Difference in Molecular Structure of Rod and Cone Visual Pigments Studied by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: To investigate the local structure that causes the differences in molecular properties between rod and cone visual pigments, we have measured the difference infrared spectra between chicken green and its photoproduct at 77 K and compared them with those from bovine and chicken rhodopsins. In contrast to the similarity of the vibrational bands of the chromophore, those of the protein part were notably different between chicken green and the rhodopsins. Like the rhodopsins, chicken green has an aspartic acid at position 83 (D83) but exhibited no signals due to the protonated carboxyl of D83 in the C═O stretching region, suggesting that the molecular contact between D83 and G120 through water molecule evidenced in bovine rhodopsin is absent in chicken green. A pair of positive and negative bands due to the peptide backbone (amide I) was prominent in chicken green, while the rhodopsins exhibited only small bands in this region. Furthermore, chicken green exhibited characteristic paired bands around 1480 cm^{-1}, which were identified as the imide bands of P189 using site-directed mutagenesis. P189, situated in the putative second extracellular loop, is conserved in all the known cone visual pigments but not in rhodopsins. Thus, some region of the second extracellular loop including P189 is situated near the chromophore and changes its environment upon formation of the batho-intermediate. The results noted above indicate that differences in the protein parts between chicken green and the rhodopsins alter the changes seen in the protein upon photoisomerization of the chromophore. Some of these changes appear to be the pathway from the chromophore to cytoplasmic surface of the pigment and thus could affect the activation process of transducin.

The visual transduction process in photoreceptor cells begins with photon absorption by a visual pigment, which is a member of a family of G-protein coupled receptors and contains 11-*cis*-retinal as a light-absorbing chromophore (*7*). In most vertebrates, different types of visual pigments are present in rod and cone photoreceptor cells, where they mediate vision under twilight and daylight conditions, respectively. On the basis of similarity in the primary structure, vertebrate visual pigments are classified into four groups of cone pigments and a single group of rod pigment rhodopsins[1] (*1*). The presence of multiple types of cone pigments with different absorption maxima is the molecular basis of color discrimination. While each cone pigment exhibits a unique absorption maximum, it exhibits a common motif of molecular properties different from rhodopsin (*8*). That is, the cone pigment has a physiologically active meta II intermediate that decays faster than that of rhodopsin. The regeneration of cone pigment from 11-*cis*-retinal and opsin is faster than that of rhodopsin. The fast decay of meta II diminishes the ability for transducin activation (*9*), and the rapid regeneration results in the rapid recovery of photosensitivity; therefore, these two properties have some relationship with the functional difference between rod and cone photoreceptor cells (*10*). Our recent investigations clearly showed that the amino acid residue at position 122 is one of the determinants responsible for these properties (*11*).

In addition to the above properties, all of the known cone visual pigments exhibit several properties different from those of rhodopsin. The most prominent is the lower stability of the protein moiety than that of rhodopsin especially in the absence of the retinal chromophore. In addition, high sensitivity of the retinal chromophore to hydroxylamine is characteristic of cone pigments.[2] That is, cone pigments are bleached in the presence of hydroxylamine even in the dark (*1*, *12*). Furthermore, our recent low-temperature UV-vis work demonstrated the highly unstable nature of the batho-intermediates of cone pigments, the red-shifted primary photobleaching intermediate, compared to that of rhodopsin

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[1] We denoted five families of pigments as group L, S, M1, M2, and Rh (*1*). In most vertebrates, pigments belonging to the group L, S, M1, M2 are present in cones, while those belonging to the group Rh (rhodopsin) are present in rods. Therefore, we refer to these pigments as rod and cone pigments. It should be noted that there are some exceptions. American chameleon (*Anolis carolinensis*) has members of all the visual pigment families (both rod and cone pigments) in its cones (*2*). Tokay gecko (*Gekko gekko*) has cone pigments (M2 and L) in its rods (*3*).

[2] Like cone pigments, American chameleon (*4*) and lamprey (*5*) rhodopsins are unstable in the presence of hydroxylamine even in the dark.

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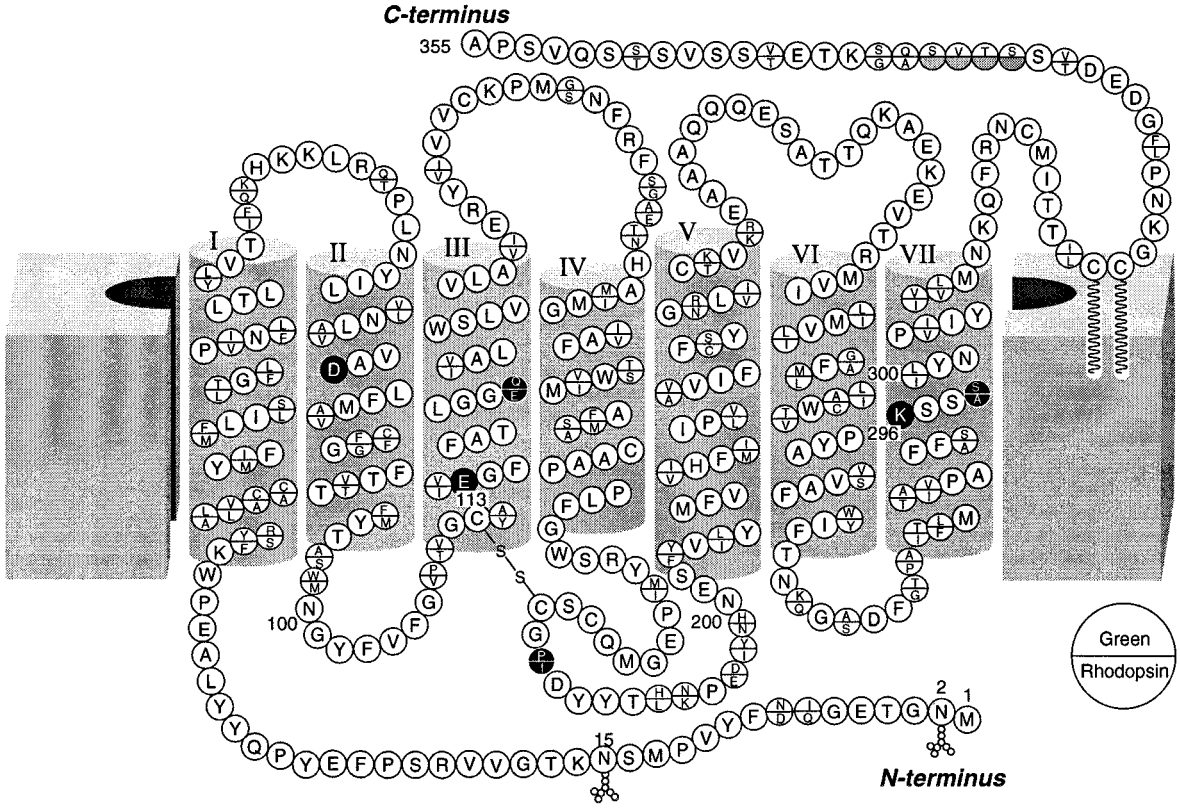

FIGURE 1: Comparison of amino acid sequences of chicken green and rhodopsin. The transmembrane topography is based on the model of Hargrave et al. (*41*). Residues common to chicken green and rhodopsin are denoted by single letters in the circle and numbered using the bovine rhodopsin numbering system. The residues that are different between chicken green and rhodopsin are denoted at the upper- and bottom-sides of the circle, respectively.

(*9*, *13*, *14*). These properties originate from the difference in local structure of the chromophore-binding site in the initial state of pigment, while the above properties are due to the difference in protein conformation of the later intermediate or opsin state. Thus to understand the difference in the resting state, it is important to investigate the nature of the cone pigments and their primary intermediates.

Chicken green, a green-sensitive cone pigment present in the chicken retina, is one of the cone pigments suitable for investigating the difference between rod and cone pigments. Although chicken green exhibits the above properties common to the cone pigments (*14*, *15*), it has an amino acid sequence and an absorption maximum highly similar to those of rhodopsin (Figure 1, refs *1* and *12*). Thus, a comparison of the molecular structure of chicken green and rhodopsin would provide detailed information on the molecular basis of the properties different between rod and cone pigments.

FTIR[3] spectroscopy is a powerful tool to elucidate the structure and light-induced events in a visual pigment (*16*). In fact, in combination with resonance Raman spectroscopy, information on chromophore structure (*17*), peptide backbone, internal water molecules (*18*), and protonated carboxyl groups (*19*) has been obtained. Thus, we applied this technique to investigate aspects of the structure of chicken green and to compare these with similar features of chicken and bovine rhodopsins. On the basis of these results, the mechanisms and the specific amino acid residue(s) responsible for the difference in molecular properties between rod and cone visual pigments will be discussed.

MATERIALS AND METHODS

Preparation of Chicken Green and Rhodopsin. Chicken green and rhodopsin were extracted from chicken retinas by a mixture of CHAPS and PC and purified by means of column chromatography (*20*, *21*). In addition to the standard procedure, the following steps were employed to prepare a film sample whose protein/lipid ratio was suitable for FTIR spectroscopy. That is, a chicken green sample purified as above was absorbed again on a ConA-Sepharose column equilibrated with buffer A [20% glycerol (w/v), 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 6.6] supplemented with 1 mM $MnCl_2$ and $CaCl_2$. After the column was washed with buffer A supplemented with 5 mM methyl α-D-mannoside to remove the other contaminating pigments, chicken green was eluted with buffer A supplemented with 200 mM methyl α-D-mannoside. It was then applied to a CM-Sepharose column (Pharmacia), followed by elution with buffer B [20% glycerol (w/v), 0.75% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 6.6].

The purified sample (1 mL) was dialyzed against 100 mL of buffer C [50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 6.6] overnight with six exchanges of the buffer

[3] Abbreviations: FTIR, Fourier transform infrared; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; ConA, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KIU, kallikrein inhibitor units.

to prepare a liposome-containing chicken green. The liposomes were collected by centrifugation, and the pellets were suspended in 40 μL of H_2O buffer (pH 7.0). The suspension was 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, sealed by another BaF_2 window with the aid of a silicon rubber O-ring and set in a brass cell holder. The FTIR measurements were done after immediate cooling to 77 K.

The wild type and mutants of chicken green were expressed in HEK293 cell lines (*22*, *23*) as reported previously (*21*). For purification, cDNA of chicken green was tagged by the monoclonal antibody Rho 1D4 epitope-sequence (ETSQVAPA) (*24*). The resulting cDNA was fully sequenced before introducing to the expression vector (*25*). The method for the construction of the chicken green mutant gene of P189I was described previously (*11*). Expressed protein was incubated with 11-*cis*-retinal to reconstitute the photoactive pigment and extracted with 1% dodecyl maltoside (DM) in buffer C and then purified with a 1D4-column as described previously (*26*). For FTIR spectroscopy, the purified pigment was supplemented with a 100-fold molar excess of PC dissolved in 0.75% CHAPS and dialyzed against buffer C for 3−4 days with at least six exchanges of the buffer. The pigment in PC vesicles was collected by centrifugation, and the pellet was suspended in 40 μL of H_2O, followed by preparing a film sample as described above.

Spectrophotometry. UV−vis absorption spectra were recorded with a Shimadzu model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The system for the measurements of absorption spectra was reported previously (*27*). FTIR spectra were recorded by a Bio-Rad FTS-40 K spectrometer according to the methods described previously (*28*, *29*). The sample was irradiated with light from a 1-kW tungsten halogen lamp (Rikagaku Seiki) that had been passed through a glass cutoff filter (VR63 and VO54; Toshiba) or an interference filter (501 nm; Nihonshinku). The obtained spectra were normalized to represent the signals of the same molar amounts of the pigments photoconverted using extinction coefficient and percentages of the photoconversions among original pigments, batho-intermediates, and isopigments at 77 K reported previously (*14*, *15*, *30*).

RESULTS

As already reported, chicken green exhibits an absorption maximum (508 nm) close to those of chicken (503 nm) and bovine (498 nm) rhodopsins in the CHAPS-PC system (*1*, *12*, *14*, *15*). The spectral shape and maximum did not change when it was incorporated with PC liposomes and dried into a film (Figure 2). The optical purity (A_{280}/A_{max}) of chicken green and rhodopsin samples was ~1.8, suggesting that these samples are sufficient for FTIR spectroscopy. To obtain information on the local structure of the pigments, we cooled the samples and measured the difference FTIR spectra between the initial states and the batho-intermediates upon irradiation of the initial states. We estimated the percentages of original pigments, batho-intermediates, and isopigments in the irradiated samples at 77 K using the method described previously (*14*). The batho/original difference spectra obtained from chicken green and rhodopsins were then normalized to represent the same amounts of original pigments photoconverted to their batho-intermediates (Figure 3). As reflected in the visible spectra, chromophore vibrational bands appearing in the <1400 cm^{-1} region of the difference FTIR spectrum from chicken green were similar to those from bovine (*29*) and chicken rhodopsins (Figure 3). However, some differences were prominent in the >1400 cm^{-1} region where the bands due to the protein part of the pigment appeared.

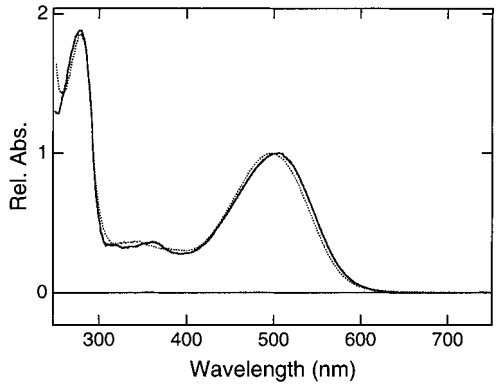

FIGURE 2: Absolute absorption spectra of chicken green and rhodopsin. Solid and dotted lines represent spectra of hydrated films of chicken green and chicken rhodopsin, respectively.

Similarity in Chromophore Bands between Chicken Green and Rhodopsins. The chicken green/batho FTIR difference spectrum was similar to those of bovine (*29*) and chicken (Figure 3) rhodopsins in the fingerprint and hydrogen-out-of-plane vibrational region. Thus the positive and negative bands of the difference spectra in chicken green and chicken rhodopsin systems were assigned by comparison with the bands in bovine rhodopsin which are shown in Table 1. The notable difference between chicken green and rhodopsins is the intensity of the negative 1237 cm^{-1} band due to the C12−C13 stretching vibration of the original state. Chicken green exhibits a band significantly larger than that for bovine or chicken rhodopsin, whereas the band position is similar (within 1 cm^{-1}). These results suggest that the chromophore conformation around C12−C13 single bond is similar between chicken green and rhodopsins, but this bond may be differently perturbed by the residue(s) nearby the bond in chicken green and rhodopsins. The ethylenic stretching bands of chicken green and its batho-intermediate (1527/1560 cm^{-1}) are also similar in frequency to those of chicken rhodopsin and its batho-intermediate (1533/1558 cm^{-1}). The negative 1544 cm^{-1} band in chicken green is likely to be an amide II band that may represent an environmental change in the α-helix in chicken green (see below).

Differences in Protein Bands between Chicken Green and Rhodopsins. Visual pigments are members of the superfamily of G-protein coupled receptors, all of which have a structural motif of seven transmembrane α-helices. Thus, the main amide bands of the difference spectra between batho and the original state should be located in the α-helix region in which the amide I and II bands show frequencies of ~1650 and ~1550 cm^{-1}, respectively. In the case of chicken green, the intense pair of negative 1663 cm^{-1} and positive 1654 cm^{-1} bands was observed in the amide I region (Figure 3, panel A) and the intense 1544 cm^{-1} band was observed in amide II region. Exposure of D_2O did not shift the bands, suggesting that these bands are probably amide I and II

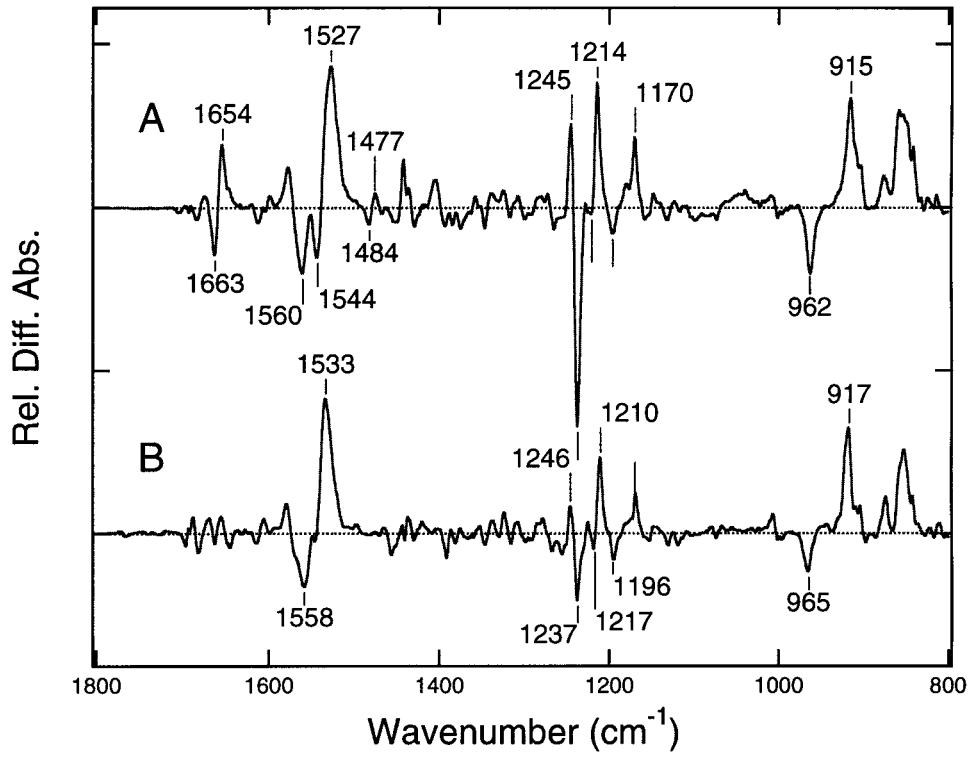

FIGURE 3: The normalized batho/original difference FTIR spectra in chicken green (A) and rhodopsin (B). The FTIR spectra of chicken green and its batho-intermediate and those of rhodopsins and batho-rhodopsins were recorded before and after irradiation with green light (501 nm) for 2 min at 77 K. The samples were then irradiated with red light (>610 nm) for 5 min to convert the batho-intermediates to the original pigments, and the spectra were recorded. Difference spectra are calculated by subtracting the spectra before irradiation with green light from those after irradiation and by subtracting the spectra after irradiation with orange light from those before irradiation. The spectral components of the isopigments produced by these irradiations were already shown to be negligible (*14*). The average of 20 spectra is shown as the difference spectra between the batho- and original states in chicken green (A) and rhodopsin (B).

Table 1: Infrared Bands (cm^{-1}) Observed in Batho/Initial Difference Spectra

	bovine rhodopsin	chicken rhodopsin	chicken green
HOOP			
C11═C12	921/967[a]	917/965	915/962
C═C str.	1535/1558	1533/1558	1527/1560
C−C str.			
C12−C13	1243/1238[b]	1246/1237	1245/1237
C8−C9	1209/1217[b]	1210/1217	1214/1218
C10−C11	1166/1098[b]	170/N. D.[f]	1170/N. D.[f]
C14−C15	1209/1192[b]	1210/1196	1214/1196
COOH			
D83	1773/1769[c,d]	1772/1766	N. D.[g]
E122	1735/1740[c,d]	1736/1740	
O−H str. (water)	3525/3538[e]	3525/3538	3527/3557
	3542/3563[d]	3546/3562	N. D.[g]

[a] Palings et al., 1989 (*39*). [b] Palings et al., 1987 (*40*). [c] Fahmy et al., 1993 (*19*). [d] Nagata et al., 1998 (*26*). [e] Nagata et al., 1997 (*31*). [f] Not Determined. [g] Not Detected.

vibrations of the membrane embedded α-helices in chicken green. On the other hand, we observed more than one bilobe bands in the amide I region of the difference spectra between batho and the original state of rhodopsins (Figure 3, panel B). These results suggest that the chromophore movement during the formation of batho-intermediate accompanies the changes in the α-helical region in chicken green, while other regions in addition to the α-helical region of rhodopsin changes upon movement of the chromophore. An alternative possibility is that the α-helix is perturbed in rhodopsin.

In bovine rhodopsin, two membrane-embedded carboxylic acids, D83 and E122, are known to be protonated, and these C═O stretching vibrations appear in the 1790−1700 cm^{-1} region (*19*). Chicken rhodopsin also exhibited two D_2O-sensitive paired bands (1772/1766 and 1735/1740) which are due to the carboxyl C═O stretching vibrations of D83 and E122, respectively (Figure 4, panel B). However, chicken green exhibited no bands in this region (Figure 4, panel A). Chicken green contains glutamine instead of glutamic acid at position 122 (*1*, *12*); therefore, the absence of the paired bands due to the E122 at about 1740 cm^{-1} is reasonable. However, the absence of the D83 bands is unusual, because chicken green also has the D83. There are two possibilities that could account for the observed result. One is that D83 of chicken green shows no environmental change in the formation process of batho-intermediate, even though the carboxyl group of D83 is protonated. The other is that D83 is not protonated in both of the chicken green and its batho-intermediate so that the signals do not appear in this region. Our preliminary experiments indicated that the clear negative band (1750 cm^{-1}) due to the protonated carboxyl group of chicken green appeared in the meta II/chicken green difference spectrum. Because the position of this band is similar to that of the D83 band of rhodopsin, it is likely that this band originates from the D83 of chicken green. Therefore, carboxyl group of D83, protonated in the initial state of chicken green, would show no change in the formation process of batho-intermediate, while it shows some changes upon formation of meta II. In other words, the structural

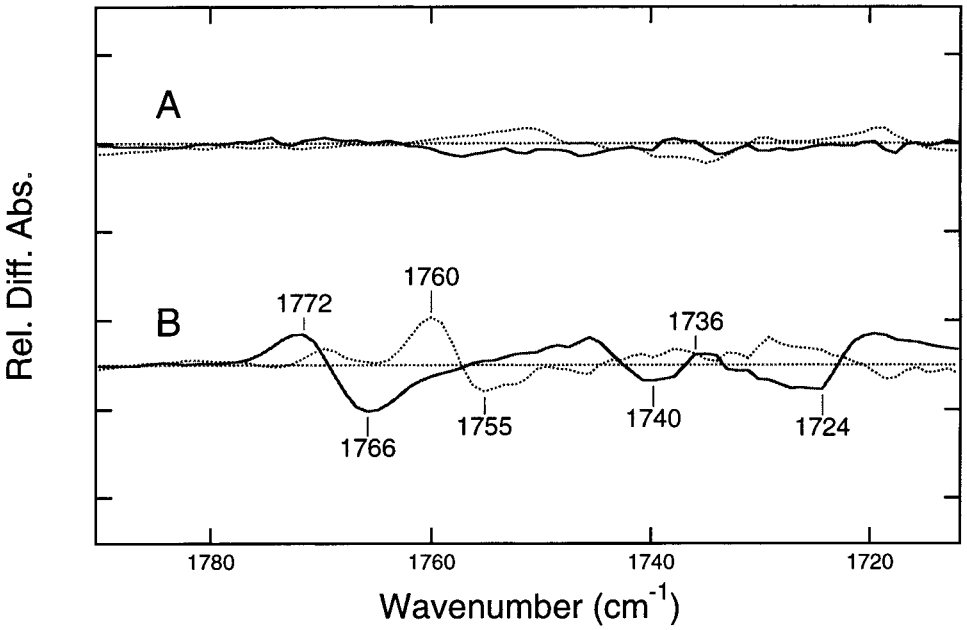

FIGURE 4: The normalized batho/original difference FTIR spectra in the 1800−1700 cm^{-1} region, which contain carboxyl C═O stretching vibrations in chicken green (A) and rhodopsin (B). The solid lines are the normalized batho/original difference FTIR spectra taken from Figure 3. The dotted lines are those obtained after hydration of the samples with D_2O.

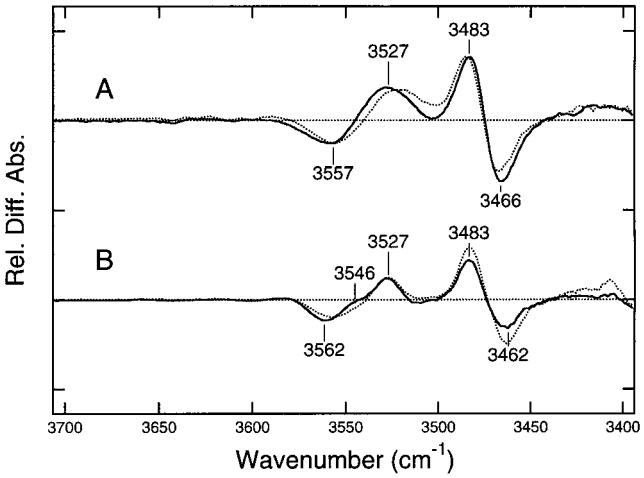

FIGURE 5: The normalized batho/original difference FTIR spectra in the 3700−3400 cm^{-1} region, which contain O−H stretching vibrations of water molecules in chicken green (A) and rhodopsin (B). The solid lines are the normalized batho/original difference FTIR spectra obtained from hydrated films. The dotted lines are those obtained after hydration of the samples with $H_2{}^{18}O$.

changes in chicken green do not extend to D83 in the batho intermediate, but do reach it in meta II.

In the high frequency (3800−3000 cm^{-1}) region, chicken green and chicken rhodopsin exhibited the O−H stretching bands of water molecule(s) (Figure 5). These bands were assigned by the isotope shifts upon hydration of the sample with D_2O or $H_2{}^{18}O$, where the bands were lower-shifted to about 900 or 10 cm^{-1}, respectively. The band profile of chicken rhodopsin (Figure 5, panel B) is similar to that of bovine rhodopsin (*29*) in this region. That is, the negative 3562 cm^{-1} and positive 3527 cm^{-1} bands with a shoulder band at 3546 cm^{-1} were observed. In bovine rhodopsin, the band profile was simulated by two overlapping paired bands (3542/3563 cm^{-1} and 3525/3538 cm^{-1}), which originate from the vibrations of water molecules existing near the counterion E113 and that between D83 and G120, respectively (*26*, *31*). In this region, chicken green exhibited broad positive and negative bands at 3527 and 3557 cm^{-1}, respectively, but it did not exhibit a shoulder band between these bands (Figure 5, panel A). The simple explanation may be that chicken green contains one water molecule whose environment is perturbed upon photoisomerization of the chromophore. However, the band profile is notably identical with that of the D83N/G120A double mutant of bovine rhodopsin and is very similar to that of the D83N mutant of bovine rhodopsin (*31*). In these mutants, two water molecules are perturbed upon photoisomerization of the chromophore, but the extent of the perturbation is different from those of wild-type rhodopsin, thereby resulting in the presence of a single pair of the positive and negative bands in this region (*31*). Therefore, we speculated that chicken green contains two water molecules similar to chicken and bovine rhodopsins, but the extent of the perturbation in forming batho is different from those of rhodopsin.

Identification of the Imide Bands of P189 in the Difference FTIR Spectrum of Chicken Green. To search for other evidence for the difference in molecular structure between chicken green and rhodopsins, we further compared the difference FTIR spectra of these pigments over the complete wavenumber region recorded and found a unique pair of bands in chicken green. That is, the pair of positive 1477 cm^{-1} and negative 1484 cm^{-1} bands was observed in chicken green (Figure 6, panel A) but not in rhodopsins (Figure 6, panels D and E). The uniqueness of these bands was emphasized by the fact that similar bands were observed in other cone visual pigments. Chicken blue exhibited bands similar to those of chicken green (Figure 6, panel B), while chicken red exhibited similar bands but the signs of the bands were opposite (Figure 6, panel C). Because these bands are situated in the frequency region of the imide II [C(O)-N stretch] vibration of the X-Pro bond, we speculated that some proline residue that is conserved in all of the cone visual pigments but not in the rhodopsins would contribute these

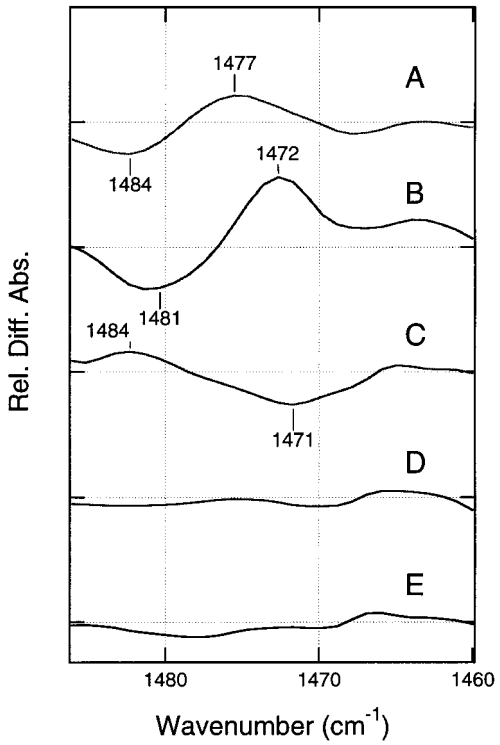

FIGURE 6: The normalized FTIR difference spectra between the batho and original states of H_2O-hydrated chicken green (A), blue (B), red (C), and rhodopsin (D) in the 1500−1460 cm^{-1} region, which contain imide II vibrations. Spectra for the red (*36*) and bovine (*29*) rhodopsin (E) were taken from the previous paper. The spectra of chicken blue and its batho-intermediate were recorded before and after irradiation of the hydrated films of chicken blue with 436 nm light at 77 K (Hirano et al., manuscript in preparation). The normalization and subtraction procedures for the isopigments present in the irradiated samples are similar to that for chicken green except for the differences in the percentage and spectrum of the intermediate and isopigment.

bands. We then searched for such residue(s) in the sequences of rod and cone pigments and found that P189 in the region[4] connecting helices IV and V (Figure 1) met the criteria. Thus, we constructed a site-directed mutant of P189I of chicken green to examine whether this residue contributes these bands. The resulting difference spectrum clearly showed that the replacement of the proline with isoleucine (the corresponding residue in chicken and bovine rhodopsins) caused the absence of these bands but had little effect on the other bands (Figure 7). Therefore, we concluded that P189 in chicken green is situated near the retinal chromophore and undergoes a structural rearrangement upon isomerization of the retinal chromophore.

DISCUSSION

In the present study, we have measured the difference FTIR spectra between chicken green and its batho-intermediate and compared them with those from chicken and bovine rhodopsins. The spectra from chicken green exhibited three features different from those of rhodopsins. First, a bilobe pair of negative and positive amide I bands at 1663/1654 cm^{-1} was observed in chicken green but not in the rhodopsins. Second, a bilobe pair of bands due to the carboxyl C═O stretching of D83 was prominent in bovine and chicken rhodopsin but was not seen in chicken green in the batho/pigment difference spectra. Third, bands due to an environmental change near P189 were observed in chicken green. Because, in the transition from the pigment to its batho-intermediate, conformational changes in the protein are probably restricted to groups near the chromophore, it is reasonable to speculate that the differences in the FTIR spectra between chicken green and chicken or bovine rhodopsin originate from differences in the amino acid residues surrounding the chromophore. Recent studies using photoaffinity-labeled retinal clearly showed that the β-ionone ring of the chromophore did not change its position upon formation of batho-rhodopsin, and therefore, the protein changes should be localized in the region near the middle of the polyene chain and Schiff base of the chromophore (*32*). Recently, the three-dimensional structure of bovine rhodopsin has been determined by X-ray crystallography (*6*). Taken together with the high similarity in amino acid sequence between chicken green and rhodopsins, these results enable us to discuss the differences in the FTIR spectra more precisely at the level of amino acids.

The Local Structure of Chicken Green is Different from That of Rhodopsin. Our observations of the unique bands due to the environmental changes in P189 showed that P189 is situated near the chromophore in chicken green. The corresponding residue in bovine rhodopsin is isoleucine. The important finding from the analysis of the three-dimensional structure of rhodopsin (*6*) is that the residues S186 to D190 including I189 (β4) form an antiparallel β-sheet with residues R177 to E181 (β3) and β4 covers the retinal chromophore from the extracellular side. The change from isoleucine to proline in chicken green might cause the destabilization of the antiparallel β-structure, resulting in a flexible conformation in the region. In fact, our preliminary experiments indicated that the P189I mutant of chicken green is more stable than that of wild-type chicken green (Imai et al., manuscript in preparation). Therefore, we speculated that the amino acid residue at position 189 is one of the determinants responsible for the protein stability of cone visual pigments.

Takeuchi and Harada reported that the imide II vibration of the X-Pro bond is greatly affected by hydrogen bonding at the imide C═O group but not affected by the trans-cis isomerization about the X-Pro bond (*33*). The frequency varies from ca. 1445 cm^{-1} in the non-hydrogen-bonding state to ca. 1485 cm^{-1} in a strongly hydrogen-binding state, while it appears around 1475 cm^{-1} upon hydrogen bonding with the solvent water molecules. According to the rhodopsin structure, the carbonyl oxygen of G188 is located at 2.7 Å from the nitrogen atom of I179 in the antiparallel β-sheet, implying a strong hydrogen bond between them. The frequencies of chicken green and its batho-intermediate at 1484 and 1477 cm^{-1} indicate that the C═O group of G188 forms a strong hydrogen bond and that photoisomerization perturbs its hydrogen bond. The strong hydrogen bond of the C═O group of G188 in rhodopsin is fully consistent with the present FTIR result for G188 in chicken green as well as in chicken blue (Figure 6). Interestingly, the imide II vibration in chicken red is much lower in frequency (1471

[4] According to the recently reported three-dimensional structure of rhodopsin (*6*), residues including I189 in this region form one of the strands of antiparallel β-sheet and are close to the chromophore. Absorption maximum of P189I mutant of chicken green is slightly blue-shifted (500 nm) from the wild-type chicken green (508 nm), suggesting that the residue perturbs the chromophore.

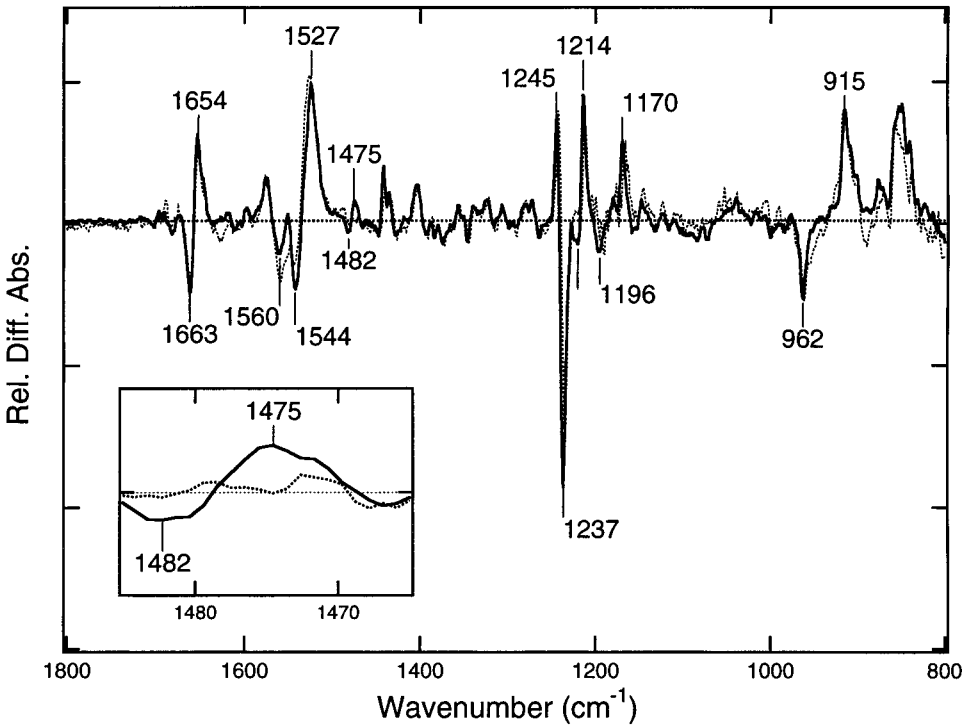

FIGURE 7: The normalized batho/original difference FTIR spectra in chicken green and its P189I mutant. The solid and dotted lines are the normalized spectra of wild-type chicken green and its mutant P189I, respectively. Inset: The normalized difference spectra in the imide II region.

cm^{-1}), and photoisomerization strengthens the hydrogen bond of the C═O group of G188. Such a difference may originate from the specific structure of the L-group to bind a chloride ion (*34*−*36*).

From the FTIR spectroscopy of rhodopsin mutants, we suggested the presence of a water molecule between D83 and G120, and this water molecule forms a hydrogen bonding network with these residues (*31*). The rhodopsin structure also indicated the presence of these interactions (*6*). Thus, the isomerization signal of the chromophore in rhodopsins propagates to D83 through G120 and the water molecule. On the other hand, chicken green contains the conserved D83 and G120, but it did not exhibit the environmental change in D83. These results suggest that there are some differences in microscopic structure near D83 between chicken green and rhodopsin. A similar band shape of the water O−H region between chicken green and the D83N/G120A double mutant of bovine rhodopsin supports this speculation. According to the three-dimensional structure of bovine rhodopsin, this region contributes to stabilization of the helix packing; therefore, it is possible that the difference in the local structure in this region affects the stability of the protein moiety and the intermediate(s).

In the amide region, chicken green showed only a regular α-helical band in the difference spectrum with the batho-intermediate, while rhodopsin showed bands in a variable frequency region. At present, there may be two possible regions that account for the change. One is the second helix region where two glycines are tandemly situated (G89 and G90) in rhodopsins, but one of the glycine (G89) changes to phenylalanine in chicken green. In rhodopsins, the presence of two glycines causes some distortion of the peptide backbone and forms a kink structure around these residues (*6*), while in chicken green, the distortion may be recovered by the replacement of glycine with phenylalanine. This region was thought to be situated near the chromophore by the fact that the glutamic acid artificially introduced at position 90 acts as a counterion in bovine rhodopsin (*37*, *38*) and was then confirmed by X-ray crystallography (*6*). Therefore, replacement of glycine with phenylalanine causes a disappearance of the kink structure in this region, resulting in the appearance of the amide I bonds in chicken green.

The other region may be around A299. In bovine rhodopsin, the peptide carbonyl of A299 interacts with N55 and/or D83 so that the helix structure in this region is greatly distorted (*6*). Thus, the introduction of a serine residue instead of alanine in chicken green might cause changes in the hydrogen-bond network around the region and could form a regular α-helix in this region, thereby resulting in the presence of the amide I signal upon isomerization of the chromophore in chicken green.

In other cone pigments, chicken blue shows a green-like amide I-band shape (Hirano et al., manuscript in preparation), while chicken red showed bands in a broad frequency region (*36*). Chicken red shows a specific behavior of the batho-intermediate, namely, the formed batho-intermediate decays to the original pigment rather than the next lumi-intermediate at low temperature. Thus, the amide I bands observed in chicken red might reflect an unique structure related to the special property. On the basis of the investigation of the difference in microscopic structure between chicken green and rhodopsins, we could speculate the mechanisms and amino acid residues responsible for the difference in properties between rod and cone. Further FTIR experiments on rod and cone pigments in combination with site-directed mutagenesis will shed light on the complete understanding of the mechanisms and the residue(s) responsible for the differences in properties between rod and cone pigments.

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