Protein Structural Changes in Bacteriorhodopsin upon Photoisomerization As Revealed by Polarized FTIR Spectroscopy

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We used polarized Fourier transform infrared (FTIR) spectroscopy to investigate the structural change of bacteriorhodopsin (BR) upon photoisomerization of the retinal chromophore. By measuring the difference spectra between the K-intermediate and BR in the whole mid-infrared region (700−4000 cm^{-1}) at 77 K, complete vibrational information was obtained on how the protein responds to the displacement of the chromophore. In particular, changes in O−H and N−H stretching vibrations, which directly probe the hydrogen bonding strength, have provided not only the relevant frequencies but also their angles to the membrane normal. Structural perturbation of the peptide backbone appears in the 3270−3320 cm^{-1} (peptide N−H stretch) and the 1650−1670 cm^{-1} (peptide C═O stretch) regions. These peptide bands are insensitive to H−D exchange, and the dipole moments of the N−H and C═O stretches are parallel to the membrane normal. In contrast, several bands are downshifted upon D_2O substitution, indicating that O−H or N−H groups that participate in a hydrogen bonding network near the chromophore change upon cis−trans isomerization.

Introduction

Bacteriorhodopsin (BR) is a light-driven proton pump in *Halobacterium salinarium* that binds all-trans retinal as a chromophore.[1−4] Recent cryoelectron microscopy and X-ray crystallography have revealed the structure of BR at atomic resolution (Figure 1).[5−8] The retinal chromophore is deeply embedded inside BR and binds covalently to Lys-216 through a protonated Schiff base. Light absorption triggers a cyclic reaction that is comprised of a series of intermediates, designated as the J-, K-, KL-, L-, M-, N-, and O-states. Protein structural changes forming these intermediate states allow proton translocation across the protein as a sum of several local proton transfers. The mechanism of these structural changes is the central question in current studies of BR.[9−11] Having the atomic structure in hand, the structural change is now required also at atomic resolution.

The primary all-trans to 13-cis isomerization after photon absorption leads to the formation of the K-intermediate[12−15] through the vibrationally hot J-intermediate.[16] Although the primary proton transfer occurs only on a sub-millisecond time scale, the complete information of the protein structural change for proton pumping must be already stored in the structure of the primary intermediates. Thus, the structural analysis of the primary intermediates will reveal the "structural programming" for proton translocation. In addition, it is well-known that photoisomerization in BR is highly selective and efficient. In solution the photoproduct of all-trans retinal with protonated Schiff base is mainly 11-cis (82% 11-cis, 6% 13-cis, 12% 9-cis in methanol),[17] while in BR it is 100% 13-cis. The quantum efficiency for isomerization in BR (∼0.6)[18,19] is much higher than that in solution (0.13[17] or 0.17[20] in methanol). The specific chromophore−protein interaction in BR that yields such selective and efficient primary reaction is also the cause of the efficient proton pumping.

How does the highly selective and efficient isomerization occur in BR? How does the structure of the primary intermediates drive the functional processes? Whereas the diffraction studies have not been applied to the primary intermediates so far, vibration spectroscopy has attempted to reveal the structural change. Earlier resonance Raman spectroscopy of the K-intermediate has revealed the distorted structure of the chromophore through analysis of hydrogen-out-of-plane (HOOP) vibrations.[21,22] This distortion could constitute one of the modes of light energy storage in K,[23] but little has been known about how the protein responds to the motion of the chromophore.

FTIR spectroscopy is a powerful tool for probing the protein structural change in BR, because difference spectra before and after light illumination can reveal even small vibrational changes.[24−26] Although the previous FTIR spectroscopy covered a limited frequency region (800−1800 cm^{-1}), we have optimized the measuring system for conversion to the primary photointermediate, as reported for visual rhodopsin.[27] The improved equipment now allows detection of an absorbance change between BR and K as small as 10^{-5}, by which we can detect a single vibration in the 1800−4000 cm^{-1} region. In addition, application of polarized FTIR spectroscopy allows measurement of the angles of the dipole moments of vibrations to the membrane normal.[28−31]

In the present article, we report infrared spectral changes between BR and the K-intermediate in the whole mid-infrared region (800−4000 cm^{-1}). The K-intermediate is stabilized at 77 K, and polarized FTIR spectroscopy is applied. The observed spectra were highly reproducible, thus providing complete vibrational information on the protein structural changes. Polarized FTIR spectroscopy further determined the angle of dipole moments of vibrations to the membrane normal. The present observation in the new frequency region promises better understanding of protein structural changes of BR by means of isotope labelings and mutations.

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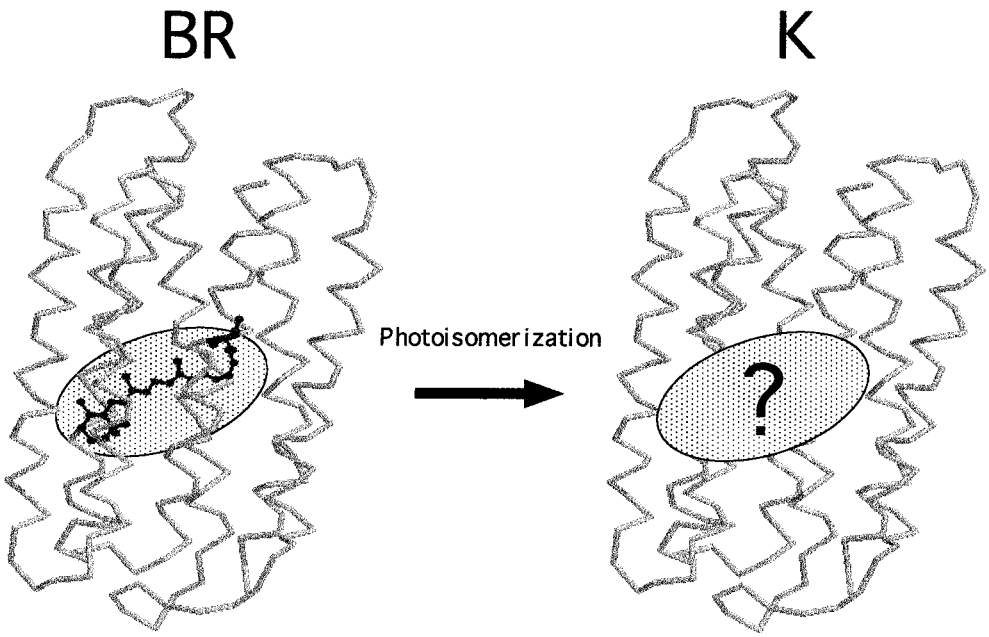

Figure 1. Structure of BR and the K-intermediate. The structure of BR is now given at the atomic resolution, where the drawing is the side view of 2BRD.[5] The membrane normal exists approximately in the vertical direction of this figure. In contrast, little is known about the K-intermediate. In particular, very little is known about the structural changes of the protein side.

Materials and Methods

Purple membranes were prepared by the method described previously.[32] The membranes were suspended in 2 mM phosphate buffer (pH 7.0), and a 120 μL aliquot was dried on a BaF_2 window with a diameter of 18 mm. After hydration by 1 μL of H_2O or D_2O, the sample was placed in a cell and then mounted in an Oxford DN-1704 cryostat. The film was illuminated with >500 nm light for 1 min at 273 K to obtain the light-adapted state of BR.

The illumination with a 501 nm light at 77 K for 2 min converted BR to the K-intermediate. Since the K-intermediate completely reverted to BR upon illumination with >660 nm light for 1 min, as evidenced by the same but inverted spectral shape, the cycles of alternative illuminations with a 501 nm light and >660 nm light were repeated a number of times. The difference spectrum was calculated from the spectra constructed with 128 interferograms before and after the illumination. Twenty-four spectra obtained in this way were averaged for each K minus BR spectrum under various conditions. The details of polarized FTIR spectroscopy are described elsewhere.[30] Briefly, a BaF_2 polarizer in the vertical *xy* plane is placed in front of a mercury−cadmium−technetium (MCT) detector in a Bio-Rad FTS/60 FTIR spectrometer. The IR probe light travels along the *z*-axis to the window with the vertical and horizontal polarizations, A_V and A_H, in the *xz*- and *yz*-planes, respectively. The window in the *xy*-plane was tilted around the vertical *x*-axis by rotation of the rod holding the window. The tilt angles (ϕ_0) were 0, 17.8, 35.7, and 53.5°. The dichroic ratio *R* is defined as

$$R = [A_H(\phi_0)/A_H(0°)]/[A_V(\phi_0)/A_V(0°)] \ldots \quad (1)$$

An increase in the effective number of the BR molecules absorbing light with tilting was corrected for the increase in the intensity of the A_V component. *R* is related to the angle of the dipole moment to the membrane normal θ_0 by the equation described previously.

$$R = 1 + \sin^2\phi_0/n^2\,[\rho\langle 9\cos^2\theta_0 - 3\rangle]/[2 - \rho\langle 3\cos^2\theta_0 - 1\rangle] \ldots \quad (2)$$

where *n*, the refractive index of the film in the IR region, was assumed to be 1.7 as used previously and ρ, the degree of orientation of the membrane, to be 0.95 as reported.[30] The assumption for the latter is rational as judged from the intensity ratio of the amide II/amide I in our unhydrated film of 1.02 in comparison with their value of 0.98.

Results

Figure 2 shows the K minus BR spectrum at the tilting angle 0°. The spectrum in the 800−1800 cm^{-1} region reproduced the previous ones.[33−37] The large spectral changes in the 800−1800 cm^{-1} region, such as the C═C and C−C stretching vibrations, are ascribable predominantly to the chromophore. In addition, reproducible change was obtained in the higher frequency region (>1800 cm^{-1}), such as a sharp positive band at 3352 cm^{-1} and a broad negative feature at 2800−3100 cm^{-1}. If we neglect the contribution of overtones or combination bands of the lower frequency modes, the chromophore has only C−H stretching vibrations in the 2800−3100 cm^{-1} region. All other chromophore modes are located below 1800 cm^{-1} except for the N−H stretch of the Schiff base. Thus, the spectral change in the high-frequency region is a good probe for resolving changes in the protein structure.

Figure 3 shows the K minus BR spectra at various window tilting angles for hydrated film with H_2O (a) and D_2O (b) in the 2720−3680 cm^{-1} region. In the spectra, only a pair of bands at 3643 cm^{-1} (−) and 3636 cm^{-1} (+) has been assigned as the O−H stretch vibration of a water molecule.[38] The spectral changes in the other region are obtained for the first time. The largest peak at 3352 cm^{-1} at 0° (Figure 2) slightly decreases upon tilting a window. In contrast, a sharp positive peak appears at 3296 cm^{-1} when tilted, which is as large as the 3352-cm^{-1} band at 53.5° (Figure 3a). The change in intensity upon tilting of the window is most prominent in the 3280−3320 cm^{-1} region, where the N−H stretching vibration of the peptide

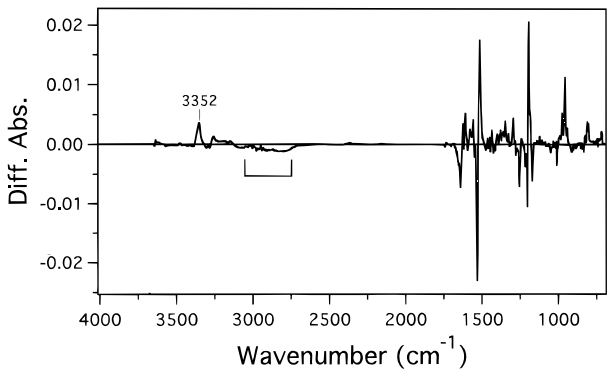

Figure 2. K minus BR difference spectrum at the tilting angle 0° measured at 77 K.

backbone (amide-A) appears.[39] The present observation is consistent with the tertiary structure of BR, where α-helices span the membrane and peptide N−H and C═O groups are parallel to the membrane normal (Figure 1). The frequency of amide-A vibration in the α-helix has been reported to be 3279 cm^{-1}.[39]

In general, both O−H and N−H stretching vibrations appear in wide frequency regions depending upon their structures and hydrogen-bonding strengths.[40] The O−H stretching appears in the 2800−3650 cm^{-1} region as is shown by water O−H vibrations. The N−H stretching appears in a somewhat narrower region between 3500 and 3000 cm^{-1}. Thus, spectral changes in Figure 3 originate from O−H and N−H stretching vibrations except for the C−H stretches in the 2800−3100 cm^{-1}. C−H groups are generally unexchangeable by D_2O substitution, whereas O−H and N−H groups are exchangeable unless the protein environment excludes the H−D exchange. Thus, the spectra upon hydration with D_2O provide further information.

Figure 3b represents the K minus BR spectra in D_2O. In the 2800−3100 cm^{-1} region, the broad negative spectral feature disappears, while the sharp peaks are similar between H_2O and D_2O. This suggests that the spectral changes in this region are mainly due to C−H stretching vibrations. Spectral changes in the amide-A region are more prominent in D_2O than in H_2O, where the positive 3296 cm^{-1} and the negative 3313 cm^{-1} bands at 53.5° are greatest. The present results show that the dipole moments of the vibrations at 3313 (−), 3296 (+), and 3280 cm^{-1} (−) tend to be parallel to the membrane normal. Spectral changes at >3500 cm^{-1} in H_2O are all sensitive to D_2O substitution. Two peak pairs at 3480 cm^{-1} (+)/3461 cm^{-1} (−) and 3415 cm^{-1} (+)/3402 cm^{-1} (−) are insensitive to D_2O substitution.

If the bands are well-separated from other vibrations, it is possible to estimate the angle of the dipole moment to the membrane normal. For instance, the angle of the dipole moments of the negative 3643 cm^{-1} and the positive 3636 cm^{-1} bands due to the water O−H group (Figure 3a) are calculated to be 66 and 85°, respectively, to the membrane normal.[41] The angle of the 3643 cm^{-1} band in BR is consistent with that obtained previously from the M minus BR spectrum at 230 K (60°).[30] The water O−H whose angle is 66° to the membrane normal slightly strengthens its hydrogen bonding and changes its angle by about 20° upon formation of the K-intermediate. On the other hand, the angle of the dipole moments of the negative 3461 cm^{-1} and the positive 3480 cm^{-1} bands in D_2O (Figure 3b) are calculated to be 63 and 43° to the membrane normal. The unidentified O−H or N−H group at 3461 cm^{-1} weakens the hydrogen bonding with altering of its angle by about 20° upon photoisomerization of the chromophore.

The difference between parts a and b of Figure 3 directly corresponds to the D_2O-sensitive bands, and the spectral feature should coincide with O−D or N−D stretching vibrations in the lower frequency region. Figure 4a shows the subtraction of the spectra hydrated with D_2O from those with H_2O in the 2700−3700 cm^{-1} region, where spectral changes of the D_2O-sensitive bands are illustrated in the O−H or N−H stretch region. As indicated, a broad negative feature is observable in the 2700−3100 cm^{-1} region. Figure 4b shows the spectra in D_2O, directly displaying the D_2O-sensitive bands. No spectral changes are present in this region that are seen with hydration with H_2O (not shown). The fine structure of the positive 2358 cm^{-1} band is due to CO_2 gas present in the spectrometer, which is overlapped onto the positive band due to the K-intermediate. There is an essentially good coincidence between spectra in Figure 4a,b, indicating that infrared spectral changes are measurable in the whole mid-infrared region.

Figure 4b shows that six D_2O-sensitive bands at 2690, 2632, 2599, 2506, 2292, and 2174 cm^{-1} change their hydrogen-bonding strengths upon formation of the K-intermediate. These are downshifted by H−D exchange from 3643, 3570, 3516, 3378, 3100, and 2796 cm^{-1}, respectively. Indeed, the angle dependence is similar for these bands in H_2O and in D_2O. The angles of the dipole moments to the membrane normal are estimated to be 58° at 2690 cm^{-1}, 60° at 2632 cm^{-1}, 35° at 2599 cm^{-1}, ∼0° at 2506 cm^{-1}, 62° at 2292 cm^{-1}, and 65° at 2174 cm^{-1} on the assumption that no spectral overlappings are present. Similarly, the angles of the positive bands are estimated to be 79° at 2684 cm^{-1}, 38° at 2568 cm^{-1}, and 36° at 2466 cm^{-1} on the same assumption. It is hard to estimate the angle of the 2674, 2495, 2358, and 2268 cm^{-1} bands because of spectral overlapping.

From the spectral comparison between Figures 3 and 4, it is likely that spectral changes in amide-A are mainly D_2O-insensitive. Although the positive bands at 3353 and 3317 cm^{-1} and a negative band at 3378 cm^{-1} could be amide-A vibrations (Figure 4a), the D_2O-insensitive bands whose dipole moments are parallel to the membrane normal (3313, 3296, and 3280 cm^{-1}) are likely to originate from amide-A vibration. In addition, other vibrations in the 3100−3400 cm^{-1} may also originate from amide-A vibration. Since the peptide N−H group (amide-A) and C═O group (amide-I) form hydrogen bonding with each other in the α-helix, similar features in spectral changes are thereby expected for the corresponding amide-A and amide-I vibrations.

Figure 5 shows the K minus BR spectra in the amide-I region. In addition, the C═N stretching vibration of the protonated Schiff base of the retinal chromophore is also involved in this region: 1641 (H_2O) and 1628 cm^{-1} (D_2O) in BR and 1608 (H_2O) and 1607 cm^{-1} (D_2O) in K.[35,36,42] The negative 1592 cm^{-1} band in H_2O is slightly upshifted in D_2O (1596 cm^{-1}). All other vibrations look insensitive to D_2O substitution, suggesting amide-I vibrations. Among them, the spectral change at 1623 cm^{-1} (+)/1618 cm^{-1} (−) has been identified as the amide-I vibration of Val49.[43] It is noted that amide-I vibration of the α-helix has a frequency at about 1658 cm^{-1}. The peptide C═O group of Val49 forms stronger hydrogen bonding than the normal α-helix in BR, and the hydrogen bonding is weakened upon K formation.

The most significant change when the window is tilted is in the 1650−1670 cm^{-1} region. Appearance of the negative 1668 cm^{-1} and the positive 1664 cm^{-1} bands reproduced the previous results.[28] From the area, a single or a few peptide groups contribute the spectral changes. The positive peak at 1664 cm^{-1}

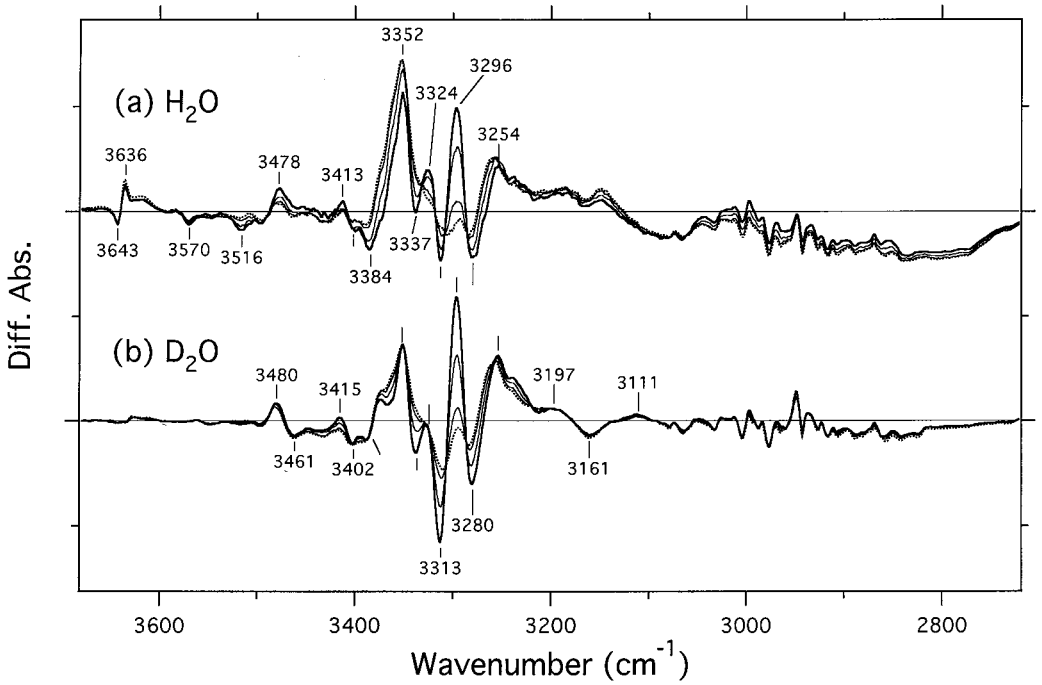

Figure 3. K minus BR spectra in the 2720−3680 cm^{-1} region. The sample was hydrated with H_2O (a) or D_2O (b). The window tilting angles (ϕ_0) are 0° (dotted line), 17.8° (thin solid line close to dotted line), 35.7° (thin solid line apart from dotted line), and 53.5° (thick solid line). One division of the *Y*-axis corresponds to 0.0025 absorbance unit.

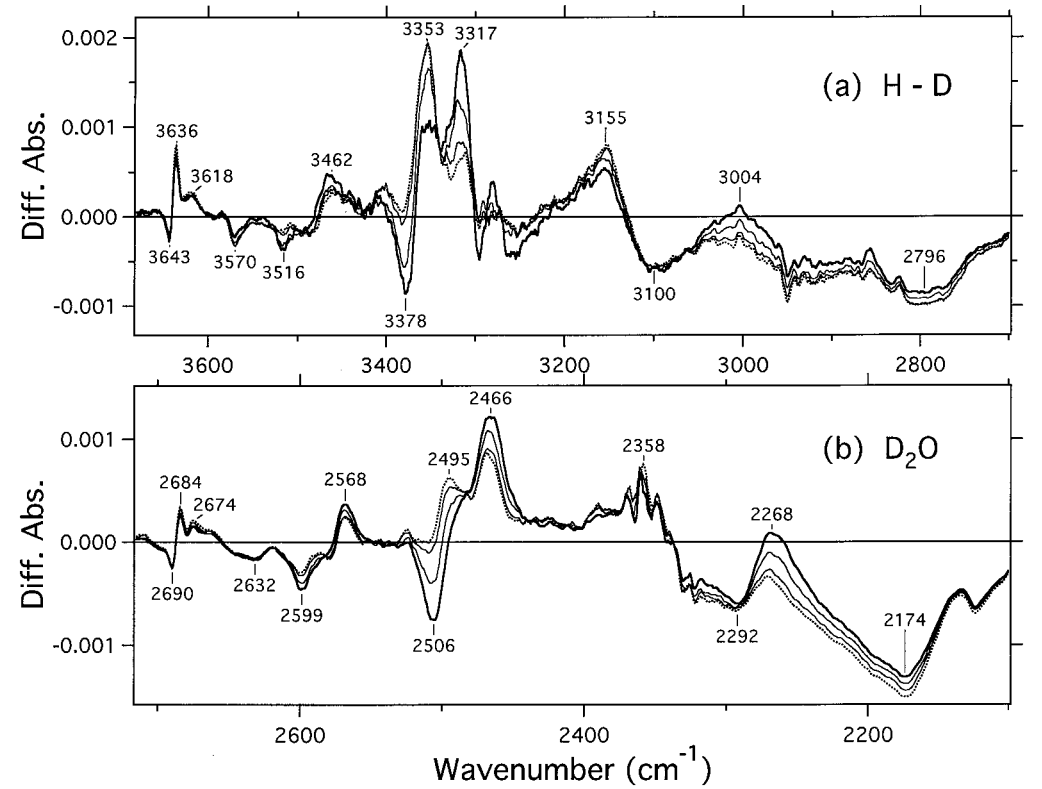

Figure 4. Spectral comparison of D_2O-sensitive bands. The difference spectra between H_2O and D_2O in Figure 3a and the spectra in D_2O Figure 3b are shown in the 2700−3680 and 2100−2715 cm^{-1} regions, respectively. The window tilting angles (ϕ_0) are 0° (dotted line), 17.8° (thin solid line close to dotted line), 35.7° (thin solid line apart from dotted line), and 53.5° (thick solid line). The fine structure at about 2358 cm^{-1} in D_2O originates from the CO_2 gas present in the spectrometer.

is at a 6 cm^{-1} higher frequency than the normal amide-I frequency of the α-helix (1658 cm^{-1}). The deviation from the normal frequency of the α-helix is the same in the positive peak at 3296 cm^{-1} (Figure 3), which is 17 cm^{-1} higher than the normal amide-A frequency of the α-helix (3279 cm^{-1}). Thus the N−H group at 3296 cm^{-1} is likely to form hydrogen bonding with the C═O group at 1664 cm^{-1} in the K-intermediate. Similarly, in BR, the amide-A band at 3313 cm^{-1} and the amide-I band at 1668 cm^{-1} appear to correspond with each other. The spectral features in Figure 5 suggest that the amide-I band at 1668 cm^{-1} in BR is downshifted to 1664 cm^{-1} in K. The corresponding amide-A band is shifted from 3313 cm^{-1} in

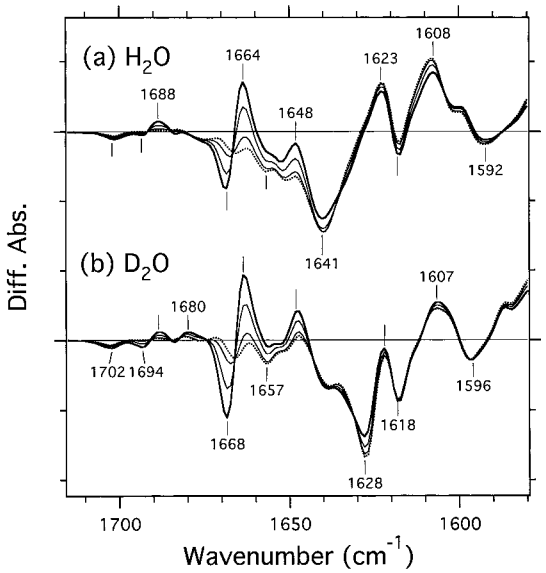

Figure 5. K minus BR spectra in the 1580−1715 cm^{-1} region. The sample was hydrated with H_2O (a) or D_2O (b). The window tilting angles (ϕ_0) are 0° (dotted line), 17.8° (thin solid line close to dotted line), 35.7° (thin solid line apart from dotted line), and 53.5° (thick solid line). One division of the *Y*-axis corresponds to the 0.005 absorbance unit.

BR to 3296 cm^{-1} in K. Interestingly, clear isosbestic points among spectra at various angles are observed for both amide-A (between 3313 and 3296 cm^{-1}; Figure 3) and amide-I (between 1668 and 1664 cm^{-1}; Figure 5) vibrations.

Discussion

The present polarized FTIR spectroscopy provides difference infrared spectra between BR and the K-intermediate with a high signal-to-noise ratio in the whole mid-infrared region (800−4000 cm^{-1}). The frequencies and angles represent the structural change before and after photoisomerization from all-trans to 13-cis. On the basis of the BR structure at atomic resolution,[5−8] detailed structural analysis is now possible. While complete understanding requires isotope-labeling and/or mutation studies that will identify the respective infrared bands in the future, various aspects can be (or should be) discussed at present.

Along with protein modes, the N−H stretching vibration of the Schiff base of the retinal chromophore is expected to appear in the high-frequency region. Although the N−H stretching frequency is a direct measure of the hydrogen-bonding strength of the Schiff base, it was not easy to detect it by Raman or infrared spectroscopy. Instead, the difference in frequency of the C═N stretch between H_2O and D_2O has been regarded as the measure.[44−46] In this case, C═N stretching in H_2O is upshifted by coupling with the N−H bending vibration from the intrinsic C═N stretching in D_2O, and as hydrogen bonding of the Schiff base is stronger, the upshift becomes larger. Since the difference between H_2O and D_2O is small in the K-intermediate (1608 and 1607 cm^{-1}, respectively) in comparison to that in BR (1641 and 1628 cm^{-1}, respectively) (Figure 5), it has been argued that hydrogen bonding in the K-intermediate is much weaker than in BR. The more direct approach in probing the N−H bending vibration (1254 cm^{-1} in BR and 1245 cm^{-1} in K) also argued that hydrogen bonding is weakened in K, though the difference was not much.[36] These results are consistent with the picture that photoisomerization breaks hydrogen bonding between the Schiff base and the counterion, Asp85.[47]

We thus expect that the N−H stretching frequency of the Schiff base in BR is upshifted upon K formation because of weakened hydrogen bonding. Since the N−H group of the Schiff base is exchanged by D_2O, the spectral change of the Schiff base mode must be contained in Figure 4b. Among negative bands, the 2690 cm^{-1} band is due to the water O−D stretch. Since the plane of the polyene chain of the retinal chromophore is parallel to the membrane normal,[5−8,48] the N−H group is also expected to be oriented parallel to the membrane normal. In this sense, the 2506 cm^{-1} band (Figure 4) may be a good candidate of the N−H stretch of the Schiff base. The frequency is present at 3377 cm^{-1} in H_2O (Figures 3 and 4). Interestingly, previous resonance Raman spectroscopy observed a peak at 3379 cm^{-1} that was assigned as the Schiff base N−H stretch in the article.[49] The frequency is coincident with the D_2O-sensitive negative band (Figure 4a). If it is, the N−H stretch of the K-intermediate must appear in the higher frequency region. The positive 2568 cm^{-1} band might correspond to it. Such a specific assignment of this band has to be from isotope labeling. We can conclude that it is the Schiff base N−H stretch only when it is so identified.

Previously, Olejnik et al. reported infrared spectral changes of BR at low temperature.[50] They observed a very broad band in the 2200−2800 cm^{-1} region upon formation of the K intermediate. They interpreted it in terms of hydrogen-bonding formation in which the proton is not well-localized. This observation, or the derived concept, called "IR continuum" became an experimental basis of further studies such as model compounds[51,52] and time-resolved spectroscopy of BR.[53,54] In the present study, however, we never observed such a broad band in the K-intermediate (positive bands), and the difference spectra coincided with the zero line in the 2200−2800 cm^{-1} region (Figure 2).[55] We observed a broad band in the 2700−3100 cm^{-1} region only in BR (negative bands). The broad band is downshifted completely by D_2O substitution. The frequency (2700−3100 cm^{-1}) indicates strong hydrogen bonding, but still within the normal O−H stretching region. Thus, at least for the K-intermediate, it is not necessary to take into account such an IR continuum.

In 1992, Maeda et al. reported a structural change of the hydrogen bonding of water molecules during the BR photocycle.[56] This opened a way to experimentally reveal the mechanism of water-mediated proton transfer in proteins. In fact, the subsequent approach by use of BR mutants implicated the specific location for water molecules and their roles in proton translocation.[57−65] The infrared study probing water molecules is particularly important, since the atomic coordinates of water molecules are given by X-ray crystallography.[7,8] However, the observed frequency region in the previous studies was limited in the >3450 cm^{-1}. A water has two O−Hs, and their frequencies distribute in the wide 2700−3650 cm^{-1} region dependent on their coupling and hydrogen-bonding strength. Experimental difficulty has forced us to limit the water O−H detection by FTIR in the >3450 cm^{-1} region. Since highly accurate measurements of the difference spectra of BR and the K-intermediate are now possible, an attempt for complete identification of water O−H stretches is an important and challenging issue.

According to the structure of BR, the hydrogen-bonding complex is present near the Schiff base.[5−8] It is reasonably considered that the O−H or N−H groups of such residues participate in the spectral changes obtained in the present study. For instance, O−H groups of Thr89 and Tyr185 form hydrogen bonding with aspartate oxygens of Asp85 and Asp212, respec-

tively. These O−H groups must play an important role in the proton pumping process of BR, which has been suggested by the great effects of their mutations on the photocycle.[66−69] Thus, identification of the O−H stretches is important, which is our future focus.[70] In addition, many other residues would contribute spectral changes observed in the present study. Although there are no glutamines in the membrane spanning region of BR, asparagine (1 amino acid is in the membrane), threonines (14 are in the membrane), serines (6 are in the membrane), and tyrosines (8 are in the membrane) possess an O−H group and tryptophans (8 are in the membrane) and arginines (4 is in the membrane) possess an N−H group. Elucidation if the hydrogen bonding of these residues is changed or not upon K formation will provide information on the extent of the protein structural change.

It has been well-known that photoisomerization in BR is selective and efficient, which is obviously different from in solution.[17−20] In this regard, an interesting question is whether cis−trans isomerization in BR is uni-directional. In solution, half isomerizes with one direction (of rotation) and the other half with the other direction. From the highly selective and efficient isomerization in the protein, one might imagine that only one direction of isomerization is allowed in BR, like one-directional electron transfer between two possible pathways in the photosynthetic reaction center.[71] Nevertheless, no experimental clue has ever been gained. The present observation opened the possibility for experimentally analyzing the directionality of isomerization in the protein by directly probing the protein modes.[72]

Photoisomerization of the retinal chromophore yields structural changes of not only the side chains but also the peptide backbone, as is shown in amide-A and amide-I bands. The change in peptide backbone is prominent when the window is tilted, indicating that the N−H and C═O groups parallel to the membrane normal mainly take part in the structural changes. From the results of amide-A, it is likely that structural change occurs for the peptide backbone buried inside the protein, where H−D exchange does not occur. Identification of amide vibrations will also reveal how the protein moiety responds to the chromophore change.

The frequency change in amide-A may be worthy of note. Krimm and Bandekar showed the linear relationship between the N−H stretching frequency and the distance from the nitrogen to the oxygen atom in the 3200−3300 cm^{-1} region, when an oxygen is the hydrogen-bonding acceptor.[39] According to the relationship, the variation corresponds to 0.0035 Å/cm^{-1}. This may raise the possibility of obtaining the distance information from the amide-A band. If this relation is applicable for BR and the 3313 cm^{-1} band of BR shifts to 3296 cm^{-1} in the K-intermediate (Figure 3b), the result shows that hydrogen bonding of the peptide backbone is shortened by 0.06 Å.

The greatest amide-A and amide-I changes appear at 3313 cm^{-1} (−)/3296 cm^{-1} (+) and 1668 cm^{-1} (−)/1664 cm^{-1} (+), respectively. These frequencies are deviated from the typical frequencies of the α-helix. Although there are spectral changes at the normal α-helix frequencies (3280 cm^{-1} for amide-A and 1657 cm^{-1} for amide-I), spectral changes in the slightly higher frequency region are more prominent. Regarding the BR structure, there has been a long discussion on whether it comprises a normal α-helix or not. The distorted α-helix structure of BR, called α_{II}, which is distinguished from normal α_I helix, was originally proposed on the basis of the infrared observation[73] and supported by various spectroscopic techniques such as infrared, Raman, circular dichroism, and NMR.[74−76] Although recent diffraction studies showed normal α-helix structure,[5−8] the present results also support the distorted α-helix (or α_{II} helix) comprised around the retinal chromophore.

In summary, the present polarized FTIR spectroscopy showed highly accurate K minus BR spectra in the whole mid-infrared region. The results on frequencies, angles, and distances will lead to better understanding of structural changes caused by light. Photoisomerization in the specific protein environment will be proven by use of isotope-labeling and mutation studies in the future.

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