Water Molecule Rearrangements around Leu93 and Trp182 in the Formation of the L Intermediate in Bacteriorhodopsin's Photocycle†

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Received August 14, 2002; Revised Manuscript Received December 9, 2002

ABSTRACT: After the chromophore's isomerization in the initial photochemical event in bacteriorhodopsin, the primary photoproduct K makes a thermal transition to the L intermediate, which prepares the pigment for Schiff base deprotonation in the following step (L → M). Substantial changes in the hydrogen bonding of internal water molecules take place upon L formation. Some of these mobile waters are probably involved in changing the p*K* of the Schiff base and perhaps that of the proton acceptor Asp85 to allow proton movement [Maeda, A. (2001) *Biochemistry (Moscow) 66*, 1555−1569]. Here we show that mutations of Leu93 and Trp182, residues close to the 13-methyl group of the chromophore, allow the formation of L at much lower temperatures than in the wild type (80 K instead of 140 K). Moreover, an intense band due to weakly bound water that is peculiar for L was already present in the initial (unphotolyzed) state of each mutant at 2632 cm^{-1} (in D_2O) but not in the wild type. This unique, intense water band is shifted compared to the L band at 2589 cm^{-1} but coincides with the band seen in L′, the all-trans photoproduct of wild-type L formed at 80 K. We propose that the L93M and W182F mutations induce changes in the hydrogen bonding of one or more water molecules in the unphotolyzed states of these pigments, which are similar to those H-bonding changes that take place upon formation of L in the wild type, and thus facilitate the formation of L even at 80 K. We infer that L formation involves perturbation of a site which includes retinal, Trp182, and Leu93, and this structure is temporarily stabilized by rearranged hydrogen bonds with water molecules.

Bacteriorhodopsin is a light-driven proton pump. Its action is initiated by the absorption of light by its all-trans retinal chromophore, linked to Lys216 of the apoprotein through a protonated Schiff base linkage [all-trans bacteriorhodopsin (BR)].[1] Light isomerizes the C_{13}═C_{14} bond of the retinal, forming the 13-cis primary photoproduct K which induces the subsequent transformations of the protein. BR cycles through a series of intermediates (K, L, M, N, O) and their substates; these were originally distinguished by their visible spectra (*1*−*3*) but can also be identified by their vibrational spectra (reviewed in refs *4* and *5*). At room temperature, the initial BR state is restored ∼20 ms after light absorption. During the photocycle, one proton is transported from the cytoplasm to the extracellular medium.

BR gives us a unique opportunity to study structural changes of very early intermediates of an enzymatic reaction, which are difficult to detect in most enzymes. At room temperature, K converts to L in ∼1 μs, but remains in equilibrium with L through its lifetime, ∼100 μs (*6*, *7*). K and L can be stabilized at cryogenic temperatures, at 80 and 170 K, respectively.

Formation of K leads to the appearance of an intense vibrational band due to the C_{15},N HOOP, which was attributed to a twisting of the chromophore around the C═N bond of the Schiff base (*8*−*10*). In K, the interaction of the Schiff base with the protein moiety is altered so that the H-bond of the Schiff base in K becomes very weak (*9*; reviewed in ref *4*). The K to L transition is accompanied by a decrease in the intensity of the HOOP band which suggests at least partial release of the constraints and a more planar conformation of the chromophore. The Schiff base in L is engaged in stronger H-bonding than BR and K, and its C═N bond is distorted (*9*, *11*). L can be assumed to be a transition state-like intermediate, in which many changes in the interactions between key groups and water molecules occur around the Schiff base as the active site of the proton-translocating enzyme. The formation of the L intermediate is a prerequisite for proton transfer from the Schiff base to Asp85, which occurs in the next transition (L → M). Amino acid residues and hydrogen-bonded water molecules which critically affect the formation of L are the focus of our study.

FTIR spectroscopy is an ideal method for detecting changes in protonation states and changes in H-bonding (*4*). A set of unique features accompanying L formation has been seen by difference FTIR spectroscopy (reviewed in ref *12*). They include the intensified O−H stretching vibration bands of water molecules (*13*) and the N−H stretching vibration band of Trp182 (*14*). Large-intensity water O−H vibrations of L at 3550−3500 cm^{-1} are reduced in magnitude by the T46V mutation and restored in the T46V/D96N mutant (*15*).

† This work was supported by NIH Grants GM 52023 (to T.G.E. and S.P.B.) and HL16101 (to R.B.G.).

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[1] Abbreviations: BR, all-trans bacteriorhodopsin; HOOP, hydrogen out-of-plane vibration; Wat46, water molecule responsible for the O−H stretching vibrations which are affected by the mutation of Thr46.

10.1021/bi020532n CCC: $25.00
Published on Web 02/11/2003

Thus, the water molecules responsible for these bands (called Wat46) were assigned to the cytoplasmic domain of BR. The intensity of vibrations due to the Schiff base changed in parallel with the intensities of the O−H stretching vibration bands of Wat46 (*15*, *16*). Hence, we suggested that the interaction of the Schiff base with the Wat46 water molecule(s) occurs in L. A vibration at 3642 cm^{-1} due to a water in the extracellular domain undergoes increasing extents of perturbation in the BR → K → L transition which is reflected in the larger intensity of this band in the L minus BR difference spectrum than in the K minus BR spectrum (*17*, *18*). Perturbations of several other residues close to the chromophore were also detected upon L formation such as the carboxyl groups of Asp96 and Asp115 (*19*), the peptide C═O bond of Val49 (*20*), and the O−H bond of Thr89 (*21*).

Similar to the K intermediate, L contains a 13-cis chromophore which can be reisomerized back to trans by light (*22*, *23*). Photoexcitation of the K intermediate results in a fast recovery of the initial BR even at liquid nitrogen temperatures (*1*, *22*, *24*). However, this is not the case for L at those temperatures. Light-induced reisomerization of the chromophore of L at 80 K leads to the all-trans photoproduct L′ (*25*, *26*) which relaxes to BR at 140 K. L′ exhibits a mixture of the features of both L and BR (*27*). The lack of a full recovery of L back to the initial state at 80 K indicates that L formation involves structural changes of the chromophore binding site, and these changes cannot be reversed immediately in a barrierless transition after the geometry of the chromophore is changed back to all-trans. Many of the groups which undergo perturbation upon L formation are located in the cytoplasmic domain. Therefore, we decided to screen mutants of cytoplasmic residues to determine their effect on the formation of L.

In the study presented here, we show that mutations of two of these, Leu93 (L93M) and Trp182 (W182F), residues that are close to the 13-methyl group of the chromophore [see Figure 1, depicted on the basis of the coordinates of Luecke et al. (*28*)], facilitate formation of L. They do this by apparently creating an L-like structure, already in the initial states of these two mutant pigments. This must include hydrogen bonding changes of key water molecules from their positions in the wild-type pigments. Our data suggest a critical role of conformational changes involving Trp182 and Leu93 and their hydrogen bonding with water molecules in the formation of L.

MATERIALS AND METHODS

Bacteriorhodopsin Mutants. Ten mutants of nine cytoplasmic residues were screened to determine their effect on the formation of L: V49A, T46V, T90V, L93M, L93V, D96N, D115N, T178N, W182F, and F219L. Two of them, L93M and W182F, had large effects as described below. In addition to the wild type, we used F219L as a control. The L93M, W182F, and F219L mutants and some of their vibrational spectral properties have been described previously (*29*−*31*). These pigment proteins were provided by J. K. Lanyi and L. S. Brown. The wild-type protein was provided by M. Lu.

FTIR Measurements. Preparation of dried films on BaF_2 windows, hydration, and mounting into the cryostat (Oxford Optistat) were carried out as described previously (*16*). In

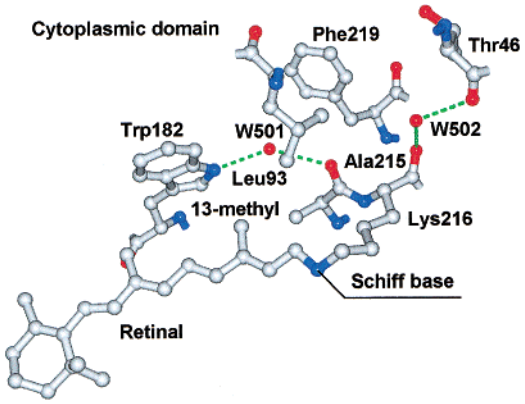

FIGURE 1: Locations of amino acid residues, water molecules, and the retinal chromophore in bacteriorhodopsin which are discussed in this text. This figure was depicted using the coordinates from PDB entry 1c3w (*28*).

the study presented here, all the measurements were taken on films hydrated with D_2O or $D_2{}^{18}O$ (purchased from ICON; 85.5% ^{18}O, 80% D). Utilization of D_2O instead of H_2O is helpful in separating the water bands from other bands that are not D_2O-sensitive. For the wild type and F219L, the replacement of exchangeable protons with deuterons was nearly as complete as expected from the deuterium content in D_2O, as judged from the intensity of the 3642 cm^{-1} band due to the O−H stretching vibration of an internal water molecule. However, in L93M and W182F, ∼45% of the O−H stretching vibration bands remained. This is not due to the presence of a fraction that is unexchangeable with added deuterated water. Approximately half of these bands shifted in $D_2{}^{18}O$, indicating that added D_2O or $D_2{}^{18}O$ was mixed with a larger than usual amount of residual water in the dry films. The results were, however, convincing enough to allow us to study the O−D stretching vibrations without further improvements of the extent of the exchange, which would require large amounts of expensive $D_2{}^{18}O$ water.

Each hydrated film was placed in the cryostat, installed in the FTIR spectrometer (Bio-Rad FTS6000), and then illuminated with yellow light (Corning 3-71 filter, >450 nm) at 273 K for 5 min to adapt the sample to light. After illumination, the sample was kept for 5 min at 273 K (to ensure the decay of N) and then cooled to 80 K. The difference spectra for the K and L intermediates were obtained in the following way. The absolute spectra (average of eight spectra, each of which is composed of 256 scans at 2 cm^{-1} resolution) were recorded at 80 K before (A) and after (B) illumination with blue light (Corning 4-96 filter, 350−500 nm) for 2 min. The sample was then warmed to 170 K, held for 10 min, and then recooled to 80 K to record the absolute spectra (C). These temperature transitions take ∼10 min. Spectrum B minus spectrum A gives the K minus BR difference spectrum and spectrum C minus spectrum A, the L minus BR spectrum at 80 K for the wild type. The sample was warmed to 273 K to return the pigment to its initial state before the next measurement cycle at low temperatures. These measurements were repeated three times and the data averaged. To record the spectra between L and its primary photoproduct L′, a sample was first illuminated with red light (2-62 filter, >600 nm) at 170 K to form L, and then cooled to 80 K. L′ was produced by illumination of L at 80 K with blue light (4-96 filter, 350−500 nm) with

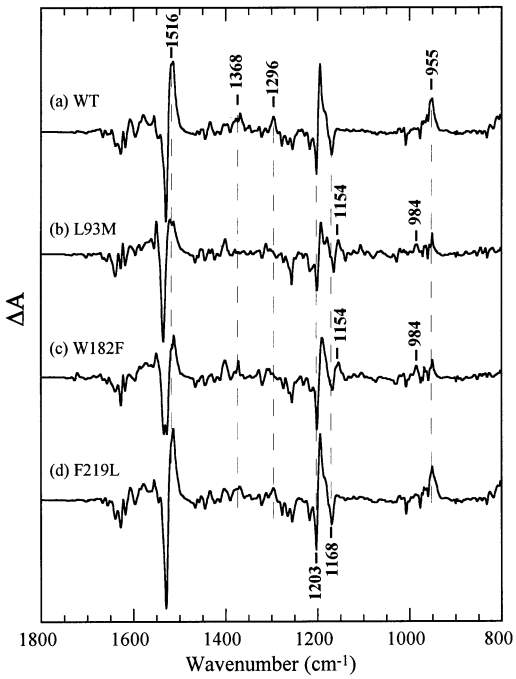

FIGURE 2: Difference spectra in the 1800−800 cm^{-1} region for the photoreaction of BR at 80 K: (a) wild type, (b) L93M, (c) W182F, and (d) F219L. The full length of the ordinate is 0.19 absorbance unit for the wild type. This value is lower by a factor of 1.5 for the mutant pigments.

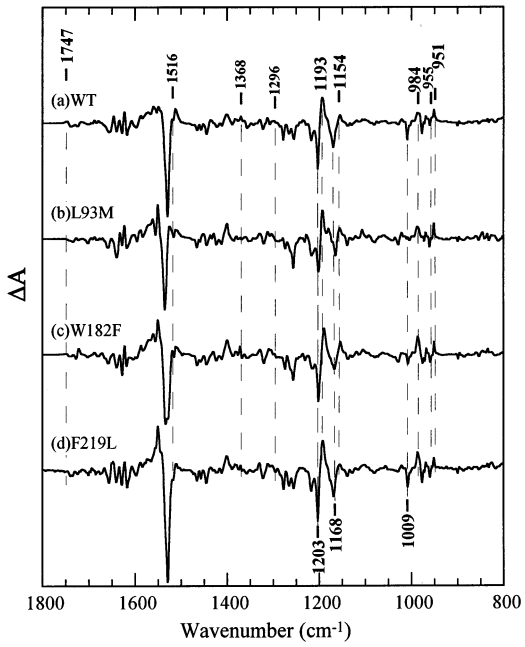

FIGURE 3: Difference spectra recorded at 80 K between BR and the species produced by illumination of BR at 80 K, after warming to 170 K, and then recooling to 80 K: (a) wild type, (b) L93M, (c) W182F, and (d) F219L. Wavenumbers for the bands which are absent in L and present in K (see Figure 2) are shown with smaller letters. The full length of the ordinate is 0.19 absorbance unit for the wild type.

subsequent deep red light illumination (2-64 filter, >640 nm) to remove K, as described previously (*27*).

RESULTS

L Is Formed at 80 K in the L93M and W182F Mutants. We first obtained the difference spectra in D_2O between wild-type BR and its photoproduct K at 80 K (Figure 2a) and then its thermal product, L, upon warming to 170 K and cooling the sample back to 80 K in the dark (Figure 3a). In the wild type, the bands at 1516, 1368, 1296, and 955 cm^{-1}, characteristic of the chromophore of K (Figure 2a), are virtually absent in L (Figure 3a). Hydration by D_2O is advantageous not only for comparing the O−D stretching vibration bands in the absence of other, D_2O-insensitive bands (see below) but also for detecting L by its diagnostic 984 cm^{-1} band, which appears only in D_2O (*32*) (Figure 3a). Bands characteristic of the chromophore of L are observed at 1154 and 984 cm^{-1} (Figure 3a). The former was assigned to the C_{14}−C_{15} stretching vibration (*33*) and the latter to the C_{15},N HOOP of the retinal (*9*, *10*). These bands were absent in K (Figure 2a). The K to L conversion occurs completely for the wild type at 170 K, as judged from depletion of the K specific bands.

These wild-type spectra were compared with the difference FTIR spectra for the mutants, obtained after illumination at 80 K (Figure 2b−d) and after subsequent warming to 170 K (Figure 3b−d). The absence of the intense negative band at 1189 cm^{-1} due to the 13-cis, 15-syn chromophore (*34*) verifies that the photoproduct in the wild type and the mutants arise from the all-trans bacteriorhodopsin (BR). The amplitudes of the L minus BR spectra of the mutants have been adjusted by matching their amplitudes for the band of L at 951 cm^{-1} to the amplitude of this band for the wild type (Figure 3). The differences between the spectra of the photoproducts formed at 80 K and their thermal products at 170 K are much smaller for the two mutant pigments, L93M (Figures 2b and 3b) and W182F (Figures 2c and 3c) than for the wild type (Figures 2a and 3a). Moreover, the light-induced spectra of these mutant pigments taken at 80 K without warming to 170 K are similar to those of L for the wild type (Figure 3a), with respect to the presence of the bands at 1154 and 984 cm^{-1}, among others, and do not resemble the K minus BR spectrum of the wild type (Figure 2a). For example, the K specific bands at 1368, 1296, and 955 cm^{-1} are missing in the spectra of L93M (Figure 2b) and W182F (Figure 2c) at 80 K. The results show that in these mutants L is produced upon irradiation already at 80 K. Nevertheless, a smaller K specific band prominent at 1516 cm^{-1} was observed for both L93M and W182F at 80 K (Figure 2b,c). This band could be removed by illumination with deep red light (Corning filter 2-64, >650 nm). The resulting spectra were the same as those seen after warming to 170 K (not shown). Thus, in these mutants, K is unstable at 80 K and mostly L is formed. As another control, the difference spectrum at 80 K of F219L (Figure 2d) is similar to that of the wild type (Figure 2a), so L is not formed at 80 K in this mutant.

Water O−D Stretching Vibrational Bands in the L minus BR Spectra of the Wild-Type and Mutant Pigments. Figure 4 shows the L minus BR spectra for the wild type (a), L93M (b), W182F (c), and F219L (d) in the region where the water O−D stretching vibrations are expected, 2750−2500 cm^{-1}. The spectra were recorded at 80 K. The spectra obtained in $D_2{}^{18}O$ are superimposed as thin lines to identify the water O−D stretching vibration bands. Since the bands of the L minus BR spectra in D_2O have not been described, below we characterize these bands in the wild-type and mutant pigments.

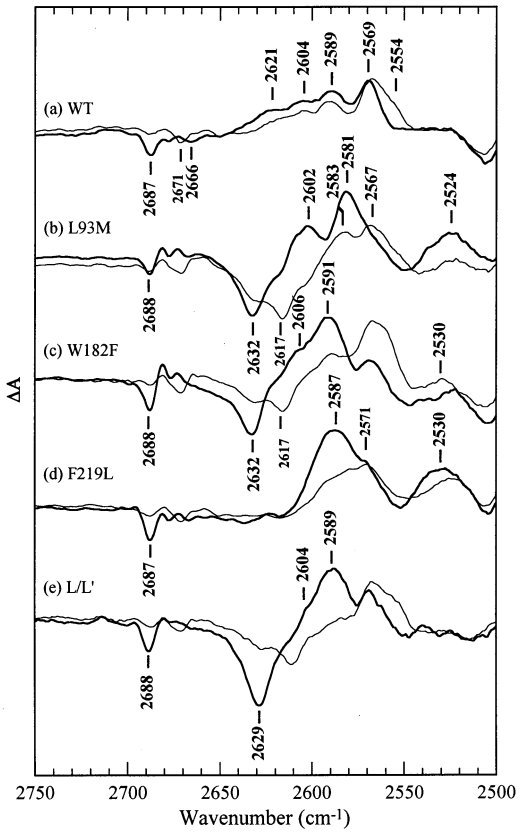

FIGURE 4: L minus BR difference spectra in the 2750−2500 cm^{-1} region recorded at 80 K in D_2O (thick lines) and in $D_2{}^{18}O$ (thin lines): (a) wild type, (b) L93M, (c) W182F, and (d) F219L. Also shown are the L minus L′ spectra of the wild type (e) in D_2O (thick line) and in $D_2{}^{18}O$ (thin line). The length of the ordinate is 0.019 absorbance unit for the wild type.

First, we examine the negative bands due to the initial state of the pigment (BR). The 2687 cm^{-1} band of the wild type in D_2O (Figure 4a) corresponds to the 3642 cm^{-1} band of the water O−H stretching vibration of BR, which disappeared in the D85N mutant (*35*). It shifts to 2671 cm^{-1} in $D_2{}^{18}O$. A 16 cm^{-1} downshift upon ^{18}O substitution is expected for water O−D stretching vibrations in this spectral region, thus identifying this as a water band. This band appeared at almost the same frequency for all the mutants that were studied (Figure 4b−d). It can be assigned to a vibration of a water molecule in the extracellular domain interacting with Asp85. The 2666 cm^{-1} band of the wild type that disappears in $D_2{}^{18}O$ (Figure 4a) probably corresponds to the 3607 cm^{-1} band in H_2O, which is depleted in T46V (*15*); it can be assigned to a water molecule in the cytoplasmic domain, presumably close to Thr46.

The most remarkable feature in the L minus BR spectrum of L93M (Figure 4b) is an intense negative band of the pigment at 2632 cm^{-1}. A similar negative band at 2632 cm^{-1} is also present in W182F (Figure 4c). These bands are largely due to water because they are shifted to 2617 cm^{-1} in $D_2{}^{18}O$ (thin lines). Such an intense negative band was not observed for the wild type (Figure 4a) or for F219L (Figure 4d).

Four positive bands at 2621, 2604, 2589, and 2569 cm^{-1} are present in the spectrum of wild-type L (Figure 4a). These four positive bands are separated by ∼16 cm^{-1} from each other. The 2621 cm^{-1} band undergoes a shift to ca. 2606 cm^{-1} in $D_2{}^{18}O$. The intensity of the band in $D_2{}^{18}O$ is similar to that in D_2O (i.e., $D_2{}^{16}O$) at 2604 cm^{-1}, suggesting the compensation of the band shifting out from 2604 cm^{-1} by the band shifting down from 2621 cm^{-1}. A similar shift might take place for the 2589 cm^{-1} band. Its shift in $D_2{}^{18}O$ will be shown below for the L minus L′ spectrum (Figure 4e). Thus, these three bands contain O−D stretching vibration modes. The 2569 cm^{-1} band is preserved even after $D_2{}^{18}O$ substitution, but also is skewed toward lower frequencies around 2554 cm^{-1}, where a shifted band in $D_2{}^{18}O$ is expected.

The L intermediate of L93M (Figure 4b) exhibited positive bands at 2602, 2581, and 2524 cm^{-1}. The first two bands moved to 2583 and 2567 cm^{-1} in $D_2{}^{18}O$ (thin line). The magnitude of the shifts ensures that these are water O−D stretching vibrations. Two similar O−D stretching bands for W182F were observed as a peak at 2591 cm^{-1} with a shoulder at 2606 cm^{-1} (Figure 4c). Thus, these mutants retained the 2604 and 2589 cm^{-1} bands of the wild type (Figure 4a), but the possible presence of the 2621 cm^{-1} band that appeared in the wild type was obscured by the large negative band at 2632 cm^{-1}. F219L exhibited positive bands at 2587 and 2530 cm^{-1} (Figure 4c). The bands around 2621 and 2604 cm^{-1}, which are present in the wild type, are absent. Only the 2587 cm^{-1} band can be ascribed to water because of its ∼16 cm^{-1} shift toward 2570 cm^{-1} in $D_2{}^{18}O$. The other bands have much smaller shifts.

Similarity of the L minus BR Spectra in L93M and W182F to the L minus L′ Spectrum of the Wild Type. The 2632 cm^{-1} O−D band is peculiar for the L minus BR spectra of L93M and W182F in D_2O (Figure 4b,c). A similar intense negative band at 3556 cm^{-1} with a paired positive band at 3503 cm^{-1} was observed for L93M in H_2O (not shown in the figures). This pair of bands in L93M is similar to a pair of bands at 3549 (−) and 3497 (+) cm^{-1} which was previously found in the L minus L′ difference spectrum of the wild type in H_2O (*27*). The L minus L′ spectra in D_2O and in $D_2{}^{18}O$ are shown in Figure 4e. L′ of the wild type exhibited an intense 2629 cm^{-1} band, which is similar to the 2632 cm^{-1} bands of the initial states of L93M (Figure 4b) and W182F (Figure 4c). Because of the cancellation by this large band, the 2621 cm^{-1} band of L could not be seen and the 2604 cm^{-1} band appears only as a shoulder. The large depletion of this band makes the shift of the 2589 cm^{-1} band in $D_2{}^{18}O$ clear, as described above.

The intense negative band at 2629 cm^{-1}appears in the spectrum of the all-trans photoproduct of L at 80 K, L′, but not in the initial state (BR) of the wild type. However, in the L93M and W182F mutants, a similar negative band is present even in the unphotolyzed state. It does not belong to a photoproduct produced by steady state illumination at 80 K. This was tested in successive partial phototransformations produced by short-term illuminations of L93M in D_2O (Figure 5). Despite some baseline distortion, it is clear that the negative band at 2632 cm^{-1} in D_2O was formed in parallel with emerging positive bands at 2602 and 2581 cm^{-1}. The progression of these bands also occurs in parallel with the generation of the chromophore bands (not shown) and the perturbation of the water O−D band at 2687 cm^{-1} (Figure 5). These results indicate that the O−D water vibration that gives the intense O−D stretching band at 2632 cm^{-1} is present in the unphotolyzed (initial) state of L93M and is not due to a photoproduct produced by illumination at 80 K.

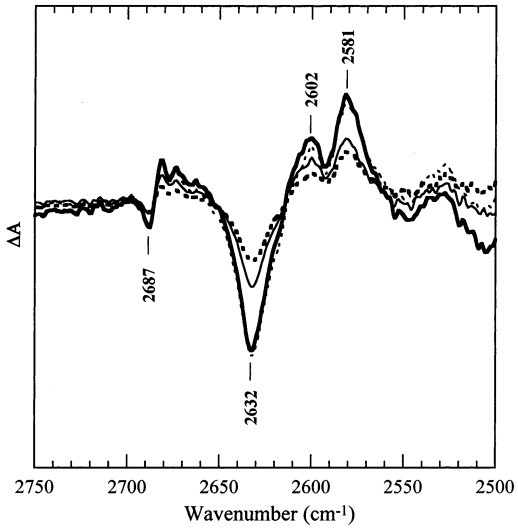

FIGURE 5: Light-induced difference spectra in the 2750−2500 cm^{-1} region recorded at 80 K for L93M. The duration for the illumination was 1, 2, 8, and 64 s for the innermost thick dotted line, the second thin line, the third thin dotted line, and the outermost thick line, respectively. The full length of the ordinate is 0.007 absorbance unit.

The L93M and W182F mutations affect the behavior not only of water but also of other bands. The 1747 cm^{-1} band, due to the perturbation of Asp96, is characteristic of the L intermediate of the wild type in D_2O (*19*, *36*) (Figure 3a); it is absent in L93M (Figure 3b) and small in W182F (Figure 3c). The 1009 cm^{-1} band due to the retinal methyl vibration (*37*) was not observed in the L minus BR spectrum of L93M (Figure 3b) and has reduced intensity in the spectrum of W182F (Figure 3c). The perturbations of Asp96 and the methyl vibration also did not occur in the L to L′ transition (*27*). The latter observation indicates the conservation of the environment around Asp96 and methyl groups of the retinal in the L to L′ transition. These results suggest that the environment of Asp96 and the methyl groups of the chromophore in L93M do not change from the initial unphotolyzed BR state to L, either because they were already perturbed in the initial state of these mutants or because these mutations decouple Asp96 from the changes in the chromophore environment upon L formation.

DISCUSSION

Understanding the mechanism of the proton pump implies elucidation of how the light energy, stored in the isomerized and twisted chromophore of the primary photoproduct K, is used for subsequent proton transfer. In turn, this requires identifying the residues and water molecules that interact with the chromophore and the interactions that are critical for the formation of the intermediates. In this paper, we pinpoint two residues, Trp182 and Leu93, mutation of which strongly affects the hydrogen bonding of internal waters and the formation of the L intermediate, the state which follows K and precedes proton transport from the Schiff base to Asp85 during the L to M transition. These residues are located close to the chromophore (see Figure 1 for this region of BR). The nitrogen of Trp182 is connected to Ala215 by Water501 in the initial BR. Mutation of Trp182 for Phe most likely destroys or weakens the hydrogen bond connecting the two residues in helices F and G. The methionine residue in L93M, which is rather apolar (*38*), introduces a sulfur atom which provides an additional site for hydrogen bonding, which might affect Water501. A high-resolution structure of L for the wild type and of the initial state of the mutants would be helpful for clarifying the changes involved in L formation. Earlier studies of the K and L intermediates trapped at low temperatures did not reveal any structural changes (*39*, *40*). Recent X-ray crystallographic studies indicate that the changes involved in the early stages of the photocycle, including K formation (*41*, *42*) and even the formation of the early M intermediate (*43*), are small; in most cases, displacements are in the range of a fraction of an angstrom, often close to the limit of resolution which is still a limiting factor along with partial occupancy of the intermediates, in gaining the exact structural information. The structure of L determined by Royant et al. (*44*) showed larger changes, including those involving Trp182; however, concerns that the occupancy of L was low were raised (*3*, *45*, *46*), and the crystal contained a large amount of M (or an M-like species). The latest structures of K and early M (*42*, *43*) reveal important structural changes in the chromophore in addition to isomerization around the C13═C14 bond and changes in the interaction of the Schiff base and Asp85 with the water between them (W402).

FTIR spectroscopy is very sensitive to small changes in hydrogen bonding or changes in bond orientation. This motivated us to extend our studies on the interaction of the chromophore with the retinal binding site in L. Earlier data indicated that formation of L involves perturbation of Trp182. This study shows that mutations of Trp182 and nearby Leu93 critically affect L formation and hydrogen bonding of nearby water molecules.

Several Weakly H-Bonding Waters Are Involved in the Formation of L. It is known that several water O−H stretching vibration bands appear in L with much larger intensities than in BR (*4*, *47*). At 170 K in H_2O, they appear as a broad diffuse band, and analysis of individual bands is further hampered by the presence of an intense N−H stretching vibration band of Trp182 which is located at the center of the broad water band. In this study, this difficulty was largely overcome by recording the spectra in D_2O-hydrated samples which shifted the water bands away from the Trp band and also enhanced their resolution. In addition, recording the L spectra at 80 K further sharpened the overlapping bands. The 2604 cm^{-1} band of L for the wild type was observed at 170 K only as a shoulder of the strong band at 2594 cm^{-1} (not shown), but became much more discernible by its 5 cm^{-1} downshift upon cooling to 80 K. Four O−D stretching bands at 2621, 2604, 2589, and 2569 cm^{-1} could be distinguished for L at 80 K. These correspond to the unresolved water O−H stretching bands distributed between 3550 and 3450 cm^{-1}. The >900 cm^{-1} downshift caused by replacing H_2O with D_2O is close to the expected value of ∼960 cm^{-1}.

A recent study (*48*) on the water molecules in a model system of a neat CCl_4−H_2O interface showed that the O−H bond that orients into CCl_4 and the other O−H bond that H-bonds with a water in the bulk phase exhibited O−H stretching vibrations with peaks at 3669 and 3444 cm^{-1} with full bandwidths at the half-maximum of 44 and 220 cm^{-1}, respectively. They further showed that the water O−H vibration that is present in the bulk phase exhibits a wider

band with a peak at 3250 cm^{-1}. These can be classified as the vibrations of very weak, weak, and strong H-bonding waters, respectively. From their somewhat higher frequencies, the water molecules for the four O−D stretching vibrations of L can all be regarded as weakly H-bonded. No water O−D stretching vibration bands were observed below 2500 cm^{-1} in the L minus BR spectrum (not shown). This does not exclude the presence of vibrations containing a water O−D stretch coupled with vibrations of other groups.

Facilitation of L Formation in L93M and W182F. L formation is greatly facilitated at low temperatures for both the L93M and W182F mutants; in both, L is formed at 80 K, where normally only K is present. The initial (unphotolyzed) states of both mutants also exhibited a large water O−D stretching band at 2632 cm^{-1}. These intense bands in L93M and W182F gave rise to the large, similarly shaped 2581 and 2591 cm^{-1} bands for the L intermediates of the mutants. The shift to lower frequencies indicates that L formation is accompanied by an increase in hydrogen bonding strength of these waters, but the H-bonds are still weak. The presence of the intense water band in the initial state of L93M and W182F indicates that the structures of the water hydrogen bonds, similar to those specific for L, are already present in the initial state of the mutants. Moreover, the L′ photoproduct of the wild-type pigment seems to have almost the same water structure as the two mutants, as judged by the large negative water O−D band that appeared at about the same frequency as that of the mutants, 2629 cm^{-1}. The chromophore of L′ is in the all-trans state like the initial state of BR. However, its conformation and environment are not identical with those of BR; the methyl vibration of the retinal retained the same state as in L, and the in-plane bending vibrations of the Schiff base did not return completely to their state in BR (*27*). These features can be explained by positing that some interactions of the chromophore with water change only slightly upon photoisomerization of the chromophore in the L to L′ transition as reflected by the shift of the 2589 cm^{-1} band in L to 2629 cm^{-1} in L′ (Figure 4e). This shift occurs without a substantial change in the intensity of the water band, which in L′ is much more intense than the water bands of BR in the L minus BR spectrum (Figure 4a). The formation of L in the L93M and W182F mutant pigments is thus similar to the L′ to L transition in the wild type which also can occur at 80 K (*23*). In all these cases, trans → 13-cis isomerization of the chromophore at 80 K yields L and is accompanied by the shift of the intense water band from 2629 to 2589 cm^{-1}. The latter indicates that the water molecule(s) responsible for this intense band must interact with the chromophore and change its hydrogen bonding in response to the isomerization.

The absence of perturbations of both Asp96 and the methyl groups of the retinal in the L intermediates of the L93M and W182F mutants is similar to that in the L ↔ L′ photoconversions (*27*). It indicates that the environment of these groups in L is similar to the environment in the initial states of these pigments.

Changes in Water Binding in the Cytoplasmic Domain in L. The negative band of the water O−D stretching vibration at 2666 cm^{-1} in the L minus BR spectrum (Figure 4a) could be due to W502 found in the X-ray structure of BR (*28*). This water is present between the peptide carbonyls of Thr46 and Lys216 and also close to the methyl group of Thr46. This band and the positive O−D band at 2589 cm^{-1} are depleted by T46V (not shown) as are the corresponding O−H stretching bands (*15*). This suggests that W502 is one of the water molecules the hydrogen bonding of which must change for L formation to occur.

L formation is accompanied by the perturbation of Trp182 (*14*, *49*). Changes in the orientation of some of the residues in contact with the retinal, like Trp182 and Leu93, were shown in the X-ray structures of M (*50*). The results presented here suggest that perturbation of these residues has already occurred in L and water molecules (probably W501 and W502, but others are also candidates) move into a space produced by the displacement of Leu93, Trp182, and the retinal, or at least change their hydrogen bonding with these groups. In the X-ray structure of BR (Figure 1), the CD1 group of Leu93 and the indole NH group of Trp182 face each other and also face the 13-methyl group of the retinal. In the L93M and W182F mutants, the space delineated by these groups might become wider to accommodate additional water molecules, or water molecules already there might acquire more freedom to interact. The W182F and L93M substitutions also change the hydrogen bonding capabilities of these sites as mentioned above. Hydrogen bonding of W501 changes during the photocycle. The oxygen atoms of Thr178 and the C═O group of Ala215 were assumed to be hydrogen bonding sites for W501 in M and BR, respectively (*51*, *52*). The Phe219 residue is separated from the retinal, and F219L does not yield an L intermediate at 80 K.

The O−D vibrations of water in L at 2589 cm^{-1}, in L′, and in the L93M and W182F mutants at ca. 2632 cm^{-1} exhibit large intensities associated with their having a strongly polar character. This could be provided by the polarization induced by the interaction with the local positive charge in the retinal and also by the interaction between water molecules.

Several observations suggest that L can be regulated by elements on the cytoplasmic side of retinal. The lifetime of the L intermediate is lengthened in W182F (*14*, *30*) and L93M (*29*). These data along with the present and previous results, that L is stabilized by the interaction of the Schiff base with Wat46 (*16*), indicate that formation and decay of L are regulated from the cytoplasmic side of the retinal. This does not exclude, of course, participation of waters in the extracellular domain in the formation of L.

Water as a Mobile Group Involved in the Formation of L. Gottschalk et al. (*53*) showed microsecond exchange of internal water molecules in bacteriorhodopsin in a multinuclear magnetic relaxation dispersion study. Recent molecular dynamics studies on water in hydrophobic channels such as aquaporin (*54*) have shown that water can penetrate into these channels within the nanosecond time domain, and subtle changes in interaction with the channels can bring a large change in water occupancy. The L intermediate is formed very quickly (sub-microseconds) at room temperature and appears to be stable at a low temperatures (170 K). These conditions probably do not allow the movement of significant parts of the protein. Photoisomerization leads to conflict or steric tension between the retinal and nearby residues, Leu93 and Trp182, causing their displacement. Water as small mobile molecules can react to these changes, for instance, by changing hydrogen bonding partners or entering into a

newly formed cavity and temporarily stabilizing the new structure, L. If the altered conformation and hydrogen bonding are present in this region before isomerization, like in the L93M and W182F mutants, L is produced more easily.

In conclusion, features of the mutants described in this study indicate that the altered residues, Leu93 and Trp182, and the water molecule(s) interacting with them from the cytoplasmic side are critically involved in the formation of the L intermediate. Formation of L is accompanied by the alteration of hydrogen bonding with water molecules. This change may be stimulated by L93M and W192F mutations that facilitate L formation and exhibit some features of L already in their initial states.

ACKNOWLEDGMENT

We are thankful to Dr. Joel E. Morgan for his help with using FTIR facilities and to Drs. Janos K. Lanyi, Richard Needleman, Leonid S. Brown, and Miao Lu for their kind gift of the mutant and wild-type pigments.

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BI020532N