Chromophore−Protein−Water Interactions in the L Intermediate of Bacteriorhodopsin: FTIR Study of the Photoreaction of L at 80 K†

Akio Maeda,* Farol L. Tomson, Robert B. Gennis, Thomas G. Ebrey, and Sergei P. Balashov

Center for Biophysics and Computational Biology, Department of Cell and Structural Biology, and Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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ABSTRACT: Using FTIR spectroscopy, perturbations of several residues and internal water molecules have been detected when light transforms all-trans bacteriorhodopsin (BR) to its L intermediate having a 13-cis chromophore. Illumination of L at 80 K results in an intermediate L′ absorbing around 550 nm. L′ thermally converts to the original BR only at >130 K. In this study, we used the light-induced transformation of L to L′ at 80 K to identify some amino acid residues and water molecules that closely interact with the chromophore and distinguish them from those residues not affected by the photoreaction. The L minus L′ FTIR difference spectrum shows that the chromophore in L′ is in the all-trans configuration. The perturbed states of Asp96 and Val49 and of the environment along the aliphatic part of the retinal and Lys216 seen in L are not affected by the L → L′ photoreaction. On the other hand, the environments of the Schiff base of the chromophore, of Asp115, and of water molecules close to Asp85 returned in L′ to their state in which they originally had existed in BR. The water molecules that are affected by the mutations of Thr46 and Asp96 also change to a different state in the L → L′ transition, as indicated by transformation of a water O−H vibrational band at 3497 cm^{-1} in L into an intense peak at 3549 cm^{-1} in L′. Notably, this change of water bands in the L → L′ transition at 80 K is entirely different from the changes observed in the BR → K photoreaction at the same temperature, which does not show such intense bands. These results suggest that these water molecules move closer to the Schiff base as a hydrogen bonding cluster in L and L′, presumably to stabilize its protonated state during the BR to L transition. They may contribute to the structural constraints that prevent L from returning to the initial BR upon illumination at 80 K.

Bacteriorhodopsin is a heptahelical transmembrane protein. The retinal polyene chain lies roughly in the center of the membrane, parallel to its plane, and is joined to a lysine residue in the seventh helix through a Schiff base linkage (reviews in refs *3* and *4*). The Schiff base is protonated in the unphotolyzed state of the pigment. The retinal chromophore can assume the all-trans and the 13-cis, 15-syn configurations that are in the thermal equilibrium; the species with all-trans retinal is active in light-dependent proton pumping. This state will be termed BR.[1] Upon absorption of a photon, the all-trans retinal isomerizes around the C_{13}=C_{14} bond to the 13-cis, 15-anti form during BR → K photoreaction, and a linear sequence of subsequent thermal reactions K → L → M → N → O → BR (*5*) results in transmembrane proton translocation. The ionic pair consisting of a positively charged Schiff base and a negatively charged Asp85 comprises the locus for the proton pumping mechanism. The L → M transition involves proton transfer from the Schiff base to Asp85 (*6*, *7*). This proton transfer is accompanied by nearly instantaneous proton release to the extracellular medium from a set of interacting groups on the extracellular side of BR, composed of Glu204, Glu194, Arg82, and one or more bound water molecules (*8*−*17*). The Schiff base is subsequently reprotonated in the M → N process by Asp96 which is located on the cytoplasmic side of the retinal (*18*, *19*).

The isomerization of the chromophore results in the displacement of the Schiff base, thus changing the relative positions of Asp85, the Schiff base, and the water molecule interacting with them (*17*). The relation among them can be different in various intermediates. The conformation of the retinal moiety around the Schiff base in L, which occurs just before proton transfer from the Schiff base to Asp85, is distorted, as detected by both FTIR and solid-state NMR spectroscopic studies (*20*, *21*). A plausible distorted structure for the L intermediate was determined by a molecular dynamics calculation (*22*). Stronger hydrogen bonds of the protonated Schiff base and a water molecule close to Asp85 relative to those in BR may contribute to this distorted conformation (*20*). The conformational changes in L, however, are not confined to the region around the Schiff base and Asp85, but also extend to several residues on the cytoplasmic side of the retinal. The perturbation of Asp96

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* To whom correspondence should be addressed: Center for Biophysics and Computational Biology, 156 Davenport Hall, 607 S. Mathews, University of Illinois, Urbana, IL 61801. Phone: (217) 333-2435. Fax: (217) 244-6615. E-mail: amaeda@life.uiuc.edu.

[1] Abbreviations: BR, all-trans bacteriorhodopsin; L′, photoproduct produced by illumination of L at 80 K (*1*, *2*); Wat85, water molecule responsible for the O−H stretching frequencies which are affected by the mutation of Asp85; Wat46, water molecule responsible for the O−H stretching frequencies which are affected by the mutation of Thr46; HOOP, hydrogen out-of-plane; λ_{max}, wavelength for the maximum absorption.

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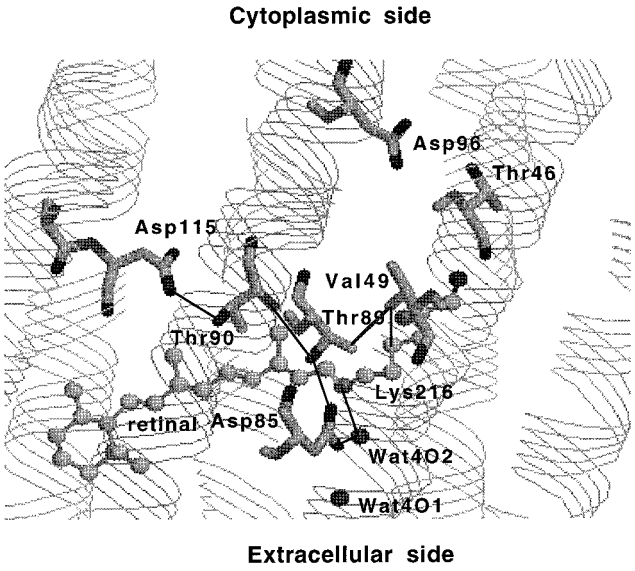

FIGURE 1: Locations and mutual relationships among some of the residues which are affected by the BR → L transition based on the coordinates of Luecke et al. (*17*). Trp182, which is located between Asp115 and Thr90 in this presentation, was omitted so the figure was not too clustered. Water molecules are depicted with dark balls. Wat402 and/or Wat401 described by Luecke et al. (*17*) may correspond to our Wat85. The positions of Wat46 are not determined in the structure of Luecke et al. (*17*). Solid lines connecting two atoms of nitrogen and oxygen represent possible hydrogen bonds. A hydrogen bonding network extends from the Schiff base to Asp115 through Thr89. Retinal and Lys216 are drawn as balls and sticks, to distinguish them from the amino acid residues near these chains.

and Asp115 is well documented (*6*, *23*, *24*). Perturbation of the indole N−H of Trp182 (*25*), the peptide carbonyl of Val49 (*26*), and water molecules that are affected by mutation of Thr46 (*27*) in the cytoplasmic region upon formation of L have also been detected. These residues along with related residues are depicted in Figure 1, drawn according to the structural coordinates of Luecke et al. (*17*). So far, only two crystallographic structures of BR (*17*, *28*) include the positions of internal water molecules. We will discuss various residues and water molecules using the latest structure (*17*) and FTIR data. One of the two carboxylate oxygens of Asp85 acts as a hydrogen bond acceptor of a water molecule (Figure 1, Wat402), and Wat402 is also a hydrogen bond acceptor for the protonated Schiff base. The other carboxyl oxygen of Asp85 is hydrogen bonded to the γ-oxygen of Thr89. It is at the correct distance for a hydrogen bond to the peptide amide of its neighbor, Thr90. Thr90's γ-oxygen forms a hydrogen bond with Asp115. Thus, these residues are connected through hydrogen-bonding interactions and a few covalent bonds. Rothschild et al. (*29*) have shown that mutation of Thr89 abolishes the perturbation of Val49 seen upon L formation. The methyl groups of these residues are located relatively close to each other in the X-ray structure (see Figure 1). Previous studies have shown that the carboxyl carbonyl of Asp96 is affected in L by mutations of Thr46 (*27*, *29*) and Val49 (*26*) but not by mutations of Thr89 (*29*), suggesting that the perturbation of Asp96 is not achieved through Thr89, but through direct interaction of the side chain of Lys216 with the side chain of Val49 (*30*), and through a conformation change around these residues in the BR → L transition.

The presence of water molecules was proposed to be crucial for stabilization of the charged states of ionizable residues (*31*, *32*) and might be essential for efficient proton transport. FTIR studies showed that upon formation of the L intermediate several water bands undergo spectral shifts caused by changes in hydrogen bonding. The O−H stretching band of water at 3642 cm^{-1}, which undergoes frequency shifts during the photocycle (*33*, *34*), is abolished in a mutant that lacks the negative charge of Asp85, D85N (*35*). The water molecules that comprise the 3642 cm^{-1} band will be referred to as Wat85 in this text. They can be ascribed to the water molecule, Wat402, or the set of two molecules, Wat401 and Wat402, which are detected in the proximity of Asp85 in the crystallographic structure (*17*) (see Figure 1).

Two water O−H stretching bands of BR at 3607 and 3577 cm^{-1} seen in the L minus BR difference spectrum are affected by mutation of Thr46 (*27*, *36*). They are not affected by mutation of the other residues except for Val49 and Asp96 (*26*). These water bands are restored by the additional mutation of Asp96 (T46V/D96N). A cavity to accommodate one or two water molecules was detected around Asp96 in the several versions of the BR structures (*17*, *28*, *37*, *38*). The water molecule(s) responsible for these two O−H stretching bands is likely to be present in the cytoplasmic region, more specifically at a site close to the pair of Thr46 and Asp96, whose side chains face each other at a distance of 0.36 nm (*17*), and will be termed Wat46 in this text. Currently, Wat46 has not been identified in the X-ray structure, and is not shown in Figure 1. However, this designation does not exclude the possibility that Wat46 occupies the Thr46-Asp96 site transiently and shuttles among other sites in the cytoplasmic region. Changes in the water bands produced by mutations of Thr46 and Asp96 correlate with changes in the rate of proton transfer from the Schiff base to Asp85 in the L → M transition (*27*).

Visible spectroscopy at low temperatures indicated that the L intermediate is photoactive (*39*). Upon illumination at 80 K, the L intermediate is transformed into a photoproduct, L′, whose absorption maximum is shifted 5 nm to the red, to ca. 555 nm, so L′ can be considered the bathoproduct of L (*1*, *2*, *40*). The L → L′ photoreaction is photoreversible and is accompanied by absorption changes in the β-bands, indicating that the 13-cis → all-trans isomerization of the chromophore takes place during the L → L′ photoreaction. The L → L′ photoreaction interrupts the photocycle, preventing deprotonation of the Schiff base which normally occurs during the L → M transition, and leads to restoration of the initial state. At temperatures above 130 K, the photoproduct L′ returns thermally to BR. The existence of L′ suggests that the formation of L is accompanied by structural changes of BR that cannot be reversed upon illumination at 80 K. In this paper, we used low-temperature FTIR spectroscopy to examine what part of the protein, including bound water molecules, retains the structure inherent in L when the chromophore is forced to return to the all-trans form. The FTIR difference spectra of the L → L′ photoreaction give further clues about the structure of L, and especially changes in hydrogen bonds of internal water molecules upon L formation.

MATERIALS AND METHODS

Recording of the FTIR Spectra. The wild type and the mutant bacteriorhodopsins were prepared by the standard method (*41*). The mutant strains of T46V, T46V/D96N, and V49A were described previously (*30*). The pigments of T46V and T46V/D96N were provided by J. K. Lanyi and L. S. Brown, and V49A was provided by Y. Yamazaki. About 50 μL of a purple membrane suspension ($A_{570nm} \sim 5$) was placed on a BaF_2 window 13 mm in diameter and dried in moderate vacuum to form a ~10 mm diameter film. The film was hydrated with 0.3 μL of H_2O, D_2O, or $H_2{}^{18}O$ (99.5% in isotope content obtained from ISOTEC Inc.), and sealed with another BaF_2 window with a 2 mm thick Teflon spacer. The sample was installed in a homemade brass cell holder attached to an Oxford cryostat Optistat, and placed into a Bio-Rad model FTS6000 FTIR spectrometer. The temperature was controlled by an Oxford Intelligent Temperature Controller. Before and after each illumination, the FTIR spectrum was constructed from 256 interferograms at 2 cm^{-1} resolution. Recording each spectrum takes about 2.5 min. The sample was illuminated by light from a 550 W slide projector. A mirror directing the light to the sample was operated from a computer. Light was passed through a 5 cm 5% $CuSO_4$ solution to remove heat and an appropriate optical filter (Corning) as described below.

Light-Induced Transformations and Registration of the L′ Minus L Spectrum. The film was illuminated at 273 K with yellow light (Corning 3-73 filter, >400 nm) for 2 min to light-adapt the sample. Light-adapted BR was then cooled to 170 K and illuminated with red light (2-62 filter, >590 nm) for 1 min to form the L intermediate. The mixture of BR and L was then cooled to 80 K and illuminated first with blue light (4-96 filter, 350−500 nm light) for 1 min to produce L′, the photoproduct of L, and then with deep red light (2-64 filter, >650 nm light) for 1 min to convert K back to BR. The complete recovery of the initial BR state was achieved by warming the sample to 0 °C, and the whole process was repeated several times to attain a higher signal-to-noise ratio. In the difference spectrum between before and after the illumination with blue light (350−500 nm) (Figure 2a), intense HOOP bands at 974 and 957 cm^{-1}, a C−C stretching band at 1194 cm^{-1}, and a C═C stretching band at 1514 cm^{-1} on the positive side were elucidated. These bands are characteristic of the K intermediate (*42*, *43*), which was formed from BR upon blue light illumination at 80 K, and were completely removed by subsequent illumination with deep red light (>650 nm), which photoreverses K back to BR (see below).

RESULTS

L Minus L′ FTIR Difference Spectrum. The difference spectrum produced by successive illumination of a mixture of L and BR at 80 K with blue and then red light reveals bands (Figure 2b) of the photoproduct L′ that must have been photochemically produced from L. This photoproduct (also called P555 and K_L) was discovered and characterized using visible spectroscopy (*1*, *2*, *40*). In this paper, we call this state L′ to avoid confusion with the frequently used name KL for a subspecies of K (*44*, *45*).

The L minus L′ spectrum (Figure 3a) can be compared with the L minus BR difference spectrum obtained at 170 K

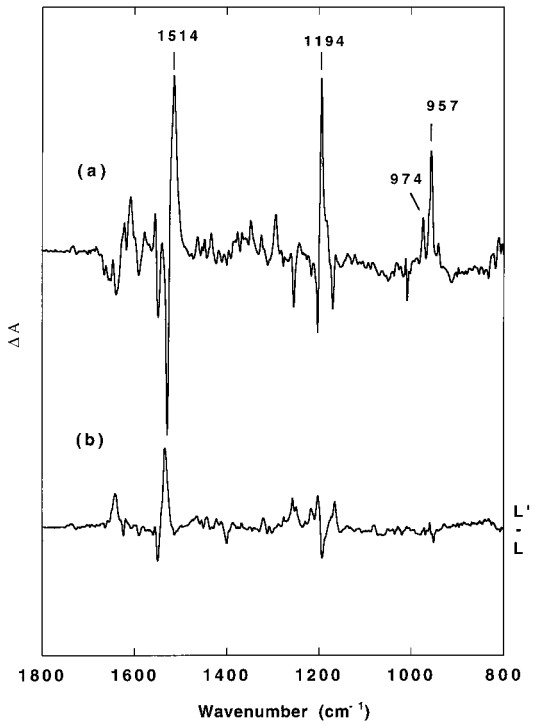

FIGURE 2: FTIR difference spectra in the 1800−800 cm^{-1} region produced by (a) illumination of a mixture of BR and L at 80 K with blue light (350−500 nm) to produce L′ and K (the K + L′ minus BR + L spectrum) and (b) additional illumination with red light at >650 nm to remove K (the L′ minus L spectrum). The full length of the ordinate corresponds to 0.05 absorbance unit for both spectra.

(Figure 3b). The amplitudes were adjusted so that the common hydrogen out-of-plane (HOOP) band of L at 951 cm^{-1} had the same amplitude; to do this, the L minus BR spectrum was multiplied by a factor for the fraction of L that undergoes the transition to L′, 0.42 under the conditions described here. The L bands appear on the positive sides and the L′ or BR bands on the negative sides in traces a and b of Figure 3, respectively. Besides the HOOP band, a C−C stretching vibration of the chromophore at 1192 cm^{-1} in L has exactly the same intensity in both difference spectra (traces a and b of Figure 3). The two bands of the C═C stretching vibration of L at 1549 and 1540 cm^{-1}, which were observed in the resonance Raman spectrum (*46*), are detected in these difference FTIR spectra as a peak and a shoulder, respectively, though the latter in the L minus L′ spectrum is obscured because of overlap with a negative band at 1534 cm^{-1}. These features clearly show that the L intermediate undergoes phototransformation at 80 K.

Evidence for the All-Trans Configuration of the Chromophore in L′. The negative C═C stretching band at 1528 cm^{-1} in BR appears in L′ at 1534 cm^{-1} and with a lower intensity. This is also the case for the bands at 1202 and 1166 cm^{-1} of the L′ (Figure 3a). These frequencies show that L′ has an all-trans chromophore (*47*), but different intensities suggest a somewhat different structure compared to the all-trans chromophore of BR. This may reflect a different electronic structure, since the HOOP band at 959 cm^{-1} has an identical shape in L′ and BR (see traces a and b of Figure 3).

The C═C stretching vibration of retinal in the L′ state at 1534 cm^{-1} (Figure 3a) is located at a higher frequency than

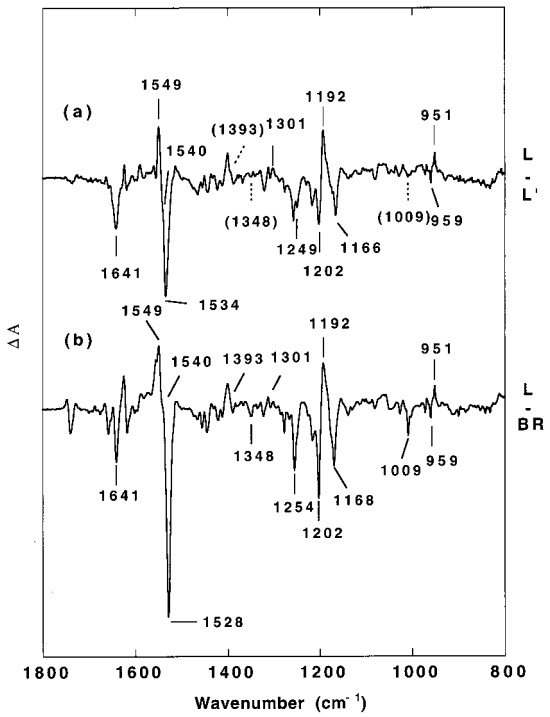

FIGURE 3: Difference spectra in the 1800−800 cm^{-1} region: (a) L minus L′ spectrum obtained at 80 K, as described in the text and the legend of Figure 2b (spectrum was additionally multiplied by −1 for convenience of comparison with the L minus BR spectrum) and (b) L minus BR spectrum obtained at 170 K. Positive bands in spectra a and b are due to the L intermediate at 80 and 170 K, respectively. Negative bands in a and b are due to L′ and BR, respectively. The amplitude of the L minus L′ spectrum was normalized using the intensity of the 951 cm^{-1}. The bands which were not observed in spectrum a but were observed at the corresponding frequencies in spectrum b are represented by dotted lines with the frequencies in parentheses. The full length of the ordinate corresponds to 0.034 and 0.080 absorbance unit for spectra a and b, respectively.

in the BR state (1528 cm^{-1}) but at a lower frequency than in L (1540 and 1549 cm^{-1}). According to the empirical inverse relation between the absorption maximum, λ_{max}, and the C═C stretching frequencies of the chromophore (*48*), this would be consistent with the absorption maximum of L′ being red shifted relative to that of L, but blue shifted relative to that of BR, as shown indeed by visible spectroscopy (*1*, *2*, *40*, *49*).

Changes in the Interaction of the Schiff Base in L′. The C═C stretching vibration frequency depends on the degree of electron delocalization in the chromophore. This is modulated through its interaction with the environment (*50*). The C═C stretching band at 1534 cm^{-1} of L′ (Figure 3a) indicates a less delocalized electronic structure compared to that for BR at 1528 cm^{-1} (Figure 3b). This is probably due to a stronger interaction of the chromophore in L′ with its environment than in BR, despite the otherwise identical chromophore configuration. A 1641 cm^{-1} band in L′ (Figure 3a), which is shifted to 1629 cm^{-1} in D_2O (data not shown), can be ascribed to the C═N stretching vibration of the Schiff base of L′. This is located at the same frequency as the corresponding band of BR (Figure 3b). The 1249 cm^{-1} band in L′ (Figure 3a) is also sensitive to D_2O substitution (data not shown). This corresponds to one of two overlapping bands at 1254 cm^{-1} in BR (Figure 3b), which was assigned to the coupled mode of the Schiff base N−H and C_{15}−H vibrations (*43*). The corresponding band in L is located at 1301 cm^{-1}. The C═N stretching vibration of L cannot be resolved in these difference FTIR spectra. It was located at 1648 cm^{-1} in the resonance Raman spectrum of L (*46*). These results indicate that these vibrational bands due to the protonated Schiff base are shifted toward higher frequencies in L, and in L′ largely return to the original frequencies seen in BR (see Table 1A).

Table 1: Changes in Vibrational Frequencies (cm^{-1}) in the BR → L and L → L′ Transitions[a]

vibration	BR	L	L′
(A) Vibration Frequencies Which Change in the L → L′ Transition			
retinal HOOP	959	951	959
retinal C−C stretching	1168, 1202	1192	1166,[b] 1202[b]
Schiff base N−H bending[c]	1254	1301	1249
retinal C═C stretching	1528	1540, 1549	1534[b]
Schiff base C═N stretching	1641	1648[d]	1641
Asp115 carboxyl C═O stretching	1737	1726[e]	1737
(B) Vibration Frequencies Which Remain Unchanged in the L → L′ Transition			
retinal C−CH_3 bending	1009	1009[b]	1009[b]
Lys216 Cα−H bending[b]	1348	1393[f]	1393[f]
Val49 amide I	1618	1625	1625
Asp96 carboxyl C═O stretching	1741	1748	1748
(C) Water O−H Stretching			
Wat85	3642	<3525	3642
Wat46	3607, 3574	3497[e]	3549

[a] See the text for details and citations. [b] Smaller in intensity than that in BR. [c] Coupled further with retinal C_{15}−H bending. [d] Observed in the resonance Raman spectrum. [e] Value at 80 K. [f] Not identified by isotope labeling of the Cα of lysine, but was assigned to a related mode with the 1348 cm^{-1} band of BR.

Changes Produced by the BR → L Transition That Are Not Reversed by the L → L′ Transition. The environment along the aliphatic chains of the retinal and Lys216 persists in the L → L′ transition. Thus, the 1348 cm^{-1} band in BR (Figure 3a), a coupled mode of C_{15}−H and Cα−H vibrations of Lys216 in BR (*43*, *51*), does not appear in L′ (Figure 3b). A shoulder at 1393 cm^{-1} in L (Figure 3a) may correspond to this band (*19*, *43*), though not identified by isotope labeling of the Cα of lysine, as was done for the 1348 cm^{-1} band by Gat et al. (*51*). It may stay at the same frequency in L′. A small dip at this position in the L minus L′ spectrum may arise from cancellation by a negative band at the same frequency in L′. The C−CH_3 in-plane bending vibration in BR at 1009 cm^{-1}, which appears by virtue of its loss of intensity in L (Figure 3b), is persistently absent in L′ (Figure 3a).

The spectra in the 1800−1500 cm^{-1} region are shown on an expanded scale in Figure 4. The pair of 1618 (−) and 1625 (+) cm^{-1} bands in the L minus BR spectrum (Figure 4b) is attributed to amide I (a coupled mode of the C═O stretching and C−N stretching vibrations of peptide bond) of Val49 (*26*). The 1618 cm^{-1} band does not appear in L′ (Figure 4a). The small intensity remaining at 1618 and 1625 cm^{-1} can be attributed to other modes, because similar bands are also detected after the amide I band of Val49 was shifted away in [1-^{13}C]Val-labeled BR (*26*). Since the side chain of Val49 is near the lysine side chain (Figure 1), this result is consistent with an unchanged environment along the aliphatic

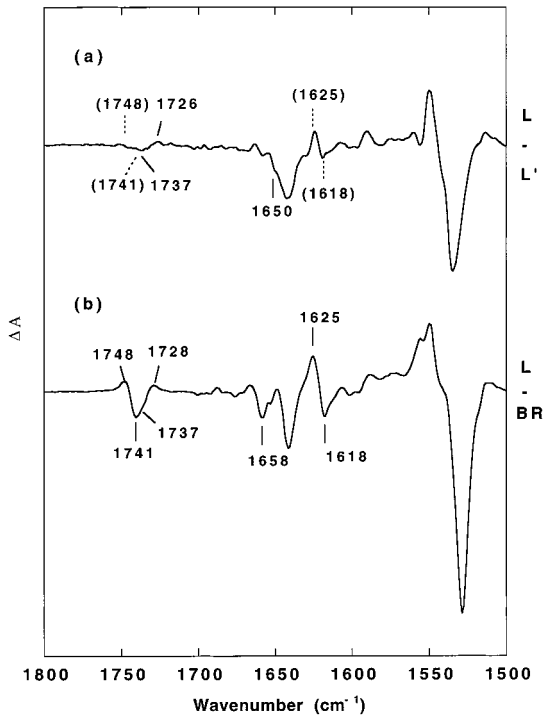

FIGURE 4: Spectra in the 1800−1500 cm^{-1} region expanded from the corresponding spectra shown in Figure 3. The L minus L′ spectrum (a) is compared with the L minus BR spectrum (b). The unobserved bands in spectrum a, which were observed at the corresponding frequencies in spectrum b, are represented by dotted lines with the frequencies in parentheses. The full length of the ordinate corresponds to 0.034 and 0.080 absorbance unit for spectra a and b, respectively.

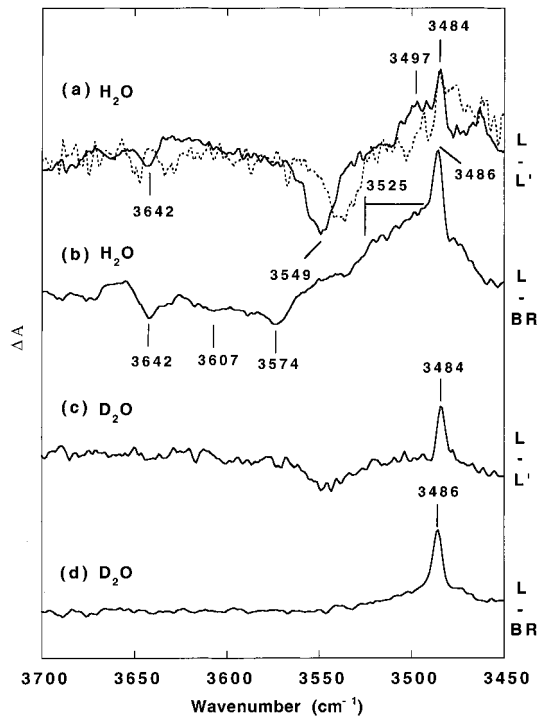

FIGURE 5: Spectra in the 3700−3450 cm^{-1} region of the corresponding spectra in Figure 3. The L minus L′ spectrum (a) was compared with the L minus BR spectrum (b). The L minus L′ spectrum represented by a dotted line in part a was recorded for films hydrated with $H_2{}^{18}O$. The corresponding L minus L′ (c) and L minus BR spectra (d) were recorded for the films hydrated with D_2O. The normalization was carried out for the HOOP band at 951 cm^{-1}. The full length of the ordinate corresponds to 0.013, 0.030, 0.010, 0.018, and 0.018 absorbance unit for spectra a−d and the dotted line in part a, respectively.

chains of the retinal and Lys216 in the transition from L to L′. Another amide I band at 1658 cm^{-1} in BR (Figure 4b), which is affected in the D115N mutant protein (*24*), does not appear in the L minus L′ spectrum at this frequency. A shoulder at 1650 cm^{-1} might be a candidate for the corresponding band in the L minus L′ spectrum (Figure 4a).

Perturbation of Asp115 Is Reversed in the L → L′ Transition but Not That of Asp96. In the L minus BR spectrum, the spectral region of the C=O stretching vibrations of carboxylic acids (Figure 4b) is composed of a bilobe with 1737 (−) and 1728 (+) cm^{-1} bands due to Asp115 and a bilobe with 1748 (+) and 1741 (−) cm^{-1} bands due to Asp96 (*6*, *24*). In the L minus L′ spectrum (Figure 4a), the former at least partially remains but the latter is almost entirely absent. This indicates that the environment of Asp115 changes in the L → L′ transition while that of Asp96 does not.

Structural Change around Trp182 Is Reversed by the L → L′ Photoreaction. The sharp band at 3484 cm^{-1} in the L minus L′ spectrum (solid line in Figure 5a), which remains unaltered in the spectrum in $H_2{}^{18}O$ (dotted line in Figure 5a), corresponds to the 3486 cm^{-1} band of the indole N−H stretching vibration of Trp182 in the L minus BR spectrum (*27*; also see Figure 5b). The extremely large molar absorbance of this band in the L minus BR spectrum was attributed to an especially polar environment as a consequence of Trp182's interaction with the 9-methyl group of the retinal in L (*25*). The amplitude of this band in the L minus L′ spectrum (Figure 5a) appears to be slightly smaller than that in the L minus BR spectrum (Figure 5b). The difference spectra obtained from films hydrated with D_2O (traces c and d of Figure 5), which eliminated all other bands in this region (*52*), exhibit nearly the same amplitude. This indicates that the L → L′ photoreaction eliminates strong interaction of Trp182 with the chromophore peculiar for the L state and that the environment of Trp182 in L′ returned to a state similar to the original BR.

Changes in Water O−H Bands in the L → L′ Transition. The 3642 cm^{-1} band of BR (Figure 5b), attributed to Wat85, persists in L′ (Figure 5a). This indicates that a water molecule close to Asp85 shifted in L below 3525 cm^{-1} returned in L′ to the same state as it had in BR. An intense negative band at 3549 cm^{-1} is a distinctive feature of L′ (Figure 5a), which is absent in the BR spectrum (Figure 5b). This band is clearly shifted in $H_2{}^{18}O$ (Figure 5a). Hence, it is assigned to a water O−H stretching vibration in L′. The origin of the 3549 cm^{-1} band will be considered below. The positive band of L around 3497 cm^{-1} shifts toward lower frequencies of 3484 cm^{-1} in $H_2{}^{18}O$ (Figure 5a); it was therefore assigned to the O−H stretching vibrations of water in L. The 3497 cm^{-1} band is sharper in shape and larger in intensity than the corresponding broad band in the L minus BR spectrum at 170 K (Figure 5b).

Effects of T46V and T46V/D96N Mutations on the Water Bands. Previous studies showed that the water O−H bands at 3607 and 3574 cm^{-1} of BR and the wide positive band of L ranging from 3525 cm^{-1} to the region beneath the 3486 cm^{-1} band (Figure 5b) are absent in the T46V mutant and are restored after an additional mutation of Asp96 in T46V/

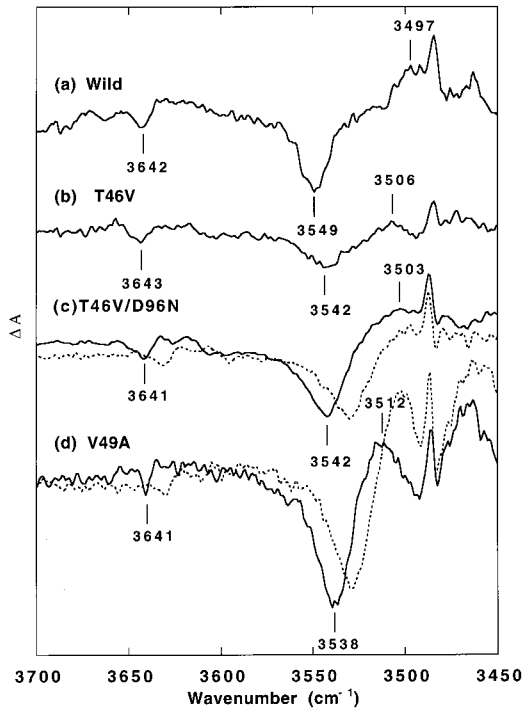

FIGURE 6: L minus L′ spectra in the 3700−3450 cm^{-1} region of T46V (b), T46V/D96N (c), and V49A (d) in comparison with that of the wild type (a). Spectra represented by dotted lines in parts c and d were recorded for films hydrated with $H_2{}^{18}O$. The spectra were normalized by the intensity of the 1192 cm^{-1} band. The full length of the ordinate corresponds to 0.0125, 0.0078, 0.0069, 0.0083, 0.0156, and 0.0067 absorbance unit for spectra a−d and the dotted lines in parts c and d, respectively.

D96N (*27*). Hence, these bands are assigned to water molecules in the Thr46−Asp96 region (Wat46). The L minus L′ spectra were recorded for these mutant proteins. The spectra in the 1800−800 cm^{-1} region are similar to that of the wild type (data not shown), indicating that the effects of the mutations are local. In Figure 6, the spectra in the 3700−3450 cm^{-1} region for these mutants were compared with that of the wild type (Figure 6a). The T46V mutation decreases the intensity of the intense negative band at 3549 cm^{-1} of L′ by half with the remaining band now absorbing at 3542 cm^{-1} (Figure 6b). In T46V/D96N, the intensity is restored to that of the wild type, but the frequency is shifted to 3542 cm^{-1} (Figure 6c). This band is entirely due to water because it is completely shifted in $H_2{}^{18}O$. Wat46 in the BR state (3607 and 3574 cm^{-1} bands in Figure 5b) likewise exhibits an intensity decrease in T46V and a restoration in T46V/D96N (*27*). Thus, half of the 3549 cm^{-1} band is due to the two (or more) water molecules of Wat46, while the other half is unidentified. Since they have the same frequency, this suggests that two water O−H bonds are present in the same environment. The band around 3497 cm^{-1} in L′ of the wild type disappears in the T46V pigment (Figure 6b). The band at 3506 cm^{-1} that remains in L of T46V may correspond to the 3542 cm^{-1} band in the L′ state. The intensity is restored in the T46V/D96N pigment, but its position has moved to the higher frequencies, relative to the 3497 cm^{-1} band of the wild type, forming a band around 3503 cm^{-1} (Figure 6c). This band is clearly shifted in $H_2{}^{18}O$.

Effect of the V49A Mutation on Water Bands. The previous measurement of the L minus BR difference spectrum of the V49A mutant protein (*26*) showed a large water O−H stretching band in BR at 3565 cm^{-1}, in addition to the other water O−H bands observed in the spectrum of the wild-type protein. This new band may arise from water molecules filling the void that was produced with the replacement of valine by alanine.

Since this frequency is very similar to that of the large water band of L′ at 3549 cm^{-1} and the position of the side chain of Val49 is close to Thr89 (see Figure 1), we obtained the L minus L′ spectrum for V49A (Figure 6d). The spectrum in the 1800−800 cm^{-1} region is similar to that of the wild type, except that the vibrations due to the Schiff base are affected (data not shown), as is also observed in the L minus BR spectrum. The spectrum in the 3700−3450 cm^{-1} region in Figure 6d exhibits a much larger O−H stretching vibration in L′ at 3538 cm^{-1} than the 3549 cm^{-1} band of the wild type (Figure 6a). This band along with the positive band at 3512 cm^{-1} shows a clear shift in $H_2{}^{18}O$, despite some baseline distortion. Hence, these bands are due to water molecules. Thus, the water molecules responsible for the 3549 cm^{-1} band of L′ in the wild type are located at the position that is affected by mutation of Val49, and the water molecules filling in the void produced by the V49A change to a single narrow band at 3538 cm^{-1} together with the water molecule whose O−H vibrations comprise the 3549 cm^{-1} band in the wild type.

DISCUSSION

Differences between L′ and BR. Table 1 shows a compilation of the frequencies of the salient vibrational modes that are detected in the BR → L → L′ transitions. The bands which are shifted in the BR → L transition and returned more or less to their original frequencies in L′ (Table 1A) are due to vibrations from the retinal side chain, the Schiff base, and Asp115. The restoration of the vibrational frequencies associated with the side chain of retinal in the L to L′ transition is due to the reisomerization of the chromophore from the 13-cis back to its all-trans form. The hydrogen bond of the protonated Schiff base, which is stronger in L than in BR (*43*), returns to a weaker state in L′, as judged by both the frequencies of the C═N stretching and N−H bending vibrations (*47*). However, these bands do not always return to exactly the same frequencies in L′ as in the initial BR, indicating a different environment around the Schiff base in BR and L′ and indicating that the chromophore in L′ does not attain a state identical to that in BR. Hydrogen bonding changes of Wat85 (Table 1C) and the carboxylic carbonyl of Asp115 (Table 1A), which occur in the BR → L process, are reversed in L′. These are connected by hydrogen bonds and a few covalent bonds as shown in Figure 1. Also, the perturbed indole N−H of Trp182 in L returned to the initial BR state in L′. On the other hand, the environment along the aliphatic part of the retinal, as reflected by the persistent loss of the intensity of the C-methyl bending vibration of retinal and the persistent shift of the coupled mode consisting of both bending vibrations of the lysine side chain and the C_{15}−H of the retinal at 1348 cm^{-1}, does not change during the L → L′ photoreaction (Table 1B). Also, the perturbations of the amide I mode (a coupled mode of the C═O and C−N stretching vibrations of the peptide backbone) of Val49 are preserved almost completely in L′. These residues are not directly influenced by photoisomerization.

Structural Changes and Movement of Water Molecules in the Schiff Base−Asp85 and the Cytoplasmic Regions in the BR → L and L → L′ Transitions. Wat85 returns completely to the initial BR state in the L → L′ transition (Table 1C), as expected from it being located close to the Schiff base. Wat46 is also altered, but to a state different from that in BR. The alteration is accompanied by the participation of other unidentified water molecules and attains a hydrogen bonding state different from that of BR. Thus, these Wat46 water molecules, which are influenced by Thr46 and Asp96 in the BR state, change in concert with the Schiff base but independently of Asp96 in the L → L′ transition. This suggests that these water molecules do not stay in the Thr46−Asp96 region in L and L′ but rather are closer to the Schiff base. In these intermediates, the two (or more) water molecules merge to the same hydrogen bonding state characterized by the same O−H stretching frequency. This could result from how they are arranged as a hydrogen-bonded cluster in a cavity, which might be created in the proximity of the Schiff base in L and persists in L′.

This notion is further supported by the fact that these water molecules are affected by the mutation of Val49, whose side chain is in close contact with the side chain of Lys216 and Thr89 (Figure 1). Furthermore, additional water molecules, which are probably present in a cavity created by the replacement of Val49 by alanine, seem to merge together with the water molecules present in the wild type. The presumed scheme of the conversion of BR to L to L′ is that the water molecules in the Thr46−Asp96 region in BR are relocated closer to the Schiff base region in L and undergo structural changes in L′ at a similar location, together with other water molecules that are derived from other unidentified locations. This may contribute to the constraint that prevents the photoreversal of L to the BR state in the photoreaction at 80 K. Notably, the changes of the water O−H stretching bands in the L → L′ transition are entirely different from those in the BR → K photoreaction at 80 K, which does not show such an intense band (*34*, *53*).

Role of Water in the Photocycle. Quantum chemical calculations show that internal water molecules may be required to stabilize the electric charges in the Schiff base and Asp85 in their apolar environment (*31*); indeed, this was found in a recent crystallographic study (*17*). The FTIR spectra suggest that in L clustered water molecules located in the cytoplasmic side of retinal play a role in stabilizing and probably connecting the protonated Schiff base with the anionic Asp85. Conversely, removal of these water molecules may stabilize the M intermediate with its uncharged unprotonated Schiff base and uncharged protonated Asp85. This notion is supported by the acceleration of the L to M conversion in T46V and its reversal in T46V/D96N (*27*), in parallel with the disappearance and reappearance of Wat46. The very slow L → M conversion in V49A (*30*) is also correlated with the additional water molecules to form the putative cytoplasmic side cavity, as proposed in this study. The small accumulation of M in Y57D and Y57N mutants (*54*−*56*) may also be explained with the same reasoning, in view of the large negative band at 3547 cm^{-1} that was observed in the L minus BR spectrum of the Y57D mutant protein (*34*). The movement of water molecules in the cytoplasmic region was suggested to take part in the stabilization of the protonated Schiff base in the N intermediate (*36*). This study suggests that the movement of water molecules to the proximity of the Schiff base stabilizes its protonated state in L. Water could function as a mobile mediator changing the protonation state of Asp85, the Schiff base, and Asp96 in the apolar environment and form a proton transfer pathway.

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