Water Rearrangement around the Schiff Base in the Late K (K_L) Intermediate of the Bacteriorhodopsin Photocycle

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Previous FTIR studies at room temperature have distinguished K_L (late K) from K (early K) as distinct photocycle intermediates of all-trans bacteriorhodopsin differentiated by the prominence of their hydrogen out-of-plane bending vibration (HOOP) bands at 985 and 957 cm^{-1}, respectively. Earlier we showed that in the next photointermediate, L, the C_{15},N-HOOP appears as a set of several bands at around 1064 cm^{-1}, which were shown to be due to the interaction of the Schiff base with water (Maeda et al. *Biochemistry* **2002**, *41*, 3803). The present study examines water rearrangement around the Schiff base in K and K_L by analysis of the HOOP bands and the water vibration bands. (1) Studies of the thermal transformation of the primary photoproduct K formed at 80 K upon warming to 135 and 170 K confirm that a K-like photoproduct formed at 135 K is an intermediate between K and L. From its HOOP band at 985 cm^{-1}, this photoproduct can be assigned to K_L. (2) In K_L, as in L, the C_{15},N-HOOP band is affected upon replacement of H_2O by $H_2{}^{18}O$ water indicating the Schiff base's interaction with water. This feature was not observed for K. (3) The water molecule that is affected by the T46V mutation undergoes a perturbation in K_L as in L, and to a smaller extent in K. However, in K_L, the water does not exhibit the intense band seen in L. (4) A water molecule near Asp85 (most likely Water401), which is perturbed only slightly in K, is more strongly perturbed in K_L and in L.

Bacteriorhodopsin from *Halobacterium salinarum* acts as a light-driven proton pump, transporting protons from the cytoplasm to the extracellular medium. The retinal chromophore of bacteriorhodopsin is linked to Lys216 through a protonated Schiff base linkage ($-C_{15}H{=}NH-$). Light absorption by the chromophore leads to trans to cis isomerization of the $C_{13}{=}C_{14}$ bond of the all-trans retinal in bacteriorhodopsin (BR), resulting in formation of a high-energy photoproduct K.[1−4] Subsequent thermal relaxation of K and restoration of the initial state occurs through a series of intermediates, usually given as K, L, M, N, and O, which can be distinguished by different visible spectra.[1] FTIR spectroscopy has been used to characterize these intermediates and to detect changes of the chromophore and the protein, including changes in the protonation state of the Schiff base and several key amino acid residues during the photocycle (see reviews in refs 5−9).

The Schiff base is an important part of the chromophore because it is directly involved in proton transfer. FTIR studies have shown that the light-induced isomerization of the chromophore to form K results in a weakening of the H-bonding of the Schiff base N−H with its acceptor, a water molecule.[10−11] Unusually strong hydrogen out-of-plane bending vibration (HOOP) bands detected in resonance Raman spectrum of K indicate a distorted conformation of the chromophore.[12] An intense C_{15}-HOOP band of K in the difference FTIR spectrum was taken as evidence for the increased polarization of the $C_{15}-H$ bond due to the out-of-plane twist of the C=N bond in the chromophore.[13] X-ray studies showed that in the initial all-trans state a water molecule (Water402) intervenes between the Schiff base and the counterion(s), Asp85 and Asp212.[14] These two residues are further bridged by two other water molecules (Water401 and Water406).[15,16] FTIR spectroscopic studies,[11] ab initio quantum mechanical/molecular mechanical calculations,[17] and the X-ray structure[18,19] have shown an out-of-plane orientation of the N−H bond toward Water402 in forming K. Besides distortion in the Schiff base−13-methyl region of the chromophore and small changes in the position of the amide of Lys216 and the side chain of Tyr 185, no significant displacements of other side chains or water molecules were detected in the most recent X-ray studies on K produced by illumination at 100 K.[18,19] These studies did not detect larger changes around the Schiff base seen at 110 K in the earlier X-ray study by Edman et al.[20] Nevertheless, FTIR studies have detected perturbations of the following: Asp115;[21] Thrl7, Thr89, and Thr90;[22,23] the interaction of the Cα of Lys216 with the Schiff base;[13] the proline C=N bond between Tyrl85 and Pro186.[24] Changes of water bands upon K formation were much smaller than the perturbations observed for L or M formation.[25,26]

In the L intermediate, H-bonding of the protonated Schiff base to water is stronger than that in BR or K.[10,27] In formation of L a number of residues undergo perturbations.[7,28] One of the most noticeable features in L is large intensity water vibration bands in the 3550−3450 cm^{-1} region; these bands are greatly diminished by a mutant of Thr46, a residue close to Asp96 in the cytoplasmic domain,[29] suggesting that they are

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due to water near these residues. The vibrational properties of these water molecules indicate that they are weakly H-bonded and more polarized than those in the initial BR state. Three HOOP bands at 1072, 1064, and 1056 cm^{-1} were observed for L.[30] These bands were depleted in T46V, partially restored in T46V/D96N, and augmented in V49A. These changes in the HOOP bands occur in parallel with the shifts of the L to M equilibrium and with the changes in the intensities of the water bands, suggesting that water molecules in the cytoplasmic domain stabilize L through their interaction with the Schiff base.[30] Some mutations of Leu93 and Trp182, which are located close to the 13-methyl group of the retinal, strongly perturb water molecules in the initial state of the pigment and facilitate the formation of L even at much lower temperatures (80 K) than in the wild type.[31] Probably one or more water molecules are present in the Schiff base−13-methyl group region of the initial state of the mutant, unlike in the wild-type, allowing for formation of L at these low temperatures.

Selective far-red light photoconversion of K to BR at 135 K revealed the presence of a K-like photoproduct with a prominent HOOP band at 985 cm^{-1}, a band that is barely seen in K.[32,33] The vibrational spectrum of this K-like photoproduct obtained at low temperatures is similar to that of K_L obtained by time-resolved spectroscopy.[33−37] Time-resolved vibrational spectroscopic studies at room temperature have presented evidence that a K-like species is formed in the submicrosecond time domain just before the formation of L.[33−39] This later K-like species has a lower intensity for the C_{15},N-HOOP band at 957 cm^{-1} than that seen in early K.[34,36,37] It was named K_L (late K) to distinguish it from the earlier species that was called K_E (early K)[37] or more commonly K and from the K intermediate formed at low temperature, around 80 K.[10,32,33,40]

Previous FTIR studies of K_L did not describe the changes in water vibrations upon the K to K_L transition nor the coupling of water vibrations with the HOOP vibrations. The present study examines the differences between K, K_L, and L. First we show that K_L observed at 135 K is an intermediate between K and L; it is formed thermally from K and decays to L. The specific features of the interaction of the Schiff base and water in K_L were then analyzed using bacteriorhodopsin of which C_{15}-position of the chromophore is deuterated (C_{15}-D bacteriorhodopsin), labeled water, and the T46V mutant, which affects water molecules in the cytoplasmic domain. The experiments show that the early conformational changes that occur during the K-to-K_L transition involve structural changes around the Schiff base and the perturbation of water molecules both in the extracellular domain (around Asp85) and in the cytoplasmic domain.

Materials and Methods

Bacteriorhodopsin films were made by air-drying a purple membrane suspension of wild-type pigment or wild-type bacteriorhodopsin reconstituted with C_{15}-deuterated retinal (C_{15}-D bacteriorhodopsin) in water[30] on a BaF_2 window. The films were hydrated by placing 0.2 μl of H_2O, D_2O, $H_2{}^{18}O$, or $D_2{}^{18}O$ at the edge of the film, and then hydrated films were placed into a cryostat in the FTIR spectrometer (BioRad FTS6000), as described previously.[30] Before cooling, the films were illuminated at 273 K by yellow light (a Corning 3-71 filter transmitting light at >450 nm) for 10 min to light-adapt them.

The difference spectra were calculated as the differences between the absolute spectra (average of eight spectra, each of which is composed of 256 scans at 2 cm^{-1} resolution). The difference spectra for K and K_L were obtained by two procedures. In procedure 1, first the absolute spectrum was measured after cooling to 135 K in the dark (spectrum A). The film was then cooled to 80 K, and the absolute spectra were measured before (spectrum B) and after (spectrum C) illumination with blue light (a Corning 4-96 filter transmitting light at 350−500 nm) for 2 min to form K. Then the film was warmed to 135 K, and the absolute spectrum was measured after keeping the sample at 135 K for 12 min (spectrum D). About 40% of K was transformed to K_L by this procedure. The absolute spectrum of BR and K_L was measured after removal of K by illumination with far-red light (Corning 2-64 filter plus a 7-59 filter transmitting light >700 nm) (spectrum E) for 5 min. The film was subsequently warmed and kept at 170 K for 10 min. The absolute spectrum was measured at 135 K (spectrum F). The difference (spectrum C − spectrum B) is the K minus BR spectrum at 80 K, (spectrum D − spectrum A) is the K + K_L minus BR spectrum at 135 K, (spectrum D − spectrum E) is the K minus BR spectrum at 135 K, (spectrum E − spectrum A) is the K_L minus BR spectrum at 135 K, and (spectrum F − spectrum A) is the L minus BR spectrum at 135 K. In procedure 2, the absolute spectrum was measured at 135 K (spectrum G). The spectrum of the film was then measured after the illumination with blue light at 135 K (spectrum H). Then the absolute spectrum was measured after removal of K by illumination with far-red light (spectrum I). (Spectrum H − spectrum I) is the K minus BR spectrum at 135 K, and (spectrum I − spectrum G) is the K_L minus BR spectrum.

Results

K_L Formed at 135 K is an Intermediate between K and L. K_L was obtained by procedure 1, in which K was produced by illuminating BR with blue light at 80 K and then warmed to 135 K. The formation of K is accompanied by a characteristic HOOP band at 957 cm^{-1}, the C−C stretching band at 1194 cm^{-1}, and the C═C stretching vibration band at 1514 cm^{-1} (Figure la), as described previously.[10,32,40] The absence of photoproducts from the 13-cis, 15-syn species was ascertained by the absence of its characteristic bands at 1346 and 1185 cm^{-1}.[41] Warming to 135 K resulted in the appearance of another HOOP band around 985 cm^{-1}, suggesting that K underwent a thermal transformation (Figure 1b). Illumination of this mixture at 135 K with far-red light depleted K. The K minus BR difference spectrum, obtained upon transformation of K to BR at 135 K, is shown in Figure lc. The spectrum is nearly identical with the K minus BR spectrum at 80 K (Figure la) and does not contain the 985 cm^{-1} band that arose by warming to 135 K (Figure lb). The difference spectrum between initial BR and the photoproduct left after removal of K contribution is shown in Figure 1d. Its shape is different from the K minus BR spectrum at 135 K (Figure lc) with respect to the main HOOP band at 985 cm^{-1}, which replaced the 957 cm^{-1} band of K, and is very similar to the K_L minus BR spectrum, which was obtained at 135 K by successive illumination of BR with blue light and then far-red light.[32,33] The K_L spectrum also has a smaller band at 1194 cm^{-1}, due to the C−C stretch, than K. The two bands at 1520 and 1515 cm^{-1}, which replace the C═C stretching band at 1514 cm^{-1} of K, are also less intense. Subsequent warming of K_L from 135 to 170 K produces L. Figure le shows the spectrum between initial BR and the state obtained upon warming the film to 170 K and recooling to 135 K. This difference spectrum is identical with the L minus BR spectrum,[6] showing a negative band at 1742 cm^{-1} due to the perturbation of Asp96[21,42] and positive bands at 1152 cm^{-1} due

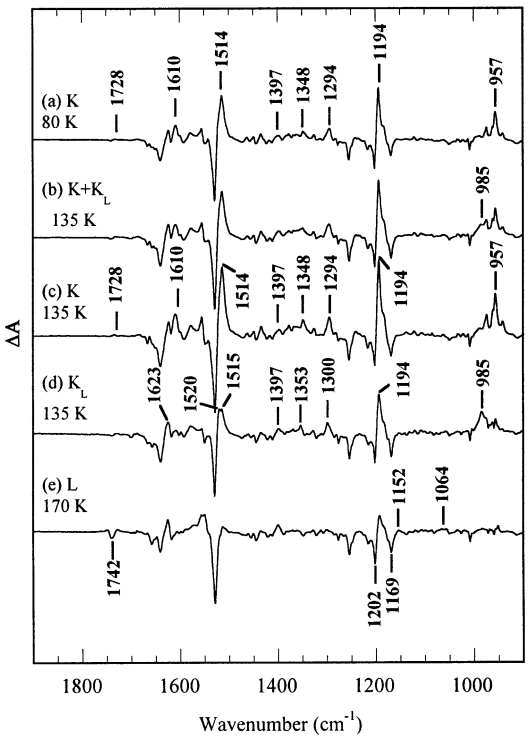

Figure 1. Spectra for the photoproducts formed by procedure 1 (see Materials and Methods) of unlabeled native bacteriorhodopsin. BR was illuminated by blue light at 80 K, giving the K minus BR spectrum at 80 K (a). This mixture of the BR and K was warmed to 135 K, giving the K + K_L minus BR spectrum at 135 K (b). The mixture was illuminated by far-red light to deplete K. The spectrum taken before minus the spectrum measured after this illumination gives the K minus BR spectrum at 135 K (c). The remainder is the K_L minus BR spectrum at 135 K (d). The spectrum after warming to 170 K and recooling to 135 K is the L minus BR spectrum (e). The amplitudes of the spectra were adjusted by normalization of the 1202 cm^{-1} band. The full length of the ordinate corresponds to 0.200, 0.140, 0.076, 0.050, and 0.074 absorbance unit for spectra a, b, c, d, and e, respectively.

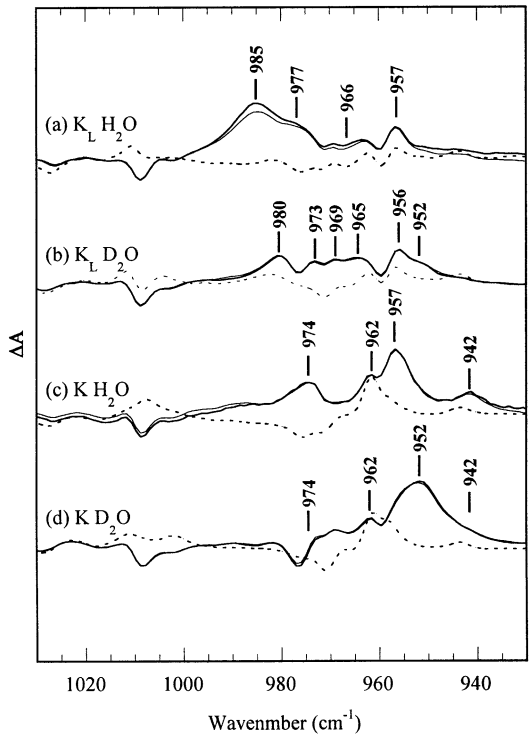

Figure 2. Comparison of HOOP vibration bands of K_L and K at 135 K. The K_L minus BR and K minus BR spectra were obtained by procedure 2 (see Materials and Methods) for native bacteriorhodopsin (thick lines) and C_{15}-D bacteriorhodopsin (dotted lines). The corresponding spectra in $H_2{}^{18}O$ (a and c) and $D_2{}^{18}O$ (b and d) were superposed as thin lines. Part a shows the K_L minus BR spectra in H_2O. Part b shows the K_L minus BR spectra in D_2O. Part c shows the K minus BR spectra in H_2O. Part d shows the K minus BR spectra in D_2O. The full length of the ordinate corresponds to 0.024 and 0.054 absorbance unit for the K_L minus BR and K minus BR spectra of the wild-type in H_2O, respectively.

to $C_{l4}-C_{15}$ stretching vibration,[43] at 1064 cm^{-1} due to the C_{15},N-HOOP,[30] and at 3486 cm^{-1} due to Trp182.[44] These results show that K_L is produced from K upon warming to 135 K and K_L is converted to L upon further warming to 170 K. Thus, the low-temperature photoproduct K_L is an intermediate between K and L. All K_L formed at 135 K changed to L at 170 K as K produced at 80 K changed completely to L upon warming to 170 K.[31] At 135 K, the K intermediate is present together with K_L (about 40%).

These spectra obtained at low temperatures are similar to those at room temperature. The early bathoproduct K_E described in the time-resolved study of Dioumaev and Braiman[37] is similar to the low-temperature bathoproduct K regarding the bands at 1728, 1610, 1514, 1397, 1348, 1294, and 1194 cm^{-1} shown in Figure la except that the 985 cm^{-1} band appeared in K_E. The FTIR spectrum of K_L at 135 K is similar to that at room temperature. The 1397, 1353, and 1300 cm^{-1} bands of K_L at 135 K (Figure ld) appear in the time-resolved spectrum of K_L at room temperature.[33,35-37] The small bands of this low-temperature K_L at 1515 and 1520 cm^{-1} (Figure 1d) are due to C═C stretching vibrations because they merged into a large band at 1512 cm^{-1} in C_{15}-D bacteriorhodopsin (not shown). The resonance Raman spectrum of a late K at room temperature[38,45] showed a band at 1520 cm^{-1}. These small differences from a C═C stretching band detected at 1512 cm^{-1} in the room-temperature spectrum[35] might be due to overlap with the C═C stretching vibration band of the initial unphotolyzed state.

Selective elimination of K by deep-red light suggests that its visible spectrum is red-shifted compared with that of K_L. However, the main band of the C═C stretching vibration of K at 1514 cm^{-1} is accompanied by a shoulder band at 1520 cm^{-1} (Figure lc). In this regard, K and K_L differ from each other only in the ratio of intensity of the two bands. It is not clear whether such a difference reflects a red shift in the visible spectrum. It should be noted that at room temperature no clear changes in the visible spectrum were detected for the K to K_L transition,[46] whereas changes were observed in time-resolved vibrational studies.[37,45]

Effects of D_2O and ^{18}O Water on K_L HOOP Vibrations. The effects of isotope substitution on the HOOPs of the Schiff base of K_L were compared with those of the HOOPs of K in Figure 2. The amplitudes of the spectra were adjusted by normalization of the 1202 cm^{-1} band, except for use of the 1169 cm^{-1} band for the spectra of the C_{15}-D bacteriorhodopsin. The C_{15}-HOOP bands of native bacteriorhodopsin (thick lines) can be identified as the bands that are depleted in C_{15}-D bacteriorhodopsin (dotted lines). The coupled C_{15},N-HOOP bands can be identified as the bands that are further shifted in D_2O (parts a vs b and c vs d in Figure 2). This is because the corresponding N-deuteron out-of-plane bending vibration bands in D_2O, which should be located at much lower frequencies (not seen in the spectra shown), are coupled only weakly with the C_{15}-HOOP. Procedure 2 was used for recording the K minus BR and K_L minus BR spectra (see Materials and Methods).

We will first examine the K minus BR spectrum. Among the positive bands of the K minus BR spectrum (Figure 2c),

the intense band at 957 cm^{-1} of K disappeared in C_{15}-D bacteriorhodopsin (dotted line) and underwent a slight shift to 952 cm^{-1} in D_2O (thick line in Figure 2d). The shifted band disappeared in the C_{15}-D bacteriorhodopsin (dotted line in Figure 2d). The smaller 974 and 942 cm^{-1} bands also disappeared in C_{15}-D bacteriorhodopsin (dotted line in Figure 2c) but persisted in D_2O.[10] The 962 cm^{-1} band persists in both D_2O and in C_{15}-D bacteriorhodopsin. Thus, the 957 cm^{-1} band is the only C_{15},N-HOOP band of K. The spectral shape and the responses to the isotopes for K at 135 K are identical to those of the K minus BR spectrum obtained by illumination at 77 K[10], indicating no contribution of K_L in the K minus BR spectrum at 135 K.

The K_L minus BR spectrum (Figure 2a,b) does not have the 942 cm^{-1} band seen in the K minus BR spectrum, indicating that K was completely reverted to BR by far-red light. The K_L minus BR spectrum in H_2O (thick line in Figure 2a) exhibited two large C_{15}-HOOP bands at 985 and 977 cm^{-1} and two small bands at 966 and 957 cm^{-1}. These multiple bands appeared in D_2O at slightly lower frequencies as a set of bands at 980, 973, 969, 965, 956, and 952 cm^{-1} (thick line in Figure 2b). Hence, those bands at 985, 977, 966, and 957 cm^{-1} (thick line in Figure 2a) are due to the C_{15},N-HOOP of K_L. All of these bands were detected at similar frequencies in the time-resolved spectrum at room temperature.[36] The shift in C_{15}-D bacteriorhodopsin was also detected for the 985 cm^{-1} band at room temperature.[35]

One of the remarkable features of the HOOP bands in K_L is the intense band at 985 cm^{-1} (Figure 2a) compared to the relatively lower intensities of the HOOP bands in D_2O (Figure 2b). Intensity increases of the C_{15}-HOOP upon coupling with the N-HOOP suggest the presence of a H-bonding interaction of the Schiff base N−H with another group, which could result in the polarization of the N−H bond. One of the likely candidates for the interacting species is water. To examine further possible interactions of the HOOP chromophore vibrations in both K and K_L with water vibrations, the effects of changing the mass of water was examined by replacing ^{16}O water with $H_2{}^{18}O$ (Figure 2a,c) or $D_2{}^{18}O$ (Figure 2b,d). Among the HOOP bands, only the band at 985 cm^{-1} of K_L in H_2O has its intensity decrease slightly in $H_2{}^{18}O$ water (thin line in Figure 2a). This effect was not observed for other less-intense C_{15},N-HOOP bands of K_L. The results suggest the coupling of the N-HOOP of the Schiff base with the overtones or the combinations of the out-of-plane vibration of H-bonding water O−H, which is expected to be located in the 300−700 cm^{-1} region.[47] The lowering in intensity of the HOOP band could result from the ^{18}O-HOOP of water having a lower intensity and more-separated frequency than the corresponding ^{16}O−H vibration. Such an effect of ^{18}O water was not observed for the HOOP bands in K (Figure 2c). The corresponding C_{15}-HOOP bands of K_L in D_2O (Figure 2b) did not show a detectable ^{18}O effect.

Multiple bands of the C_{15}-HOOP bands of K_L are difficult to explain by the couplings of only the HOOP modes of the chromophore. They may arise from extensive interactions of the Schiff base with nearby groups, such as water O−H. However, this explanation cannot be applied to the C_{15}-HOOP in D_2O, which should be uncoupled. Molecular dynamics calculations suggested that the C_{15}−H part of the Schiff base approaches the carboxylate of Asp212.[17] The actual partners for the interactions could be determined in future isotope studies.

Another vibration mode of the Schiff base of K_L, the N−H in-plane bending vibration coupled with the C_{15}−H in-plane bending vibration, was identified as bands at 1354 and 1298 cm^{-1} that disappeared in D_2O and were affected by C_{15}D-bacteriorhodopsin, respectively. They also decreased the intensity in ^{18}O water (data not shown).

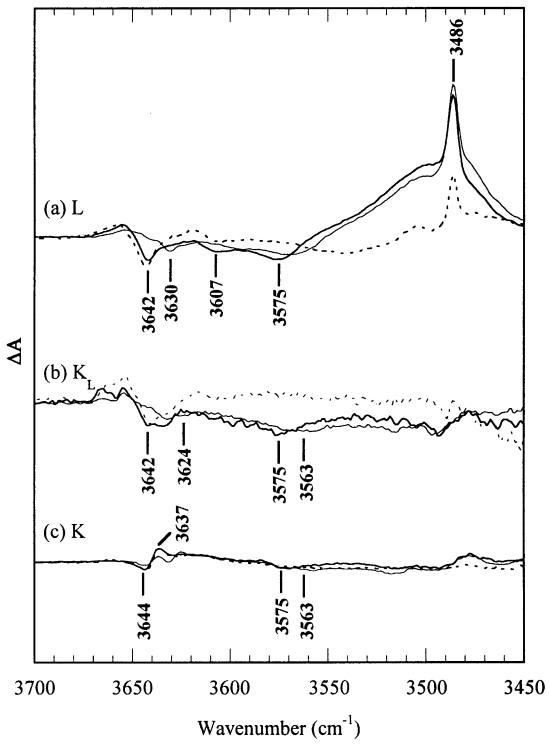

Figure 3. Water O−H stretching vibration bands of BR, K, K_L, and L. The water bands in the K_L minus BR spectra of the wild-type (b) were compared with those of the L minus BR spectra (a) and K minus BR spectra (c). The K_L minus BR and K minus BR spectra in the 3750−3400 cm^{-1} region were obtained by procedure 1 (see Materials and Methods) for native bacteriorhodopsin in H_2O (thick lines) and in $H_2{}^{18}O$ (thin lines). The L minus BR spectra were obtained by illumination of BR at 170 K. The spectra of the T46V mutant were superposed (dotted lines). The full length of the ordinate corresponds to 0.02 absorbance unit for the K minus BR spectrum in H_2O. The amplitudes of the other spectra were adjusted to this spectrum by normalization of the 1202 cm^{-1} band.

Perturbation of Internal Water in the K to K_L Transition. The water O−H stretching vibration bands of BR, K, K_L, and L were examined (Figure 3). The bands in the K_L minus BR difference spectrum were compared with those of the K minus BR and L minus BR spectra. These spectra were obtained by procedure 1. A contribution of about 10% due to the L minus BR spectrum to the K_L minus BR spectrum was subtracted by adjusting the L-specific band at 3486 cm^{-1}. To identify the water O−H bands, the spectra in H_2O (thick lines) were superimposed with the spectra in $H_2{}^{18}O$ (thin lines). If due to water, the latter bands should be shifted by ca. 12 cm^{-1} to lower wavenumbers in this spectral region. The intense water O−H stretching bands of L in the 3550−3450 cm^{-1} region, along with the negative band at 3575 cm^{-1} due to the depletion of BR (Figure 3a), disappeared in the T46V mutation[29] (dotted line). Hence, these bands are due to water molecules on the cytoplasmic side of the chromophore; they were named Wat46. The negative (BR) side of the K_L minus BR spectrum (Figure 3b) showed a band at 3575 cm^{-1}, which shifted toward a lower frequency to 3563 cm^{-1} in $H_2{}^{18}O$ (thin line) and was depleted in T46V (dotted line). This indicates a perturbation of Wat46 in K_L; a similar perturbation was seen for L. Though to a lesser extent, the K minus BR spectrum (Figure 3c) exhibited a similar band. On the other hand, the positive side due to K_L did not show any bands, unlike L. In K_L Wat46 is perturbed but does not seem to undergo bond polarization, unlike in L. This is consistent

with the observation that the T46V mutation does not deplete the C_{15},N-HOOP bands of K_L and K (not shown), in contrast to its effect on the C_{15},N-HOOP bands of L.[30]

The L minus BR spectrum exhibits (Figure 3a) a second water band at larger wavenumber (3642 cm^{-1}). It is due to an extracellular water because it is depleted in D85N[48] but not by T46V.

In the K_L minus BR spectrum (Figure 3b), a negative band at 3642 cm^{-1} is observed (Figure 3b), as in the L minus BR spectrum (Figure 3a). In the K minus BR spectrum, the corresponding water band appears only as a bilobe of much smaller bands at 3644 (−) and 3637 (+) cm^{-1}, as shown previously.[25,26]

Discussion

The changes in the FTIR spectrum produced when a sample containing K has its temperature increased from 80 to 135 to 170 K (Figure 1) indicate that a distinct species is formed at 135 K and is an intermediate between K and L. It has very similar spectral properties to the K_L species observed at room temperature.[33,35−37] In the present paper, the changed interactions of water around the Schiff base, which might be crucial for the formation of L and proton transfer, were investigated for K_L at 135 K.

Weak Schiff Base/Water Interaction in K_L. Earlier we showed that the L minus BR spectrum has multiple C_{15},N-HOOP bands.[30] They decreased their intensities in ^{18}O water and were depleted in T46V. These features of the bands indicate an interaction of the chromophore with water molecules and in particular those waters that are affected by the T46V mutation.[44] The HOOP bands in L are located at much higher frequencies, 1072, 1064, and 1056 cm^{-1}, than the uncoupled C_{15}-HOOP at 985 cm^{-1}. This must result from strong coupling of the N-HOOP with the C_{15}-HOOP and the interaction of the Schiff base with water. The stronger H-bonding of the Schiff base in L might be responsible for these effects.

The coupling of the N−H in-plane bending vibration with the C═N stretching vibration leads to a band in the 1650−1600 cm^{-1} region, though it is difficult to identify in the FTIR difference spectra even with the use of C_{15}-D bacteriorhodopsin because of overlap with the BR bands and amide I bands. The coupled band of K_L seems to be located at 1628 cm^{-1} in H_2O and 1612 cm^{-1} in D_2O (not shown). The difference between them, which is a measure of the H-bonding strength of the Schiff base (see the review in ref 6), is 16 cm^{-1}. The corresponding value for K was estimated to be 3−12 cm^{-1} [10] and for L to be 24 cm^{-1}.[49] More direct comparison of the H-bonding strength of the Schiff base could be done on the N−H in-plane bending vibrations of K_L at 1354 and 1298 cm^{-1}. They are located at higher frequencies than the corresponding bands of K at 1348 and 1245 cm^{-1} (ref 10) and at lower frequencies than the bands of L at 1400 and 1301 cm^{-1}. X-ray results showed that the Schiff base in K is almost free from the H-bonding interaction.[18,19] The Schiff base of K_L has moderate H-bonding, which is weaker than that in L. The partner for the interaction could be water, as in L.

Evidence for the interaction of the Schiff base with water upon the formation of K_L from K can be seen in the spectra of both the Schiff base HOOPs vibrations and the water vibrations. Intensity increases upon coupling with the N-HOOP seen for K_L could arise from coupling between the vibrations of the chromophore and those of one or more interacting water molecules. The C_{15},N-HOOP band of K_L at 985 cm^{-1} showed a slight decrease in intensity in $H_2{}^{18}O$ water. The same effect of ^{18}O water was observed also for the 1354 and 1298 cm^{-1} bands of the Schiff base N−H in-plane bending vibration. These effects were not observed for K. The intense bands of polarized water, which appear in L, were not detected in K_L. We propose that the depletion of the C_{15},N-HOOPs of L in T46V[30] is due to the decrease in intensity that these HOOP bands get from the polarization of the Schiff base by its interaction with Wat46. Thus the absence of an effect of the T46V mutation for the 985 cm^{-1} band of K_L (not shown) could be due to the absence of the polarization of the Schiff base by its interaction with the Wat46 water molecule. This absence could be due to a very weak interaction of the Schiff base with Wat46; rather the Schiff base might be involved in an interaction with another water molecule like Water402, which seems to still be present close to the Schiff base in K[18,19] and is not affected by the T46V mutation.

Perturbation of Water around Asp85 in K_L. The 3642 cm^{-1} water O−H stretching vibration of BR is very small in K (Figure 3c) and appears with greater amplitude in K_L (Figure 3b), and its amplitude persists in L (Figure 3a) and in M.[50] This water O−H is free from H-bonding, as deduced from its relatively high frequency.[51,52] This band was assigned to a water close to Asp85 because it is absent in D85N[48] and in Y57D[25] and changes its frequency to 3636 cm^{-1} in D212N.[53] The water molecule(s) responsible for this band interacts more strongly with Asp212 in L[53] and is perturbed in the photoreaction of M to M′ at 80 and 100 K in parallel with the perturbation of Asp85.[54] The apparent absence of a protonated Asp85 band in the M minus BR spectrum of D212N/R82Q[55] suggests that the protonated Asp85 of M is stabilized by Asp212. The water of which the OH is at 3642 cm^{-1} in BR may be involved by intervening between Asp85 and Asp212. Of the three water molecules of BR near Asp85 and Asp212, which are seen in the X-ray structure, Water401 has a free O−H (see review in ref 56). Hence, it is most likely to be the water molecule giving rise to the 3642 cm^{-1} band. All of the X-ray structures of M[57−61] invariably show that at least one water molecule hydrates protonated Asp85.

To transfer a proton toward Asp85 from the Schiff base, the pK_a of Asp85 has to be increased to >10.5.[62] The interaction of Asp85 with a water leading to the 3642 cm^{-1} band in M could be one of the factors that stabilizes the protonated form of Asp85. Thus one of the environment factors for increasing the pK_a of Asp85 is already being prepared as early as K_L.

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