra in the 1900−900 cm^{-1} region of T46V (Figure 3b) and T46V/D96N (Figure 3c) are very similar to the N-minus-N′ spectrum of wild type (Figure 3a), except for the 1700−1500 cm^{-1} region where large amide bands in the absolute spectrum cause uncertainty in the difference spectrum. All the characteristic bands at 1743 (+), 1747 (−), 1303 (+), 1273 (−), 1201 (−), 1189 (+), 1164 (−), and 990 (+) cm^{-1} seen in wild type (Figure 3a) are present in both T46V (Figure 3b) and T46V/D96N (Figure 3c), as indicated by vertical dotted lines. This result is in agreement with an earlier observation that the N-minus-BR spectrum of T46V in the 1900−900 cm^{-1} region is similar to that of wild type (*32*).

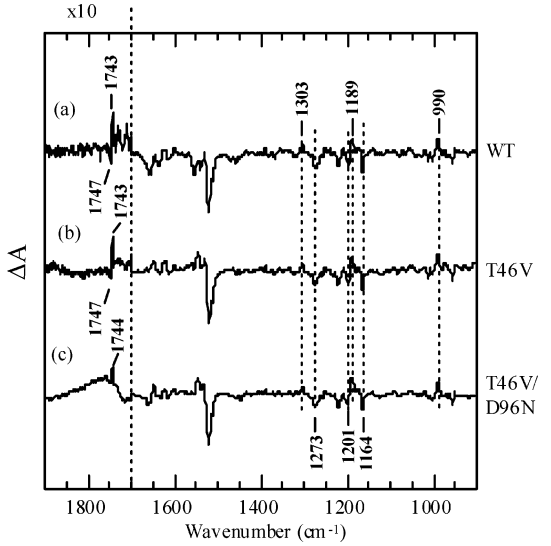

FIGURE 3: Comparison of the N-minus-N′ spectra of the mutants with that of wild type. The amplitudes of the spectra in the 1900−1700 cm^{-1} region were expanded 10-fold. (a) Wild type reproduced from Figure 1c. (b) T46V. (c) D96N. The full length of the ordinate is 0.0041, 0.016, and 0.094 absorbance unit for spectra a, b, and c, respectively. These numbers should be multiplied 10-fold in the 1900−1700 cm^{-1} region.

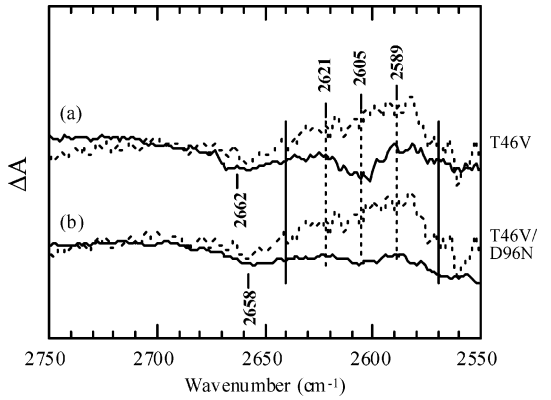

FIGURE 4: The effects of the T46V and T46V/D96N mutations on the water O−D bands in the spectra for the N-to-N′ transition. Each spectrum was superimposed by the spectrum of wild type (dotted lines). (a) T46V. (b) T46V/D96N. A broad feature due to a cluster of waters is delineated by two vertical solid lines at 2640 and 2570 cm^{-1}. The full length of the ordinate is 0.00055, 0.0022, and 0.0126 absorbance unit for the spectra of both dotted lines and the solid lines in parts a and b, respectively.

In Figure 4 the water O−D features seen in the N-minus-N′ difference spectra of these mutants (solid lines) were compared with that of wild type (dotted line). The spectra of the mutants are less noisy because larger amounts of N are trapped, due to the longer lifetime. As discussed above for Figure 2, the broad feature of wild-type N in the 2640−2570 cm^{-1} region looks like it is composed of three water

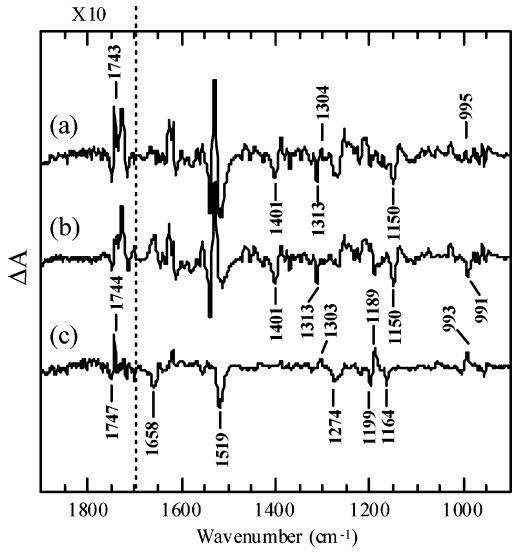

FIGURE 5: The N-minus-N′ spectrum of V49A. The difference spectra for the region showing the bands characteristic of N. The amplitudes of spectra in the 1900−1700 cm^{-1} region were expanded 10-fold. (a) The spectrum is obtained as for Figure 1a. (b) The corresponding spectrum of a sample without N (as obtained for Figure 1b). (c) The N-minus-N′ spectrum obtained by subtraction of spectrum b from spectrum a. The subtraction was done by scaling the bands at 1401 and 1313 cm^{-1}. The difference spectrum in part b contains bands due to BR and X on the positive side and X′ and L, and possibly L′ on the negative side. In addition to these the spectrum in part a contains bands due to N on the positive side and N′ on the negative side. The full length of the ordinate is 0.016 absorbance unit for all the spectra. These numbers should be multiplied 10-fold in the 1900−1700 cm^{-1} region.

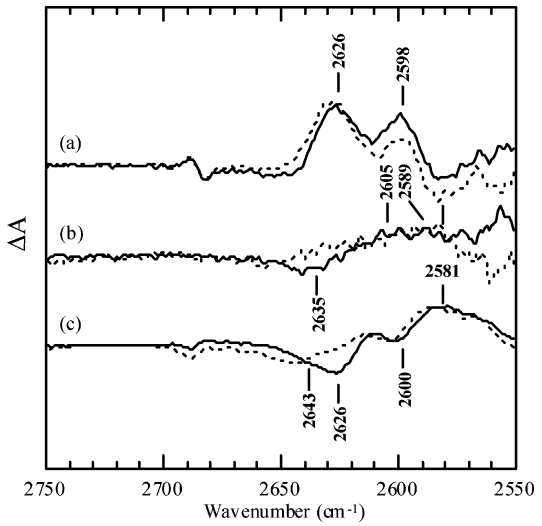

FIGURE 6: The water O−D bands of N of V49A. (a) This spectrum containing N was obtained as for Figure 1 (solid line), and the corresponding spectrum without N was obtained as for Figure 1b (dotted line). (b) The N-minus-N′ spectrum of V49A (solid line) and wild type (dotted line). (c) The L-minus-L′ spectrum (solid line) and the L-minus-BR spectrum (dotted line) of V49A. The full length of the ordinate is 0.0025 absorbance unit for spectra a and b.

O−D bands around 2621, 2605, and 2589 cm^{-1} similar to L of wild type. It can safely be concluded that, in T46V, the bands around 2621 and 2589 cm^{-1} are partially depleted and the band around 2605 cm^{-1} is severely depleted, though tilting of the baseline makes the peak positions somewhat ambiguous. Similar changes of the water O−D bands for T46V/D96N suggest that the deprotonation of Asp96 is not the main cause for the effect of T46V. The negative bands at 2662 cm^{-1} of T46V (solid line in Figure 4a) and at 2658 cm^{-1} of T46V/D96N (solid line in Figure 4b) are very weak in intensity as that of wild type at 2657 cm^{-1} (Figure 2c). The effect on the water O−D bands of the T46V mutation suggests that the water cluster detected in N is located between the Schiff base and Thr46, as in L.

Water Bands for the N and N′ Intermediates of V49A. It is interesting to obtain the FTIR spectrum of the N of V49A, because this mutant was used for an X-ray crystallographic study of N (*22*). We trapped N by irradiation of the V49A mutant at 260 K and subsequent cooling to 80 K in the dark, as was done for wild type and the other mutants. After cooling to 80 K, this sample was irradiated with 520-nm light followed by >640-nm light, giving the spectrum in Figure 5a. This spectrum shows bands at 1743, 1304, and 995 cm^{-1} on the positive side, which are similar to the bands of N in the N-minus-N′ spectrum of wild type (Figure 1c). The spectrum, however, also exhibits the chromophore bands at 1401, 1313, and 1150 cm^{-1} on the negative side, which are similar to the bands of L of wild type (*31*, *33*). The 1401- and 1313-cm^{-1} bands may have contributions from X′, which appeared at 1405 and 1315 cm^{-1} for wild type (Figure 1b). The corresponding spectrum for a sample that was not irradiated at 260 K before cooling to 80 K also shows these L-specific bands along with the HOOP band at 990 cm^{-1} (Figure 5b). The results show that, upon irradiation at 80 K, V49A converts partially to L, as the L93M and W182F mutants also do at this temperature (*15*). In addition, the irradiation converts the L to L′, as expected for L (at 80 K). L and L′ remain even after removal of K by >640-nm light. The subtraction of the spectrum containing L and L′ in addition to X and X′ (Figure 5b) from that which also includes N and N′ (Figure 5a) yields the spectrum of Figure 5c. The positive side of the spectrum in Figure 5c shows the N-specific bands at 1744, 1303, and 993 cm^{-1}, and the negative side contains bands at 1747, 1274, 1199, and 1164 cm^{-1}. These bands are common to the N-minus-N′ spectrum of wild type (Figure 1c), except for very small (1−2 cm^{-1}) differences in frequency.

The spectra of the water bands of V49A corresponding to the pigment states given in Figure 5 are shown in Figure 6. The spectrum for the sample without N (dotted line in Figure 6a) exhibited intense bands due to X of V49A at 2626 and 2598 cm^{-1} on the positive side. The shape of these bands in the spectrum for the sample with N (solid line in Figure 6a) is similar to, but not coincident with, those in the dotted-line spectrum in Figure 6a. The difference between them should be due to the N-minus-N′ spectrum, and is shown in Figure 6b by a solid line. The broad feature thus obtained for V49A in the 2640−2570 cm^{-1} region nearly coincides with the 2605- and 2589-cm^{-1} bands of the N-minus-N′ spectrum of wild type (dotted line in Figure 6b; duplicated from Figure 2c), though with some tilting of the baseline. An intense negative band due to N′ is clearly observed at 2635 cm^{-1} in the N-to-N′ spectrum of V49A (solid line in Figure 6b), in contrast to the absence of such an intense band in N′ of wild type (dotted line in Figure 6b). This bilobed feature, together with the accompanying negative band in the N-minus-N′ spectrum of V49A, is similar to the L-minus-L′ spectra of V49A (a solid line in Figure 6c) and wild type

spectrum (a solid line in Figure 2d), though the frequencies differ slightly and the bands in the L-minus-L′ spectrum of V49A are more intense. These results suggest that waters in the N of V49A interact with the apoprotein in a similar way as waters in the L's of wild type and V49A.

DISCUSSION

Strategy To Obtain the Water O−D Vibration Bands of N. In the present study we investigated the water O−D vibrations of the N intermediate by applying two additional irradiation protocols at 80 K to the mixture of photoproducts initially produced by irradiation at 260 K. The spectral features of N seen in the N-minus-N′ spectrum produced in this way (Figure 1c) exhibited the same features as those seen in the N-minus-BR spectrum obtained at 260 K (*28*). At the same time, irradiation of N at 80 K, which induces photoisomerization of the chromophore and formation of N′, provides novel information about the water molecules in N.

Due to the small fraction of the N intermediate present in the sample after cooling from 260 K, another species which was present in the sample, X, makes a significant contribution to the spectrum. The contribution of X is smaller in the N spectra of T46V and T46V/D96N, probably because of larger amounts of trapped N in these mutants compared to that trapped in wild type. In studying the V49A mutant, the formation of L (and L′) upon irradiation at 80 K (this study) presents an additional problem. These difficulties were remedied by subtracting a second, appropriately scaled, spectrum from a control sample that was not irradiated at 260 K, and genuine N-minus-N′ spectra were obtained.

Specific Structures Accommodating Water in L. The FTIR spectrum of the L intermediate of the wild-type pigment exhibits three intense water O−D bands (dotted line in Figure 2c,d). These are attributed to a cluster of more than three water molecules forming a hydrogen bonded network connecting the Schiff base and the Thr46-Asp96 region in L (*13*). The intensity of these water vibration bands probably is due to the polarization of the water molecules by their interactions with polar bonds of the protein, such as those of Thr46 and Asp96, and the chromophore. The generally apolar environment on the cytoplasmic side of the Schiff base, including Leu93, Val49, Ala215, retinal, and other hydrophobic residues (see Figure 7), may play a role in enhancing the interaction of polar groups with water and so increasing the polarity of the water O−D bonds. Also, the long side chain of Lys216 may contribute to hydrophobicity in this region. The irradiation of the L intermediate at 80 K to produce L′ showed that the intense water bands of L are almost unchanged in L′ with only a slight frequency shift (*14*). In this L-to-L′ conversion, vibration bands of Asp96 and Val49 were unchanged, while those due to a H-bonding network from the Schiff base to Asp115 through a water molecule hydrated to Asp85 returned to their original state (*11*, *14*). These observations suggest that the L intermediate contains a specific structure of the protein formed by Leu93, Val49, Lys216, Gly220, Thr46, and Asp96, which accommodates this string of polarized water molecules. This particular structure containing water molecules is rigid enough to be preserved upon the isomerization of the chromophore from the 13-cis to all-trans form in the L-to-L′ conversion at 80 K.

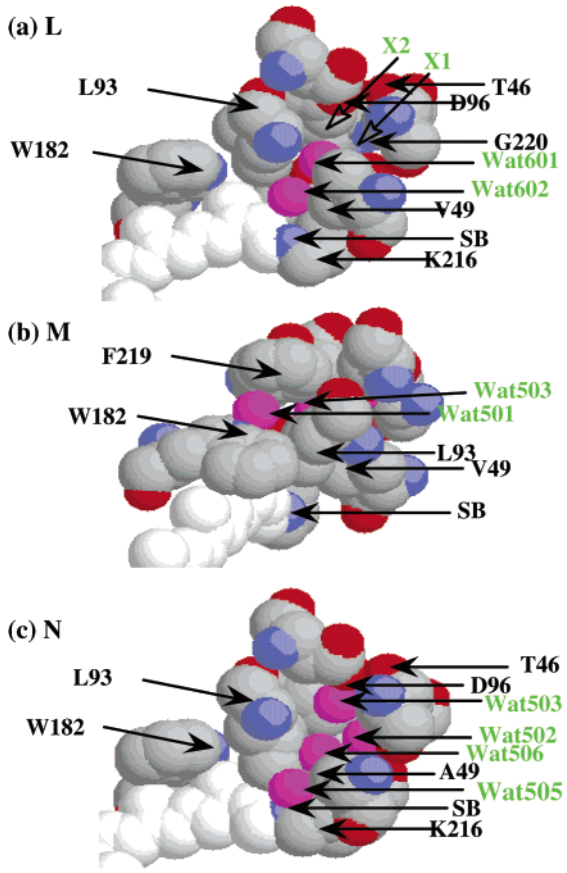

FIGURE 7: X-ray crystallographic structures of the region between the Schiff base and the Thr46−Asp96−Phe219. Oxygen and nitrogen atoms were shown by red and blue, respectively. Water oxygens are shown by pink balls. Gray and white balls are carbons of amino acids and retinal, respectively. SB stands for the Schiff base. (a) The L intermediate depicted on the basis of protein data bank entry 1ucq (*16*). The approximate positions of the two water molecules, X1 and X2, which are described in ref *16* but omitted in 1ucq, were shown by open arrows at the positions close to the backbone carbonyl of Lys216 and carboxyl oxygen of Asp96, respectively. Only an amide nitrogen part can be seen for Gly220, which is involved in the interaction with the water molecule at X2. (b) The M intermediate of E204Q depicted on the basis of protein data bank entry 1f4z (*38*). This picture was shown from another direction in order to show the relation of Wat501 with Wat503 (c) The N intermediate of V49A on the basis of the protein data bank entry 1p8u (*22*).

The various X-ray structures of L are not in agreement with each other (see review by Lanyi (*34*)). Of them, the structure by Kouyama et al. (*16*) coincides more with our FTIR data than the structures of Lanyi and Schobert (*35*) or Edman et al. (*36*) with respect to a string of water molecules from the Schiff base (*9*) to the Thr46−Gly220 region (*13*), the closer approach of Trp182 to the 9-methyl group of the retinal (*37*), and a perturbation of Leu93 (*15*). The X-ray crystallographic structure of the protein data bank entry 1cuq by Kouyama et al. (*16*) (Figure 7a) showed two fixed water molecules, Wat602 and Wat601, linking to the Schiff base, and two positive electron densities, presumably due to partially occupied water molecules. These two are located at the positions close to the backbone carbonyl of Lys216 (X1) and to the carboxyl oxygen of Asp96 (X2), which is H-bonding with Thr46. We suggest that the waters in these sites give rise to the O−D bands at 2621, 2605, and 2589 cm^{-1} in the L-minus-BR spectrum, which were assigned to

the water cluster from the Schiff base to the Thr46−Gly220 region (*13*).

Val49 faces Leu93 on the cytoplasmic side of the Schiff base (Figure 7). Though to a lesser extent than the L93M and W182F mutants (*15*), V49A exhibits L-like intense water O−D bands in its initial state (dotted line in Figure 6c), and at 80 K this state can be converted to L by irradiation (Figure 5b). The features observed with V49A probably arise from the presence of an L-like structure in the initial unphotolyzed state of V49A, in which the water molecules are polarized in a manner which occurs for the wild type only in L. L-like waters have also been observed in the initial states of L93M and W182F (*15*). All three mutations apparently lead to cavities on the cytoplasmic side of the Schiff base. The X-ray structure of V49A, however, does not indicate any additional water molecules filling the cavity produced by the loss of two methyl groups of valine side chain (*22*). Water molecules in the cavity of V49A may not be well-fixed, and hence may be less polarized than the corresponding water molecules of L93M and W182F.

Relocation of Water Molecules in the L-to-M Transition. Previous FTIR studies on M have shown that it has two water O−H bands at 3671 and 3567 cm^{-1} (*10, 23*). In the presence of D_2O two O−D bands at 2714 and 2638 cm^{-1} are observed (not shown). The 3671-cm^{-1} band, along with its side band at 3662 cm^{-1}, is influenced severely by F219L and slightly by T46V (*23*). The X-ray crystallographic structure of M by Luecke et al. (*38*) (Figure 7b; protein data bank entry 1f4z) shows one water molecule (Wat501) between Phe219 and Trp182 and one (Wat503) between Phe219 and Thr46. A theoretical study predicts a large cavity in the region containing Asp96 and Phe219 of M (*39*). The other broad band in the 3600−3550 cm^{-1} region is probably due to two water molecules H-bonding to Asp85 in the crystallographic structure of M (not shown in Figure 7b, discussed in refs *11* and *40*). The intensities of the water O−H vibration bands of M are nearly preserved and their frequencies changed only slightly after isomerization of the chromophore by irradiation of M at 80 K (*10*), as in L. This further suggests that these water molecules are present in cavities surrounded by a protein structure specific for M and are not involved in direct interaction with the lone-pair nitrogen of the Schiff base, which changes its orientation after irradiation. In agreement with this, no water molecules are detected close to the cytoplasmic side of the Schiff base of M (Figure 7b). These features are similar in other crystallographic structures of M (*22, 38, 41*−*43*).

Displacement of waters from near the Schiff base may be responsible for the deprotonation of the Schiff base in M. Alternatively, the loss of the polar character of the chromophore upon the deprotonation of the Schiff base in the L-to-M conversion may be responsible for the water relocation from the Schiff base. These events are accompanied by the closure of the cavity close to the Schiff base with the widening of another cavity around Phe219. The water cluster would primarily be involved in modulating the pK_a's of the Schiff base. It may also be involved in a proton transfer pathway, though there is no experimental evidence for this.

Interaction of Water with the Schiff Base in N. The string of waters in the X-ray crystallographic structure of the N of V49A (*22*), shown in Figure 7c, is similar to that in L reported by Kouyama et al. (*16*). In both structures, there is a water cluster between the Schiff base and the Thr46−Asp96 region. Intense O−D vibrations due to polarized water molecules in N are detected, similar to what was observed for L (Figure 6). Thus, the water cluster observed in the crystallographic structure for N of V49A (*22*) correlates with the present FTIR results. In V49A, the intense water bands in N, similar to those in L, are nearly unchanged upon reorientation of the Schiff base in N′. The N of L93M also preserved the water O−D bands upon the isomerization of the chromophore (not shown), as is also the case with V49A. In the V49A mutant, a cavity to accommodate water molecules (probably Wat505 and Wat506) in the region surrounded with Leu93 and Ala49 may form. In this region, the L of wild type may have a structure with some features similar to V49A. Such a cavity could stabilize the L-like water cluster even after the orientation change of the protonated Schiff base in L′. The L-like water cluster in N of V49A also is stabilized in N′.

The N intermediate of wild type exhibits intense water O−D bands, as in L. The water bands of N are also similar to those of N of V49A. However, these intense bands observed with the wild type are not preserved upon change in the orientation of the Schiff base which occurs in forming N′. These results suggest that the polarized water molecules in N of the wild type interact strongly with the Schiff base, but not with other parts of the protein. This is the opposite to what is observed with L. Previous FTIR studies on N and M (*23*) showed that the water O−H band of N at 3654 cm^{-1}, which is not as well-resolved in the present N-minus-N′ spectrum, is affected more by the V49A mutation than the corresponding water band of M at 3671 cm^{-1}. Furthermore, in N there is a perturbation of the Val49−Pro50 backbone imide bond, a perturbation that is absent in M (*23*). Together, these observations suggest a relocation of water molecules to the Schiff base in the M-to-N transition. The increased hydration may be necessary to keep the Schiff base positively charged in N. The water cluster in N may also help modulate the pK_a of the Schiff base.

One of the long-standing problems for N is to show the existence of a H-bonding string of water molecules between the Schiff base and Asp96, which is thought to be necessary for the deprotonation of Asp96 and subsequent transfer of its proton to the Schiff base in the M-to-N transition. Such a water cluster was detected by X-ray crystallography for the N intermediate of V49A, though under conditions when Asp96 is largely protonated (*22*). The water cluster we infer for V49A is in line with this X-ray result. A similar water cluster is detected in our FTIR spectrum of wild type, but the interaction of this cluster with the protein moiety is absent or weak, unlike in V49A. Thus, Asp96 may not be involved in the interaction with the water cluster for wild type, at least in its protonated state (in late N). The N intermediate of T46V, in which Asp96 is unprotonated, has an impaired water cluster (Figure 4b) with more rapid N formation (*8*). Further studies are necessary in order to understand the relation of the deprotonation of Asp96 to the water cluster.

L-to-M-to-N Transition. The L-to-M-to-N transition in the photocycle ensures unidirectional proton transfer by modulating the pK_a of the Schiff base. The deprotonation−reprotonation of the Schiff base is facilitated by the relocation of cluster-forming water molecules. The process is shown

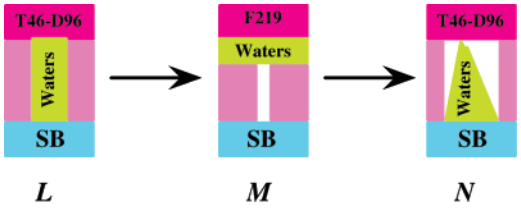

FIGURE 8: Schematic presentation of structure changes between the Schiff base and the Thr46−Asp96−Phe219 region in the L-to-M-to-N transition. Yellow rectangles (in L and M) and triangle (in N) represent water clusters, blue rectangles are the Schiff base (SB), and purple rectangles are surrounding apolar residues (the side chains of Val49, Leu93, Ala215, and Lys216). Thr46, Asp96, and Phe219 are included in red rectangles.

as a cartoon in Figure 8. In L, the region of the protein moiety extending from the Schiff base to the Thr46−Asp96 region forms a cavity, which is filled by a cluster of water molecules. This organized structure stabilizes the protonated form of the Schiff base. In the formation of M this cavity is moved to the Thr46−Asp96−Phe219 region. A different water cluster may be formed in this cavity, while the water cavity close to the Schiff base disappears. This change destabilizes the protonated Schiff base. The water cluster returns to the Schiff base region in N, helping in the reprotonation of the Schiff base. An increase in the pK_a of the Schiff base and conformation changes around the Schiff base to accommodate the water molecules may prompt the returning of waters. The protein does not form a rigid cavity to accommodate the water cluster in N.

In conclusion, the study of the behavior of the water bands in N upon the photoisomerization of the chromophore during the N to N′ transition at 80 K showed that the Schiff base of the N intermediate interacts with a polarized water molecule in N as in L. In wild type, but not in the V49A mutant, this interaction and the state of the water molecules in N are not preserved and they nearly returned to their initial state after isomerization of the chromophore. A set of waters in a cavity extending from the Schiff base to Thr46 is unique to L. In N a similar water cluster, probably extending to Thr46, interacts with the Schiff base but not with other parts of the protein cavity. Water molecules in such a cavity probably modulate the proton affinity of the Schiff base. The V49A and L93M mutants stabilize N as well as L by forming a larger cavity to accommodate more water molecules in the region surrounded by the Schiff base, Val49 and Leu93.

The water molecules around the Schiff base in N may be ascribed to a general hydration of the charged residues. The pK_a of the Schiff base in N of D96N (∼8.3) (*21*) is only slightly higher than that of the Schiff base in aqueous solution (∼7). In contrast the water cluster in L is more extensively stabilized by the protein moiety from the Schiff base to Thr46. The L intermediate is formed rapidly in the microsecond time range, and so probably only slight movements of protein residues occur. These changes may be limited only to cavity formation with small mobile water molecules helping to stabilize the cavity.

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REFERENCES

1. Fodor, S. P. A., Pollard, W. T., Gebhard, R., van den Berg, E. M. M., Lugtenburg, J., and Mathies, R. (1988b) Bacteriorhodopsin's L550 intermediate contains a C_{14}−C_{15} s-*trans* retinal chromophore, *Proc. Natl. Acad. Sci. U.S.A.* 85, 2156−2160.
2. Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., and Rothschild, K. J. (1988) Vibrational spectroscopy of bacteriorhodopsin mutants: Light-driven proton transport involves protonation changes of aspartic acid residues 85, 96 and 212, *Biochemistry 27*, 8516−8520.
3. Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B., Needleman, R., and Lanyi, J. K. (1992) Structures of Asp-96 in the L and N intermediates of bacteriorhodopsin: Analysis by Fourier transform infrared spectroscopy, *Biochemistry 31*, 4684−4690.
4. Govindjee, R., Balashov, S. P., Ebrey, T. G., Oesterhelt, D., Steinberg, G., Sheves, M. (1994) Lowering the intrinsic pK_a of the chromophore's Schiff base can restore its light-induced deprotnation in the inactive Tyr-57→Asn mutant of bacteriorhodopsin, *J. Biol. Chem. 269*, 14353−14354.
5. Braiman, M. S., Dioumaev, A. K., and Lewis, J. R. (1996) A large photolysis-induced pK_a increase of the chromophore counterion in bacteriorhodopsin: Implication for ion transport mechanism of retinal proteins, *Biophys. J. 70*, 939−947.
6. Váró, G., and Lanyi, J. K. (1991) Thermodynamics and energy coupling in the bacteriorhodopsin photocycle, *Biochemistry 30*, 5016−5022.
7. Chizhov, I., Chernavskii, D. S., Engelhard, M., Mueller, K.-H., Zubov, B. V., and Hess, B. (1996) Spectrally silent transitions in the bacteriorhodopsin photocycle, *Biophys. J. 71*, 2329−2345.
8. Yamazaki, Y., Hatanaka, M., Kandori, H., Sasaki, J., Karstens, W. F. J., Raap, J., Lugtenburg, J., Bizounok, M., Herzfeld, J., Needleman, R., Lanyi, J. K., and Maeda, A. (1995) Water structural changes at the proton uptake site (the Thr46-Asp96 domain) in the L intermediate of bacteriorhodopsin, *Biochemistry 34*, 7088−7093.
9. Maeda, A., Balashov, S. P., Lugtenburg, J., Verhoeven, M. A., Herzfeld, J., Belenky, M., Gennis, R. B., Tomson, F. L., and Ebrey, T. G. (2002) Interaction of internal water molecules with the Schiff base in the L intermediate of the bacteriorhodopsin photocycle, *Biochemistry 41*, 3803−3809.
10. Maeda, A., Tomson, F. L. Gennis, R. B., Kandori, H., Ebrey, T. G., and Balashov, S. P. (2000) Relocation of internal bound water in bacteriorhodopsin during the photoreaction of M at low temperature: an FTIR study, *Biochemistry 39*, 10154−10162.
11. Maeda, A. (2001) Internal water molecules as mobile polar groups for light-induced proton translocation in bacteriorhodopsin and rhodopsin as studied by difference FTIR spectroscopy, *Biochemsitry (Moscow) 66*, 1256−1268.
12. Maeda, A., Sasaki, J., Shichida, Y., and Yoshizawa, T. (1992) Water structural changes in the bacteriorhodopsin photocycle: Analysis by Fourier-transform infrared spectroscopy, *Biochemistry 31*, 462−467.
13. Maeda, A., Herzfeld, J., Belenky, M., Needleman, R., Gennis, R. B., Balashov, S. P., and Ebrey, T. G. (2003) Water-mediated hydrogen-bonded network on the cytoplasmic side of the Schiff base of the L photointermediate of bacteriorhodopsin, *Biochemistry 42*, 14122−14129.
14. Maeda, A., Tomson, F. L. Gennis, R. B., Ebrey, T. G., and Balashov, S. P. (1999) Chromophore-protein-water interactions in the L intermediate of bacteriorhodopsin: FTIR study of the photoreaction of L at 80 K, *Biochemistry 38*, 8800−8807.
15. Maeda, A., Tomson, F. L., Gennis, R. B., Balashov, S. P., and Ebrey, T. G. (2003) Water molecule rearrangements around Leu93 and Trp182 in the formation of the L intermediate in bacteriorhodopsin's photocycle, *Biochemistry 42*, 2535−2541.
16. Kouyama, T., Nishikawa, T., Tokuhisa, T., and Okumura, H. (2004) Crystal structure of the L intermediate of bacteriorhodopsin: Evidence for vertical translocation of a water molecule during the proton pumping cycle, *J. Mol. Biol. 335*, 531−546.
17. Fodor, S. P. A., Ames, J. B., Gebhard, R., van den Berg, E. M. M., Stoeckenius, W., Lugtenburg, J., and Mathies, R. (1988) Chromophore structure in bacteriorhodopsin's N intermediate: implication for the proton pumping mechanism, *Biochemistry 27*, 7097−7101.
18. Lakshmi, K. V., Farrar, M. R., Raap, J., Lugtenburg, J., Griffin, R. G., and Herzfeld, J. (1994) Solid state ^{13}C and ^{15}N NMR

investigations of the N intermediate of bacteriorhodopsin, *Biochemistry 33*, 8853−8857.
19. Balashov, S. P., Lu, M., Imasheva, E. S., Govindjee, R., Ebrey, T. G., Othersen, B., III, Chen, Y., Crouch, R. K., and Menick, D. R. (1999) The proton release group of bacteriorhodopsin controls the rate of the final step of its photocycle at low pH, *Biochemistry 38*, 2026−2039.
20. Zscherp, C., Schlessinger, R., Tittor, J., Oesterhelt, D., and Heberle, J. (1999) In situ determination of transient pK_a changes of internal amino acids of bacteriorhodopsin by using time-resolved attenuated total reflection Fourier transform infrared spectroscopy, *Proc. Natl. Acad. Sci. U.S.A. 96*, 5498−5503.
21. Brown, L. S., and Lanyi, J. K. (1996) Determination of the transiently lowered pK_a of the retinal Schiff base during the photocycle of bacteriorhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 93*, 1731−1734.
22. Schobert, B., Brown, L. S., and Lanyi, J. K. (2003) Crystallographic structures of the M and N intermediates of bacteriorhodopsin: Assembly of a hydrogen-bonded chain of water molecules between Asp-96 and the retinal Schiff base, *J. Mol. Biol. 330*, 553−570.
23. Yamazaki, Y., Kandori, H., Needleman, R., Lanyi, J. K., and Maeda, A. (1998) Interaction of the protonated Schiff base with the peptide backbone of valine 49 and the intervening water molecule in the N photointermediate of bacteriorhodopsin, *Biochemistry 37*, 1559−1564.
24. Balashov, S. P., Imasheva, E. S., Litvin, F. F., and Lozier, R. H. (1990) The N intermediate of bacteriorhodopsin at low temperatures, *FEBS Lett. 271*, 93−96.
25. Balashov, S. P., Imasheva, E. S., Karneyeva, N. V., Litvin, F. F., and Ebrey, T. G. (1992) in *Structures and Functions of Retinal Proteins* (Rigaud, J. L., Ed.) p 119, John Libbey Eurotext Ltd., Paris.
26. Brown, L. S., Gat, Y., Sheves, M., Yamazaki, Y., Maeda, A., Needleman, R., and Lanyi, J. K. (1994) The retinal Schiff base-counterion complex of bacteriorhodopsin: Changed geometry during the photocycle is a cause of proton transfer to aspartate 85, *Biochemistry 33*, 12001−12011.
27. Yamazaki, Y., Tuzi, S., Saitô, H., Kandori, H., Needleman, R., Lanyi, J. K., and Maeda, A. (1996) Hydrogen bonds of water and C═O groups coordinate long-range structural changes in the L photointermediate of bacteriorhodopsin, *Biochemistry 35*, 4063−4068.
28. Pfefferlé, J.-M., Maeda, A., Sasaki, J., and Yoshizawa, T. (1991) Fourier transform infrared study of the N intermediate of bacteriorhodopsin, *Biochemistry 30*, 6548−6556.
29. Ormos, P., Chu, K., and Mourant, J. (1992) Infrared study of the L, M, and N intermediates of bacteriorhodopsin using the photoreaction of M, *Biochemistry 31*, 6933−6937.
30. Weidlich, O., and Siebert, F. (1993) Time-resolved step-scan FT-IR investigations of the transition from KL to L in the bacteriorhodopsin photocycle: Idnetification of chromophore twist by assigning hydrogen-out-of-plane (HOOP) bending vibrations, *Appl. Spectrosc. 47*, 1394−1399.
31. Maeda, A. (1995) Application of FTIR spectroscopy to the structural study on the function of bacteriorhodopsin, *Isr. J. Chem. 35*, 387−400.
32. Dioumaev, A. K., Brown, L. S., Needleman, R., and Lanyi, J. K. (2001) Coupling of the reisomerization of the retinal, proton uptake, and reprotonation of Asp-96 in the N intermediate of bacteriorhodopsin, *Biochemistry 40*, 11308−11317.
33. Gerwert, K., and Siebert, F. (1986) Evidence for light-induced 13-*cis*, 14-s-*cis* isomerization in bacteriorhodopsin obtained by FTIR difference spectroscopy using isotopically labelled retinals, *EMBO J. 5*, 805−811.
34. Lanyi, J. (2004) What is the real crystallographic structure of the L photointermediate of Bacteriorhodopsin?, *Biochim. Biophys. Acta 1658*, 14−22
35. Lanyi, J. K., and Schobert, B. (2003) Mechanism of proton transport in bacteriorhodopsin from crystallographic structures of the K, L, M_1, M_2, and M_2' intermediates of the photocycle, *J. Mol. Biol. 328*, 439−450.
36. Edman, K., Royant, A., Larsson, G., Jacobson, F., Taylor, T., van der Spoel, D., Landau, E. M., Pebay-Peyroula, E., and Neutze, R. (2004) Deformation of helix C in the low temperature L-intermediate of bacteriorhodopsin, *J. Biol. Chem. 279*, 2147−2158.
37. Yamazaki, Y., Sasaki, J., Hatanaka, M., Kandori, H., Maeda, A., Needleman, R., Shinada, T., Yoshihara, K., Brown, L. S., and Lanyi, J. K. (1995) Interaction of tryptophan-182 with the retinal 9-methyl group in the L intermediate of bacteriorhodopsin, *Biochemistry 34*, 977−982.
38. Luecke, H., Schobert, B., Cartailler, J.-P., Richter, H.-T., Rosengarth, A., Needleman, R., and Lanyi, J. K. (2000) Coupling photoisomerization of retinal to directional transport in bacteriorhodopsin, *J. Mol. Biol. 300*, 1237−1255.
39. Friedman, R., Nachiel, E., and Gutman, M. (2003) The role of small intraprotein cavities in the catalytic cycle of bacteriorhodopsin, *Biophys. J. 85*, 886−896.
40. Kandori, H. (2000) Role of internal water molecules in bacteriorhodopsin, *Biochim. Biophys. Acta 1460*, 177−191.
41. Facciotti, M. T., Rouhani, S., Burkard, F. T., Betancourt, F. M., Downing, K. H., Rose, R. B., McDermott, G., and Glaeser, R. M. (2001) Structure of an early intermediate in the M-state phase of the bacteriorhodopsin photocycle, *Biophys. J. 81*, 3422−3455.
42. Sass, H. J., Büldt, G., Gessenich, R., Hehn, D., Neff, D., Schlessinger, R., Berendzen, J., and Ormos, P. (2000) Structural alterations fro proton translocation in the M state of wild-type bacteriorhodopsin, *Nature 406*, 549−653.
43. Takeda, K., Matsui, Y., Kamiya, N., Adachi, S., Okumura, H., and Kouyama, T. (2004) Crystal structure of the M intermediate of bacteriorhodopsin: Allosteric structural changes mediated by sliding movement of a transmembrane helix, *J. Mol. Biol. 341*, 1023−1037.

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