Structural Change of Threonine 89 upon Photoisomerization in Bacteriorhodopsin As Revealed by Polarized FTIR Spectroscopy†

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ABSTRACT: The all-trans to 13-cis photoisomerization of the retinal chromophore of bacteriorhodopsin occurs selectively, efficiently, and on an ultrafast time scale. The reaction is facilitated by the surrounding protein matrix which undergoes further structural changes during the proton-transporting reaction cycle. Low-temperature polarized Fourier transform infrared difference spectra between bacteriorhodopsin and the K intermediate provide the possibility to investigate such structural changes, by probing O−H and N−H stretching vibrations [Kandori, Kinoshita, Shichida, and Maeda (1998) *J. Phys. Chem. B 102*, 7899−7905]. The measurements of [3-^{18}O]threonine-labeled bacteriorhodopsin revealed that one of the D_2O-sensitive bands (2506 cm^{-1} in bacteriorhodopsin and 2466 cm^{-1} in the K intermediate, in D_2O) exhibited ^{18}O-induced isotope shift. The O−H stretching vibrations of the threonine side chain correspond to 3378 cm^{-1} in bacteriorhodopsin and to 3317 cm^{-1} in the K intermediate, indicating that hydrogen bonding becomes stronger after the photoisomerization. The O−H stretch frequency of neat secondary alcohol is 3340−3355 cm^{-1}. The O−H stretch bands are preserved in the T46V, T90V, T142N, T178N, and T205V mutant proteins, but diminished in T89A and T89C, and slightly shifted in T89S. Thus, the observed O−H stretching vibration originates from Thr89. This is consistent with the atomic structure of this region, and the change of the S−H stretching vibration of the T89C mutant in the K intermediate [Kandori, Kinoshita, Shichida, Maeda, Needleman, and Lanyi (1998) *J. Am. Chem. Soc. 120*, 5828−5829]. We conclude that all-trans to 13-cis isomerization causes shortening of the hydrogen bond between the OH group of Thr89 and a carboxyl oxygen atom of Asp85.

Bacteriorhodopsin (BR) is a light-driven proton pump in *Halobacterium salinarum* that contains *all-trans*-retinal as chromophore (*1*−*4*). The retinal chromophore binds covalently to Lys216 through a protonated Schiff base linkage. Absorption of light triggers a cyclic reaction that comprises a series of intermediates, designated as the J, K, KL, L, M, N, and O states (*1*−*4*). Protein structural changes in these intermediate states cause proton translocation across the protein. The mechanism of these structural changes is the central question in current studies of BR (*5*−*7*).

The all-trans to 13-cis isomerization after absorption of a photon leads to the formation of the K intermediate (*8*−*11*) through the vibrationally hot J intermediate (*12, 13*). Although the proton transfers occur much later, on the microsecond and millisecond time scales, the complete information of the protein structural change for proton transport must be stored in the structure of the primary intermediates as their high-energy states. Thus, 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, ref *14*), while in BR it is 100% 13-cis. The quantum efficiency for isomerization in BR (∼0.6) (*15, 16*) is much higher than that in solution [0.13 (*14*) or 0.17 (*17*) in methanol]. This efficiency is closely correlated with the rate constant of isomerization, since the isomerization occurs in the femtosecond regime (*8*−*11, 18*). The protein environment thus certainly facilitates the specific reaction of the retinal chromophore.

Recent determinations of the structure of BR at the atomic level by electron cryomicroscopy (*19, 20*) and X-ray crystallography (*21*−*23*) allow renewed studies of the mechanism

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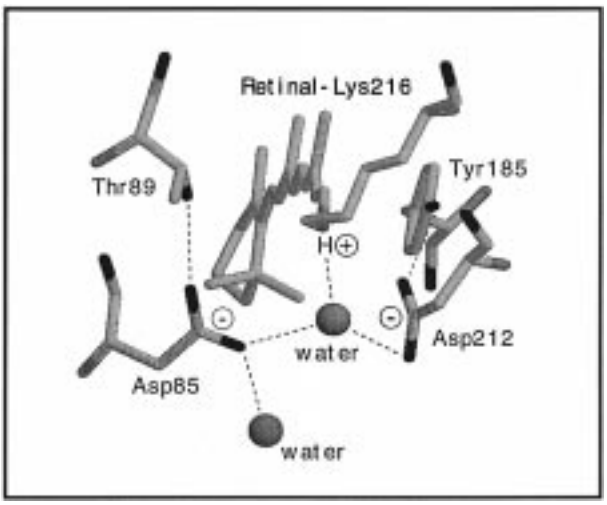

FIGURE 1: Structure of the Schiff base region in bacteriorhodopsin. This is the side view of 1BRX (*22*). The membrane normal is approximately in the vertical direction of this figure. Dotted lines represent supposed hydrogen bonds.

of isomerization. Although various differences between the structural models have been pointed out, particularly at the surface and in the location of bound water molecules, the amino acid residues that surround the retinal chromophore are more or less in agreement. The chromophore is sandwiched by two tryptophan residues: Trp86 from the extracellular side and Trp182 from the cytoplasmic side. It is likely that the presence of such bulky groups is related to the selective formation of the 13-cis form. Significant changes of protein structure and its characteristics by mutations of Trp86 (*24*, *25*) and Trp182 (*26*−*28*) have been reported. The structure of BR shows that two negatively charged carboxylates, Asp85 and Asp212, are present at a similar distance from the retinal Schiff base (Figure 1). It is thus an interesting and important question why the Schiff base proton is transferred only to Asp85. A water molecule in the active center (*22*) must play a substantial role (*29*, *30*). Figure 1 also illustrates the presence of two hydrogen bonds near the Schiff base; one is between an OH group of Thr89 and an oxygen of Asp85, while the other is between an OH group of Tyr185 and an oxygen of Asp212. Although the two carboxylates are symmetrically arranged at the Schiff base, other residues that make direct contact with them are not symmetrically located. Such a structure with broken symmetry might be the cause of the unidirectional isomerization, which could further yield unidirectional proton transfer. Thus, protein structural changes before and after isomerization are of interest, particularly changes in the hydrogen bonds in this region.

The protein structural changes that accompany the photoisomerization of retinal can be studied in early intermediates, like the K state. Time-resolved (*31*−*35*) or low-temperature (*36*−*40*) infrared spectroscopy has provided evidence for such structural changes of the protein as well as the chromophore change. Despite such studies of the K intermediate, however, the atomic picture of structural changes has not been well established. Recently we optimized the Fourier transform infrared (FTIR)[1] measuring system so that the K minus BR difference spectra are measurable with high signal-to-noise ratio in the 4000−700 cm^{-1} region (*41*). Polarized FTIR spectroscopy further allowed determining the angles of dipole moments of vibrations to the membrane normal (*41*−*43*). Various spectral features are newly observed in the so-called high-frequency region (>1800 cm^{-1}). It is noted that measurements in this frequency region can probe structural changes in hydrogen bonds, because O−H and N−H vibrations are involved. Replacement of water with D_2O causes splitting of stretching bands into D_2O-sensitive and -insensitive bands. Several, most likely involved in the hydrogen bonding network near the Schiff base, are downshifted in D_2O. Changes of D_2O-insensitive bands could originate from those around β-ionone and the polyene chain, because these are hydrophobic. Once these spectra are in hand, the bands need to be assigned.

The first implication of actual protein movements was from studies of the T89C mutant (*44*), using the cysteine SH as a hydrogen bonding probe. The S−H stretching vibration was observed at 2523 cm^{-1} in BR, and it shifted to 2477 cm^{-1} in the K state. A model study showed that the S−H stretching frequency should be in the 2580−2525 cm^{-1} region dependent upon its hydrogen bonding conditions (*45*). These facts indicate that the SH group at position 89 forms a strong hydrogen bond, probably with an oxygen of Asp85, and this hydrogen bond is further strengthened upon photoisomerization. Thus, the distance between the sulfur of Cys89 of T89C and the oxygen of Asp85 is likely to become shorter upon photoisomerization. This idea had to be examined in the wild-type protein.

According to the recent report by Liu et al. (*46*), the O−H stretching vibration at 3496 cm^{-1} was assigned to the threonine side chain at position 89. They measured the M minus BR spectra of [3-^{18}O]threonine-labeled BR and observed the isotope shift of the 3496 cm^{-1} band of BR (*46*). Because the band disappeared in the T89N mutant, they concluded that the 3496 cm^{-1} band is due to Thr89. The O−H stretching frequencies of secondary alcohols as the model of threonine have been reported as follows: free O−H stretches at 3626−3629 cm^{-1} [CH_3−CH(OH)−CH_3, 3626.1 cm^{-1}; CH_3−CH(OH)−C_2H_5, 3627.0 cm^{-1}, measured in CCl_4 solution] and hydrogen-bonded O−H stretches at 3340−3355 cm^{-1} [CH_3−CH(OH)−CH_3, 3342 cm^{-1}; CH_3−CH(OH)−C_2H_5, 3343 cm^{-1}, measured in neat liquid] (*47*). The O−H stretching vibration of threonine thereby ranged from ∼3630 to 3350 cm^{-1}, dependent on its hydrogen bonding conditions. The O−H stretch frequency at ∼3500 cm^{-1} reported by Liu et al. (*46*) would indicate rather weak hydrogen bonding, in contradiction to the results with T89C.

In the present study, we prepared [3-^{18}O]threonine-labeled BR, and used polarized FTIR spectroscopy to investigate the possible involvement of hydrogen bonding alterations in the photoisomerization of the retinal chromophore. The present results clearly show that one of the D_2O-sensitive bands is from the O−H stretching vibration of a threonine side chain. The mutant studies reveal that the threonine in question is Thr89. The frequency of the O−H (O−D) stretching vibration at 3378 (2506) cm^{-1} is very different from the previously reported value (*46*), and indicates strong hydrogen bonding of the O−H group of Thr89. Strong hydrogen bonding of Thr89 with Asp85 oxygen, the counterion of the Schiff base, must play an important role in weakening the

[1] Abbreviations: FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

interaction between the Schiff base and the counterion. Further strengthening of the hydrogen bonding upon photoisomerization implies that the distance between Thr89 and Asp85 becomes shorter. The structural changes of Thr89 and their possible role in the primary processes are discussed in relation to the atomic structure of BR.

MATERIALS AND METHODS

Preparation of Samples. L-[3-^{18}O]Threonine was synthesized as described previously (*48*). [3-^{18}O]Threonine-labeled bacteriorhodopsin was prepared by growing *Halobacterium salinarum* (JW-3) in a defined medium similar to that of Gochnauer and Kushner (*49*), except that the D-amino acids and NH_4Cl were omitted and the L-threonine was replaced by 0.25 g/L L-[3-^{18}O]threonine. Under these conditions, lipid extraction and amino acid analysis with radiotracers show that typically about two-thirds of the threonine residues are labeled, with no scrambling of the label. The mutated genes for T46V, T89S, T89A, T89C, T90V, T142N, T178N, and T205V were constructed and introduced into *Halobacterium salinarum* as described previously (*50*). The purple membrane was isolated by the method of Oesterhelt and Stoeckenius (*51*).

Polarized FTIR Spectroscopy. Polarized FTIR spectroscopy was applied as described previously (*41*). A 120 μL aliquot of the sample in 2 mM phosphate buffer (pH 7.0) was dried on a BaF_2 window with a diameter of 18 mm. After hydration by 1 μL of 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 bacteriorhodopsin (BR).

Illumination with 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 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 (*41*, *42*). 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-40 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

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

Increase in the intensity of the A_V component from the increased effective number of BR molecules absorbing light with tilting was corrected in eq 1. *R* is related to the angle of the dipole moment to the membrane normal θ_0 by the equation described previously:

$$R = 1 + \sin^2\phi_0/n^2 \times [\rho<9\cos^2\theta_0 - 3>]/[2 - \rho<3\cos^2\theta_0 - 1>] \quad (2)$$

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, was 0.95 as reported before (*41*, *42*). The latter assumption is reasonable, as judged from the intensity ratio of 1.02 for amide II/amide I in our unhydrated film in comparison with the earlier value of 0.98.

RESULTS

Identification of the D_2O-Sensitive O−H Stretching Vibrations (O−D Stretches). OD or ND stretching vibrations appear in the 2700−2000 cm^{-1} region, downshifted from higher frequencies. The dotted lines in Figure 2 show K minus BR difference spectra in D_2O with the window tilting angles at 0° (a) and at 53.5° (b). The present results reproduced the previous ones (*41*). Solid lines in Figure 2 show the spectra of [3-^{18}O]threonine-labeled BR. The solid and dotted lines coincide well, except for the 2500−2450 cm^{-1} region. The intensity of the sharp peak-pair of 2506 cm^{-1} (−) and 2466 cm^{-1} (+) in Figure 2b is remarkably reduced, probably because of downshift to 2490 cm^{-1} (−) and 2445 cm^{-1} (+), respectively. These bands are thus assigned to the O−D stretching vibration of a threonine. The corresponding O−H stretching vibrations are located at 3378 and 3317 cm^{-1}, respectively (*41*). Since no other bands are shifted by isotope labeling of all threonines, the negative 2506 cm^{-1} band and the positive 2466 cm^{-1} bands correspond with each other. Namely, the threonine O−D group whose stretching frequency is at 2506 cm^{-1} in BR changes its O−D stretching at 2466 cm^{-1} upon formation of the K intermediate. This spectral downshift upon photoisomerization presumably represents the formation of a stronger hydrogen bond. The change is more prominent at 53.5° than at 0°, indicating that the dipole moment of the O−D stretch is closer to the membrane normal.

More detailed spectral changes are shown in Figure 3. Figure 3a shows the K minus BR spectra of unlabeled BR at various tilting angles. Both negative and positive peaks are observed at 2506 and 2466 cm^{-1}, respectively. Although there is a single isosbestic point at 2482 cm^{-1}, other spectral components are also present as reflected by the positive 2495 cm^{-1} band at 0° (dotted line in Figure 3a). The corresponding frequency in H_2O is located at 3353 cm^{-1} (*41*). Figure 3b shows the K minus BR spectra of [3-^{18}O]threonine-labeled BR at various tilting angles. As clearly seen in the spectrum at 53.5° (thick solid line of Figure 3b), negative and positive bands appear at 2490 and 2445 cm^{-1}, respectively, which must be the downshifted O−D stretching vibrations of the threonine side chain.

The spectral features are very complex in this frequency region. For instance, the negative signal at 2506 cm^{-1} in Figure 3a is not present at 0° (dotted line) and emerges upon tilting, suggesting that the threonine O−H group is mostly parallel to the membrane normal. However, the difference spectrum of [3-^{18}O]threonine-labeled BR at 0° has a positive signal at this frequency (dotted line in Figure 3b). This fact indicates that there is a positive signal other than threonine O−H at 0° in the 2506 cm^{-1} region. Similarly, the positive 2466 cm^{-1} band (Figure 3a) loses intensity upon labeling of

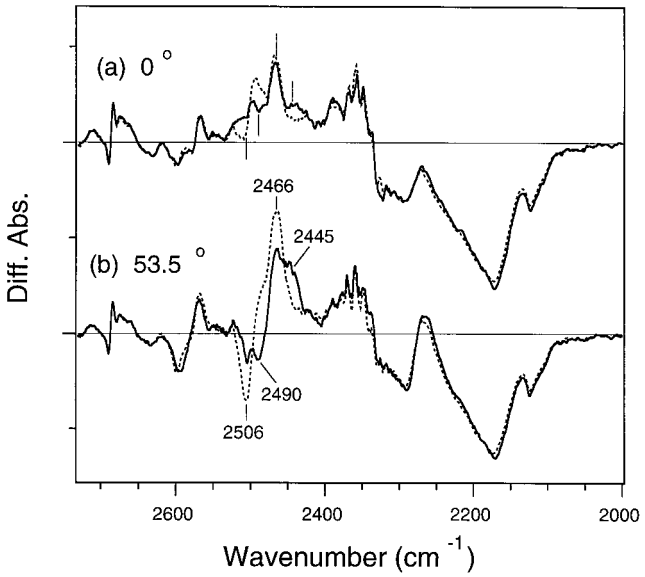

FIGURE 2: K minus BR difference spectra of unlabeled (dotted lines) and [3-^{18}O]threonine-labeled (solid lines) bacteriorhodopsin in the 2730−2000 cm^{-1} region. The sample was hydrated with D_2O. The window tilting angles (ϕ_0) are 0° (a) and 53.5° (b). One division of *y*-axis corresponds to 0.001 absorbance unit. The fine structure in the positive band in the 2380−2330 cm^{-1} region is due to CO_2 gas present in the spectrometer.

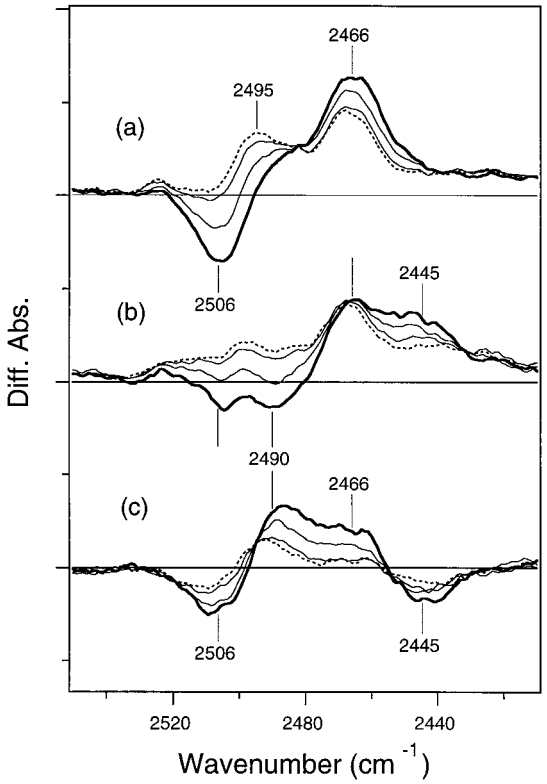

FIGURE 3: K minus BR difference spectra of unlabeled (a) and [3-^{18}O]threonine-labeled (b) bacteriorhodopsin in the 2550−2410 cm^{-1} region. The sample was hydrated with D_2O. The window tilting angles (ϕ_0) are 0° (dotted lines), 17.8° (thin solid lines close to dotted lines), 35.7° (thin solid lines apart from dotted lines), and 53.5° (thick solid lines). One division of *y*-axis corresponds to 0.001 absorbance unit. The subtractions of (b) from (a) at respective tilting angles are shown in (c).

the threonines, whereas an angle-independent positive signal remains at 2466 cm^{-1} (Figure 3b). These facts suggest the presence of positive bands at ∼2506 and at ∼2466 cm^{-1}, the latter of which is the band in the magic angle. In general, they make it difficult to determine the angle of the O−H group to the membrane normal.

The spectral complexity in the 2520−2420 cm^{-1} region can be simplified by subtracting the spectra between labeled and unlabeled BR, because signals other than threonine O−H stretches are then canceled. Figure 3c shows the results of the subtraction. The 2506 and 2466 cm^{-1} bands represent threonine O−D stretching vibrations of BR and the K intermediate, respectively. They are downshifted to 2490 and 2445 cm^{-1}, respectively, upon isotope labeling. Thus, among 18 threonine residues, 1 threonine undergoes hydrogen bonding change upon photoisomerization, whose O−H group is D_2O-exchangeable.

K minus BR Spectra of Mutants. The 2506 cm^{-1} band of BR originates from a threonine O−D stretching frequency, and it shifts to 2466 cm^{-1} upon formation of the K intermediate. To identify the threonine residue, we measured the K minus BR spectra of various threonine mutants. BR has 18 threonines, with 13 in the trans-membrane region. These are Thr17 and Thr24 in helix A, Thr46, Thr47, and Thr55 in helix B, Thr89 and Thr90 in helix C, Thr107 and Thr121 in helix D, Thr142 in helix E, Thr170 and Thr178 in helix F, and Thr205 in helix G. Thus, threonine resides are distributed over all of the helices, and located in both extracellular and cytoplasmic regions. For the present study, we prepared mutants for six threonines: Thr46, Thr89, Thr90, Thr142, Thr178, and Thr205. We expected that replacement of Thr would yield the loss of the O−H stretching vibration in question, although the mutations could possibly alter also a hydrogen bonding network, which could lead to misidentification.

Figure 4 shows the K minus BR spectra of the wild type (a) and various mutants (b−i) in the 1820−750 cm^{-1} region. The dotted and solid lines represent the spectra whose window tilting angles are 0° and 53.5°, respectively. In all cases, the K intermediate is formed as the product (positive bands), as shown by the spectral downshift of the ethylenic C═C stretching vibrations at around 1530−1510 cm^{-1} (a−i). The spectra of T46V (b) and T205V (i) were identical to that of the wild type (a) in the 1820−750 cm^{-1} region, indicating that replacement of these threonine residues does not affect the structural change. The spectrum of T142N (g) is also similar to that of the wild type (a) except that the C═C stretching vibrations of BR and the K intermediate are at a 1 cm^{-1} lower frequency. In contrast, T178N (h) exhibits a new negative band at 1695 cm^{-1}. In addition, C═O stretching vibrations of protonated Asp115 are at 1728 (−)/1722 (+) cm^{-1} while for the wild-type BR they appeared at 1725 (−)/1720 (+) cm^{-1}. The C═C stretching vibration of BR appeared at 1528 cm^{-1} in T178N (h), being 1 cm^{-1} downshifted from the wild type, while that of the K intermediate was identical. The spectral features of fingerprint and hydrogen out-of-plane vibrations were similar between T178N (h) and the wild type (a).

In contrast to the mutants at positions 46, 142, 178, and 205, mutants at position 89 and 90 exhibited differences from the wild type in the chromophore bands. In the wild type, the hydrogen out-of-plane (HOOP) vibration at position C15 appears at 951 cm^{-1} for the K intermediate in D_2O (*52*). This band appeared at 950 and 938 cm^{-1} in T89S (c) and T89A (d), respectively. The peak frequency was not changed

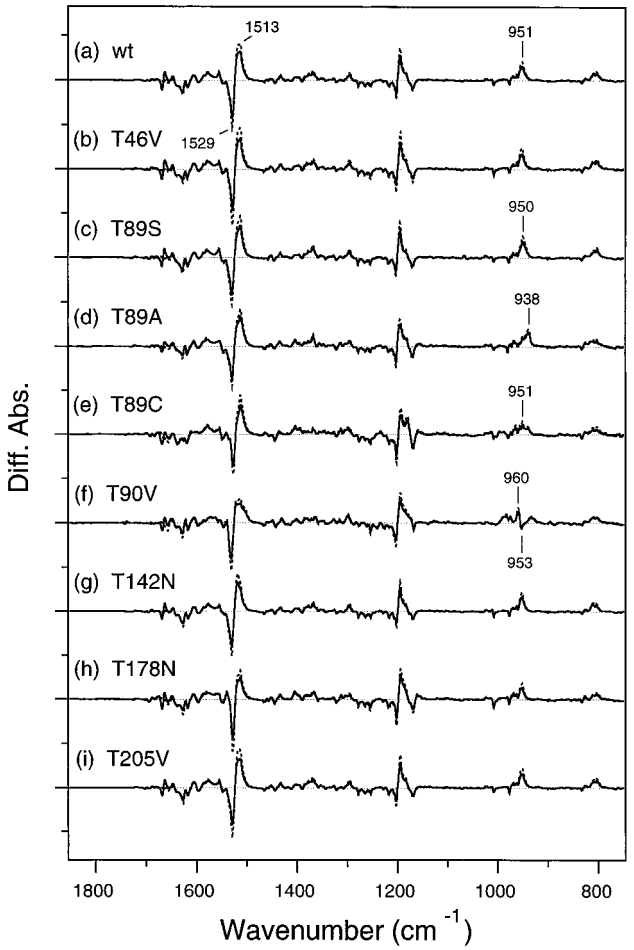

FIGURE 4: K minus BR difference spectra in the 1820−750 cm^{-1} region of the wild type (a), T46V (b), T89S (c), T89A (d), T89C (e), T90V (f), T142N (g), T178N (h), and T205V (i). The sample was hydrated with D_2O. The window tilting angles (ϕ_0) are 0° (dotted lines) and 53.5° (solid lines). One division of *y*-axis corresponds to 0.025 absorbance unit.

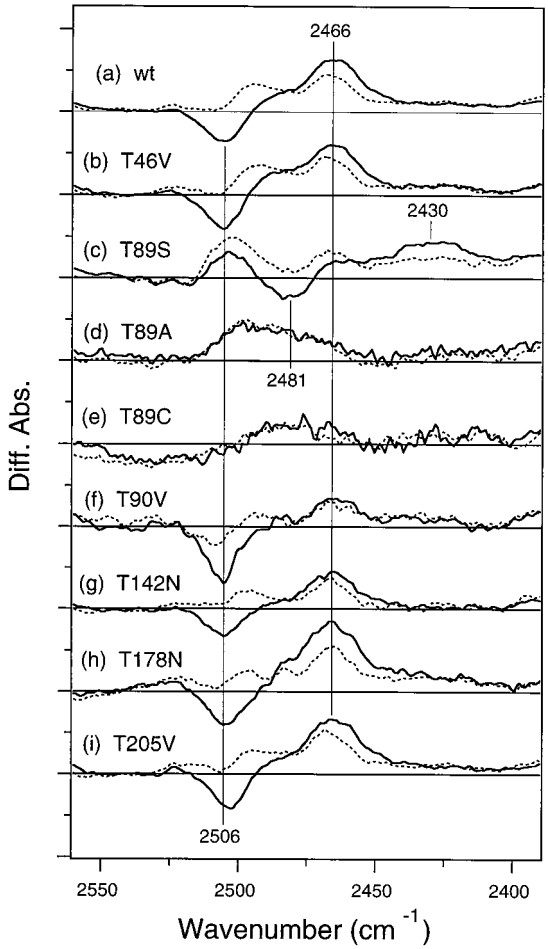

FIGURE 5: K minus BR difference spectra in the 2560−2390 cm^{-1} region of wild type (a), T46V (b), T89S (c), T89A (d), T89C (e), T90V (f), T142N (g), T178N (h), and T205V (i). The sample was hydrated with D_2O. The window tilting angles (ϕ_0) are 0° (dotted lines) and 53.5° (solid lines). One division of *y*-axis corresponds to 0.001 absorbance unit.

in T89C (e), whereas overall spectral features of HOOP bands were different from the wild type. In T90V (f), a negative band was observed at 953 cm^{-1} as well as a positive 960 cm^{-1} band. Thus, structural alteration at the T89-T90 position affects the local structure of the chromophore of the K intermediate at the Schiff base region. Among three mutants at position 89, spectral features of T89S were very similar to those of the wild type (a), implying the importance of the hydrogen bond with the side chain of Asp85.

Figure 5 shows the K minus BR difference spectra in the 2560−2390 cm^{-1} region of the wild type (a) and various mutants (b−i) in D_2O. Both negative 2506 cm^{-1} and positive 2466 cm^{-1} bands are clearly present in T46V (b), T90V (f), T142N (g), T178N (h), and T205V (i) like in the wild type (a), indicating that the threonine O−D stretching vibrations do not originate from these residues. In contrast, the spectral features are remarkably changed in the T89 mutants. Neither the negative 2506 cm^{-1} band nor the positive 2466 cm^{-1} band was observed for T89A (d) and T89C (e), whereas a broad positive feature was visible in the 2500−2450 cm^{-1} region. This strongly suggests that the O−H stretching vibration of threonine originates from Thr89.

In the case of T89S (c), a negative band was observed at 2481 cm^{-1}, and positive bands appeared at about 2500 cm^{-1} and at about 2430 cm^{-1}. The positive spectral feature at about 2500 cm^{-1} was observed for the wild type (a) and T89A (d), while for T89S only a broad positive feature at about 2430 cm^{-1} was observable. The vibration at 2430 cm^{-1} possesses a dipole moment parallel to the membrane normal, which is consistent with the threonine O−H stretch at 2466 cm^{-1} of the wild type (a). In addition, it has been reported that the hydrogen-bonded O−H stretching frequencies of primary and secondary alcohols as the model of serine and threonine are 3332 cm^{-1} (CH_3-CH_2-OH) and 3342 cm^{-1} ($CH_3-CH(OH)-CH_3$) (*47*), suggesting that the O−H stretch of serine would be intrinsically lower than that of threonine.

On the basis of these observations, we concluded that the 2506 (−)/2466 (+) cm^{-1} bands (Figures 1 and 2) originate from Thr89. It is likely that the O−D stretches of serine in T89S are at 2481 and 2430 cm^{-1} for BR and the K intermediate, respectively. As described in Figure 2, the presence of broad positive features due to other vibrations at 2520−2420 cm^{-1} makes it impossible to determine the angle of the dipole moment of the threonine O−D stretch to the membrane normal. If we assume that positive bands of T89A (Figure 5d) are preserved in the wild type, the angle of the dipole moment can be calculated. Based on such an assumption, the dipole moment of the O−D stretching

vibration of Thr89 was found to be 21° and 29° for BR and the K intermediate, respectively.

DISCUSSION

According to the structure of BR, a hydrogen bonding complex is present near the Schiff base (*19*−*23*, *53*). It is reasonable to consider that the O−H or N−H groups of the residues in this complex participate in the spectral changes after photoisomerization. For instance, the O−H groups of Thr89 and Tyr185 form hydrogen bonds with aspartate oxygens of Asp85 and Asp212, respectively (Figure 1). In the present study, structural changes of threonine OH groups have been investigated by use of [3-^{18}O]threonine-labeled BR. Among 18 threonines contained in BR, only 1 (i) exchanges with D_2O AND (ii) changes its hydrogen bonding upon photoisomerization. The mutant study clearly indicated that the residue is Thr89. We note that structural changes in the OH groups of other threonine residues with D_2O-insensitive bands were observed also (not shown). Identification of these bands is in progress.

The O−D stretch of Thr89 is at 2506 and 2466 cm^{-1} for BR and the K intermediate, respectively. The corresponding O−H stretch lies at 3378 cm^{-1} for BR and at 3317 cm^{-1} for the K intermediate (*41*). The angle between the O−H (O−D) group and the membrane normal was estimated to be 21° (BR) and 29° (K). The free O−H stretching frequencies of secondary alcohols as the model of threonine are at 3626−3629 cm^{-1}, as measured in CCl_4 solution, while hydrogen bonded O−H stretches measured in neat liquid are at 3340−3355 cm^{-1} (*47*). The O−H stretching vibration of threonine is thereby located from ∼3630 to 3340 cm^{-1}, dependent on its hydrogen bonding conditions. The observed frequency of BR (3378 cm^{-1}) is close to those in neat liquid, showing that the hydrogen bonding of the O−H group is fully formed. According to the crystallographic structure, this hydrogen bonding is with one of the oxygens of Asp85 (Figure 1). Interestingly, the frequency of the K intermediate (3317 cm^{-1}) is even lower than that in neat liquid. The unusually strong hydrogen bond of the O−H group of Thr89 could reflect the specific structure of the K intermediate as a higher energy state. Relaxation of the K intermediate to the L intermediate could be accompanied by weakening of the hydrogen bond of the O−H group of Thr89.

In a previous article (*44*), we introduced a cysteine S−H as a hydrogen bonding probe. In that study, we measured the frequency change of the S−H stretch at position 89 in the T89C mutant directly after photoisomerization (in the K state) and after primary proton transfer (in the M state). A model study showed that the S−H stretching frequency appears in the 2580−2525 cm^{-1} region dependent upon its hydrogen bonding conditions (*45*). The S−H stretching vibration was observed at 2523 cm^{-1} in BR, at 2477 cm^{-1} in the K intermediate, and at 2576 cm^{-1} in the M intermediate (*44*). The results showed that hydrogen bonding was very strong in BR, unusually strong in K, and lost in M. The unusually strong hydrogen bonding of residue 89 indicated by the behavior of the S−H stretch frequency in K is now confirmed in the wild-type protein.

Our value for the O−H stretch frequency of Thr89 at 3378 cm^{-1} contradicts the value (3496 cm^{-1}) reported by Liu et al. (*46*). In fact, the frequency difference is >100 cm^{-1}. They measured the M minus BR spectra of [3-^{18}O]threonine-labeled BR and observed the isotope shift of the 3496 cm^{-1} band of BR. They assigned the 3496 cm^{-1} band to Thr89, because it disappeared in the T89N mutant. However, many regions of the protein can be altered in the M intermediate (*54*), which was stabilized at 250 K. Such global structural changes are restricted in the K intermediate (77 K) (*55*) in this work. In fact, we observed the D_2O-insensitive 3495 cm^{-1} (−) band as well as the D_2O-sensitive 2502 cm^{-1} (−) band in the M minus BR spectrum, both of which originate from threonine O−H (O−D) stretches (manuscript in preparation). The negative 3495 cm^{-1} band is absent in the K minus BR spectrum, while the negative 2502 cm^{-1} band seems to be identical to the band of Thr89.

In the previous article (*41*) we argued that the 2506 cm^{-1} (−) band could be the N−D stretch of the retinal Schiff base because: (i) the N−H group of the Schiff base is D_2O-sensitive and expected to be oriented parallel to the membrane normal according to the BR structure, and these features agree with those of the 2506 cm^{-1} band; (ii) the corresponding O−H stretch at 3377 cm^{-1} coincides with the value by resonance Raman spectroscopy (3379 cm^{-1}), where it was assigned as the Schiff base N−H stretch. Nevertheless, the present results clearly show that the 2506 cm^{-1} band originates from the O−D group of Thr89, not the Schiff base N−D. The angle of the N−H group to the membrane normal is of interest, particularly in the K intermediate, because the position of the Schiff base hydrogen is important in view of subsequent proton transfer to Asp85.

Since the atomic positions are constrained in a protein, all possible hydrogen bonds are not necessarily formed. They form only when the distance and angle between hydrogen bonding donor and acceptor are matched. Regarding the local structure of the O−H group of Thr89, the present FTIR study shows that the hydrogen bonding is strong and the O−H group is parallel to the membrane normal. The distance between the oxygen atom of Thr89 and the closer carboxyl oxygen atom of Asp85 is 3.32 Å (*19*) and 3.94 Å (*20*) by electron cryomicroscopy, and 4.35 Å (*21*), 2.74 Å (*22*), and 2.96 Å (*23*) by X-ray crystallography.[2] The distances in the last two structural models are compatible with hydrogen bonding. The angle of the internuclear vector between the two oxygens to the membrane normal is 23.2° (*19*) and 29.2° (*20*) by electron cryomicroscopy, and 18.0° (*21*), 34.5° (*22*), and 53.3° (*23*) by X-ray crystallography.[2] In general, the present results are consistent with those structural studies. It is, however, noted that the position of the hydrogen atom of Thr89 is not determined in these studies, and these parameters are not strictly comparable to the present ones.

In the present study, threonine mutants are used for assignment of its O−H stretching vibration. There were no spectral differences in T46V and T205V from the wild type, the former of which reproduced the previous report (*56*). This indicates that the structural changes in the K intermediate do not involve these residues, although various changes have been reported for the late intermediates of the mutants of Thr46 (*48*, *56*−*58*). According to the structure by Luecke et al. (*22*), three threonines form the retinal binding pocket

[2] More recent structural models provide the following values: T. Kouyama's group, distance 3.57 Å and angle 31.0° (T. Kouyama, private communication); Luecke et al., distance 2.67 Å and angle 31.2°.

through possible direct van der Waals contact: (i) the oxygen atom of Thr89 is located at 4.00 Å from the Schiff base nitrogen and 3.68 Å from Cϵ of Lys216 (Figure 1); (ii) the Cγ atom of Thr90 is located at 3.69 and 3.50 Å from C11 and C12 of retinal, respectively; and (iii) the oxygen atom of Thr142 is located at 3.90 Å from the carbon atom of fifth methyl group. In contrast to the significant spectral changes of the mutants of Thr89 and Thr90, the spectrum of T142N was similar to that of the wild type (Figure 4). These results suggest that structural change around the β-ionone ring is much smaller than that in the Schiff base region. The remarkable mutant effect of the two threonines at positions 89 and 90 indicates the important role to constitute the active center of BR.

The direction of the bond rotation of the C13−C14 double bond in the isomerization is an interesting issue, because selectivity and efficiency in protein (*14*−*17*) could be given by such reaction. The present study provides structural information of the stable states, before and after the isomerization reaction, but does not reveal the course of the reaction itself. Direct observation of the protein change during isomerization is difficult, because reaction occurs in the femtosecond regime and protein change has to be probed by protein modes. However, we are able to modify the protein environment by mutation, and systematic study of the mutant proteins with various spectroscopic methods will provide experimental evidence on the directionality of bond rotation. In fact, T89C was less efficient in forming the K intermediate than the wild type, while no clear difference in T89S (not shown) was found. These results suggest the possible role of the bulk of the side chain (OH vs SH) at position 89.

In conclusion, we identified the O−H stretching vibration of Thr89 in BR by polarized FTIR spectroscopy of isotope-labeled and mutant proteins. Hydrogen bonding is almost fully formed with Asp85, and photoisomerization yields an even stronger interaction.[3] Relaxation of the K state will be coupled to local structural change of the C-helix, which produces the subsequent L state.

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[3] The hydrogen bonding partner of Thr89 in K is most likely Asp85. However, the other possibility is not excluded at this moment. A water molecule could be the partner.

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