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Low-Temperature FTIR Study of *Gloeobacter* Rhodopsin: Presence of Strongly Hydrogen-Bonded Water and Long-Range Structural Protein Perturbation upon Retinal Photoisomerization

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ABSTRACT: *Gloeobacter* rhodopsin (GR) is a light-driven proton-pump protein similar to bacteriorhodopsin (BR), found in *Gloeobacter violaceus* PCC 7421, a primitive cyanobacterium. In this paper, structural changes of GR following retinal photoisomerization are studied by means of low-temperature Fourier-transform infrared (FTIR) spectroscopy. The initial motivation was to test our hypothesis that proton-pumping rhodopsins possess strongly hydrogen-bonded water molecules in the active center. Water O−D stretching vibrations at < 2400 cm^{-1} in D_2O have been regarded as coming from such strongly hydrogen-bonded water, and there is a strong correlation between the proton-pumping activity and the presence of such water molecule. Since GR pumps protons, we expected that GR also possesses strongly hydrogen-bonded water molecule(s), and the FTIR results clearly show that this is indeed the case. In addition, another unexpected finding was gained from the frequency region of protonated carboxylic acids in the GR_K minus GR spectra at 77 K, where we observed the unique bands of a protonated carboxylic acid at 1735 (+)/1730 (−) cm^{-1}. Comprehensive mutation study revealed that the vibrational bands originate from the carboxylic C=O stretch of Glu132 at the position corresponding to Asp96 in BR. Glu132 presumably functions as an internal proton donor for the retinal Schiff base, but they may be located far apart (ca. 12 Å in BR). The present study demonstrates the long-range structural changes of GR along the proton pathway, even though the protein matrix is frozen at 77 K.

Four archaeal-type rhodopsins [bacteriorhodopsin (BR)[1], halorhodopsin (HR), sensory rhodopsin I (SRI), sensory rhodopsin II (SRII); also called phoborhodopsin (pR)] were discovered in the cytoplasmic membrane of *Halobacterium salinarum* (*1*−*4*). The former two rhodopsins (BR and HR) function as light-driven proton and chloride pumps, respectively, while the latter two rhodopsins (SRI and SRII) function as photosensors responsible for attractive or repellent phototaxis, respectively. They have been extensively studied as model systems converting light energy to chemical potential or environmental signal (*5*). Recently, the genome sequencing projects and the environmental genomics have revealed that rhodopsins of the archaeal type also exist in Eukarya and Eubacteria (*6*).

Gloeobacter rhodopsin (GR) was found in the genome of *Gloeobacter violaceus* PCC 7421, a primitive cyanobacterium, which lacks the thylakoid membrane and the distinct shape of phycobilisomes used for efficient photosynthesis (Figure 1) (*7*). Since GR has a light-driven proton-pumping activity like BR (*8*), GR probably helps photosynthetic energy production by compensating the lack of the sophisticated membrane structure. In fact, recent spectroscopic study of GR revealed the features common to proton-pump retinal proteins (*9*). Through the comprehensive low-temperature FTIR spectroscopy, we found that strongly hydrogen-bonded water molecules (water O−D stretching vibrations in D_2O at < 2400 cm^{-1}) are found in the proton-pumping rhodopsins (*10*−*17*) but not in the non-proton-pumping rhodopsins (*18*−*26*). This suggests that a strongly hydrogen-bonded water molecule that bridges the Schiff base and its counterion is essential for the proton-pumping function (*27*). Then, is this empirical rule applicable to GR?

In this paper, we studied low-temperature Fourier-transform infrared (FTIR) characteristics of the K intermediate of GR, comparing it with those of BR and paying special attention to the bands of bound water. We observed the signal from a strongly hydrogen-bonded water molecule similar to other proton-pumping rhodopsins. In addition, another unexpected finding was gained from the frequency region of protonated carboxylic acids in the GR_K minus GR spectra at 77 K. Namely, the present study reports the structural perturbation at the position of the cytoplasmic proton donor to the Schiff base, homologous to Asp96 in BR, at the very early stage of the proton-pumping process in GR.

MATERIALS AND METHODS

The wild-type and mutant GR proteins with a histidine tag at the C-terminus were expressed in *Escherichia coli* strain BL21, as described previously (*9*). For mutagenesis, a QuickChange site-directed mutagenesis kit (Stratagene) was used according to the standard protocol. The GR proteins were solubilized with 1.0%

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[1]Abbreviations: BR, bacteriorhodopsin; HR, halorhodopsin; SRI, sensory rhodopsin I; SRII, sensory rhodopsin II; pR, phoborhodopsin; GR, *Gloeobacter* rhodopsin; PR, proteorhodopsin; LR, *Leptosphaeria* rhodopsin; NR, *Neurospora* rhodopsin; ASR, *Anabaena* sensory rhodopsin; FTIR, Fourier-transform infrared; XR, xanthorhodopsin.

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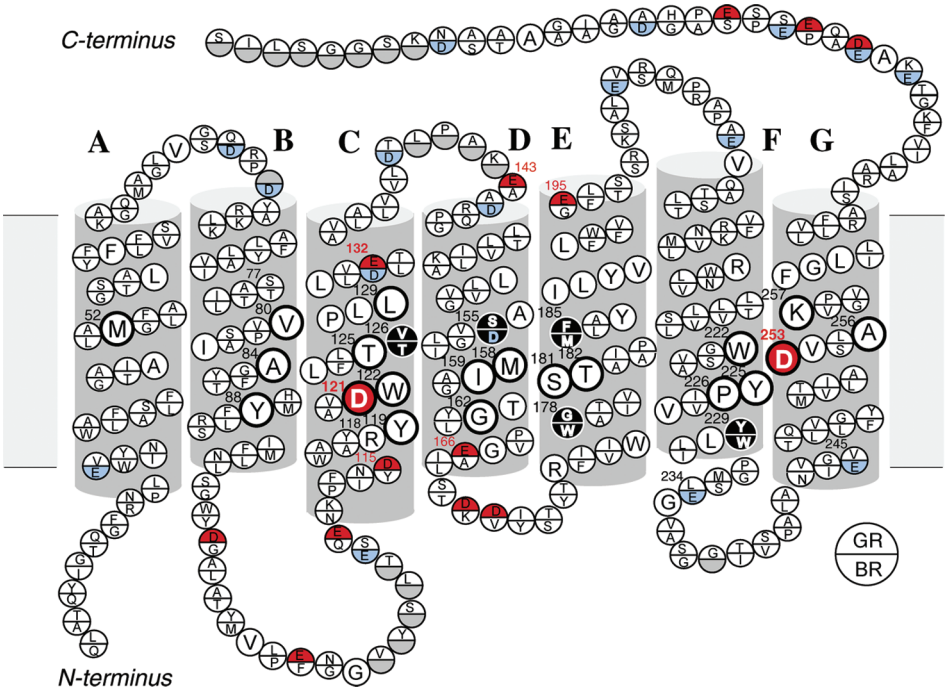

FIGURE 1: Comparison of the amino acid sequences of GR and BR. The transmembrane topology is based on the crystallographic three-dimensional structure of BR. The sequence alignment was done using CLUSTAL W (*46*) with the default settings. Single letters in a circle denote residues common to GR and BR. The residues that are different in GR and BR are denoted at the top and bottom of the circles, respectively. The residues forming the retinal binding site within 5 Å of the chromophore are shown by bold or filled circles. Carboxylates of GR and BR are colored by red and blue, respectively. There are seven carboxylates possibly located in the transmembrane region of GR: Asp115, Asp121, Glu132, Glu143, Glu166, Glu195, and Asp253.

n-dodecyl β-D-maltoside and purified by a Ni^{2+} column. The purified GR sample was then reconstituted into L-α-phosphatidylcholine (PC) liposomes by the removal of the detergent with Bio-Beads, where the molar ratio of the added PC to GR was 30:1. The GR protein in PC liposomes was washed three times with a buffer [2 mM sodium phosphate (pH 7.5)]. [ξ-^{15}N]Lys-labeled GR was prepared according to the method described previously (*28*).

Low-temperature FTIR spectroscopy was performed as described previously (*10*−*26*). A 60 μL aliquot was deposited on a BaF_2 window of 18 mm diameter and dried in a glass vessel that was evaporated by an aspirator. After hydration by putting about 2 μL of H_2O, D_2O, or $D_2{}^{18}O$ and sealing with a silicon rubber O-ring and another BaF_2 window, the sample was placed in a cell, which was mounted in an Oxford DN-1704 cryostat in the Bio-Rad FTS-40 spectrometer.

Illumination with 500 nm light at 77 K for 2 min converted GR to the red-shifted K intermediate (GR_K). Normally, subsequent illumination at longer wavelength light reverts the red-shifted K state to the original state in most rhodopsins, and repeated forward and backward photoreactions increase the signal-to-noise ratio of difference FTIR spectra. In the case of GR, however, the extent of reversion was small compared to other rhodopsins, and some spectral features of the back-photoreaction (GR_K to GR) differ from those of the forward photoreaction (GR to GR_K). This observation, no clear photochromism, was unusual for rhodopsins, and it is interesting to study in more detail. However, the photochemistry under the photoequilibrium conditions at 77 K was so complicated and now under investigation. To avoid complexity in the structural analysis, here we only averaged the difference spectra of the forward GR to GR_K photoreaction for the GR_K minus GR spectra, where the single photon event of the functional photocycle was monitored. Each difference spectrum was calculated from the spectra constructed from 128 interferograms before and after the illumination, and 4, 10, and 12 spectra obtained in this way were averaged to obtain the GR_K minus GR spectra in H_2O, D_2O, and $D_2{}^{18}O$, respectively. Because the protein molecules in the films were oriented randomly, the IR polarizer was not used. The obtained difference spectra were compared with those for BR with the window tilting angle of 53.5° in the polarized measurement, where all vibrational bands are observed in the highly oriented BR molecule.

RESULTS

Comparison of the Difference Infrared Spectra Obtained by the Photoreactions of Gloeobacter Rhodopsin (*GR*) *at 77 K with Those of Bacteriorhodopsin* (*BR*). Figure 2 shows the GR_K minus GR (a) and the BR_K minus BR spectra (b), which were measured at 77 K after the hydration with H_2O (solid lines) and D_2O (dotted lines). The HPLC analysis revealed that GR contains 90% all-*trans* and 10% 13-*cis* form of retinal (data not shown), and we estimated that the contribution of the photoreaction of the 13-*cis* form is less than 10% by use of the marker band (1178 cm^{-1}) under our illumination conditions.

Two negative bands at 1548 and 1534 cm^{-1} and a positive band at 1525 cm^{-1} in Figure 2a correspond to the ethylenic stretching vibration of the all-*trans* chromophore in GR and the red-shifted K state (GR_K), as is the case for BR at 1530 (−)/1514 (+) cm^{-1} (Figure 2b). A sharp negative band at 1445 cm^{-1} was observed only for GR, not BR, and it was insensitive to H−D exchange. Similar negative bands were observed for ASR at 1457 and 1451 cm^{-1} but remained unidentified thus far (*23*).

In the C−C stretching vibrations region of the retinal, similar bands were observed for GR and BR. A negative band at 1206 cm^{-1} in GR corresponds to that at 1203 cm^{-1} in BR,

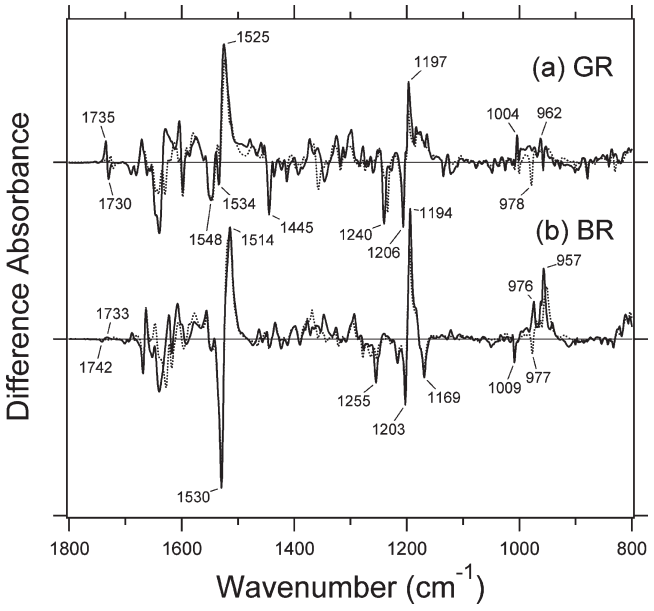

FIGURE 2: The GR_K minus GR (a) and the BR_K minus BR (b) spectra in the 1800−850 cm^{-1} region measured at pH 7 and 77 K upon hydration with H_2O (solid line) and D_2O (dotted line), respectively. In the hydrated film, GR molecules are oriented randomly, while BR molecules are highly oriented. The spectrum in (b) is reproduced from Kandori et al. (*47*), where the sample window is tilted by 53.5°. One division of the *y*-axis corresponds to 0.01 absorbance unit.

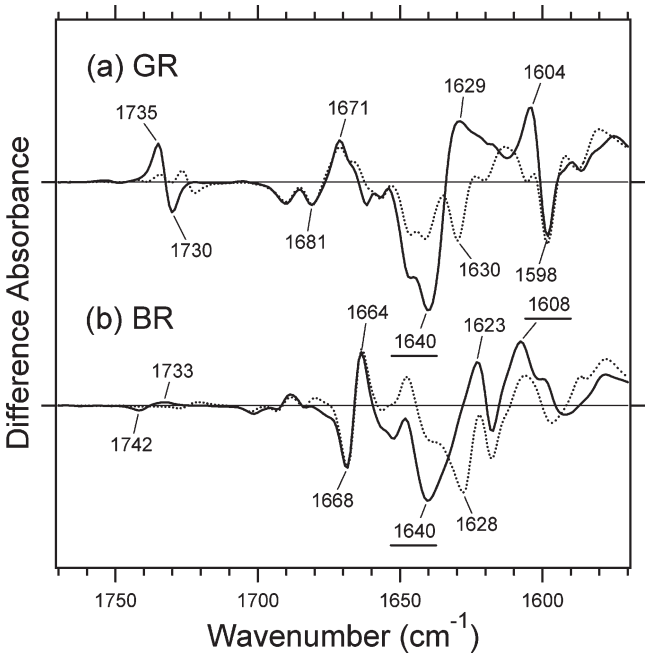

FIGURE 3: The GR_K minus GR (a) and the BR_K minus BR (b) spectra in the 1770−1570 cm^{-1} region, most of which are ascribable to vibrations of the protein moiety. The underlined peaks are C=N stretching vibrations of the chromophore. The sample was hydrated with H_2O (solid lines) or D_2O (dotted lines). One division of the *y*-axis corresponds to 0.007 absorbance unit.

assignable to the C14−C15 stretching vibration of all-*trans*-retinal (*21*). A positive band at 1197 cm^{-1} in GR and at 1194 cm^{-1} in BR is assigned to the C10−C11 and C14−C15 stretching vibrations of the 13-*cis*-retinal after formation of the K state. A negative band at 1169 cm^{-1} in BR, C10−C11 stretch, is absent in GR. This is also the case in *pharaonis* phoborhodopsin (*pp*R; also called *pharaonis* sensory rhodopsin II) (*29*) and PR (*30*). The H−D exchangeable band at 1255 cm^{-1} was assigned to one of the modes containing N−H in-plane bending vibration of the Schiff base of BR (*31*), while similar negative bands appear at 1240 cm^{-1} in the spectra of GR. This band is assignable to the modes of the Schiff base because of the shift to 978 cm^{-1} upon hydration with D_2O, as in BR to 977 cm^{-1}.

Hydrogen-out-of-plane (HOOP), N−D in-plane bending, and methyl rocking vibrations are observed in the 1110−890 cm^{-1} region, and the presence of strong HOOP modes represents the distortion of the retinal molecule. The most intense HOOP band in the BR_K minus BR difference spectra (Figure 2b) was observed at 957 cm^{-1} (in H_2O) and 951 cm^{-1} (in D_2O), which were assigned to the C15−H HOOP vibration of BR_K (*31*). In the case of GR, a broad spectral feature at 1000−950 cm^{-1} was observed. The negative band at 977 cm^{-1} in Figure 2b was assigned to the N−D in-plane bending vibrations of BR (*31*), and similar band was also observed at 978 cm^{-1} for GR (Figure 2a).

C=N stretching vibrations of the protonated retinal Schiff base are observed in the 1650−1600 cm^{-1} region (Figure 3). The C=NH and C=ND stretching vibrations of BR were observed at 1640 and 1628 cm^{-1}, while those of BR_K were observed at 1608 and 1606 cm^{-1}, respectively (Figure 3b) (*29*). The former difference in frequency is 12 cm^{-1} and the latter is 2 cm^{-1}, suggesting that the protonated Schiff base forms a hydrogen bond in BR and it is broken upon retinal isomerization. The C=N stretchings were observed at 1640 (C=NH) and 1630 cm^{-1} (C=ND) in GR, and its difference is 10 cm^{-1}, suggesting that the hydrogen-bonding strength is similar but slightly weaker than that of BR. On the other hand, it is difficult to assign the positive bands because of more complicated spectral features. The N−D stretching vibration of the Schiff base in D_2O provides additional and more direct information on the hydrogen bond strength of the Schiff base as described below.

The band pairs at 1668 (−)/1664 (+) cm^{-1} and at 1623 (+)/1617 (−) cm^{-1} in the BR_K minus BR difference spectrum were assigned to the C=O stretching vibrations of amide I. The former was assigned to the amide I of α_{II} helix (*32*) and the latter to the amide I of Val49 (*33*). In the case of GR, the band pair at 1681 (−)/1671 (+) cm^{-1} could have similar origin to that of the bands at 1668 (−)/1664 (+) cm^{-1} in BR.

In the BR_K minus BR difference spectra (Figure 3b), the bands at 1742 and 1733 cm^{-1} were assigned to the C=O stretching vibrations of the protonated Asp115, which are downshifted upon hydration with D_2O (*34*). In the GR_K minus GR difference spectra (Figure 3a), more intense bands were observed at 1735 (+)/1730 (−) cm^{-1}. It should be noted that the intensity is much stronger than in other rhodopsins. More importantly, GR has a serine residue at the position corresponding to Asp115 in BR (Figure 1). Below we study these bands in greater detail.

Comparison of the X−D Stretching Vibrations in GR and BR. X−D stretching vibrations of protein and water molecules appear in the 2800−2000 cm^{-1} region (Figure 4). A spectral comparison of the samples hydrated with D_2O and $D_2{}^{18}O$ identifies O−D stretching vibrations of water molecules which change their frequencies upon retinal photoisomerization. Green-tagged bands in Figure 4 are assignable to the O−D stretching vibrations of water because of the isotope shift. In BR, six negative peaks at 2690, 2636, 2599, 2323, 2292, and 2171 cm^{-1} were assigned to vibrations of three water molecules in the Schiff base region (Figure 4b) (*12*), among which the 2171 cm^{-1} band

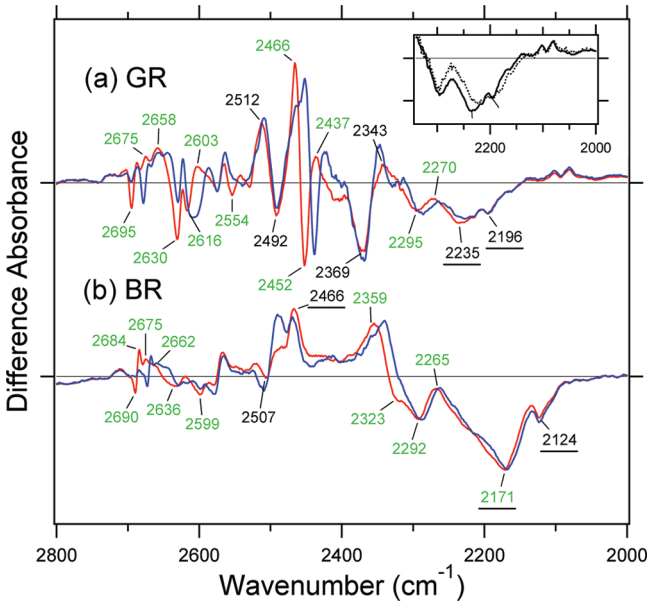

FIGURE 4: Comparison of the difference infrared spectra of GR (a) and BR (b) hydrated with D_2O (red lines) or $D_2{}^{18}O$ (blue lines) in the 2800−2000 cm^{-1} region. Green-labeled frequencies correspond to those identified as water stretching vibrations. In BR (b), the bands at 2507 (−)/2466 (+) cm^{-1} in (b) were assigned to the O−D stretches of Thr89 (*38*, *39*), while the underlined frequencies are N−D stretches of the Schiff base (*30*). In (a), the negative bands at 2235 and 2196 cm^{-1} can be assigned to the N−D stretches of the Schiff base because the peaks (solid line in inset) exhibit spectral downshift for [ζ-^{15}N]Lys-labeled GR (dotted line in inset). Spectra in (b) are reproduced from Tanimoto et al. (*48*), where the sample window was tilted by 53.5°. One division of the *y*-axis corresponds to 0.0012 absorbance unit.

was assigned to the O−D group of water 402 bridging the Schiff base and Asp85 (*18*). Water stretching vibrations of BR_K tend to be higher in frequency, implying that the overall hydrogen bonding of water becomes weaker upon photoisomerization.

The GR_K minus GR spectra (Figure 4a) exhibit sharper peaks compared to those of BR. From the isotope effect of ^{18}O water, we identified six negative and six positive peaks of water O−D stretch: at 2695 (−), 2675 (+), 2658 (+), 2630 (−), 2616 (−), 2603 (+), 2554 (−), 2466 (+), 2452 (−), 2437 (+), 2295 (−), and 2270 (+) cm^{-1}. Numbers of water bands are similar between GR and BR, whereas their frequencies are different. The O−D stretching frequency of water is distributed in the 2700−2150 cm^{-1} region, and the frequency is lowered if the hydrogen bond is strengthened. Nevertheless, the water O−D stretches in BR are clustered in the weak hydrogen bond region (at 2690−2599 cm^{-1}) and in the very strong hydrogen bond region (at 2323−2171 cm^{-1}) (Figure 4b). By use of mutants, we previously reported that each of the three water molecules in the pentagonal cluster possesses separate O−D stretches at 2690−2599 and 2323−2171 cm^{-1} (*12*). The large separation (300−470 cm^{-1} for O−D stretches and 500−770 cm^{-1} for O−H stretches) indicates weak vibrational coupling of the stretches in each water, being presumably due to strong association of the waters to negative charges of Asp85 and Asp212 in BR. In contrast, water O−D stretches in GR distribute more evenly than those in BR. The water O−D stretch at 2695 cm^{-1} indicates lack of a hydrogen bond, while the O−D stretches at 2630 and 2616 cm^{-1} correspond to a weak hydrogen bond of water. The water O−D stretches at 2554 and 2452 cm^{-1} correspond to moderate and relatively strong hydrogen bonds, respectively. On the other hand, the water O−D stretch at 2295 cm^{-1} corresponds to a very strong hydrogen bond. The negative band at 2235 cm^{-1} may also contain a water O−D stretching vibration, because spectral difference between D_2O and $D_2{}^{18}O$ was reproducible.

These water signals at 77 K strongly suggest that GR possesses the BR-like water-containing hydrogen-bonding network in the Schiff base region, while vibrational decoupling of two O−D stretches in each water is likely to be small in GR. This indicates that the hydrogen-bonding network is formed less ideally in GR, where hydrogen bonds are weaker than in BR. On the other hand, the negative water band at 2295 cm^{-1} (and possibly at 2235 cm^{-1}) should be particularly noted. By using various rhodopsins and their mutants, we have discovered a correlation between proton pumping activity and the presence of strongly hydrogen-bonded water molecule(s), whose O−D stretches in D_2O are located at $<$2400 cm^{-1} (*27*). It is likely that strongly hydrogen-bonded water and transient weakening of its bonding play an important role in the light-energy storage for proton-pumping function. The presence of the water band at 2295 cm^{-1} in GR further supports the correlation.

The frequency region shown in Figure 4 also contains X−D stretching vibrations other than those of water molecules. In the BR_K minus BR spectrum, the bands at 2507 (−)/2466 (+) cm^{-1} were assigned to the O−D stretching vibrations of Thr89 (Figure 4b) (*35*, *36*). In the GR_K minus GR spectrum, the bands at 2512 (+)/2492 (−) cm^{-1} do not exhibit isotope shift of [^{18}O]water and thus are strong candidates for the O−D stretching vibrations of Thr125 (Figure 1). It is interesting that the frequency upshift indicates weakening of the hydrogen bond of this O−D group upon retinal photoisomerization in GR, being in contrast to the case of BR. This feature is unique for GR. The BR-like spectral changes of threonines were observed for other rhodopsins such as *p*SRII (*11*), NR (*21*), ASR (*23*), and SrSRI (*17*), whereas such bands are absent in LR (*13*) and PR (*30*).

The bands at 2466 (+), 2171 (−), and 2124 (−) cm^{-1} in the BR_K minus BR spectrum were assigned to the N−D stretching vibrations of the retinal Schiff base (*37*). Thus, the negative 2171 cm^{-1} band contains both O−D stretch of water and N−D stretch of the Schiff base. The corresponding negative bands at 2235 and 2196 cm^{-1} are the candidates for the N−D stretching vibrations of the Schiff base in GR (Figure 4a). Indeed, the two negative peaks exhibit spectral downshift for [ζ-^{15}N]Lys-labeled GR (Figure 4, inset). The N−D stretches of the Schiff base at 2235 and 2196 cm^{-1} in GR presumably correspond to those at 2171 and 2124 cm^{-1} in BR, respectively, and the upshifts by ~150 and ~70 cm^{-1} indicate a slightly weaker hydrogen bond of the protonated Schiff base in GR. Unfortunately, the purity of [ζ-^{15}N]Lys-labeled GR was not sufficient to identify the N−D stretch in GR_K, though the positive band at 2466 cm^{-1} was assigned as the N−D stretch of the Schiff base in BR_K (*37*).

A Protonated Carboxylic Acid in GR. The C=O stretches of protonated carboxylic acids appear in the 1780−1700 cm^{-1} region, being well isolated from other vibrations. As shown in Figure 3, the obtained GR_K minus GR spectrum possesses intense bands in this frequency region. Therefore, we compared the signal with those published previously for other microbial rhodopsins. Figure 5 shows light-induced spectral changes of BR (a), proteorhodopsin (PR) (b), *Leptosphaeria* rhodopsin (c), GR (d), *pp*R (*p*SRII) (e), *Neurospora* rhodopsin (NR) (f), and *Anabaena* sensory rhodopsin (ASR) (g). As published previously (*13*, *14*, *21*, *23*, *29*, *38*), these difference spectra reflect

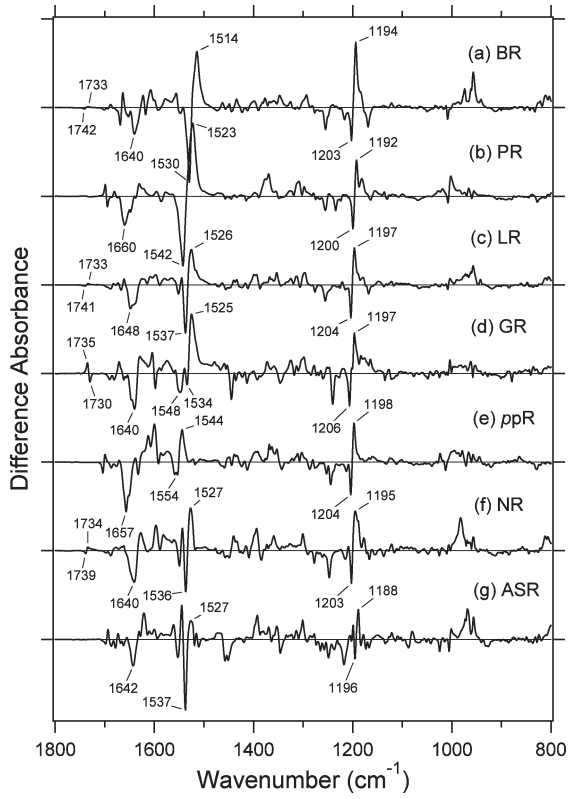

FIGURE 5: Light minus dark difference FTIR spectra in the 1800−800 cm^{-1} region for various microbial rhodopsins measured at 77 K. The spectra in (a), (b), (c), (e), (f), and (g) are reproduced from refs *38*, *14*, *13*, *29*, *21*, and *23*, respectively, while that in (d) was obtained in the present study.

the light-induced changes in the K intermediates compared to the respective unphotolyzed states. The spectra are normalized by the negative peak at 1206−1200 cm^{-1} except for ASR (Figure 5g). In hydrated film samples, only BR molecules are highly oriented, while other rhodopsins are randomly oriented as described previously (*13*, *14*, *21*, *23*, *29*, *38*). Thus, we used the BR_K minus BR spectrum with the sample tilting angle of 53.5° in the polarized FTIR spectroscopy. Since the transition dipole moment of the C=O group of Asp115 is oriented close to the magic angle, the bands at 1742 (−)/1733 (+) cm^{-1} are never dichroic (*39*, *40*). It should be noted that the present comparison is not strictly quantitative. Nevertheless, the comparison here should be reasonable, because the signal intensity of protonated carboxylic acids is much stronger in GR.[2]

Figure 6 enlarges the frequency region of protonated carboxylic acids of Figure 5. The BR_K minus BR spectrum (Figure 6a) possesses the bands at 1742 (−)/1733 (+) cm^{-1} owing to Asp115 nearby the retinal, while the absence of other bands indicates that Asp96 is not involved in the protein structural changes in BR_K. This is reasonable, because Asp96 is located far (about 12 Å) from the retinal (*41*) and formation of BR_K is a local photoisomerization-induced conformational event. Among the other rhodopsins, only LR and NR possess Asp at the position corresponding to Asp115 in BR (Asp169 and Asp161, respectively), and the bands

[2]After submission of the present paper, Dioumaev et al. reported the difference FTIR spectra of xanthorhodopsin (XR) at 80 K (*49*). Similar intense bands were observed at 1739 (+)/1731 (−) cm^{-1} in the XR_K minus XR spectra. Since XR has Glu and Ala at the positions corresponding to Asp96 and Asp115 in BR, respectively, the bands possibly originate from Glu (Glu107) in XR.

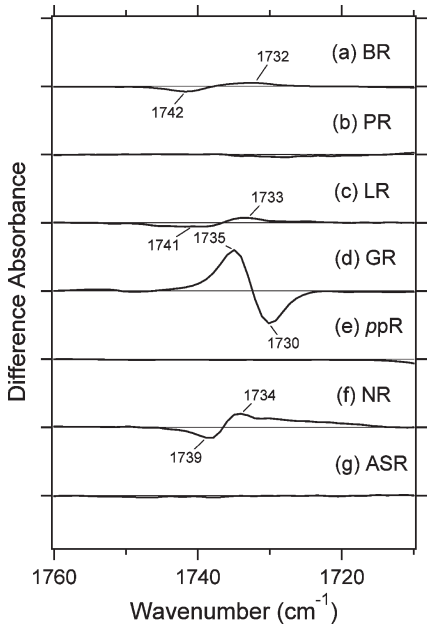

FIGURE 6: Light minus dark difference FTIR spectra in the 1770−1710 cm^{-1} region for the wild-type (a) and mutant (b−g) proteins of GR measured at 77 K. Each spectrum is scaled as in Figure 5.

at 1741 (−)/1733 (+) cm^{-1} (Figure 6c) and 1739 (−)/1734 (+) cm^{-1} (Figure 6f) can be assigned to these groups. Thus, no carboxylic vibrations other than those of Asp115 (in BR) have been known to change in the K formation of microbial rhodopsins, though PR, LR, and NR possess Glu or Asp at the position corresponding to Asp96 in BR.

In the frequency region of protonated carboxylic acids in the GR_K minus GR spectra, prominent peaks were observed at 1735 (+)/1730 (−) cm^{-1} (Figure 6d). It should be noted that GR has Ser (S155) at the position corresponding to Asp115 in BR (Figure 1), so that the signal cannot come from its homologue. In fact, the frequency is upshifted upon retinal photoisomerization in GR unlike in BR, LR, and NR, indicating that the hydrogen bond of the carboxylic C=O group is weakened only in GR.

Then, what is the origin of this unique band? There are three Asp and four Glu in the membrane region of GR: Asp115, Asp121, Glu132, Glu143, Glu166, Glu195, and Asp253 (Figure 1). Among them, Asp121, Glu132, and Asp253 correspond to Asp85, Asp96, and Asp212 in BR, respectively. To identify the C=O stretch, we prepared the entire set of D-to-N and E-to-Q mutants for the seven carboxylates. However, D121N and E132Q exhibited predominantly the photoreaction of the 13-*cis* form at 77 K, as suggested by the replacement of the all-*trans* specific bands at 1205 (−)/1197 (+) cm^{-1} of the wild type (Figure 7a, right panel) by the 13-*cis* specific bands at 1186 (−)/1174 (+) cm^{-1} (Figure 7h,i, right panel). Therefore, we next prepared D121E and E132D mutants, but the expression of the former was not sufficiently high for the IR analysis.

Figure 7, left panel, shows the GR_K minus GR spectra in the region of frequencies of protonated carboxylic acids, where the bands at 1735 (+)/1730 (−) cm^{-1} were perfectly preserved for the five mutants: D115N (Figure 7b), E143Q (Figure 7d), E166Q (Figure 7e), E195Q (Figure 7f), and D253N (Figure 7g). In contrast, the bands disappeared in E132D, while the new bands appeared at 1753 (+)/1748 (−) cm^{-1} (Figure 7c). Thus, the most straightforward interpretation is that the frequencies of the C=O

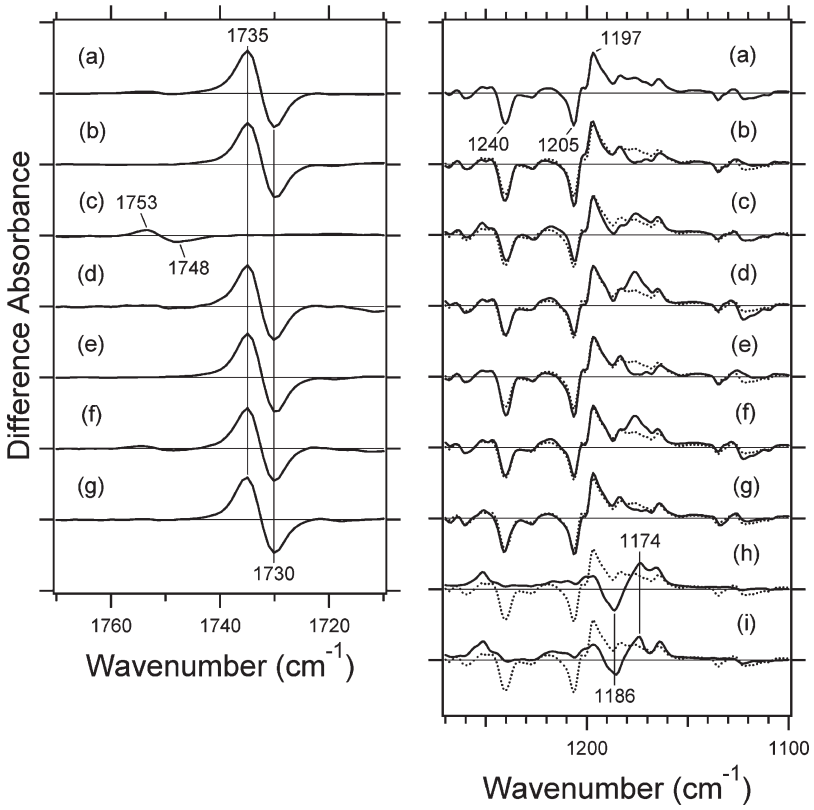

FIGURE 7: Light minus dark difference FTIR spectra in the 1770−1710 cm^{-1} region (left) and in the 1270−1110 cm^{-1} region (right) for the wild-type (a) and mutant (b−i) proteins of GR measured at 77 K. The mutants are D115N (b), E132D (c), E143Q (d), E166Q (e), E195Q (f), D253N (g), D121N (h), and E132Q (i). Dotted lines in the right panel (b−i) represent the spectra of the wild type. One division of the *y*-axis corresponds to 0.002 and 0.008 absorbance unit for the left and right panels, respectively.

stretching vibrations are at 1730 and 1748 cm^{-1} for Glu132 (wild-type GR) and Asp132 (E132D mutant), respectively.

The present study could not exclude the possibility of Asp121 as the origin of the bands at 1735 (+)/1730 (−) cm^{-1}, because the photoreaction from the functional all-*trans* form was not obtained for either D121N or D121E. However, it is unlikely, because Asp121 presumably constitutes the counterion complex similar to Asp85 in BR and thus should be deprotonated. In fact, the pK_a of Asp121 was estimated to be around 5 from the pH titration of absorption maxima (data not shown). It is thus concluded that the bands at 1735 (+)/1730 (−) cm^{-1} in GR originate from the C=O stretch of Glu132. At present, it is unclear why the signal is much smaller in E132D (Figure 7c) than in the wild type (Figure 7a). Size of the amino acid side chain may significantly influence the local structural perturbation at position 132 in GR.

DISCUSSION

In this paper, we studied structural changes of GR following retinal photoisomerization by means of low-temperature FTIR spectroscopy. The initial motivation was to reconfirm our hypothesis that proton-pumping rhodopsins possess strongly hydrogen-bonded water molecules. Water O−D stretching vibrations at <2400 cm^{-1} in D_2O have been regarded as markers of strongly hydrogen-bonded water (*5*), and there has been strong correlation between the proton-pumping activity and the presence of such water molecules (*27*). Since GR pumps protons, we expected that GR also possesses strongly hydrogen-bonded water molecule(s). The FTIR results clearly show that this is indeed the case. In addition, the low-temperature FTIR experiments presented in this work provided another entirely unexpected finding. This study reports the structural perturbation at the position homologous to Asp96 in BR at the early stage of the proton-pumping process in GR. Below we discuss these two issues, an expected observation on water and an unexpected observation on the long-range structural perturbation.

Strongly Hydrogen-Bonded Water Molecules as the Determinant of Proton-Pumping Activity of Rhodopsins. We previously reported that among various BR mutants only D85N, D85S, and D212N lack strongly hydrogen-bonded water molecules (O−D stretch at <2400 cm^{-1}) and proton-pumping activity (*12*, *18*, *26*). Therefore, we proposed a hypothesis that the presence of a strong hydrogen bond of water is a prerequisite for proton pumping in rhodopsins (*27*). We have extensively tested this hypothesis for various rhodopsins by measuring O−D stretches of water. We found a perfect correlation between the presence of strongly hydrogen-bonded water molecule(s) and proton-pumping activity. For example, strongly hydrogen-bonded water molecules are observed for *pp*R (*11*), LR (*13*), PR (*14*), azide-bound *pharaonis* HR (*15*), and *Salinibacter* SRI (*17*), which all pump protons. Strongly hydrogen-bonded water molecules were not observed for bovine rhodopsin (*19*), *salinarum* HR (*20*), NR (*21*), *pharaonis* HR (*20*, *22*), ASR (*23*, *25*), and squid rhodopsin (*24*), which have no proton-pumping acitivity. Cl^--pumping D85S has no strongly hydrogen-bonded water molecules (*26*), whereas H^+-pumping D212N(Cl^-) has a strongly hydrogen-bonded water molecule (*16*). Thus, hydrogen-bonding strength of water molecules is very important for the proton-pumping function. It should be noted that photon energy absorbed by rhodopsins is stored first in the photoisomerized form of the retinal. In addition to the distorted

shape of the chromophore, importance of changes in its hydrogen-bonding network, which includes water, has to be emphasized for light-energy storage. Since only about 10% of light energy is spent on the electrochemical potential of the transported proton (*42*), a small energy difference in hydrogen-bonding stabilization may be crucial for the pumping function.

Our hypothesis and its successful application to various rhodopsins have provided a concept for the new roles of internal water molecules. One of the important roles of internal water molecules is to occupy the empty space inside protein that may be energetically unfavorable. Internal water molecules may assist the transport of ions inside protein by raising the dielectric constant (*38*). In addition, we showed the possibility that the hydrogen bond of water carries energy for the proton-pumping function, which can be monitored by FTIR spectroscopy. A strongly hydrogen-bonded water molecule is observed for GR, which is consistent with our hypothesis that such water molecule is a prerequisite for proton-pumping activity of rhodopsins.

Long-Range Structural Perturbation at the Position Homologous to Asp96 in BR in the K Intermediate of GR. This study reports the structural perturbation at the position homologous to Asp96 in BR at the early stage of the proton-pumping process in GR. One possible reason might be the shorter distance between Glu132 and the retinal in GR than in BR (about 12 Å). The overall sequence identity is 21% between GR and BR, whereas 20 amino acids are identical among the 25 amino acids in the retinal binding pocket. The GR_K minus GR spectra were similar to the BR_K minus BR spectra (Figure 5), indicating that the all-*trans* to 13-*cis* isomerization takes place normally. Thus, the protein core architecture of GR must be similar to other microbial rhodopsins, suggesting the existence of the long-range interaction between the retinal and the cytoplasmic proton donor region.

From the amino acid sequence, GR is classified into the same cluster as xanthorhodopsin (XR), the carotenoid-associated proton pump from *Salinibacter ruber* (*43*, *44*). While the structure of GR is unknown, the X-ray structure of XR (*45*) provides a reasonable explanation. In BR, Asp96 forms a hydrogen bond with Thr46, and the Asp-Thr pair is replaced by the Glu-Ser pair in XR and GR. In XR, there is no hydrogen bond between Glu and Ser, but the glutamate residue (Glu107) is hydrogen-bonded to a water (water 502) that connects it to the chromophore region. Consequently, the distance between the Schiff base nitrogen and the side chain of Glu107 in XR is closer (about 10 Å) than that of Asp96 in BR (about 12 Å), though the distance between the Schiff base nitrogen and the Cα carbon of the carboxylate is identical (*41*, *45*). It is thus possible that Glu132 in GR is similarly connected to the chromophore region through the hydrogen-bonding network in the cytoplasmic side. Miranda et al. reported an unusually strong coupling between Glu132 and the chromophore region, as the replacement of Glu132 changed color and isomeric composition (*9*). The structural perturbation at the position homologous to Asp96 in BR at the K state of GR is presumably explained in terms of the unique hydrogen-bonding network in the cytoplasmic side.

The present study reports the vibrational signals of internal water molecules of GR (Figure 4), where the water molecules are possibly located near the retinal chromophore. It should be however noted that the location of such water molecules has to be examined by use of mutant proteins (*38*). Previous comprehensive mutation analysis of BR revealed that six water O−D stretches (green-tagged negative peaks in Figure 4b) originate from three water molecules in the Schiff base region constituting a pentagonal cluster (*12*). This implies that the structural changes in BR are highly localized at the Schiff base region at 77 K, which is consistent with no structural perturbation of Asp96 in the cytoplasmic side at the stage of BR_K. Although six negative peaks were similarly observed as water O−D stretches in GR, the origin could be different because their vibrational features differ considerably between GR and BR (Figure 4). Interestingly, the X-ray structure of XR observed only a single water molecule, not three, in the Schiff base region (*45*). The bridged water molecule (water 402) between the proton donor (the Schiff base) and acceptor (Asp96 in XR) is preserved, whose strong hydrogen bond must be a prerequisite for the proton-pump function. On the other hand, the other two water molecules (waters 401 and 406) in the pentagonal cluster of BR (*41*) are absent in the XR structure (*45*). Instead, water 502 forms hydrogen bonds to Glu107 and the peptide carbonyl of Lys240. If the water structure is similar between GR and XR, some water signals in Figure 4a must originate from the water molecules in the cytoplasmic region of GR. This interpretation is reasonable, because the present study observed the structural perturbation of the hydrogen-bonding network in the cytoplasmic side of GR at 77 K that is extended to Glu132, the position homologous to Asp96 in BR. Mutational analysis of GR will provide the direct evidence, as was shown for BR (*12*).

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