Isomer-Specific Interaction of the Retinal Chromophore with Threonine-118 in Rhodopsin†

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The retinal binding pocket in rhodopsin accommodates three isomeric states at 77 K: 11-cis (rhodopsin), all-trans (bathorhodopsin), and 9-cis (isorhodopsin) forms. A previous Fourier transform infrared study of bovine rhodopsin observed an isomer-specific protein band, which appears at 3463, 3487, and 3481 cm^{-1} for rhodopsin, bathorhodopsin, and isorhodopsin, respectively [Kandori, H.; Maeda, A. *Biochemistry* **1995**, *34*, 14220−14229]. The present infrared study of the rhodopsin mutants revealed that the band originates from the O−H stretching vibration of threonine at position 118. The frequency is highly sensitive to various mutations, suggesting that the O−H group of Thr118 is located at a crucial position in the interaction with the retinal chromophore. The recent crystallographic structure of rhodopsin indeed showed that the O−H group directly interacts with the 9-methyl group [Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. *Science* **2000**, 289, 739−745]. On the basis of the present results and the structure of rhodopsin, isomer-specific interaction and the isomerization process in rhodopsin are discussed.

Introduction

Rhodopsin is one of the G protein-coupled receptors (GPCRs) that has diverged into a photoreceptive protein in retinal visual cells.[1−3] It is a membrane protein consisting of a single polypeptide, opsin, and a light-absorbing chromophore, 11-*cis*-retinal. The opsin contains seven transmembrane α-helices, the structural motif typical of the GPCRs.[4] The 11-*cis*-retinal is bound to Lys296 in the transmembrane helix VII through a protonated Schiff-base linkage. The primary event of rhodopsin is a highly efficient cis−trans photoisomerization of the retinal chromophore,[5,6] which occurs in a femtosecond regime[7−10] with coherent production of the primary intermediate.[11,12] Because the efficiency is facilitated by the protein environment,[13,14] the amino acid residues surrounding the chromophore have been of great interest.

One of the characteristics of the protein environment in rhodopsin is the ability to accommodate three isomeric states of the chromophore, 11-cis, all-trans, and 9-cis forms, even at very low-temperature such as 4 K, and these states are interconverted by light.[15] This fact suggests that the protein environment facilitates retinal isomerization without large motion of protein itself. How is such motion achieved in rhodopsin? To elucidate the mechanism, information about the structural change of the protein environment is required. An isomer-specific vibrational band of protein was previously observed by extending the frequency region toward those of X−H stretching vibrations (4000−1800 cm^{-1}) in the low-temperature Fourier transform infrared (FTIR) spectroscopy of bovine rhodopsin.[16] The frequencies of the 11-cis (rhodopsin), all-trans (bathorhodopsin), and 9-cis (isorhodopsin) forms are at 3463, 3487, and 3481 cm^{-1}, respectively, and it was not exchangeable for D_2O.[16] Structural changes of the X−H group seem to be preserved in late intermediates such as lumirhodopsin, metarhodopsin-I, and metarhodopsin-II.[17,18]

In this article, we attempted to identify the isomer-specific stretching vibration by use of various mutants. As the candidates of the bands, N−H and O−H stretching vibrations are possible. The frequency (3490−3460 cm^{-1}) and character (D_2O-insensitive intense band) are very similar to the indole N−H stretch (3486 cm^{-1}) of Trp182 in the L intermediate of bacteriorhodopsin,[19] suggesting that the band of rhodopsin may originate from the N−H stretch of the tryptophan indole. Therefore, we first tested tryptophan mutants. Nevertheless, the results clearly showed that all tryptophan mutants possess the X−H stretch. Further studies were conducted to test the amide-A vibration (N−H stretch) and the O−H stretches of protonated carboxylic acids, tyrosine, and threonine. As the results, we assigned the 3463-cm^{-1} band of rhodopsin to the O−H stretching vibration of threonine at position 118 (Thr118). The O−H stretching vibration of threonine ranged between ∼3630 and 3350 cm^{-1}, dependent on its hydrogen-bonding conditions from the literature.[20,21] The frequency at 3463 cm^{-1} of rhodopsin corresponds to a moderate hydrogen bond of the O−H group, and the hydrogen bond is weaker in bathorhodopsin and isorhodopsin.

After the present experiments had been completed, the X-ray crystallographic structure of bovine rhodopsin was reported.[22] The structure showed Thr118 being in contact with the retinal chromophore. The side-chain oxygen is 3.0 Å from the 9-methyl group and 3.5 Å from the C11 atom of retinal, certainly locating at a critical position. In addition, it is in hydrogen-bonding distance (2.8 Å) with the peptide carbonyl of Ala114. On the basis of the present observation and the atomic structure of rhodopsin,[22] we discuss the mechanism of molecular motion of the retinal chromophore in the restricted protein environment.

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Experimental Section

The wild-type and its mutant proteins were prepared as described previously.[23,24] The recombinant proteins were expressed in 293S cells and reconstituted to rhodopsin by mixing the membrane fraction in the disrupted cell lyaste with 11-*cis*-retinal. All procedures were carried out under dim red light at 4 °C. Rhodopsin was extracted in 1% dodecyl maltoside (DM) in buffer P (0.05 M HEPES, 0.14 M NaCl, pH 6.5) and then diluted 2-fold for incubation with rho 1D4 antibody-linked agarose. After washing with 0.02% DM in buffer P, rhodopsin was eluted in the same solution containing the C-terminal octadecapeptide of rhodopsin at room temperature. Absorption spectra of the elution samples were measured with a Shimadzu MPS-2000 spectrophotometer at room temperature.

For infrared spectroscopy, a solubilized rhodopsin sample was supplemented with a 100-fold molar excess of L-α-phosphatidylcholine from egg yolk (Sigma type XI-E) dissolved in 0.75% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate and dialyzed against buffer P for 3−4 days. The sample in PC vesicles has absorbance in the range from 0.02 (W265F/Y) to 0.1. Rhodopsins were collected by centrifugation, and the pellets were suspended in 40 μL of 1 mM phosphate buffer (pH 5.7). The whole suspensions were placed on a BaF_2 window and dried to a film under vacuum by an aspirator. About 1 μL of either H_2O or D_2O was put beside the film for humidification, and the samples were sealed by another BaF_2 window with the aid of a silicon rubber O-ring and set in a brass cell holder.

Low-temperature FTIR spectroscopy was applied to the hydrated films as described previously.[16,23,24] Briefly, the sample film was mounted in an Oxford cryostat (DN-1704) attached to the FTIR spectrometer (Bio-Rad FTS-40). The temperature of the sample was cooled to 77 K and controlled within 0.1 K by the cryostat equipped with an Oxford temperature controller (ITC 4) with liquid nitrogen as coolant. Illumination conditions to obtain the B/R and B/I spectra were described previously.[16,23,24] One hundred twenty-eight interferograms in a 2 cm^{-1} resolution were recorded before and after illumination, and 10−20 recordings were averaged. The B/I difference spectra were calculate from the B/R and B/I spectra to cancel the bathorhodopsin-characteristic 921-cm^{-1} band.

Results

A. Isomer-Specific Protein Bands of Rhodopsin. Figure 1 shows the bathorhodopsin minus rhodopsin (B/R; a), bathorhodopsin minus isorhodopsin (B/I; b), and isorhodopsin minus rhodopsin (I/R; c) difference infrared spectra of the wild-type rhodopsin expressed from 293S cells. The three isomeric states possess characteristic peaks at 3463 cm^{-1} (rhodopsin), 3487 cm^{-1} (bathorhodopsin) and 3480 cm^{-1} (isorhodopsin), all of which are insensitive to D_2O substitution. These bands are identical to those of the native rhodopsin in rod outer segments,[16] indicating that the mutational studies could be applicable for identification of these bands. As noted in the difference spectra of the native rhodopsin,[16] the B/R and B/I spectra display a remarkably similar feature, particularly in the high frequency region. This fact indicates the structural similarity of the protein moiety in rhodopsin and isorhodopsin. It is clearly shown in the difference spectrum between isorhodopsin and rhodopsin (Figure 2). Strong peaks in the low-frequency side originate from the chromophore bands such as the C═C stretch (1557 cm^{-1}), C−C stretch (1237, 1207, and 1193 cm^{-1}), and hydrogen out-of-plane (967 and 959 cm^{-1}) vibrations. Spectral changes

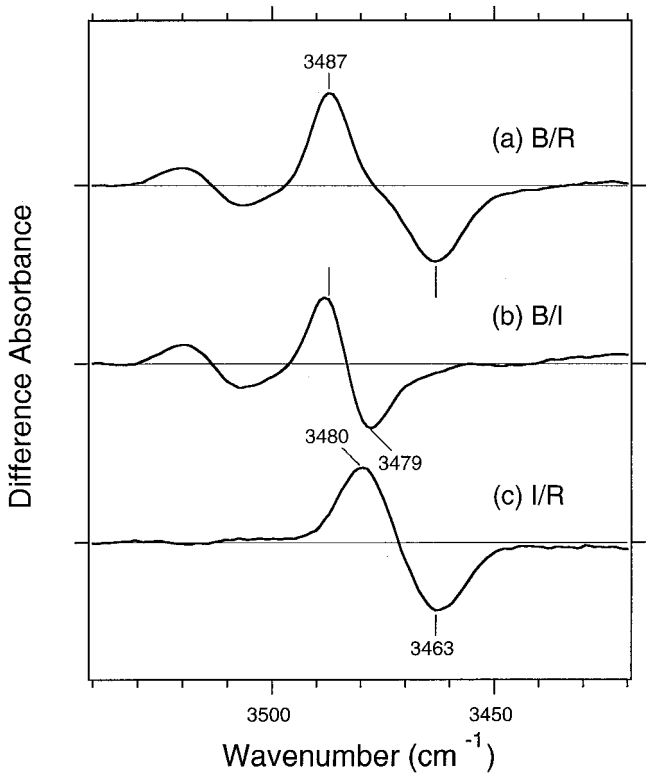

Figure 1. Bathorhodopsin minus rhodopsin (B/R; a), bathorhodopsin minus isorhodopsin (B/I; b), and isorhodopsin minus rhodopsin (I/R; c) spectra in the 3540−3420 cm^{-1} region. Difference spectra were measured in D_2O at 78 K for the wild-type protein of bovine rhodopsin. One division of the *Y* axis corresponds to 0.002 absorbance unit. D_2O-insensitive bands at 3463, 3487, and 3480 cm^{-1} are characteristic for rhodopsin (11-cis), bathorhodopsin (all-trans), and isorhodopsin (9-cis).

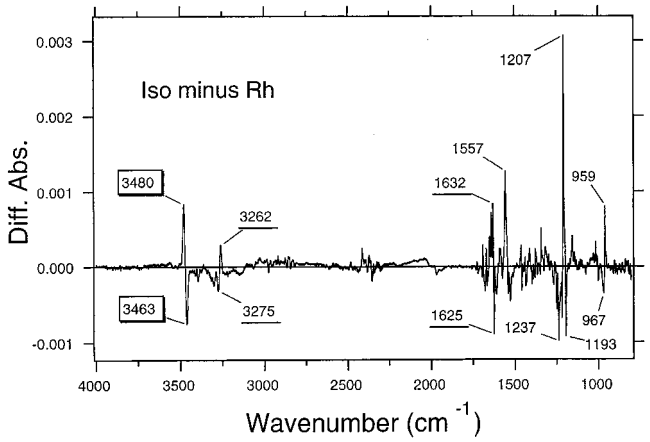

Figure 2. Difference absorption spectra between 9-cis (isorhodopsin) and 11-cis (rhodopsin) forms of the wild-type protein of bovine rhodopsin measured in D_2O at 78 K. The spectra are identical to those for the native bovine rhodopsin. The 3480 (+)/3463 (+) band is the strongest one in the stretching vibrations of the difference spectrum.

of the chromophore bands are reasonable because the retinal chromophore changes its configuration between them (9-cis and 11-cis).

In contrast, protein bands are much less shown in Figure 2. Possible protein bands are observed at 1632 (+)/1625 (−), 3275 (−)/3262 (+), and 3480 (+)/3463 (−) cm^{-1}. The frequencies at 1632 (+)/1625 (−) and 3275 (−)/3262 (+) cm^{-1} suggest that they originate from amide-I (C═O stretch) and amide-A (N−H stretch) vibrations of the peptide backbone, respectively.[16] The 3480 (+)/3463 (−) cm^{-1} bands are the most intense bands as the X−H stretching vibrations. What is the origin of this

band? It is known that O−H and N−H stretching vibrations distribute at 3700−3200 and 3500−3200 cm^{-1}, respectively, and that the frequency lowers as the hydrogen bond is strengthened.[25] If it is an O−H stretch, side chains of (i) protonated carboxylic acids, (ii) tyrosine, (iii) serine and (iv) threonine are candidates. The O−H stretch of water molecules is excluded because the band does not exhibit isotope shift in $H_2{}^{18}O$.[16] On the other hand, if it is the N−H stretch, those frequencies are located at the almost upper limit of N−H stretches, indicating that the putative N−H group would be free from hydrogen bonds. The peptide backbone has an amide N−H group (amide-A vibration), while tryptophan, asparagine, glutamine, histidine, lysine, and arginine have N−H groups in their side chains. Among the latter, the N−H group of the histidine side chain may not be a candidate because the N−H stretch (3200−2799 cm^{-1}) is lower than the observed X−H stretch.[20] Similarly, those of lysine and arginine side chains are also unlikely because charged N−H groups have generally a low stretching frequency. As the free N−H group, (i) a peptide amide, (ii) a tryptophan side chain, (iii) an asparagine side chain and (iv) a glutamine side chain are the candidates. In the present study, we examined these possibilities as follows.

B. Infrared Spectral Analysis of Tryptophan Mutants. Among various possibilities, the indole N−H stretch of tryptophan is the strong candidate because one of the tryptophan residues in the protein moiety (Trp265) is situated near the β-ionone ring of the chromophore, which was elucidated by the cross-linking experiments using a retinal analogue having a photoaffinity substituent in its β-ionone ring.[26,27] In addition, a similar band was observed in bacteriorhodopsin. Formation of the L intermediate in bacteriorhodopsin accompanies appearance of a D_2O-insensitive strong peak at 3486 cm^{-1}, which was assigned as the indole N−H stretch of Trp182.[19] Thus, the observed band in rhodopsin may be ascribable to the indole N−H stretch of a tryptophan residue.

There are five tryptophan residues at positions 35, 126, 161, 175, and 265 in the primary structure of bovine rhodopsin. Thus, we prepared the tryptophan-to-phenylalanine mutants at the all five positions and measured the FTIR difference spectra. Figure 3 shows the B/R spectra hydrated with D_2O in the 3580−3430 cm^{-1} region. As is clearly seen, peaks were conserved in all tryptophan mutants. The results indicate that the band does not originate from the indole N−H of tryptophan. On the other hand, the peak pair at 3487 (+) and 3463 cm^{-1} (−) was considerably shifted among mutants. The rhodopsin band at 3463 cm^{-1} was downshifted in W126F and W265Y and upshifted in W265F, while the bathorhodopsin band at 3487 cm^{-1} was downshifted in W126F, W161A, and W175F and upshifted in W265F. These facts imply that the X−H group is present in the crucial protein moiety for the interaction with the retinal chromophore and is highly sensitive to the chromophore−protein interaction.

Spectral half widths of the X−H stretching bands at 3487, 3480, and 3463 cm^{-1} are 10, 12, and 14 cm^{-1}, respectively (Figure 1). In contrast, that of the indole N−H stretch of Trp182 in the L intermediate of bacteriorhodopsin at 3486 cm^{-1} is 6.5 cm^{-1} (H. Kandori, unpublished result). Therefore, the present X−H stretching band is considerably broader than the N−H stretch of Trp182 in bacteriorhodopsin.

C. Infrared Spectral Analysis of the Peptide N−H Stretch by Proline Mutants. Unlike bacteriorhodopsin, the bands in the 3490−3460 cm^{-1} region do not originate from tryptophan. Therefore, peptide amide or the side chains of asparagine and glutamine are the remaining candidates as the N−H stretch, while side chains of protonated carboxylic acids, tyrosine, serine, and threonine are the candidates as the O−H stretch. Next, we examined these possibilities by use of mutants.

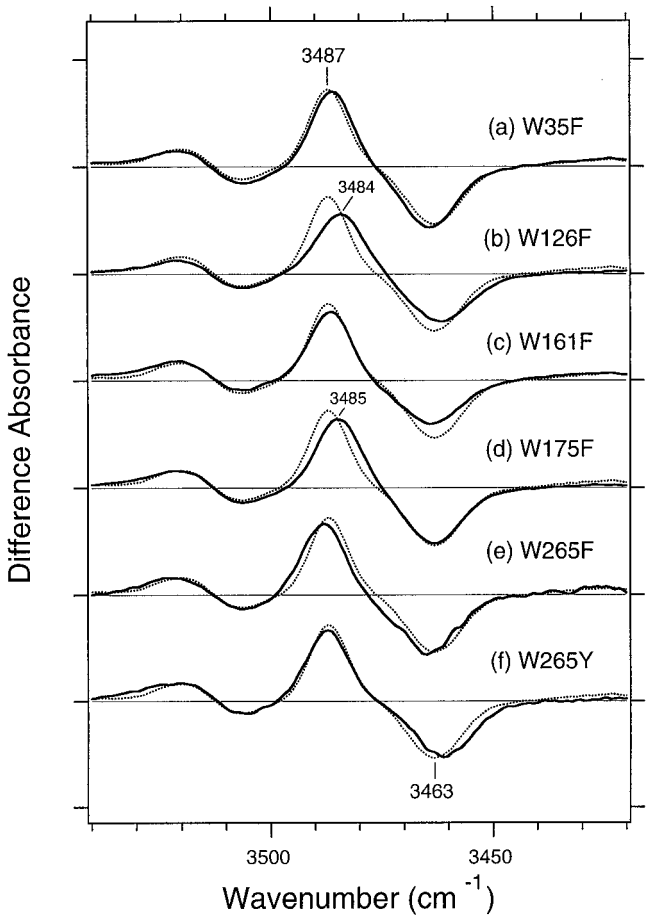

Figure 3. B/R spectra of the wild-type (dotted lines) and tryptophan-mutant (solid lines) proteins in the 3540−3420 cm^{-1} region. The sample was hydrated with D_2O. All spectra were normalized by their bathorhodopsin-specific HOOP bands at ~921 cm^{-1}. One division of the *Y* axis corresponds to 0.0015 absorbance unit.

The N−H stretching vibration of the peptide amide, so-called amide-A, appears near the 3320−3270 cm^{-1} region,[20,28] which does not include the present X−H stretch. However, if the peptide N−H group has no hydrogen-bonding acceptor, the frequency can be upshifted. Indeed, the N−H group in vapor phase and diluted solution is located at 3482−3460 cm^{-1} and 3480−3440 cm^{-1},[20] which coincides well with the X−H stretch. The peptide N−H group of an amino acid residue in an α-helix generally forms a hydrogen bond with the peptide carbonyl group (C═O) of the 4th residue behind. Thus, local perturbation of the α-helix may result in the lack of a hydrogen bond of the peptide N−H group. In particular, Sakmar's group proposed a specific interaction of Gly121 with the retinal chromophore based on their mutation studies.[29−33] Such an interaction may break the hydrogen bond of the N−H with the C═O group of Ala117. Two glycine residues at positions 120 and 121 are possible candidates. In the present study, we mutated the glycine to proline (G120P, and G121P), which does not have the amide N−H group. We also tested alanine mutations (G120A, and G121A).

Figure 4a−d shows the B/R spectra of the glycine mutants. The X−H band was identical in G120A (Figure 4a). The bands were reduced and broadened in G120P (Figure 4b), but the bilobic feature was preserved. The frequency of bathorhodopsin is upshifted by 5 cm^{-1} in G121A (Figure 4c). The X−H bands were reduced and broadened in G121P (Figure 4d), but the

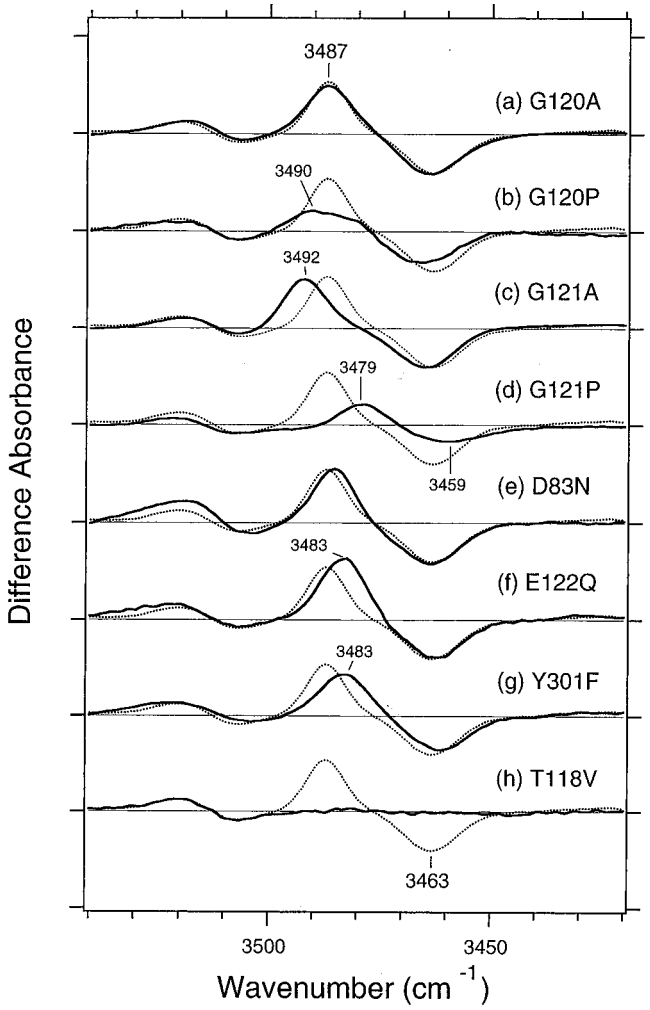

Figure 4. B/R spectra of the wild-type (dotted lines) and mutant (solid lines) proteins in the 3580−3420 cm^{-1} region. The sample was hydrated with D_2O. All spectra were normalized by their bathorhodopsin-specific HOOP bands at ∼921 cm^{-1}. One division of the *Y* axis corresponds to 0.002 absorbance unit.

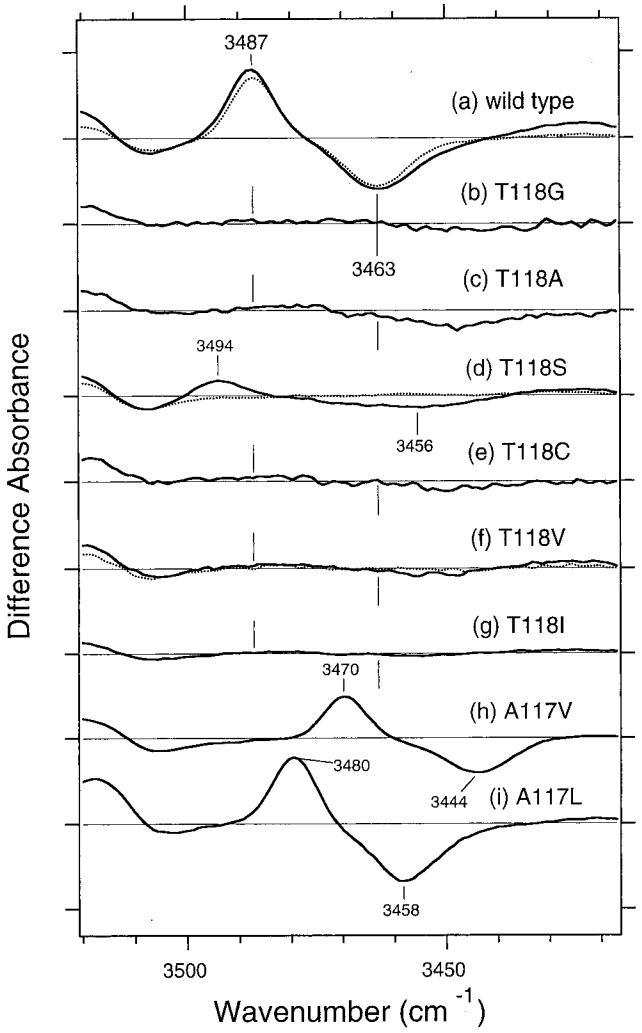

Figure 5. B/R spectra of the wild-type (a) and mutant (b−i) proteins in the 3580−3420 cm^{-1} region. The sample was hydrated with H_2O (solid lines) or D_2O (dotted lines). All spectra were normalized by their bathorhodopsin-specific HOOP bands at ∼921 cm^{-1}. One division of the *Y* axis corresponds to 0.0015 absorbance unit.

bilobic feature was preserved. Thus, these results showed that the X−H stretch does not originate from the peptide amide of Gly120 and Gly121. It is noted that proline mutants, but not alanine mutants, displayed reduced and broadened X−H stretches, suggesting that the lowered degree of freedom of motion in these proline mutants caused perturbed structures of isomeric states.

D. Infrared Spectral Analysis of the O−H Stretch. We next examined the possibilities of the O−H stretches. Side chains of protonated carboxylic acids, tyrosine, serine, and threonine are the candidates. Previous studies showed that membrane-embedded Asp83 and Glu122 are protonated,[24,34] the O−H groups of which are possible candidates of the X−H group because both experience structural changes of the carboxylic C═O stretches upon photoisomerization.[16,24] However, Figure 4e,f shows that the X−H bands remain in D83N and E122Q, respectively. Thus, the X−H band does not originate from the O−H group of protonated carboxylic acids. Similarly, Figure 4g shows that the X−H band does not originate from the O−H group of Tyr301.

In contrast, the X−H band completely disappeared in T118V (Figure 4h). This result strongly suggests that the O−H stretch of Thr118 is the X−H group. The O−H stretching frequencies of secondary alcohols as models of threonine have been reported as follows: free O−H stretches at 3626−3629 cm^{-1} and hydrogen-bonded O−H stretches at 3340−3355 cm^{-1}.[20,21] The O−H stretching vibration of threonine thereby ranged from ∼3630 to 3350 cm^{-1}, dependent on its hydrogen-bonding conditions. The frequency at 3463 cm^{-1} of rhodopsin corresponds to a moderate hydrogen bond of the O−H group, and the hydrogen bond is weaker in bathorhodopsin (3487 cm^{-1}) and isorhodopsin (3480 cm^{-1}).

In Figure 5, we further confirmed the assignment. All mutants of Thr118[35] yielded a lack of any bands in the 3490−3460 cm^{-1} region except for T118S (Figure 5d). In T118S, a small positive and a broad negative band appeared at 3494 and 3456 cm^{-1}, respectively. These bands are interpreted in terms of the O−H stretching vibrations of serine at position 118. Interestingly, the O−H stretch of Ser118 is exchangeable for D_2O, as shown in the lack of the band in D_2O (Figure 5d). This result in T118S is in contrast to that of the wild type, where the O−H stretch of Thr118 is not exchangeable for D_2O (Figure 5a). It suggests that the methyl group at position 118 prevents pathways to the aqueous phase from forming. Figure 5h, shows that the bands are preserved in the A117 mutants, while the frequencies are greatly altered. In particular, A117V exhibits about 20 cm^{-1} downshift of the O−H stretch in rhodopsin (Figure 5h). These results also support assignment of the O−H stretch of Thr118.

TABLE 1: Frequencies of the O−H Stretching Vibration of Thr118[a]

	λ_{max} (nm)[a]	rhodopsin, 11-cis	bathorhodopsin, all-trans	isorhodopsin, 9-cis
wild type	499	3463	3487	3480
W35F	499	+1	−1	±0
D83N	497	±0	−2	−2
D83N/E113Q	499	+8*	+5*	−3*
D83N/G120A	497	−3	−1	−2
E113Q	499	±0	+4	±0
E113Q/G120A	499	+9*	+5*	±0*
A117V	496	−18	−16	NP
A117L	497	−5	−8	NP
G120A	499	±0	±0	±0
G120P	490	+3*	+3*	NM
G121A	499	+2	+5	+1
G121P	506	−4*	−8*	NM
E122Q	484	−1	−5	−4
W126F	496	−1	−3	−2
W161F	498	+1	−1	±0
W175F	497	±0	−2	−1
W265F	479	+1	+1	ND
W265Y	482	−2	±0	ND
Y301F	499	−2	−4	−4

[a] The frequencies of the wild-type and the difference in the mutants are shown in cm^{-1}. NP indicates that isorhodopsin was hardly populated under any illumination conditions. NM indicates that the value was not measured. ND indicates that the value was not determined because the signal-to-noise ratio was not good. Asterisks represent much broader bands presumably because of an inhomogeneous environment. [b] Absorption maxima of the 11-cis form measured at room temperature.

In conclusion, we identified the isomer-specific stretching vibrations as the O−H stretch of Thr118.

Discussion

A. Characteristics of the O−H Stretch of Thr118. The present FTIR spectroscopy provided experimental evidence that the isomer-specific band in the 3490−3460 cm^{-1} region originates from the O−H stretching vibration of Thr118 in bovine rhodopsin. The frequency is highly sensitive to various mutations (Table 1), suggesting that the O−H group of Thr118 is located at a crucial position in the interaction with the retinal chromophore. The O−H stretching frequencies of secondary alcohols as a model of threonine have been reported as follows: free stretches at 3626−3629 cm^{-1} in CCl_4 solution and hydrogen-bonded O−H stretches at 3340−3355 cm^{-1} in neat liquid.[20] The O−H stretching vibration of threonine thereby ranged from ∼3630 to 3350 cm^{-1}, dependent on its hydrogen-bonding conditions. The O−H stretches of the threonine side chain in the restricted protein environment have been reported for bacteriorhodopsin. The observed frequencies are 3462 cm^{-1} for Thr17,[36] 3402 cm^{-1} for Thr121[36] and 3378 cm^{-1} for Thr89.[21] The O−H stretch of rhodopsin (3463 cm^{-1}) is close to that of Thr17 in bacteriorhodopsin, which is located at 2.7 Å from the peptide carbonyl of Leu13.[36] Thus, the result of bacteriorhodopsin[21,36] suggested that the O−H group of Thr118 in rhodopsin forms a moderate hydrogen bond.

B. Location of Thr118 in the Crystallographic Structure of Rhodopsin and its Possible Role in the Photobleaching Processes. During our writing of this manuscript after the experiment, the X-ray crystallographic structure of bovine rhodopsin appeared.[22] Determination of the first atomic structure as GPCRs was achieved by successful crystallization of bovine rhodopsin by Okada et al.[37] Figure 6 shows the structure of the retinal chromophore and surrounding amino acids in helix III. Unlike the predicted models by Sakmar's group[29−33] and Pogozheva et al.,[38] Gly121 is not located at the contact position

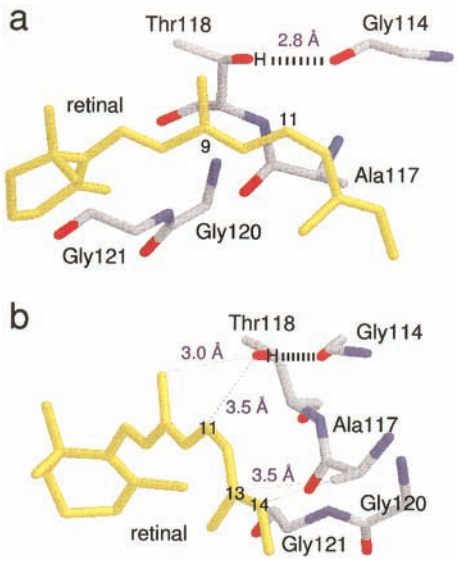

Figure 6. Crystallographic structure of bovine rhodopsin (PDB entry 1F88).[22] The retinal chromophore is colored yellow, and the side chains in helix III are shown (panel a). The distance between the side-chain oxygen of Thr118 and the peptide carbonyl of Gly114 is 2.8 Å. The distance between the amide nitrogen of Thr118 and the peptide carbonyl of Gly114 is 3.2 Å. Panel b shows the structure in panel a rotated by 60° along the vertical axis.

TABLE 2: Oxygen Atoms of Amino-Acid Side Chains within 4.0 Å from the Retinal Chromophore

amino acid	atom of side chain	atom of retinal	distance in Å
Glu113	O	C15	3.9
		N	3.9
	O	N	3.2
Thr118	O(H)	9-Me	3.0
		C9	3.5
		C10	3.6
		C11	3.5
		C12	3.9
Glu122	O(H?)	C6	3.8
Ser186	O(H)	C14	3.3
		C15	3.6
		N	3.7
Tyr268	O(H)	C10	3.6
		C11	3.5
		C12	3.9

with the methyl group at position C9. Rather, the closest atom from the C9 methyl group is the oxygen atom of the side chain of Thr118. In addition, the side-chain oxygen is located at 2.8 Å from the peptide carbonyl oxygen of Gly114. The present FTIR results imply that they form a hydrogen bond with each other (Figure 6). The present results also showed that the hydrogen-bonding strength is weaker in bathorhodopsin and isorhodopsin than in rhodopsin, suggesting that other isomeric forms possess perturbed local structure of Thr118. The rhodopsin structure shows that both the side-chain O−H and amide N−H of Thr118 are in hydrogen-bonding distance from the carbonyl oxygen of Gly114, the latter of which is 3.2 Å.[22] Therefore, strength of the two hydrogen bonds may be altered among different isomeric states.

The structure of rhodopsin[22] also implicates an interesting structural feature around the retinal chromophore. Thr118 and its hydrogen bond with the peptide carbonyl of Gly114 constitutes one side of the polyene chain (Figure 6). On the other hand, Tyr268 is located at the other side of Thr118. As shown in Table 2, Thr118 and Tyr268 sandwich the middle of the polyene chain of the retinal chromophore, while Glu122 and Ser186 interact with the β-ionone ring and the Schiff-base

region, respectively. Interestingly, Figure 2 shows that the O−H stretch of Thr118 was only observed in the difference spectrum between rhodopsin and isorhodopsin, suggesting that the other stretching vibrations were similar between 11-cis and 9-cis forms. Table 1 shows that the distances from C10, C11, and C12 are similar between oxygen atoms of Thr118 and Tyr268. This may suggest the important role in interaction of the 9-methyl group with Thr118. Alternatively, the structure of the side chain of Tyr268 may be more rigid because the O−H group of Tyr268 is in the hydrogen-bonding distances (2.9 and 2.8 Å) with Glu181 and Tyr191, respectively. No band of protonated carboxylic acids other than Asp83 and Glu122 in the B/R spectrum[21,24] strongly suggests that Glu181 is negatively charged like Glu113. Strong association among Try268, Glu181, and Tyr191 may lead to the insensitivity of the O−H stretch of Tyr268. On the other hand, the Ala117−Thr118 region is presumably stabilized by intrahelical hydrogen bonds, with the regions of Glu113−Gly114 and Gly120−Gly121. The presence of glycine residues possibly gives flexibility in motion to the helix III. Spectral broadening of proline mutants at positions 120 and 121 (G120P and G121P; Figure 4) supports this argument because introduction of a proline residue should lower the degree of freedom in motion by ring fixation into the peptide backbone. Our comprehensive mutational analysis of T118 clearly showed that the bands were observed only when the amino acid residue at position 118 contains an O−H group. However, we could not exclude a possibility that the phenolic O−H of Try268 is a candidate of the bands that might disappear by the absence of the O−H group at position 118. The mutational analysis that examines that possibility is our future research.

Possible isomerization at low temperature[15] requires structural flexibility for the surrounding protein environment, which can accommodate three isomeric states. The rhodopsin structure[22] showed that Thr118 and Tyr268 form possible contacts with the middle portion of the retinal chromophore from both sides. The flexible structure of helix III, suggested by the present results, may be correlated with the mechanism of efficient isomerization in rhodopsin, and structural changes of helix III probably lead to global motion of the cytoplasmic surface for transducin activation.

C. Threonine Side Chain in Other Visual Pigments. A threonine residue at this position within the glycine-rich region (Glu113−Gly114, Ala117−Thr118, and Gly120−Gly121 in bovine rhodopsin) is considerably conserved among vertebrate visual pigments. Among the four families (short, middle 1, middle 2, and long) of cone visual pigments and rhodopsins,[39] middle groups (M1 and M2) possess identical amino acid residues to those of rhodopsins. A family of short-wavelength-sensitive visual pigments (S-group) has similar but more various sequences; the corresponding position of Thr118 is threonine, serine, or cysteine, possessing a polar O−H or S−H group. Glycine residues are conserved in the corresponding positions of Ala117 and Gly121. A family of long-wavelength-sensitive visual pigments (L-group) has conserved sequences in the region; Glu−Gly, Val−Ser, and Cys−Gly. In this case, the corresponding position of Thr118 has the O−H group (serine), and glycine residues are conserved in the corresponding positions of Gly114 and Gly121. Therefore, a common structural motif of helix III in interacting with the retinal chromophore is suggested, while the unique differences are possibly correlated with their absorption characteristics.

Our recent studies showed that chicken green- and red-sensitive visual pigments exhibited similar bands in the 3450−3490 cm^{-1} region.[40,41] The frequencies in chicken green are located at 3484 cm^{-1} for the bathointermediate and at 3466 cm^{-1} for the original pigment, while those of chicken rhodopsin are located at 3483 cm^{-1} for bathorhodopsin and 3462 cm^{-1} for rhodopsin.[40] On the other hand, those of chicken red (iodopsin) are located at 3485 cm^{-1} for the bathointermediate and at 3430 cm^{-1} for the original pigment. Thus, the O−H groups situated at the corresponding position of bovine rhodopsin are likely to form a hydrogen bond, and the hydrogen bond is weakened upon photoisomerization of the retinal chromophore. The interesting observation is that the bands observed in the chicken red system are also D_2O-insensitive as those observed in chicken green and rhodopsin systems are, even though the chicken red has a serine residue instead of a threonine residue at this position. Because the replacement of threonine with serine at this position changes the bands to D_2O-sensitive in bovine rhodopsin (Figure 5d), these facts imply the structural difference between bovine rhodopsin and chicken red. The 3-D structure of bovine rhodopsin shows that the methyl group of Thr118 could be in van der Waals contact with the methyl group of Ala168 (distance, 3.8 Å). Because chicken red has a threonine residue at the corresponding position of alanine in bovine rhodopsin, the methyl group (or the O−H group) of the threonine could occupy the space that is produced because of the presence of the Ser118, thereby resulting in no accessibility of water to the Ser118 in chicken red. This speculation also supports the idea that the 3-D structure of chicken red as well as the other cone visual pigments would be similar to that of bovine rhodopsin and the differences in characteristics among visual pigments could originate from the differences in amino acid residues among them. Thus, the vertebrate visual pigments would share a common mechanism in molecular motion regardless of various colors.

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