Existence of Two L Photointermediates of Halorhodopsin from *Halobacterium salinarum*, Differing in Their Protein and Water FTIR Bands†

Young-Shin Chon,‡,§ Hideki Kandori,*,‡ Jun Sasaki,‡,|| Janos K. Lanyi,⊥ Richard Needleman,# and Akio Maeda‡,&

Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, Department of Physiology and Biophysics, University of California, Irvine, California 92697, and Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

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ABSTRACT: FTIR difference spectra were recorded for the photoreactions of halorhodopsin from *Halobacterium salinarum* at 170 and 250 K. Obvious differences at the two temperatures were noted in neither the visible spectra nor the FTIR bands of the chromophore. However, perturbation of Asp141 is observed in the L intermediate at 250 K but not at 170 K. We named these photoproducts La (at 170 K) and Lb (at 250 K). The spectrum of Lb is distinct from that of La also in the different shifts of water O−H stretching bands, and larger changes in the bands from the protein backbone with different sensitivities to varying the halide. These results suggest that the photocycle of halorhodopsin contains two L states, La and Lb, in which the structure of protein and internal water molecules is different but chloride stays at the same site close to the Schiff base.

Halorhodopsin is a heptahelical membrane protein (*1*) which functions as a light-driven chloride pump in *Halobacterium salinarum*. Like the homologous proton pump, bacteriorhodopsin, halorhodopsin contains a retinal chromophore bound through a protonated Schiff base to a lysine residue in the seventh helix. Different halorhodopsins were also found in *Natronobacterium pharaonis* (*2*) and other microbial species (*3*). By using light energy, these proteins pump chloride ions from the extracellular medium to the cytoplasm (*4*, *5*), in the opposite direction from the proton translocation in bacteriorhodopsin, and thus generate an outside-positive membrane potential like that in bacteriorhodopsin. The two halorhodopsins and bacteriorhodopsin have similar primary sequences (*6*, *7*), but the most important residue for the proton-transfer reaction in bacteriorhodopsin, Asp85, is replaced in the halorhodopsins with threonine. When the Asp85 of bacteriorhodopsin is replaced with threonine, the mutant transports chloride with a photocycle similar to that of *N. pharaonis* halorhodopsin (*8*).

In bacteriorhodopsin, photoisomerization of the all-trans chromophore to the 13-cis form initiates a cycle of photochemical reactions in which the consecutive intermediates are identified and named K−O (*9*). One proton moves from the Schiff base to Asp85 of the extracellular side of the retinal Schiff base in the L-to-M process, followed by proton transfer from Asp96 of the cytoplasmic side of the Schiff base in the M-to-N process. The unique structural features of the L state are revealed by Fourier transform infrared (FTIR)[1] spectroscopy (*10*, *11*), as well as by solid state NMR (*12*). The blue-shifted L intermediate is also important for the anion transport function of halorhodopsin in *H. salinarum* (*13*). It forms only from the all-trans species, and only in the presence of chloride or another transportable anion. The photocycle of halorhodopsin of *H. salinarum* does not contain the M intermediate, obviously because the acceptor of the proton from the Schiff base is missing. The most recent study by Váró et al. (*14*) shows that the photocycle is described by a series that contains a K intermediate, two kinetically distinguishable L species with the same visible spectra, and the N intermediate that decays with the same time constant as the second L state. In the *H. salinarum* protein, at least, the previously observed O intermediate was ascribed to the photoproduct from the 13-cis species, and shown not to be involved in the photocycle of the all-trans species. According to the proposed chloride transport model (*14*, *15*), the transition from the first L state to the second changes the access of the chloride ion from the extracellular to the cytoplasmic side.

So far, no studies have been available that would distinguish the FTIR spectra of the two L substates. Previous low-temperature FTIR studies with the photointermediates of halorhodopsin focused only on the intermediates produced at 77 K and at 250 K; these were ascribed to the K and L states, respectively (*16*). For bacteriorhodopsin, the spectrum of the L intermediate has been recorded at 170 K (*10*). Visible spectroscopy of halorhodopsin also shows the forma-

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* To whom correspondence should be addressed: Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. Telephone: 81 75 753 4211. Fax: 81 75 753 4210. E-mail: kandori@photo2.biophys.kyoto-u.ac.jp.

‡ Kyoto University.

§ Present address: Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea.

|| Present address: Department of Earth and Space Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560, Japan.

⊥ University of California.

Wayne State University School of Medicine.

& Present address: Center for Biophysics and Computational Biology, Universtiy of Illinois, Urbana, IL 61801.

[1] Abbreviations: FTIR, Fourier transform infrared; HR, light-adapted halorhodopsin that possesses the all-trans retinal chromophore; HOOP, hydrogen out-of-plane; λ_{max}, wavelength of maximum absorption.

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tion of the L intermediate at 170 K (*17*), but FTIR spectra have not been measured for halorhodopsin at this temperature. The FTIR study we report here for halorhodopsin from *H. salinarum* suggests that in the two L states that are described the protein assumes two different conformations but the chromophore structure is the same.

MATERIALS AND METHODS

Preparation of Halorhodopsin. Membrane fragments containing halorhodopsin were prepared from *H. salinarum* strain L33, which was transformed with a plasmid containing the *H. salinarum* hop structural gene under the control of the bop promoter, by the method described previously (*14*). Halorhodopsin, enriched in the membrane fraction, was finally purified by discontinuous sucrose gradient centrifugation, and suspended in the buffer containing 0.2 M NaCl and 10 mM phosphate (pH 7.0).

Manipulation of the Sample for FTIR Spectroscopy. For the FTIR measurements, the membranes containing halorhodopsin were centrifuged and suspended in 10 mM NaCl, NaBr, or NaI in 2 mM phosphate buffer (pH 7.0). A film was prepared by drying a 60 μL aliquot of the suspensions on a BaF_2 window (18 mm in diameter) under mild vacuum, and hydrated with H_2O, $H_2{}^{18}O$, or D_2O as described previously by Chon et al. (*18*). The absorbance ratio at 274 and 574 nm recorded in the film was ~2.0.

FTIR and Visible Spectroscopic Methods. The sample holder was mounted in an Oxford cryostat DN1704, equipped with an Oxford ITC-4 temperature controller. FTIR spectra were recorded in a Bio-Rad FTIR FTS60A/896 spectrometer at 2 cm^{-1} resolution. The spectra in the visible region were recorded for the hydrated films in a Shimadzu MPS-2000 spectrometer. Light for illumination of the samples was provided by a 1 kW halogen−tungsten lamp through one of several Toshiba cutoff filters, V42 and R62 which pass >400 and >600 nm light, respectively. Light adaptation was performed by illumination with the V42 filter for 15 min at 273 K. Measurements of the spectrum under continuous illumination at 250 K were taken as described previously (*19*). The sample window was tilted 45° relative to the probe light, and the >600 nm light from a slide projector was focused on the sample at an angle of 90° with respect to the probe light, with a germanium window to protect the detector. The spectra were recorded by collecting 166 interferograms both before and during the illumination for 2 min. The same procedure was repeated 10 times at 250 K. All the successively recorded difference spectra exhibited the same shape. Difference spectra at 170 K were obtained as reported by Chon et al. (*18*). All the photoproducts from illumination at 170 K decayed completely by incubating at 273 K for 20 min, as revealed by the absence of any bands in the difference FTIR spectrum between the spectrum after warming to this temperature and that measured before cooling (data now shown). The illumination regime was repeated five to eight times for averaging. The results also indicate that the stable 13-cis state produced by red light illumination at ambient temperature (*20*, *21*) is not included in the photoproducts at 170 and 250 K.

RESULTS

Photocycle Intermediates at 170 and 250 K. Figure 1 shows spectral changes in the visible region upon illumination of light-adapted halorhodopsin (HR) with >600 nm light at 170 (a) and 250 K (b). At 170 K (a), illumination for 1 min (curve 1) is sufficient to attain a photosteady state, as confirmed by lack of additional spectral shifts upon further illumination for 2 min (curve 2). The difference spectrum of the photosteady state photoproduct does not change after 10 min in the dark at the same temperature (curve 3), indicating that the photoproduct is stable at this temperature. On the other hand, at 250 K (b), the spectral change proceeds more slowly, and does not attain a photosteady state even after the illumination for 16 min (curve 2; compare with curve 1 at 1 min). This is because the photoproduct is not stable and decays to the HR state, as is seen in the reduced amplitude recorded at 10 min after cessation of the illumination (curve 3). To compare the difference spectra of the photoproducts minus the unphotolyzed state under the identical conditions, the sample after the illumination at 250 K for 16 min (corresponding to curve 2 in part b) was rapidly cooled to 170 K and a difference spectrum was calculated relative to the spectrum that was recorded at 170 K before the illumination at 250 K (c). The shape of this difference spectrum (dotted line in part c), with a maximum at 501 nm and a minimum at 592 nm, is almost identical with that from the photoreaction at 170 K (solid line in part c). These spectral shapes reflect the formation of the blue-shifted L intermediate (*14*). Contributions from the K and N intermediates, which exhibit red-shifted spectra, are seen to be negligible.

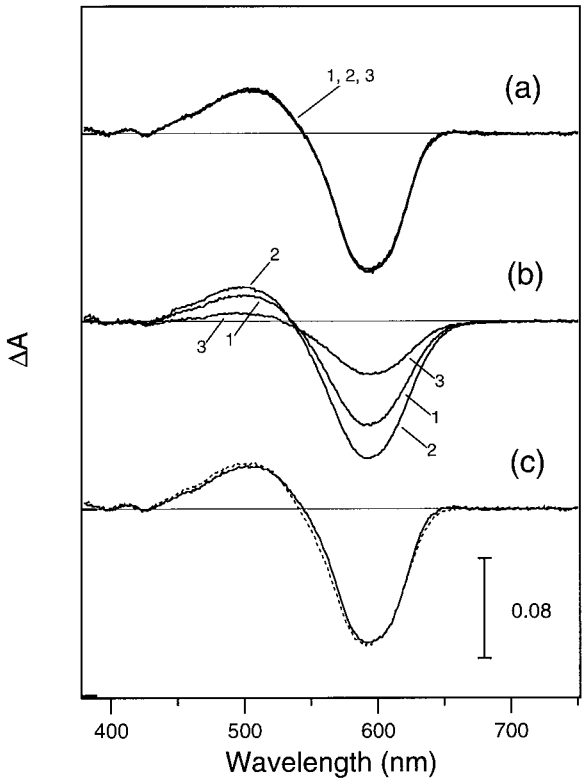

FIGURE 1: Difference spectra in the visible region after the illumination of HR with >600 nm light at 170 (a) and 250 K (b). Curves 1−3 in part a are spectra after illumination for 1 and 2 min and after incubation in the dark for a further 10 min, respectively. Curves 1−3 in part b are spectra after illumination for 1 and 16 min and after incubation in the dark for a further 10 min, respectively. The difference spectra recorded at 170 K for samples illuminated at 170 (—) and 250 K (···) are overlapped in part c.

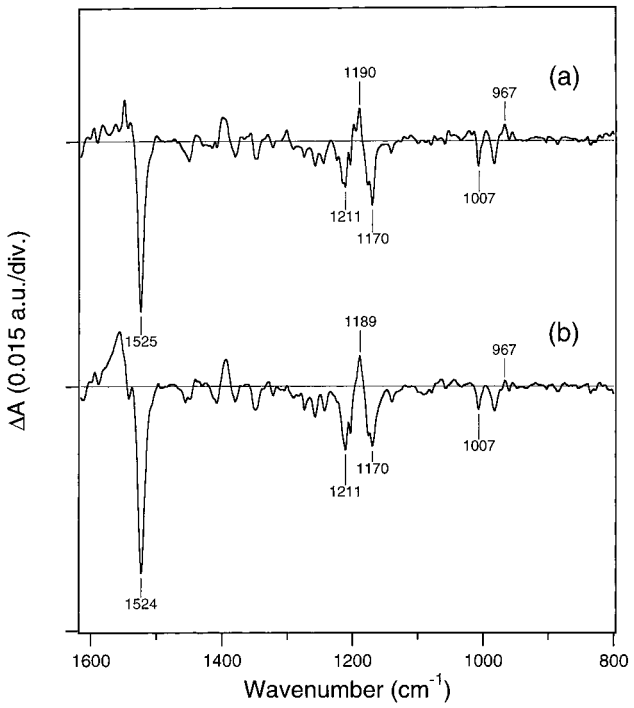

FIGURE 2: Difference FTIR spectra in the 1600−800 cm^{-1} region after the illumination of HR with >600 nm at 170 (a) and 250 K (b). The details for the illumination are described in the text. The amplitudes were normalized to the bands at 1525 and 1170 cm^{-1}. The multiplication factor to part a is 0.96 for part b.

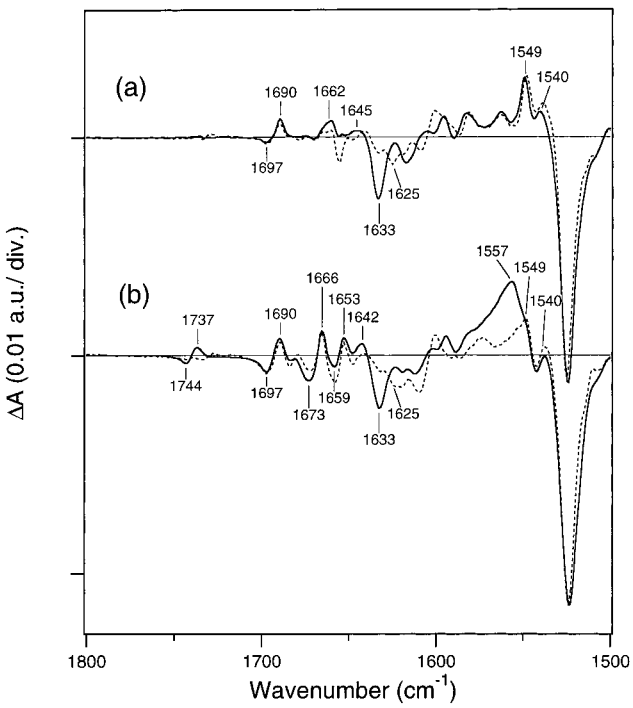

FIGURE 3: Difference FTIR spectra in the 1800−1500 cm^{-1} region after the illumination of HR in H_2O (—) and D_2O (•••) with >600 nm light at 170 (a) and 250 K (b).

Same Chromophore Structure in the Two L States. The difference FTIR spectra in the 1600−800 cm^{-1} region corresponding to these illumination regimes are shown in Figure 2. The spectrum at 170 K (a) was recorded as the difference before and after the illumination with >600 nm light. The spectrum for the relatively unstable photoproduct at 250 K (b) was recorded as the difference before the illumination and the photosteady state under illumination with the same light as at 170 K. The spectrum at 250 K is very similar to those recorded previously under similar conditions (*16*, *22*, *23*). Several features in the spectrum at 170 K are different, however, from those at 250 K. This difference does not arise from the different method for the recording because the same difference spectrum was obtained for the spectrum recorded by the constant illumination at 170 K (data not shown), as was done for the spectrum at 250 K. Also, the spectrum recorded without constant illumination at 250 K exhibited the same shape, although with lower amplitudes (data not shown).

Light-adapted halorhodopsin contains about 20% of the 13-cis species (*24*). Its contribution to these difference spectra is negligible, from the absence of both a negative band around 1185 cm^{-1} characteristic of the 13-cis species and a positive C═C stretching band below 1525 cm^{-1} which would correspond to the exclusively red-shifted photoproducts from the 13-cis species (*14*). A positive C−C stretching vibration band is located at 1190 cm^{-1} in the spectrum at 170 K (a), at a very slightly higher frequency than 1189 cm^{-1} at 250 K (b). The smaller intensity of the 1211 cm^{-1} band in the negative side at 170 K (a) may be a result of cancellation by this positive band which is located closer to the 1211 cm^{-1} band at 250 K (b) than at 170 K (a). In bacteriorhodopsin, the corresponding positive band is observed at 1192 cm^{-1} for L and at 1186 cm^{-1} for N (*10*). The negative band at 1007 cm^{-1} of HR, due to the methyl in-plane bending vibration of the retinal, which is present at 1009 cm^{-1} in the L intermediate of the light-adapted form of bacteriorhodopsin (BR) but absent in its N intermediate (*25*), is present in the spectra recorded at both 170 and 250 K. The 967 cm^{-1} band, which may contain the N−HOOP vibration, in view of its sensitivity to D_2O substitution (not shown), is also present in both spectra. As a whole, the structure of the chromophore is very similar at 170 (a) and 250 K (b). Their spectral features resemble more those of the L intermediate of bacteriorhodopsin than its N intermediate, as expected from their similarity to the L of bacteriorhodopsin in the visible region.

Different Protein Structure in the Two L States. Remarkable differences were noticed, however, between the spectra at 170 and 250 K in the 1800−1500 cm^{-1} region (parts a and b of Figure 3, respectively, solid lines), which are shown with corresponding spectra of the films hydrated with D_2O (dotted lines). The negative band at 1633 cm^{-1} in both spectra (a and b), which shows a clear shift in D_2O by about 8 cm^{-1}, is due to the C═N stretching vibration of the protonated Schiff base, as was previously shown by resonance Raman spectroscopy (*24*, *26*, *27*). This band is invariant in both spectra, and exhibits the features of the chromophore that persist at both temperatures. About 20% of the band intensity that remained in D_2O could be due to H_2O retained in the dried films containing salts. The C═N stretching vibration band of the halorhodopsin L intermediate in resonance Raman spectra was shown to be at 1648 (*28*) or 1644 cm^{-1} (*29*). The 1645 cm^{-1} band at 170 K (a) and the 1642 cm^{-1} band at 250 K (b) are shifted in D_2O by 2−3 cm^{-1}. However, such a small shift does not constitute proof that this band is from C═N stretching vibrations. The C═N stretching band in L may be very small and hardly distinguishable from

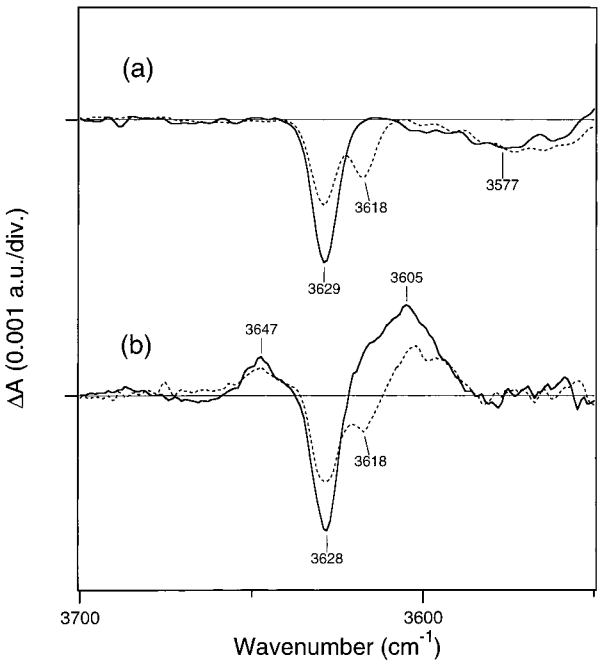

FIGURE 4: Difference FTIR spectra in the 3700−3550 cm^{-1} region after the illumination of HR in H_2O (—) and $H_2{}^{18}O$ (•••) with >600 nm light at 170 (a) and 250 K (b).

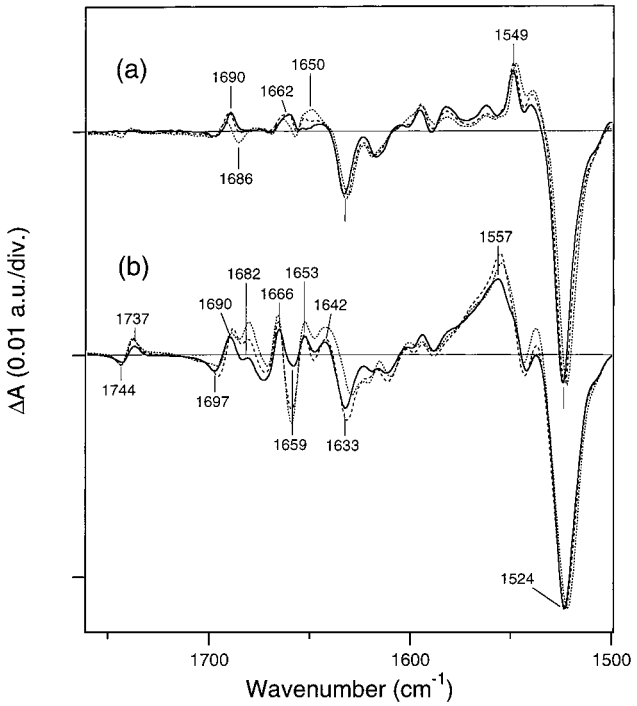

FIGURE 5: Difference FTIR spectra of HR in the 1760−1500 cm^{-1} region after the illumination of HR in the presence of chloride (—), bromide (− − −), and iodide (•••) at 170 (a) and 250 K (b).

amide I bands as judged by the much smaller intensity of the C═C stretching bands of the retinal of L, relative to the corresponding band of HR. The bands at 1744 (−) and 1737 (+) cm^{-1} in the spectrum at 250 K (b), which shift by ∼10 cm^{-1} in D_2O, are assigned to the C═O bond of a carboxylic acid. Asp141 is only a candidate for these bands as the only intramembrane carboxylic residue, which may be present as a protonated carboxylic residue as its homologue Asp115 in bacteriorhodopsin (*30*). These bands are absent in the spectrum from the photoreaction at 170 K (a), except for the spectrum in iodide with small intensities (see also Figure 5a). These frequencies are slightly higher than the corresponding bands at 1735 (−) and 1728 (+) cm^{-1} of Asp115 in the L minus BR spectrum of bacteriorhodopsin (*30*, *31*).

The 1700−1600 cm^{-1} region contains amide I (a coupled mode of the C═O stretching and N−H bending vibrations of the peptide backbone) and a coupled mode of the C═N stretching and N−H bending vibrations of the guanidium group in arginine (*22*), both of which can be shifted in D_2O, if the respective groups are exposed to the solvent. The spectrum at 170 K (a) exhibits positive bands at 1690 and 1662 cm^{-1}. The spectrum at 250 K (b) exhibits more bands, positive bands at 1690, 1666, and 1653 cm^{-1} and negative bands at 1697, 1673, and 1659 cm^{-1}. The bands at 1697 (−) and 1690 (+) cm^{-1} in the spectrum at 250 K, which were previously ascribed to the C═N stretching vibrations of Arg108 (*22*, *23*), did not show any shifts in D_2O, which would be expected for the protons of arginine N−H at this position. The 1697 cm^{-1} band at 170 K (a) appears with a very much smaller intensity than at 250 K (b).

The bands in the 1600−1500 cm^{-1} region are mostly due to amide II (a coupled mode of the C═N stretch and N−H bending vibrations of the peptide backbone) and the C═C stretching vibrations of the retinal. A positive band at 1557 cm^{-1} at 250 K (b) disappears from this region in D_2O (dotted line in b), and therefore can be assigned to amide II. This band is absent at 170 K (a). The positive bands at 1549 and 1540 cm^{-1} at 170 K (a), which are only slightly affected by D_2O, might be due to the C═C stretching vibration (*28*, *29*). The corresponding bands are revealed only in the spectrum in D_2O at 250 K (b) because of overlapping amide II bands.

The observed features of amide I and amide II bands in the spectrum at 250 K (b) are similar to those of the M minus BR spectrum of bacteriorhodopsin (*30*). In contrast to the unchanged chromophore, many differences are detected in the protein moiety between the photoproducts at 170 (a) and 250 K (b). These two photoproducts are named La and Lb, respectively. The intensity changes of amide I and amide II in the 1800−1500 cm^{-1} region correspond to one or a few bonds. These changes might be the results of differences of hydrogen bond strengths in a few peptide carbonyls and amides of the backbone between La and Lb.

Structural Changes of Water in the Two L Intermediates. Figure 4 shows the spectra in the 3700−3550 cm^{-1} region at 170 (a) and 250 K (b). Part of a negative band at 3628−3629 cm^{-1}, due to the depleted HR state (solid lines) in both spectra a and b, is shifted by ∼10 cm^{-1} upon hydration with $H_2{}^{18}O$, in common for both spectra (dashed lines). The level of exchange with $H_2{}^{18}O$ is less than half for the bands at 3628−3629 cm^{-1}, suggesting a contribution from nonexchangeable water or a protein O−H bond to these bands. This resembles the O−H stretching band at 3637 cm^{-1} in a chloride-binding mutant of bacteriorhodopsin (*18*). A broad negative feature with a trough at 3577 cm^{-1} in the spectrum at 170 K, which shifts in $H_2{}^{18}O$ (a), is not detected in the spectrum at 250 K (b). Instead, the spectrum at 250 K (b) exhibits positive bands at 3647 and 3605 cm^{-1}. The former band, which does not shift in $H_2{}^{18}O$, must be ascribed to a hydroxyl group of the protein. The latter band shows a clear shift in $H_2{}^{18}O$, even if one considers the cancellation due to the nearby negative band. These positive bands are absent

in the spectrum at 170 K (a). It appears that these internal water molecules experience different structural changes in La and Lb. For bacteriorhodopsin, the locations of water molecules were determined by the FTIR spectra of relevant mutant proteins. This approach has not yet been applied to identify the locations of those water molecules in halorhodopsin.

Different Halide Effects for La and Lb. Braiman et al. (*22*) have reported on bands in the FTIR spectra that were affected by replacing chloride with bromide or iodide. Figure 5 shows the effects of halides on the bands in the spectra at 170 (a) and 250 K (b) in the 1760−1500 cm^{-1} region, where the C═N stretching vibration of the Schiff base, the amide I and amide II bands, and the C═N stretching bands of arginine (*22*) are present, in three halides, chloride (solid lines), bromide (dashed lines), and iodide (dotted lines). It is obvious that the negative band at 1524−1525 cm^{-1} in chloride is shifted slightly toward a lower frequency upon replacement with bromide, and further with iodide. This halide sensitivity for the C═C stretching bands reproduces the previously reported spectrum at 250 K (*33*). The 1549 cm^{-1} band due to the C═C stretching vibration of La at 170 K exhibits similar halide sensitivity. The halide-dependent shift of the positive band at 1557 cm^{-1} in Lb at 250 K (b) could be due to either the shift of amide II or the overlapped C═C stretching vibration in Lb. A negative band in chloride at 1633 cm^{-1} in the spectrum at 250 K (b), which is due to the C═N stretching vibration band of the HR state, shifts progressively toward lower frequencies in bromide at 1632 cm^{-1} and iodide at 1631 cm^{-1}, also as reported previously (*33*). The same trend was observed for the same band in the spectrum at 170 K (a). Since the C═N stretching vibration of L could not be identified (see above), the effects of halide species on it cannot be evaluated.

The negative band at 1697 cm^{-1} in the spectrum at 250 K (b) shifts toward a lower frequency in bromide and iodide, as reported by Braiman et al. (*22*). These replacements appear to shift also the positive band at 1690 cm^{-1} slightly toward a lower frequency, although the previous authors regarded the latter shift to be within an experimental error. In the spectrum at 170 K (a), the corresponding negative band is very faint, indicating no changes of this mode. Hence, the 1690 cm^{-1} band of La is due to a different mode from the 1690 cm^{-1} band of Lb. This band shifts slightly to a higher frequency upon replacement of chloride by bromide. However, the effect of iodide in La is more complex because it causes the appearance of a negative band at 1686 cm^{-1} (a). Iodide probably causes a slight conformation change in addition to that caused by simple replacement of chloride, as reflected also by the appearance of a bilobe at 1744 (−) and 1737 (+) cm^{-1}, which is not observed with chloride and bromide, in the La minus HR spectrum (a). A new band appears when chloride is replaced with either bromide or iodide around 1650 cm^{-1} on the positive side of the spectra at 170 K (a). Intensity increases are detected for bands at 1682 (+), 1666 (+), 1659 (−), 1653 (+), and 1642 (+) cm^{-1} in the spectra at 250 K (b). These intensity increases might be regarded as protein conformation changes associated with different halide species. As a whole, different modes are affected differently by halides in the two states.

DISCUSSION

Two L Substates in the Photocycle. The difference FTIR spectra measured for the photoproducts of halorhodopsin of *H. salinarum* at 170 and 250 K, named here La and Lb, respectively, are distinguished by several specific features in the vibration modes ascribed to the protein and internal water molecules. These photoproducts share a common visible spectrum and FTIR spectral features ascribed to the chromophore in L. Previously, time-resolved visible spectroscopy (*14*) revealed two sequential L states (L1 and L2) in equilibrium with the preceding K intermediate and the subsequent N intermediate, respectively. The latter evolved and decayed in parallel with the late L. The two L states exhibited the same blue-shifted maxima in the visible spectra, in contrast to the red-shifted K and N intermediates. In this regard, we tentatively assigned the two different FTIR spectra at cryogenic temperatures to the two L states defined by room-temperature time-resolved visible spectroscopy (*14*). If this assignment is correct, the observed differences between La and Lb would provide important insights into the mechanism of chloride transport (see below). However, direct evidence supporting it is difficult to produce. On one hand, the same shape of the Lb minus HR spectrum appeared in the spectrum recorded at room temperature where N would be present (not shown). The absence of any contribution of N is probably due to the fact that the illumination with red light depleted the N intermediate with a visible absorption band similar to that of HR, and accumulated the equally stable Lb.

Recently, time-resolved FTIR spectroscopy of halorhodopsin produced a tentative L minus HR spectrum for the photoproduct in the time domain when the majority of L should be L2 (*34*). The Lb minus HR spectrum that we measured is very similar to it with respect to the prominent bands at 1744 (−), 1737 (+), 1697 (−), 1633 (−), 1557 (+), 1189 (+), and 967 (+) cm^{-1}. The agreement between the low-temperature photoequilibrium spectra and the time-resolved transient spectra strongly argues that Lb is equivalent to L2. On the other hand, time-resolved FTIR spectroscopy (*34*) did not show the presence of L1. According to the time-resolved visible spectroscopic study (*19*), L1 should be present in a mixture with K. The signals due to La may be masked by the intense bands of K present in the same time domain. The bilobe with 1737 and 1741 cm^{-1} bands characteristic of Lb produced by the photoreaction at 250 K did not appear by simply warming of La produced at 170 K to about 250 K, where about one-third of La decayed to HR (data not shown). However, these results do not necessarily exclude a possibility that La is not a direct precursor to Lb. Probably for kinetic reasons, L of bacteriorhodopsin produced at 170 K which is similar to L at ambient temperature (*35*) also decays to all-trans bacteriorhodopsin without conversion to the next intermediate, M, by warming (*36*). In view of the lower temperature range of its stability, La must be produced earlier than Lb in the photocycle of halorhodopsin. The presence of K and N as the equilibrium partners might cause the complication in the relationship of La and Lb. This must be solved in future studies.

Different Protein Structure in La and Lb. Although the chromophore is unchanged, the two L states exhibit differences in protein structure. The changes of protein bands in

the 1800−1500 cm^{-1} region containing amide I, amide II, and protonated carboxylic acid were more extensive in Lb than in La (Figure 3), suggesting structural changes of the protein moiety between the Lb and La. The perturbation of the C═O stretching vibration at 1744 (−) and 1737 (+) cm^{-1} in Lb ascribed to Asp141 is completely absent in La (Figure 3). Thus, the C═O stretching frequency of Asp141 in halorhodopsin at 1744 cm^{-1} remains unchanged in the La state, but shifted to 1737 cm^{-1} in Lb. This difference between La and Lb reflects the progression of the perturbation in the cytoplasmic domain.

Possible sites for the interaction with halides might be evaluated from the effects of different halides on specific vibration bands for halorhodopsin (Figure 5). Previously, the 1697 (−) and 1690 (+) cm^{-1} bands were assigned to Arg108 from a specific mutant protein (*22*, *23*). In this respect, the different effects of halide on the 1690 cm^{-1} band between La and Lb and the absence of the negative band of the halide sensitive band at 1697 cm^{-1} might be ascribed to the different interactions of chloride with Arg108. However, this conclusion is not completely justified. The absence of an L intermediate in the photocycle of the R108Q mutant makes it impossible to test whether these bands are due to Arg108, as previously proposed (*23*). The bands did not exhibit the shifts in D_2O (Figure 3) expected for the C═N stretching vibrations of the guanidium group of an arginine, i.e., for Arg108. Also, the equivalent group, Arg82, in bacteriorhodopsin mutants that bind chloride, is not involved in the chloride binding site (*37*, *38*). The R108Q mutant of halorhodopsin is inactive in chloride transport (*23*), but this is not evidence for the direct binding of chloride to Arg108, because the possibility that the imbalance of electric charge in R108Q perturbs the global structure necessary for the transport cannot be excluded. In bacteriorhodopsin, the normal proton release absent in the mutant R82Q is recovered by the additional mutation in the R82Q/D212N double mutant (*39*), probably through restoration of the balance of electrical charges. Actually, the photocycle of the R108Q halorhodopsin mutant is too different from that of the wild type to attribute the inactivation of transport simply to replacement of Arg108. Another site for chloride binding, Thr203 in the cytoplasmic domain (*40*), is still a candidate. To relate the two L states to the different accessibilities as was proposed previously (*14*, *15*) or even to different functional roles, firm assignments of these vibrational bands to specific residues are required.

Differences between La and Lb were also seen in their water O−H stretching vibrations (Figure 4). The location of these water molecules will be discussed on the basis of the results on bacteriorhodopsin, because they have not been assigned by use of mutants of halorhodopsin, as was done for bacteriorhodopsin (*11*). The O−H stretching band at 3628 cm^{-1} of a water molecule does not change between La and Lb. This originates most likely from a water molecule that hydrates the bound chloride ion, as observed for the chloride binding form of the D85T mutant protein of bacteriorhodopsin (*18*), though other possibilities cannot be excluded. In contrast, the depleted HR band at 3577 cm^{-1} and the Lb band at 3605 cm^{-1} are specific to each transition. The former is similar to the band due to a water molecule close to Thr46 in bacteriorhodopsin (*41*).

Possible Site for the Interaction with Chloride. The C═C stretching frequency, which is inversely proportional to the wavelength for the maximum absorption (λ_{max}) in the visible spectrum (*42*), is dependent on the electron delocalization in the conjugated double bond system of the retinal chromophore (*43*). The changes in the electron delocalization might be achieved mainly through the electrical interaction of the retinal Schiff base with its counterion, as was evidenced by the halide size-dependent shift of the λ_{max} in the visible spectrum of the retinal Schiff base in organic solvent (*44*) and the mutant pigment of bovine rhodopsin, in which the counterion of the Schiff base was replaced with a neutral residue (*45*). The halide size-dependent shift of the C═C stretching frequency of HR and La shows that chloride is present at a site close enough to exert an electric effect on the retinal, probably through the Schiff base. The identical spectra in the visible region between La and Lb (Figure 1) further suggest that chloride stays at the same site through the La and Lb states. A recent X-ray crystallographic study with bacteriorhodopsin (*46*) showed that Asp85 is linked to the Schiff base through an intervening water molecule hydrogen bonded to one of its carboxyl oxygens, and its other carboxyl oxygen forms a hydrogen bond to the OH of Thr89, which is located across the retinal. In halorhodopsins, Thr89 and Asp85, which belong to the same helix and might protrude in the same direction, are replaced with smaller residues, Ser115 and Thr111, respectively (*3*). Thus, halorhodopsin may have enough space to accommodate chloride ion at the site for the negative charge of Asp85 in bacteriorhodopsin. The site of chloride in L must be closer to the Schiff base than in HR, in view of the blue-shifted λ_{max} value in the visible spectrum.

Relation of the Function of the Two L States. No specific processes between the intermediates that depend on the concentration of chloride were detected for halorhodopsin of *H. salinarum* (*14*), in contrast to the chloride-transporting D85T mutant protein of bacteriorhodopsin (*8*), and halorhodopsin of *N. pharaonis* (*19*) whose O decay is suggested to be a step for the uptake of chloride. Photovoltage experiments with *N. pharaonis* halorhodopsin show ion movement across the membrane with a time constant of ∼1 ms, which may correspond to chloride release and occur after or during the L-to-N decay. No charge translocation in the 100 μs time range corresponding to the L1-to-L2 transition was observed (*47*). Although no such study on halorhodopsin of *H. salinarum* has been published, here also, chloride release may occur during the decay of the Lb−N equilibrium state. Protein structural changes for preparation of the movement of the chloride, which is initially bound close to the Schiff base on the extracellular side and must gain access to the cytoplasmic side (*15*), may be induced without changes in the chromophore. This is similar to the protein changes observed without a change of the protonation state of the Schiff base in the M_N state of bacteriorhodopsin, the protein having progressed to the N state with the chromophore remaining in the M state (*48*).

In the photocycle of bacteriorhodopsin, the L intermediate was suggested to be a state with a constrained and strongly hydrogen-bonded protonated Schiff base (*11*, *12*). The structure of the chromophore inherent to the L intermediate relaxes upon the deprotonation of the Schiff base in the M intermediate, and the subsequent N intermediate with the

reprotonated Schiff base is different from the L intermediate with respect to the conformation of the chromophore, even though both are in the 13-cis form, as detected by FTIR and NMR spectra (*12*, *25*, *49*). In halorhodopsin, on the other hand, the photocycle lacks the M intermediate with the unprotonated Schiff base, because of the absence of a proton-accepting residue. By low-temperature spectroscopy of bacteriorhodopsin, the spectrum of the L intermediate is recorded at 170 K but not above 230 K where the M and N intermediates are stabilized. In halorhodopsin, the persistent presence of the proton at the Schiff base would not produce the relaxed chromophore, and thus, the L state of the chromophore would be preserved. Indeed, Lb of halorhodopsin assessed at 250 K (Figures 1 and 2) was completely identical with La with respect to the structure of the chromophore as exemplified by the identical visible spectra. The protein part acquires structural changes, while chloride is present at the same site close to the Schiff base.

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