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FTIR study of color and twilight visual pigments

色覚および薄明視に関わる視物質の赤外分光研究

2013
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Chapter 1

1-1 Introduction and Background

Humans have two kinds of vision: twilight vision mediated by rhodopsin (Rh) in rod photoreceptor cells and color vision achieved by multiple color pigments in cone photoreceptor cells (1) (Figure 1-1). Humans possess three color pigments: red-, green-, and blue-sensitive proteins maximally absorbing at 560, 530, and 425 nm, respectively (2) (Figure 1-1), and specific perception of light by the RGB sensors is the origin of color vision.

All visual pigments share common structural motif of apoprotein (opsin) and contain a common chromophore molecule, 11-cis-retinal (Figure 1-2). The opsin apoprotein consists of a ~40kDa polypeptide domain folded into seven transmembrane (TM) helical segments. An 11-cis-retinal binds covalently to a conserved lysine residue on TM helix 7 through a protonated Schiff base (PSB) bond (3-5). An 11-cis-retinal PSB model compound dissolved in an organic solvent such as ethanol has $\lambda_{\text{max}}$ at ~440 nm. However, the $\lambda_{\text{max}}$ of the PSB-linked chromophore in visual pigments range from ~400 to 600 nm due to different chromophore-protein interactions. Such specific chromophore-protein interactions modulate the ground-to-excited state ($S_0$-$S_1$) electronic transition energy of the retinal PSB (Figure 1-3). The difference in the $\lambda_{\text{max}}$ between a visual pigment and PSB in solution has been called the “opsin shift” (6): (1) a difference in the strength of the electrostatic interaction between the PSB group of the chromophore and its counterion or hydrogen-bond acceptor (7, 8); (2) a replacement of full or partial charges along the polyene (8, 9); and (3) an alteration in the polarity or polarizability of the environment of the chromophore-binding site (10, 11).

The retinal chromophore and its protein environment have been studied most extensively in bovine Rh using site-directed mutagenesis and biophysical characterization of expressed recombinant pigments. The key residue in defining the chromophore-binding pocket in Rh is the glutamic acid at position 113 on TM helix 3.
Figure 1-1  Photoreceptors. Visible absorption spectra of primate blue, green, red color visual pigments and rhodopsin, the rod pigment. Rhodopsin that mediates twilight vision in the primate eye absorbs at 498 nm, while three color visual pigments in primate cone cells are responsible for trichromatic (color) vision peal at 425 (blue), 530 (green), and 560 nm (red), and thus constitute the basis for color vision.
Figure 1-2  Top: Crystallographic structure of bovine Rh (PDB; 1U19 (27)). Bottom; Retinal chromophore. All visual pigments contain a common chromophore molecule, an 11-cis-retinal. 11-cis-retinal binds covalently to a conserved lysine residue on TM helix 7 through a protonated Schiff base (PSB) linkage (3-5).
Figure 1-3 Mechanism of color tuning. The inherent conjugation of the retinal polyene chain allows delocalization of the positive charge of the protonated Schiff base (PSB). The absorption spectrum of rhodopsin represents a difference in energy between the ground state ($S_0$) and the first excited state ($S_1$). In the $S_0$-$S_1$ transition, charge distribution significantly changes as in the figure. Since a net positive charge appears at $\beta$-ionone ring in the $S_1$ state, hydroxyl groups near the ring stabilize the $S_1$ state, but not the $S_0$ state, leading to a spectral red shift.
Glu113 is ionized in the ground state of Rh and functions as the counterion to stabilize the positive charge of the PSB chromophore (12-14) (Figure 1-2). Most of the opsin shift in Rh (i.e., the shift from 440 nm to 500 nm) can be explained by weaker electrostatic interaction resulting from greater separation between the PSB group and the counterion compared with that of retinal PSB model compounds in solution (15). The mechanism of the opsin shift between green- and red-sensitive visual pigments have been addressed in detail. Old world primates, including humans, acquired green and red pigments, both of which belong to the L (long-wavelength absorbing) group, by gene duplication (1) (Figure 1-4a). They exhibit ~30 nm difference in the $\lambda_{\text{max}}$ and have 15 amino acid sequence difference (2) (Figure 1-4b). Previsously, the replacement of seven of these 15 variant residues in the MG sequence by those in the MR sequence is necessary to shifts the $\lambda_{\text{max}}$ of the hybrid green pigment all the way to 560 nm (16). However, substitutions of three specific polar amino acids at TM helices 4 and 6 (A180S, F277Y, A285T) account for the majority of this ~1000 cm$^{-1}$ opsin shift (17, 18). The substitutions of these polar residues at analogous positions in bovine Rh (A169S, F261Y, A269T) also shifts its $\lambda_{\text{max}}$ to the red by ~700 cm$^{-1}$ (19). To elucidate the color tuning mechanism, theoretical calculations are important (20-24), and homology modeling based on the Rh structure is also useful (25, 26), but experimental structural data are required for further understanding. In fact, on the molecular level, studying Rh is highly advantageous because large amounts of protein can be obtained from vertebrate and invertebrate native cells. Consequently, X-ray structures of bovine (27) and squid (28) Rh were determined. In the case of bovine Rh, the structures have been further determined for photointermediates (29, 30), the active state (31, 32) and the active state complexed with the C-terminus peptide of the $\alpha$ subunit of G-protein (33). These structures provided insights into the mechanism of the chromophore-protein interaction and activation. On the other hand, structural studies of color pigments lag far behind those of Rh, and none of color pigments was crystallized. The structural analysis on
Figure 1-4  (a) A phylogenetic tree of visual pigments constructed on the basis of the amino acid identity. The tree is represented with branch lengths calculated from the evolutionary distances (k). (b) Comparison of amino acid sequences of MR and MG. Residues common to MR and MG are denoted by single letters in the circle and the residues that are different between MR and MG are denoted at the upper- and bottom-sides of the circle, respectively.
the green and red pigments was only reported by resonance Raman spectroscopy, where the observed vibrational bands were very similar between human green and red, indicating similar chromophore-protein interactions (34).

It should be noted that resonance Raman spectroscopy provides only vibrational signals from the chromophore, but not from the protein. Involvement of water dipoles was discussed (34), but no experimental evidence has been obtained so far. In contrast, IR spectroscopy is able to provide vibrational signals not only from chromophore, but also from protein and water molecules (35) (Figure 1-5). In previous paper, difference FTIR spectra of the chicken red-sensitive pigment that were prepared from >2000 chicken retinas was reported (36), but identification of vibrational bands of protein is difficult for native proteins.

I thus attempted to express MG and MR in HEK293 cell lines for structural analysis using Fourier Transform InfraRed spectroscopy (FTIR spectroscopy). As Kandori’s group reported earlier, light-induced difference FTIR spectra of visual and archaeal rhodopsins at 77 K gave the insight on the changes in vibrational modes of the retinal chromophore and surrounding protein and water molecules (35, 37). Thus, information on local structural perturbation of protein upon retinal photoisomerization can be obtained. In addition, the information on hydrogen bonds is provided from the frequency region of 4000-1800 cm\(^{-1}\) that monitors X-H stretching vibrations. The measurements in D\(_2\)O further isolate H-D non-exchangeable and exchangeable vibrations at 4000-2700 and 2700-1800 cm\(^{-1}\), respectively (37-39). In this thesis, I showed the FTIR spectral comparison of MG and MR. I used 40-times larger cultures of MG and MR than of monkey Rh, because the expression level was much lower for the former two. Each protein expressed in HEK293 cell lines, was solubilized by a detergent, purified by antibody column, and reconstituted into PC liposomes. In addition, on the basis of the highly accurate FTIR spectra, I identified protein-bound water molecules of MG and MR, so the role of protein-bound water molecules would be discussed on the basis of my FTIR observations. Moreover, I
Figure 1-5  Low-temperature FTIR spectroscopy by use of Bio-Rad FTS-40 FTIR spectrometer. A 30-50 μL aliquot of the sample was dried on a BaF$_2$ window with the diameter of 18 mm. After hydration by 1 μL of H$_2$O, D$_2$O or D$_2^{18}$O, the sample was placed in a cell and then mounted in an Oxford DN-1704 cryostat.
applied FTIR measurement to several mutant pigments, which are considered to be important for color regulation. These comprehensive structural analysis of primate color pigments will enable us to discuss about color tuning mechanism on the molecular level in details.

On the molecular level, studying Rh is progressing, unlike color pigments. However, dynamical analysis during activating G protein upon retinal photoisomerization in native condition is incoming challenging problem because crystal structure is obtained motionless state of protein consistently. On the other hand, previously, an interesting observation was reported on opsin (the protein moiety without retinal) using light-induced difference FTIR spectroscopy by Vogel and Siebert (40). The techniques of Vogel and Siebert for obtained difference spectra between opsin and Rh allowed important things because hydroxyalmine was used to extract the retinal chromophore from opsin (41). Hydroxyamine has been a useful reagent in the structure-function study of Rh, because it interacts with the retinal Schiff base and forms a retinal oxime. Since oxime has its absorption maximum at about 360-370 nm, Rh loses its color upon formation of retinal oxime. The Schiff base is not reactive to hydroxylamine in the dark, which is consistent with the well-protected retinal binding site (27). In contrast, upon illumination, all-trans retinal oxime forms easily, resulting in the loss of color. This suggests that activation of Rh creates a specific reaction channel for hydroxylamine, or loosens the chromophore binding pocket. So that, I studied hydroxylamine effects on the photoactivated Rh by FTIR spectroscopy in this thesis.
1-2 Materials and Methods of expression for color visual pigments

The cDNA of MG and MR were tagged by the Rho1D4 epitope sequence and introduced into expression vectors pcDLSRa296. Point mutations were introduced using the kits QuikChange (Stratagene). All coding regions were sequenced by the dideoxy termination method. Plasmids were purified by means of a large-scale ion-exchange column (QIAGEN) and transfected into HEK293T cells a suspension-adapted variant of a human embryonic kidney cell line using the calcium phosphate method (42) (Figure 1-6). Color visual pigments were harvested 48 hr after the transfection because the amounts of expressed pigments were reduced during incubation from 48 to 72 hr. The cells were collected by washing the plates with phosphate-buffered saline (PBS) containing 5 mM EDTA. The cells were centrifuged at 1000 g for 10 min, and resuspended with buffer A [50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)] (43, 44).

All manipulations involving 11-cis-retinal were performed under dim red light. 11-cis-retinal (stored dissolved in ethanol in the dark at 193 K) was added to the opsins solution and incubated >30 min at 277 K. Then the regenerated pigments were extracted with buffer B [2 % (w/v) n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)] and quantitated the amount of pigments by hydroxylamine bleached experiment using UV-visible absorption spectra recording spectrophotometer (V-650, JASCO, Tokyo, Japan). Hydroxylamine was added to 10 mM to convert free retinal to retinal oxime (45). Spectra were recorded before and after photobleaching (Figure 1-7). After that, pigments were purified by absorption on an antibody-conjugated column and eluted with buffer C [0.06 mg/mL 1D4 peptide, 0.02 % n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)]. For the FTIR analysis, solubilized samples were reconstituted into phosphatidylcholine liposomes with a 100-fold molar excess.
Figure 1-6  (a) Culture room for expression of color pigments, which includes microscope, CO₂-incubator, and clean bench.  (b) Experiment of cell split.  (c) Image of HEK293 cells.
Figure 1-7  Typical absorbance spectra before and after photobleaching of a Rh expressed in HEK293T cells. The black curve in the visible region is before photobleaching, to contrast the red curve in the visible region is after photobleaching. The large absorbance in the ultraviolet region is due to retinal oxime. The inset corresponds to difference spectra between before and after photobleaching of Rh (black line) and MR (red line).
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Chapter 2

An FTIR Study of Monkey Green- and Red-Sensitive Visual Pigments

Introduction

Humans have two kinds of vision: twilight vision mediated by Rh in rod photoreceptor cells and color vision achieved by multiple color pigments in cone photoreceptor cells (1). Humans possess three color pigments: red-, green-, and blue-sensitive proteins maximally absorbing at 560, 530, and 425 nm, respectively (2), and specific perception of light by the RGB sensors is the origin of color vision. Rh and color-pigments both contain a common chromophore molecule, 11-cis retinal, whereas different chromophore-protein interactions allow preferential absorption of different colors (3). On the molecular level, studying Rh is highly advantageous because large amounts of protein can be obtained from vertebrate and invertebrate native cells. Consequently, X-ray structures of bovine (4) and squid (5) rhodopsins were determined. In the case of bovine Rh, the structures have been further determined for photointermediates (6, 7) and the active-state complexed with the C-terminus peptide of the α subunit of G-protein (8). These structures provided insights into the mechanism of the chromophore-protein interaction and activation. On the other hand, structural studies of color pigments lag far behind those of Rh. In fact, none of color pigments was crystallized.

Catarrhini, including Old World monkeys and Hominoids, acquired green and red pigments, both of which belong to the L (long-wavelength absorbing) group, by gene duplication (1). They exhibit ~30 nm difference in the $\lambda_{\text{max}}$ and have 15 amino acid sequence difference (2). Figure 2-1a illustrates the chromophore and surrounding 27 amino acids (within 5 Å) in bovine Rh. While monkey Rh has identical amino acids, about half of them are replaced in MG and MR (Figure 2-1b). E113 is the common counterion of the protonated Schiff base, but E181 is replaced by histidine that functions as a chloride binding site in the L group (9). Between MG and MR, 3 amino acids are different near the retinal
Figure 2-1  (a) X-ray crystallographic structure of the chromophore-binding site of bovine Rh (Protein Data Bank entry: 1U19 (4)), which is viewed from the helix VI side. The upper and lower regions correspond to the extracellular and cytoplasmic sides, respectively. The retinal chromophore, which is bound to Lys296, is shown by yellow space-filling model. Side chains of the 27 amino acids within 5 Å from the retinal chromophore are shown by stick drawings, though some residues behind the retinal are hidden. Ribbon drawings illustrate the secondary structures around the retinal. Corresponding amino acids in MG and MR are identical except for 3 amino acids shown by orange boxes.  (b) Partial amino acid sequences of Rh (bovine and monkey), MG and MR. 27 amino acids within 5 Å from the retinal chromophore in bovine Rh are shown. The amino acids are identical between bovine and monkey Rh. The 3 different amino acids between MG and MR are highlighted orange. The residue numbers are based on the bovine Rh sequence.  E113 is the common counterion of the protonated Schiff base, and E181 is replaced by H that functions as a chloride binding site in the L group.
chromophore, where O-H bearing residues are introduced in MR such as Ser, Tyr, and Thr (Figure 2-1b). This is also the case in human green and red pigments. This strongly suggests that these hydroxyl groups are responsible for the different $\lambda_{\text{max}}$ between green and red. This hypothesis was indeed confirmed by the previous site-directed mutagenesis (10-12), whereas distant hydroxyl groups are also responsible for color tuning (12). To elucidate the color tuning mechanism, theoretical calculations are important (13-17), and homology modeling based on the Rh structure is also useful (18, 19), but experimental structural data are required for better understanding. The structural analysis on the green and red pigments was only reported by resonance Raman spectroscopy, where the observed vibrational bands were very similar between human green and red, indicating similar chromophore-protein interactions (20). It should be noted that resonance Raman spectroscopy provides only vibrational signals from the chromophore, but not from the protein. Involvement of water dipoles was discussed (20), but no experimental confirmation has been obtained so far. In contrast, IR spectroscopy is able to provide vibrational signals not only from chromophore, but also from protein and water molecules (21). We previously reported difference FTIR spectra of the chicken red-sensitive pigment that were prepared from >2000 chicken retinae (22), but identification of vibrational bands of protein is difficult for native proteins.

I thus attempted to express MG and MR in HEK293 cell lines for structural analysis using FTIR spectroscopy. As we reported earlier, light-induced difference FTIR spectra of visual and archaeal rhodopsins at 77 K report on the changes in vibrational modes of the retinal chromophore and surrounding protein and water molecules (21, 23). Thus, information on local structural perturbation of protein upon retinal photoisomerization can be obtained. In addition, the information on hydrogen bonds is obtainable from the frequency region of 4000-1800 cm$^{-1}$ that monitors X-H stretching vibrations. The measurements in D$_2$O further isolate H-D non-exchangeable and exchangeable vibrations at 4000-2700 and 2700-1800 cm$^{-1}$,
respectively (23-25). In the present study, I report the FTIR spectral comparison of MG and MR. I used 40-times larger cultures of MG and MR than of monkey Rh, because the expression level was much lower for the former two. Each protein expressed in HEK293 cell lines, was solubilized by a detergent, purified by antibody column, and reconstituted into PC liposomes. Since the sample amounts for FTIR spectroscopy were very limited, I was not able to optimize the preparation conditions, and I followed the methods applied for bovine Rh (26, 27). In the present study, the FTIR signals for MR were smaller than those for MG, which yielded noisier spectra for X-H and X-D stretching frequencies. Nevertheless, I was able to obtain the difference spectra of MG and MR in the entire mid-IR region.

Materials and Methods

Sample Preparation. The cDNAs of MG, MR and Rh were tagged by the Rho1D4 epitope sequence and introduced into expression vectors pcDLSRα296. They were expressed in the HEK293T cell line and regenerated with 11-cis-retinal as previously reported (25-27). The reconstituted pigments were extracted with buffer A [2 % (w/v) n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3mM MgCl₂ (pH 6.5)] and purified by adsorption on an antibody-conjugated column and eluted with buffer B [0.06 mg/mL 1D4 peptide, 0.02 % n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3mM MgCl₂ (pH 6.5)]. For the FTIR analysis, solubilized samples were reconstituted into phosphatidylcholine liposomes with a 100-fold molar excess.

FTIR Spectroscopy. A 30 μL aliquot of the pigments suspension, which contains 0.3 mg protein of MR, MG or Rh, was deposited on a BaF₂ window with a diameter of 18 mm and dried in the glass vessel that was evacuated by an aspirator. The dry film was then hydrated by placing, 1 μL of water (H₂O) or heavy water (D₂O) next to the film. The sample was sealed by use of another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The film sample was cooled about 10 min after hydration.
The experimental setup was the same as described previously (28-30), where the cryostat was mounted in a Bio-Rad FTS40 FTIR spectrometer. The cryostat was connected with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The FTIR spectra were recorded with 2 cm⁻¹ resolution and constructed from 128 interferograms. For the formation of bathointermediates, the samples were irradiated with 543, and 501 nm light (by use of an interference filter) for MR, and MG, respectively at 77 K (30). For the reversion from bathointermediates to the original states, the samples were irradiated with >660, >610, and >610 nm light, respectively. For each measurement, 128 interferograms were accumulated, and 200, 64, and 24 recordings were averaged for MR, MG and Rh, respectively. Reproducibility of the difference FTIR spectra was confirmed by different sample preparations. Linear dichroism experiments revealed random orientation of the pigments molecules in the film, so I have not applied polarized FTIR measurements.

**Results and Discussion**

Figure 2-2 compares light-induced difference FTIR spectra measured at 77 K in D₂O. Formation of the bathointermediate is clear from the down-shifted ethylenic C=C stretches of the retinal chromophore at 1561 (-)/1536 (+), 1534 (-)/1509 (+), and 1527 (-)/1500 (+) cm⁻¹ for monkey Rh, MG, and MR, respectively, which correspond to the red-shift in the visible region. The spectra of MG and MR are very similar (Figure 2-2a), but it should be noted that the spectra are also similar to that of monkey Rh (Figure 2-2b). The reason is probably that vibrational signals of the retinal chromophore dominate in Figure 2-2, such as C=C stretch at 1570-1500 cm⁻¹, C-C stretches at 1250-1150 cm⁻¹, and hydrogen-out-of-plane vibrations at 1000-800 cm⁻¹. Between MG and MR, the vibrations due to the retinal chromophore were similar, being consistent with the previous resonance Raman results (20). On the other hand, a clear spectral difference was seen in the amide-I region. MG possesses the bands at 1665 (-)/1659 (+) cm⁻¹, which are absent in MR (Figure 2-3). Since the frequency
Figure 2-2  Light-induced difference FTIR spectra of monkey green (green dotted lines in a,b), monkey red (red line in a) and monkey rhodopsin (black line in b) in the 1770-800 cm\(^{-1}\) region. The spectra are measured at 77 K in D\(_2\)O. Positive and negative bands originate from the bathointermediate and unphotolyzed states, respectively. The obtained spectra of monkey red, green, and rhodopsin were scaled by 1, 0.41, and 0.2, respectively. One division of the y-axis corresponds to 0.0006 absorbance unit.
Figure 2-3 Light-induced difference FTIR spectra of monkey green (green dotted line) and monkey red (red line) in the 1690-1630 cm$^{-1}$ region. This figure is enlarged from Figure 2-2a. One division of the y-axis corresponds to 0.00005 absorbance unit.
is characteristic of $\alpha_{II}$-helix (31), retinal isomerization accompanies helical structural perturbation in MG, but not in MR.

Although the difference FTIR spectra are similar for the 3 pigments in the 1770-800 cm$^{-1}$ region, the situation is entirely different in the X-D (Figure 2-4) and X-H (Figure 2-5) stretching regions. H-D exchangeable vibrations such as O-D and N-D stretches appear at 2700-2000 cm$^{-1}$ in D$_2$O. The spectral features are identical between MG and MR, though the latter was noisier (Figure 2-4a). In contrast, they are very different between MG and Rh (Figure 2-4b). These facts indicate that hydrogen-bonding network involving water molecules is similar between MG and MR, but different in Rh. One noticeable band is a sharp positive peak at 2210 and 2214 cm$^{-1}$ in MG and MR, respectively, which is absent in monkey Rh. It may originate from (i) N-D or O-D stretch of protein, or (ii) N-D stretch of the retinal Schiff base. The negative bands at 2670 and 2583 cm$^{-1}$ appear at the characteristic frequencies of water O-D stretches (25), and lower frequency shifts correspond to formation of stronger hydrogen bond. If 3 hydroxyl groups belong to the retinal binding pocket in MR, but not in MG, one may expect their vibrational bands only in MR. Nevertheless, we did not observe MR specific bands in the X-D stretch region.

Spectral comparison in the X-H stretch region (Figure 2-5) led to the same conclusion as for the X-D stretch (Figure 2-4) in view of the similarity between MG and MR, but not to Rh. The sharp peaks at 3485 (+)/3467 (-) cm$^{-1}$ in monkey Rh (Figure 2-5b) are similar in frequency to those at 3487 (+)/3463 (-) cm$^{-1}$ in bovine Rh (32), thus probably originating from an O-H stretch of Thr118. Similarly, the peaks at 3485 (+)/3428 (-) cm$^{-1}$ in MG and MR are likely to originate from an O-H stretch of the corresponding Ser (Figure 2-1b). Although MG and MR exhibit similar bands in the X-H stretch region, a negative band at 3359 cm$^{-1}$ in Figure 2-5a was only observed for MR, not for MG. This band was noisy, but reproduced for 3 independent measurements. A possible candidate for the 3359-cm$^{-1}$ band is one of the 3 amino acids possessing O-H groups (orange box in Figure 2-1b). We previously identified
Figure 2-4 Light-induced difference FTIR spectra of monkey green (green dotted lines), monkey red (red line) and monkey rhodopsin (black line) in the 2750-1900 cm\(^{-1}\) region. The spectra are measured at 77 K in D\(_2\)O. The scaling factors are the same as in Figure 2-2. One division of the y-axis corresponds to 0.000035 absorbance unit.
Figure 2-5  Light-induced difference FTIR spectra of monkey green (green dotted lines), monkey red (red line) and monkey rhodopsin (black line) in the 3630-3150 (bottom panel) cm$^{-1}$ region. The spectra are measured at 77 K in D$_2$O. The scaling factors are the same as in Figure 2-2. One division of the y-axis corresponds to 0.00005 absorbance unit.
O-H stretching frequencies of Thr at 3500-3300 cm\(^{-1}\) in bacteriorhodopsin (24) and pharaonis phoborhodopsin (33), and the frequency at 3359 cm\(^{-1}\) suggests considerably strong hydrogen bond for an O-H stretch. Thus, the MR specific X-H stretch may be the key to understand the unique chromophore-protein interaction in the red pigment. It should be noted that position of the corresponding positive peak is not clear because of the overlap with a noisy spectral feature. Therefore, future FTIR analysis by use of mutant proteins will be needed to provide more detailed information.

In summary, I report the first FTIR spectral comparison of the green and red sensitive color visual pigments in the L group. For this aim, I established the sample preparation procedure based on the HEK293 cell line. The obtained FTIR spectra of the color pigments were similar to those of Rh in the conventional 1800-800 cm\(^{-1}\) region, whereas the spectra were entirely different in the X-D (2700-2000 cm\(^{-1}\)) and X-H (3800-2800 cm\(^{-1}\)) stretching regions. In addition, some spectral differences between MG and MR were observed. Since X-H and X-D stretches are the direct probes of hydrogen-bonding environment, the present FTIR study opened a new window to understanding of the specific chromophore-protein interaction in color pigments.
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Chapter 3
Protein-Bound Water Molecules in Primate Red- and Green-Sensitive Visual Pigments

Introduction
Humans have two kinds of vision: twilight vision mediated by Rh in rod photoreceptor cells and color vision achieved by multiple color pigments in cone photoreceptor cells (1). Humans possess three color pigments: red-, green-, and blue-sensitive proteins maximally absorbing at 560, 530, and 425 nm, respectively (2), and specific perception of light by the RGB sensors is the origin of color vision. Rh and color-pigments both contain a common chromophore molecule, 11-cis retinal, whereas different chromophore-protein interactions allow preferential absorption of different colors (3). On the molecular level, studying Rh is highly advantageous because large amounts of protein can be obtained from vertebrate and invertebrate native cells. Consequently, X-ray structures of bovine (4) and squid (5) Rh were determined. In the case of bovine Rh, the structures have been further determined for photointermediates (6, 7), the active state (8, 9) and the active state complexed with the C-terminus peptide of the α subunit of G-protein (10). These structures provided insights into the mechanism of the chromophore-protein interaction and activation. On the other hand, structural studies of color pigments lag far behind those of Rh, and none of color pigments was crystallized.

Old world primates, including humans, acquired green and red pigments, both of which belong to the L (long-wavelength absorbing) group, by gene duplication (1). They exhibit ~30 nm difference in the $\lambda_{\text{max}}$ and have 15 amino acid sequence difference (2). Previous site-directed mutagenesis studies revealed that 3 amino acids are mainly responsible for color discrimination between green and red (11-13). This finding suggests that these side chains may be the direct determinants of color, or these groups may perturb the chromophore-protein interaction indirectly. Involvement of distant groups for color tuning is also reported (13).
Another important element in color tuning may be protein-bound water molecules that can alter the dielectric environment of the retinal chromophore. We have shown an important role of H-bonds of protein-bound water molecules in proton-pumping microbial rhodopsins (14). In fact, proton transfer via a transient linear water-molecule chain in a membrane protein, especially microbial rhodopsins were discussed recently (15). Protein-bound water molecules to Rh are experimentally monitored by X-ray crystallography (4, 5), FTIR spectroscopy (16-18) and radiolitic labeling method (19, 20). Structural analysis of the protein-bound waters in our color visual pigments is thus intriguing, though indeed challenging.

I recently succeeded the first structural analysis of MR and MG using low-temperature Fourier-transform infrared (FTIR) spectroscopy (21). The obtained FTIR spectra of MR and MG in D₂O at 77 K were similar to those of Rh in the conventional 1800-800 cm⁻¹ region, suggesting that the chromophore structure and light-induced structural changes of chromophore upon retinal photoisomerization are similar among these visual pigments. In contrast, the spectra were entirely different between color pigments and Rh in the X-D (2700-2000 cm⁻¹) and X-H (3800-2800 cm⁻¹) stretching regions (21), indicating structural difference of the protein side for color pigments. Successful detection of the accurate FTIR spectra in the X-D stretching region motivated us to identify protein-bound water molecules in MR and MG. How are water structures different between color pigments and Rh? Although we concluded that X-D stretching vibrations at 2700-1800 cm⁻¹, the frequency of protein-bound water molecules in D₂O, are similar between MR and MG, the reported spectra contain considerable noise, specially for MR (21). The detailed spectral analysis on water should be performed for less-noisy spectra. In this paper, based on the highly accurate FTIR spectra, I identify protein-bound water molecules of MR and MG, which are localized near the retinal chromophore because the protein environment is frozen at 77 K excepting for retinal which can be isomerized by irradiation. This means that the confirmed structural change correspond to only
around the retinal. The water signals differ not only from Rh, but also between MR and MG.

To identify the position of water molecules, we normally use mutant proteins. In fact, previous comprehensive FTIR study of various mutants revealed the position of protein-bound water molecules in bacteriorhodopsin (BR), a light-driven proton pump (22, 23). Unfortunately, such measurements are very difficult at present because of the low expression level of MR and MG (one hydrated film sample contains 0.3 mg protein, which corresponds to 300 culture plates of HEK293 cells). Instead, here I estimated the location of the water molecules using the measurements of the 9-cis form, which can be photochemically formed at 77 K. Role of protein-bound water molecules are discussed on the basis of the present FTIR observation.

Materials and Methods

Sample Preparation. The cDNAs of MR and MG were tagged by the Rho1D4 epitope sequence and introduced into expression vectors pcDLSR D296. They were expressed in the HEK293T cell line and regenerated with 11-cis retinal as previously reported (21, 24-26). The reconstituted pigments were extracted with buffer A [2 % (w/v) n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)] and purified by adsorption on an antibody-conjugated column and eluted with buffer B [0.06 mg/mL 1D4 peptide, 0.02 % n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)]. For the FTIR analysis, solubilized samples were reconstituted into phosphatidylincholine liposomes with a 100-fold molar excess, followed by suspension in buffer C [2 mM phosphate and 10 mM NaCl (pH 7.25)].

In the previous paper, all the procedures for MR and MG were performed under dim red light conditions using an R-69 filter (>670 nm) (21). The dim red light does not bleach MG, but MR is possibly bleached, because we were able to see our surroundings under the light conditions. MR and human red-sensitive pigment possess identical absorption, so that both absorb the light. In fact, spectral change in
MR was smaller than in MG in the previous paper, though the expression levels were similar (0.3 mg protein from 300 culture plates of HEK293 cells) (21). This fact suggests considerable bleaching of MR during sample preparation, which yields noisier FTIR spectra of MR than those of MG. Therefore, in the present study, I used night-vision goggle (NOG-7-2, Night Optics, USA) for the sample preparation of MR.

**FTIR Spectroscopy.** A 30 μL aliquot of the pigments suspension, which contains 0.3 mg protein of MR or MG, was deposited on a BaF_2 window with a diameter of 18 mm and dried in the glass vessel that was evacuated by an aspirator. The dry film was then hydrated by placing, 1 μL of water (H_2O) or heavy water (D_2O or D_2^{18}O) next to the film. The sample was sealed by use of another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The film sample was cooled about 10 min after hydration.

The experimental setup was the same as described previously (16, 17, 21, 23), where the cryostat was mounted in a Bio-Rad FTS40 FTIR spectrometer. The cryostat was connected with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The FTIR spectra were recorded with 2 cm\(^{-1}\) resolution and constructed from 128 interferograms. For the formation of bathointermediates, the samples were irradiated with 543, and 501 nm light (by use of an interference filter) for MR, and MG, respectively at 77 K (21). For the reversion from bathointermediates to the original states, the samples were irradiated with >660, >610, and >610 nm light, respectively. On the other hand, for the reversion from bathointermediates to the Iso (9-cis retinal) states, the samples were irradiated with >560 and >530 nm light, respectively. For each measurement, 64 recordings were averaged to obtain the difference FTIR spectra between the all-trans minus 11-cis forms, and between the all-trans minus 9-cis forms. Reproducibility of the difference FTIR spectra was confirmed by different sample preparations. Linear dichroism experiments revealed random orientation of the pigments molecules in the film, so we have not applied polarized FTIR measurements.
Results and Discussion

Difference FTIR Spectra of the 11-cis and 9-cis Forms of MR and MG in the Low Frequency Region. Figure 3-1 shows the light-induced difference FTIR spectra of MR (a), the 9-cis form of MR (b), MG (c), and the 9-cis form of MG (d) in the 1800-800 cm\(^{-1}\) region measured at 77 K. The difference spectra of MR (a, b) and MG (c, d) in H\(_2\)O (red curves) are measured for the first time, while blue spectra in Figure 1a and c (hydrated with D\(_2\)O) reproduced those reported previously (21). In the difference spectra, negative signal originates from 11-cis or 9-cis forms, and positive signal originates from bathointermediate (Batho) possessing all-trans retinal. Formation of Batho state is clearly seen by the strong HOOP vibrations of the retinal chromophore at 900-800 cm\(^{-1}\). In addition, lowered C=C stretching band of retinal is characteristic of formation of Batho state, as seen for the pair bands at 1526 (-)/1500 (+) cm\(^{-1}\), 1538 (-)/1500 (+) cm\(^{-1}\), 1534 (-)/1509 (+) cm\(^{-1}\), 1536 (-)/1509 (+) cm\(^{-1}\) in Figure 1a, b, c and d, respectively. The negative 1207-cm\(^{-1}\) band in Figure 1b and d is assignable for C-C stretching vibration of the retinal chromophore characteristic of the 9-cis form (27).

I then measured light-induced difference FTIR spectra of MR and MG by using hydrated films of D\(_2\)\(^{18}\)O. The obtained spectra of MR (blue curve in Figure 3-2a) and MG (blue curve in Figure 3-2c) in D\(_2\)\(^{18}\)O are identical to those in D\(_2\)O (red curves in Figure 3-2) in the 1800-800 cm\(^{-1}\) region. In fact, double difference spectra of MR (Figure 3-2b) and MG (Figure 3-2d) are coincident with the baseline. Identical spectra between D\(_2\)O and D\(_2\)\(^{18}\)O indicate that water vibrations are not observable at 1800-800 cm\(^{-1}\). It should be noted that O-D bending vibration of water appears at \(~1200\) cm\(^{-1}\). This implies that bending vibration is less sensitive to H-bonding alteration between 11-cis and all-trans states, presumably because of small atomic motion of oxygen, which is highly contrast to the case for O-D stretching vibrations as shown below.
Figure 3-1 Light-minus-dark difference FTIR spectra of MR (a, b) and MG (c, d) in the 1800-800 cm\(^{-1}\) region, which are measured at 77 K in H\(_2\)O (red line) and D\(_2\)O (blue line). Positive bands originate from the all-trans form (bathointermediate; Batho), while negative bands originate from the 11-cis (a, c) or 9-cis (b, d) form. One division of the y-axis corresponds to 0.0006 absorbance unit.
Figure 3-2  Light-minus-dark difference FTIR spectra of MR (a) and MG (c) in the 1800-800 cm\(^{-1}\) region, which are measured at 77 K in D\(_2\)O (red line) and D\(_2^{18}\)O (blue line). Positive bands originate from the all-trans form (bathointermediate; Batho), while negative bands originate from the 11-cis form. Double difference FTIR spectra between D\(_2\)O (a, c; red line) and D\(_2^{18}\)O (a, c; blue line) of MR (b) and MG (d) in the 1800-800 cm\(^{-1}\) region. One division of the y-axis corresponds to 0.0012 absorbance unit.
Identification of Water Signal in MR and MG. Red curves in Figure 3-3a and b show the light-minus-dark difference FTIR spectra of MR and MG (11-cis form) in D$_2$O, respectively, in the 2750-1800 cm$^{-1}$ region measured at 77 K. In the spectra, positive and negative signals originate from all-trans (Batho) and 11-cis (unphotolyzed state) forms, respectively. As reported previously, the spectra are similar between MR and MG, though being entirely different from that of Rh (red curve in Figure 3-3c) (21). Nevertheless, the present measurements with the improved signal/noise ratio provide clear difference even between MR and MG. Blue curves in Figure 3-3 correspond to the measurements in D$_2$O$^{18}$O, and the spectral changes at 2700-2450 cm$^{-1}$ mostly originate from water stretching vibrations because of the downshift by isotope water.

The spectrum of MR in D$_2$O exhibits peaks at 2684 (+), 2671 (-), 2632 (-), 2612 (+), 2594 (-), 2583 (-), 2530 (+), 2517 (-), 2504 (+), 2474 (-), 2466 (+) and 2303 (+) cm$^{-1}$, which downshift by 5-17 cm$^{-1}$ in D$_2$O$^{18}$O (Figure 3-3a). Similarly, the spectrum of MG in D$_2$O exhibits peaks at 2682 (+), 2670 (-), 2646 (-), 2630 (+), 2621 (-), 2615 (+), 2611 (-), 2596 (+), 2583 (-), 2530 (+), 2524 (-), 2510 (+), 2479 (-), 2470 (+) and 2308 (+) cm$^{-1}$, which downshift by 5-17 cm$^{-1}$ in D$_2$O$^{18}$O (Figure 3-3b). If a water O-D stretch is composed of only a single O-D group, the expected isotope shift is 17 cm$^{-1}$ as for the bands at ~2670 cm$^{-1}$. Coupling with other vibrations reduces the magnitude of the isotope shift so that the observed shifts exhibit large variations.

From Figure 3-3a and b, MR shows 6 positive and 6 negative water bands, while MG shows 8 positive and 7 negative water bands. The numbers are comparable or slightly greater than those of bovine Rh (Figure 3-3c) (17). Negative bands for the 11-cis form appear at 2700-2450 cm$^{-1}$ for all MR, MG and bovine Rh. While these peaks seem to be distributed randomly, the averaged frequencies are 2579 and 2591 cm$^{-1}$ for MR and MG, respectively. Since MR and MG possess the $\lambda_{\text{max}}$ at 560 and 530 nm, respectively, it is likely that the red-shifted pigments possess protein-bound water molecules at low frequency, i.e. under strong H-bonds. Such correlation may be also applicable for bovine Rh ($\lambda_{\text{max}}$: 500 nm), because the averaged frequency is
Figure 3-3 Light-minus-dark difference FTIR spectra of MR (a), MG (b), and bovine Rh (c), which possess 11-cis retinal as the chromophore, in the 2750-1800 cm\(^{-1}\) region measured at 77 K. Red and blue lines represent the spectra in D\(_2\)O and D\(_2\)\(^{18}\)O, respectively, and green labeled frequencies correspond to those identified as water-stretching vibrations. The results for bovine Rh are reproduced from Ref. 16. The grayish curve in the 2700-2000 cm\(^{-1}\) region represents O-D stretching vibrations of D\(_2\)O at room temperature. The obtained spectra of MR, MG, and bovine Rh are scaled by 1, 1, and 0.28, respectively. One division of the y-axis corresponds to 0.00014 absorbance unit.
2600 cm\(^{-1}\) (17). It should be noted that the frequencies of internal water molecules are determined by their local environments, so that the obtained correlation may be accidental. Nevertheless, it is also possible that the observed averaged frequencies of water reflect from the dielectric environment of the chromophore binding site in MR, MG and Rh.

Positive bands for the all-tarns form after photoisomerization appear at 2700-2500 cm\(^{-1}\) in bovine Rh (Figure 3-3c). In contrast, the frequencies of the positive 2303-cm\(^{-1}\) and 2308-cm\(^{-1}\) bands for MR and MG, respectively, are much lower than the others, indicating that the water molecule forms a very strong H-bond. Since MR and MG do not have strongly H-bonded waters for the unphotolyzed state, photoisomerization yields spectral downshift of water stretching vibration at >150 cm\(^{-1}\). The frequencies are lower than that of pure deuterated water in the ice form (~2400 cm\(^{-1}\)), which forms a tetrahedral H-bonding network. This fact suggests the specific H-bonding donation of water as seen for the interaction with anions and/or charged amino acids. Among many studies of visual and microbial rhodopsins, we experienced similar observation only for halorhodopsin (HR) from Natronomonas pharaonis (28), whose frequencies of water are at 2700-2450 cm\(^{-1}\) for the unphotolyzed state, but a water band appears at 2263 cm\(^{-1}\) after photoisomerization. Interestingly, MR, MG and HR all possess a Cl\(^{-}\)-binding site near the retinal chromophore (28, 29). In the previous paper on HR, we discussed Cl\(^{-}\) is weakly hydrated by internal waters, but photoisomerization perturbs the local structure of the binding site, resulting in a strong H-bond of the water (28). It is possibly the case in MR and MG.

**Spectral Comparison of the Water Signal between the 11-cis and 9-cis Forms.** By using preferential illumination wavelengths at 77 K, we can selectively accumulate the 9-cis form (16, 30), and I established the experimental conditions for MR and MG in the present study. Figure 3-4a and b show the corresponding difference FTIR spectra of MR and MG, respectively, in the 2750-1800 cm\(^{-1}\) region, where the positive and negative sides are all-trans and 9-cis forms, respectively. As reported previously,
Figure 3-4  Light-minus-dark difference FTIR spectra for the 9-cis form of MR (a), MG (b), and bovine Rh (c) in the 2750-1800 cm\(^{-1}\) region measured at 77 K. Red and blue lines represent the spectra in D\(_2\)O and D\(_2\)\(^{18}\)O, respectively, and green labeled frequencies correspond to those identified as water-stretching vibrations. The results for bovine Rh are reproduced from Ref. 16. The grayish curve in the 2700-2000 cm\(^{-1}\) region represents O-D stretching vibrations of D\(_2\)O at room temperature. The obtained spectra of MR, MG, and bovine Rh are scaled by 1, 1, and 0.28, respectively. One division of the y-axis corresponds to 0.00014 absorbance unit. Note that no water band is observed at 2612 cm\(^{-1}\) in the difference between the all-trans and 9-cis forms of MR (a), whereas a positive band appears at 2612 cm\(^{-1}\) in the difference between the all-trans and 11-cis forms of MR (Fig. 3-3a). We thus interpret both all-trans and 9-cis forms of MR possess a water band at 2612 cm\(^{-1}\).
Figure 3-5  A; Light-minus-dark difference FTIR spectra of MR (top), MG (middle), and bovine Rh (bottom) in the 2710-2530 cm⁻¹ region measured at 77 K. Red and orange lines represent the spectra of Batho-minus-11-cis form, and Batho-minus-9-cis form in D₂O, respectively. Green labeled frequencies correspond to those identified as water-stretching vibrations for the spectra of Batho-minus-11-cis form in Figure 3-3. B; Double difference FTIR spectra of Figure 3-5A, which correspond to the 11-cis minus 9-cis form of MR (top), MG (middle), and bovine Rh (bottom) in the 2710-2530 cm⁻¹ region. Red and blue lines represent the spectra in D₂O and D₂¹⁸O, respectively. One division of the y-axis corresponds to 0.00011 absorbance unit.
the difference signals of water are identical for bovine Rh, between Batho and 11-cis form (Figure 3-3c) and between Batho and 9-cis form (Figure 3-4c) (17). This is not the case for MR and MG, specially at >2500 cm\(^{-1}\).

Figure 3-5A compares the all-trans/11-cis (red curves) and all-trans/9-cis (orange curves) difference FTIR spectra at 2710-2530 cm\(^{-1}\) for MR (top), MG (middle) and bovine Rh (bottom). As is clearly seen, both spectra are identical for bovine Rh, but considerably different for MR and MG. Red curves in Figure 3-5B represent the double difference spectra of Figure 3-5A, which correspond to the 11-cis/9-cis spectra. The spectrum in Figure 3-5B(bottom) coincides the baseline, indicating no difference between 11-cis and 9-cis forms of bovine Rh. On the other hand, Figure 3-5B (top) and (middle) show the peaks at 2710-2530 cm\(^{-1}\), which originate from water because of the downshift in D\(_2^{18}\)O (blue curves). This indicates that MR and MG possess water vibrations differing between the 11-cis and 9-cis forms, but not for Rh.

**DISCUSSION**

The present FTIR spectroscopic study identified the frequencies of the O-D stretching vibrations of internal water molecules in MR and MG, which is summarized in Figure 3-6. In the case of MR, I observed 6 O-D stretches for the original 11-cis form, while 6 and 7 O-D stretches were identified for the all-trans and 9-cis forms, respectively. In the case of MG, 7 O-D stretches were observed for the original 11-cis form, while 8 and 6 O-D stretches were identified for the all-trans and 9-cis forms, respectively. Regarding the slight discrepancy in numbers of water stretching vibrations, one observed band may be composed of multiple O-D stretches. In summary, the observed water vibrations are 6-8 for MR and MG, suggesting the presence of 3-8 water molecules near the retinal chromophore. In the case of bovine Rh, we reported 6 O-D stretches for all 11-cis, all-trans and 9-cis forms (17). Thus, color visual pigments possess essentially similar protein-bound water molecules, though numbers may be slightly more than those of Rh.

For bovine Rh, we reported the lack of water bands under strong H-bonding
**Figure 3-6** The observed O-D stretching vibrations of water molecule for MR (red) and MG (green). Top, middle, and bottom panels represent the results of all-trans, 11-cis and 9-cis forms, respectively. Identical frequencies of water between MR and MG represent as a unique marker, which illustrates the half color of red and green.
Figure 3-7 X-ray crystallographic structure of the chromophore-binding site of bovine Rh (Protein Data Bank entry: 1U19), which is viewed from the helix VII side. The upper and lower regions correspond to the cytoplasmic and extracellular sides, respectively. Three helices (Helix 3, 4 and 6) are illustrated by cylindrical drawing, and the retinal chromophore and side chains of several important amino acids are shown by stick drawing. Numbers of amino acids originate from bovine Rh and MR/MG (in parenthesis). Three important regions are highlighted; (i) the Schiff base moiety (Region I; yellow circle), (ii) Cl\(^{-}\)-binding site (Region II; purple circle), and (iii) β-ionone ring moiety (Region III; blue circle). According to the structure of bovine Rh, Region I, II and III contain one, three and one water molecules, respectively.
conditions (17), which is entirely different from the case of microbial rhodopsins. Our comprehensive FTIR analysis of microbial rhodopsins revealed that proton-pumping rhodopsins possess strongly H-bonded water molecules, whose O-D stretch is located at <2400 cm⁻¹ (3, 14). Many microbial rhodopsins like BR possess an electric quadrupole in the Schiff base region, and three water molecules participate in a roughly planar pentagonal cluster structure that must stabilize the quadrupole. In the case of BR, we identified strongly H-bonded water molecules being located at the pentagonal cluster (23). The functional role of such strongly H-bonded water molecules is to stabilize the H-bonding network in the unphotolyzed state, whose destabilization is used to store light energy upon retinal photoisomerization (14, 31). Therefore, strongly H-bonded water molecules are energetically important for proton-pumping rhodopsins. In contrast, absence of such water molecules under strong H-bonding conditions is common among Rh and color pigments.

Figure 3-7 illustrates the structure of bovine Rh (4), which includes three important regions of color pigments; (i) the Schiff base moiety (Region I), (ii) Cl⁻-binding site (Region II), and (iii) β-ionone ring moiety (Region III). Region I is composed of an ion pair of the protonated Schiff base and the counterion, where one water molecule forms a H-bond with Glu113 in bovine Rh. Water molecule is involved in the Schiff base region, whereas the absence of the bridged water between the Schiff base and counterion is consistent with no observation of strongly H-bonded water molecules in bovine Rh (17). Similar results for MR and MG in the present study suggest that the water structure near the Schiff base region in MR and MG is similar to that of bovine Rh.

Although there are no strongly H-bonded water molecules in MR and MG, a water band appears at ~2300 cm⁻¹ in MR and MG after retinal isomerization, which is not the case in Rh (Figure 3-3). This indicates that photoisomerization yields spectral downshift of water stretching vibration at >150 cm⁻¹ for MR and MG. Among many studies of visual and microbial rhodopsins, we experienced similar observation only
for a light-driven Cl⁻ pump HR (28). Therefore, the following interpretation for HR can be similarly applicable for MR and MG. According to the previous neutron diffraction study of aqueous hydrochloric acid solutions, water molecules around Cl⁻ take the configuration to orient the vector which bisects the D-O-D angle on a straight line joining an oxygen atom and Cl⁻ (32). Hydrogen bonds of water are weak under this configuration. On the other hand, photoisomerization in protein enforces the structural perturbation at the Cl⁻ binding site, causing the O-D group located on a straight line between the oxygen and Cl⁻, by which strong H-bond is formed in the K state or Batho for HR or MR/MG, respectively.

Figure 3-7 shows that His and Lys in Region II constitute the Cl⁻-binding site in both MR and MG (29). In the structure of bovine Rh, Glu181 is located at 4.8 Å to the C11 atom of the retinal chromophore, while the position of Gln184 is far distant. This suggests the Cl⁻-binding site in MR and MG, largely modified from Rh, whereas the location is probably along the polyene chain. It is likely that the retinal moiety near the C11 position and β-sheet region including His197 and Lys200 constitute the Cl⁻-binding site (Region II) in MR and MG. I infer that the water vibration at ~2300 cm⁻¹ is the result of retinal photoisomerization in the restricted protein environment.

In the case of HR, the counterpart of the strongly H-bonded water (OD stretch at 2263 cm⁻¹) in the unphotolyzed state is located at 2690-2400 cm⁻¹, being under weaker H-bonding condition. Regarding the counterpart of the strongly H-bonded water (~2300 cm⁻¹) in MR and MG, the results of Figure 3-5 are highly suggestive. Water bands at >2550 cm⁻¹ differ in frequency between the 11-cis and 9-cis forms, which is the case for MR and MG, but not for Rh (Figure 3-5). The straightforward interpretation is that MR and MG possess internal water molecules near the C11 and C9 position. Interestingly, X-ray structures of bovine Rh (4) and 9-cis Rh (33) showed no water molecules near the C11 and C9 position of the retinal chromophore. In contrast, as mentioned above, the Cl⁻-binding site is possibly located near the C11 position of the retinal chromophore (Region II in Figure 3-7). Thus, the results in
Figure 3-5 suggest that MR and MG possess internal water molecules near the C11 and C9 position. According to the X-ray structures of bovine Rh (4) and 9-cis Rh (33), the nearest atom of the retinal chromophore from Glu181 (Cl- binding His in MR and MG; Figure 3-7) is the C11 (4.8 Å) and C12 (4.9 Å) atoms, respectively. Previous FTIR study of chicken red-sensitive pigment suggests the Cl- binding site near the C14 atom of the retinal chromophore (34). Therefore, it is likely that Cl-binding site is located along the polyene chain, which is hydrated by internal water molecules in MR and MG, but not in Rh. More numerous water molecules in MR and MG than Rh may originate from the water molecules in this region (Region II).

Region III in Figure 3-7 contains the three amino acids differing between MR and MG, which has been believed to be important in color discrimination. In the structure of bovine Rh, they are in the hydrophobic environment near the β-ionone ring, containing only one water molecule near Phe261 (Figure 3-7).

How about color visual pigments such as MR and MG? Two water bands at 2550-2450 cm⁻¹ are coincident in frequency between the 11-cis and 9-cis forms (Figure 3-6), suggesting that the water molecules are located distant from the C9 and C11 position in MR and MG. Such water molecules may be located in Region III, though we cannot conclude at present.

Further experiments using mutant proteins are needed for clarification.

Finally it was unexpected that I observed correlation between the averaged frequencies of water and color of visual pigments. The averaged frequencies of water for MR, MG and bovine Rh are 2579, 2591 and 2600 cm⁻¹, respectively. The averaged frequencies of water in the 9-cis form are calculated to be 2561, 2586 and 2600 cm⁻¹ for MR, MG and bovine Rh, respectively. The averaged frequencies of water in Batho (the all-trans form) are calculated to be 2517, 2543 and 2608 cm⁻¹ for MR, MG and bovine Rh, respectively. Therefore, the red-shifted pigments possess protein-bound water molecules at low frequency for the three isomeric forms. What does this mean? Although protein-bound water molecules are probably distributed randomly, this correlation between the averaged water frequency and $\lambda_{\text{max}}$ may
suggest that water molecules participate in color tuning, whose mechanism is entirely unknown. Color tuning mechanism has been theoretically investigated on the basis of homology modeling of Rh (35-38), whereas role of internal water molecules has never been taken into account. The present observation of protein-bound water molecules in our color visual pigments thus stimulate further experimental and theoretical studies, leading to better understanding of the color tuning mechanism in our vision.
REFERENCES


Chapter 4

FTIR study of mutants of primate color pigments

Introduction

Humans possess three color pigments: red-, green-, and blue-sensitive proteins maximally absorbing at 560, 530, and 425 nm, respectively (1), and specific perception of light by the RGB sensors is the origin of color vision. Rhodopsin and color-pigments both contain a common chromophore molecule, 11-cis retinal, whereas different chromophore-protein interactions allow preferential absorption of different colors (2). Old world primates, including humans, acquired green and red pigments, both of which belong to the L (long-wavelength absorbing) group, by gene duplication (3). They exhibit ~30 nm difference in the absorption maxima and have 15 amino acid sequence difference (1).

On the basis of retinal analog studies (4, 5), calculations (6, 7), and sequence comparison (2, 8, 9, 10, 11), several chromophore-protein interaction have been postulated to regulate the opsin shift. These include four factors; (1) the strength of interaction between protonated Schiff base of retinal (PSB) and counterion, (2) the replacement of full or partial charges or polarizable groups close to the polyene chain, and (3) the planarization of the polyene chain caused by the protein environment. To understand how such color tuning mechanism works in visual pigments, we need to obtain the structural information. Vibrational spectroscopy like resonance Raman spectroscopy or FTIR spectroscopy are strong tools for the study on molecular structure, because they give much information about chemical bonds. Previously, Prof. Mathies group reported resonance Raman study of human color visual pigments, and concluded that the structure of retinal chromophore and Schiff base hydrogen-bond are similar between green and red pigments (12). The results are fully consistent with the mutational study (11). They evaluated the Schiff base hydrogen-bond using C=NH and C=ND stretching vibrations. It is well established that the difference infrequency reflects hydrogen-bonding strength of the Schiff base,
because coupled N-H bending frequency is larger if the Schiff base hydrogen-bond is stronger. Between green and red pigments, three different amino acids (Ala, Phe, Ala for green and Ser, Tyr, Thr for red, respectively) are located at the β-ionone ring side of the retinal chromophore. Therefore, together with the Raman results, the difference in polarity near the ring is the determinant of color tuning between green and red pigments. Is this idea really true? Does color tuning originated from only local chromophore-protein interaction near the ring?

I had started low-temperature FTIR spectroscopy of MG and MR sensitive pigments. Vibrational analysis of FTIR spectroscopy is a powerful technique, being sensitive not only to the retinal conformational changes but particular to hydrogen-bonding changes of amino acid side chains or water molecules. According to the first structural analysis of MG and MR (13, Chapter 2), the obtained FTIR spectra of MG and MR in D₂O at 77 K were similar in the 1800-800 cm⁻¹ region, suggesting that the chromophore structure and light-induced structural changes in the chromophore upon photoisomerization are similar among these color pigments. In contrast, there were at least two different signals between MG and MR on the protein side; green-specific structural perturbation of α-helix, and red-specific X-H stretch which indicates the unique chromophore-protein interaction in the red pigment, not for green pigment. In addition, I identified protein-bound water molecules of MG and MR (14). Interestingly, the averaged frequencies of the observed water O-D stretches for MG and MR are lower as the λ_max is red shifted, suggesting that water molecules are involved in the color tuning (Chapter 3). Here, I have investigated low-temperature FTIR spectroscopy of mutant proteins to elucidate the key residues causing different vibrational bands between MG and MR, and the location and/or distribution of internal water molecules. Focused amino acids for mutation correspond to three different amino acids near the retinal chromophore (Ala180, Phe277, Ala285 for green and Ser180, Tyr277, Thr285 for red, respectively) and Asn318 (one of the NPxxY motif).
Materials and Methods

Sample Preparation. The cDNAs of MR and MG were tagged by the Rho1D4 epitope sequence and introduced into expression vectors pcDLSRα296. The cDNA of site-directed mutants were constructed using the QuikChange mutagenesis kit (Stratagene). The wild-type and mutant opsins were expressed in the HEK293T cell line and regenerated with 11-cis retinal as previously reported (13, 15-17). The reconstituted pigments were extracted with buffer A [2 % (w/v) n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)] and purified by adsorption on an antibody-conjugated column and eluted with buffer B [0.06 mg/mL 1D4 peptide, 0.02 % n-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.5)]. For the FTIR analysis, solubilized samples were reconstituted into phosphatidylcholine liposomes with a 100-fold molar excess, followed by suspension in buffer C [2 mM phosphate and 10 mM NaCl (pH 7.25)].

Synthesis of D-Labeled Retinal Derivatives. All-trans-C₁₅-D-labeled retinal was synthesized according to previously reported methods (18-20). 11-cis isomer was separated by HPLC from the photoisomerized mixtures of the corresponding all-trans retinal.

FTIR Spectroscopy. A 30 μL aliquot of the pigments suspension, which contains 0.3 mg protein of MR or MG, was deposited on a BaF₂ window with a diameter of 18 mm and dried in the glass vessel that was evacuated by an aspirator. The dry film was then hydrated by placing, 1 μL of water (H₂O) or heavy water (D₂O or D₂²¹⁸O) next to the film. The sample was sealed by another window and a rubber O-ring and mounted in an Oxford DN-1704 cryostat. The film sample was cooled about 10 min after hydration.

The experimental setup was the same as described previously (13, 21-23), where the cryostat was mounted in a Bio-Rad FTS40 FTIR spectrometer. The cryostat was connected with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The FTIR spectra were recorded with 2 cm⁻¹
resolution and constructed from 128 interferograms. For the formation of bathointermediates, the samples were irradiated with 543, 501, and 501 nm light (by use of an interference filter) for MR, MG, and mutants, respectively at 77 K (13). For the conversion from bathointermediates to the original states, the samples were irradiated with >660, >610, and >610 nm light, respectively. On the other hand, for the reversion from bathointermediates to the Iso (9-cis retinal) states, the samples were irradiated with >560 >530 and >530 nm light, respectively. For each measurement, 64 recordings were averaged to obtain the difference FTIR spectra between the all-trans minus 11-cis forms, and between the all-trans minus 9-cis forms. Reproducibility of the difference FTIR spectra was confirmed by different sample preparations. Linear dichroism experiments revealed random orientation of the pigments molecules in the film, so I have not applied polarized FTIR measurements.

Results

Photoreactions of the Mutants at 77 K. Figure 4-1 shows the light-minus-dark difference FTIR spectra of MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f) in the 1800-800 cm\(^{-1}\) region measured at 77 K in D\(_2\)O. Since photoisomerization is the primary event in vision, the chromophore structures of the positive and negative sides are all-trans and 11-cis forms, respectively. These spectra were normalized at the negative 1238 cm\(^{-1}\) band, a characteristic C12-C13 single bond stretch of retinal chromophore. The bands due to the C=C stretching mode of retinal in the difference FTIR spectra for each pigments were appeared at 1526 (-)/1500 (+), 1527 (-)/1501 (+), 1527 (-)/1502 (+), 1530 (-)/1504 (+), 1534 (-)/1509 (+), 1534 (-)/1509 (+) cm\(^{-1}\), respectively in Figure 4-1. It is widely accepted that the C=C stretching (ethlenic) frequency of the retinal chromophore is inversely correlated with the absorption maximum of the pigment (24-26). In fact, the observed frequencies are correlated well with the absorption maxima of the pigments measured by UV-vis spectroscopy for purified samples (Figure 4-2). Unlike the C=C
Figure 4-1 Light-minus-dark difference FTIR spectra in the 1800-800 cm\(^{-1}\) region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f) which are measured at 77 K in D\(_2\)O. The spectrum of the wild type among MR and MG was from Katayama et al. (13). One division of the y-axis corresponds to 0.0022 absorbance units.
stretching mode, C-C stretching mode and the hydrogen-out-of-plane (HOOP) mode were almost the same in shape to MR-wt (Figure 4-1a), suggesting that single substitution of amino acid in the vicinity of retinal chromophore has little effect on the chromophore structure in the MR system. This suggests that difference in color between green and red pigment is not caused by distortions, being consistent with previous resonance Raman spectroscopic results (12).

**Effect of the Mutations on the Peptide Backbone and Carboxylic Acids.** The 30 nm difference between MR and MG are presumably accompanied by local protein perturbations on the retinal chromophore between MR and MG. We thereby examined the amide-I band frequency regions including protonated carboxylates for each pigment. We reported MG specific amide-I pair bands at 1665 (-)/1658 (+) cm⁻¹, which was absent in MR (13). Figure 4-3 shows the light-minus-dark difference FTIR spectra of each pigment in the 1760-1590 cm⁻¹ region measured at 77 K in D₂O. Each MR mutant has no amide-I pair band at 1665 (-)/1658 (+) cm⁻¹ unlike MG-wt (Figure 4-3f). Spectral feature of MR-T285A (Figure 4-3d) is slightly changed in comparison to MR-wt (Figure 4-3a) at amide-I band regions. On the other hand, unexpectedly, signal intensity of amide-I pair band decreased about 40 % in MG-N318Q although the position of N318 was located far from retinal chromophore (~10 Å according to the structure of bovine Rh (27)). The protein environment is frozen at 77 K, but structural perturbation accompanying with photoisomerization of retinal chromophore propagates toward the cytoplasmic side.

Pair bands at 1735 (-)/1730 (+) cm⁻¹ of MR-wt (Figure 4-3a) probably originates from C=O stretching vibration of a protonated carboxylate (-COOH) because it shifts to 1748 (-)/1741 (+) cm⁻¹ upon hydration with D₂O (data not shown). These bands did not change upon point mutation, indicating that the carboxylic acid is not involved in the color discrimination between red and green. In contrast, these pair bands were slightly down shifted at 1733 (-)/1727 (+) cm⁻¹ in MG-N318Q. Therefore, one possible candidate of these pair bands is C=O stretching vibration of carboxylic acid in the
Figure 4-2  A. Light-minus-dark difference FTIR spectra in the 1555-1470 cm$^{-1}$ region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D$_2$O. The results are taken from Figure 4-1. One division of the y-axis corresponds to 0.0025 absorbance unit. B. UV-vis absorption spectra of the wild-type and mutant proteins after purification. One division of the y-axis corresponds to 0.02 absorbance unit. C. Correlation between $\lambda_{max}$ in visible and the C=C bond frequencies for the unphotolyzed 11-cis form (negative peaks in a-f).
Figure 4-3  Light-minus-dark difference FTIR spectra in the 1760-1580 cm\(^{-1}\) region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D\(_2\)O. The results are taken from Figure 4-1. One division of the y-axis corresponds to 0.00045 absorbance unit.
vicinity of NPxxY motif including N318. The D$_2$O-insensitive 1703 (+)/1696 (-) cm$^{-1}$ bands of MR-wt are likely due to the carbonyl C=O stretching vibration band, which did not change for mutants.

**Effect of the Mutations on the specific hydrogen-bonded amino acid.** In general, H-D unexchangeable vibrations such as O-H and N-H stretches of amino acids or water molecules appear at 3600-3100 cm$^{-1}$ in D$_2$O. In previous paper (13), we have detected a negative band at 3359 cm$^{-1}$ for only MR-wt, not for MG-wt, whose band may originate from one of the 3 amino acids possessing O-H groups for MR; S180, Y277, T285. Since O-H stretching frequencies of Thr were reported at 3500-3300 cm$^{-1}$ in bacteriorhodopsin (28) and *pharaonis* phorborhodopsin (29), the frequency at 3359 cm$^{-1}$ suggests considerably strong hydrogen bond for an O-H stretch. On the other hand, position of the corresponding positive band was not clear because of the overlap with a noisy spectral feature in the previous paper (13). Thereby, we next investigated the MR specific H-D unchangeable X-H stretches at 3600-3100 cm$^{-1}$ region for each pigment. Figure 4-4 shows the light-minus-dark difference FTIR spectra of each pigment in the 3600-3100 cm$^{-1}$ region at 77 K in D$_2$O. It should be noted that a positive band at 3386 cm$^{-1}$ appears clearly for MR-wt, not for MG-wt. Thus, positive and negative bands at 3386 (+)/3359 (-) cm$^{-1}$ corresponds with each other indicating that the X-H stretch does not originate from O-H stretch of S180, Y277 and T285. Intriguingly, the spectrum of MG-N318Q exhibits the same pair bands at 3388 (+)/3364 (-) cm$^{-1}$, though the signal intensity was small.

**Effect of the Mutations on Internal Water Molecules.** Figure 4-5 shows the light-minus-dark difference FTIR spectra of each pigment in the 2700-2440 cm$^{-1}$ region measured at 77 K in D$_2$O. According to the previous paper (14), light-minus-dark difference FTIR spectrum of MR-wt shows six negative and six positive water bands in the 2800-1800 cm$^{-1}$ region as follows: 2671 (-), 2632 (-), 2594 (-), 2583 (-), 2517 (-), 2474 (-), 2684 (+), 2612 (+), 2530 (+), 2504 (+), 2466 (+), and 2303 (+) cm$^{-1}$. From Figure 4-5, the difference FTIR spectra of MR-specific single
Figure 4-4 Light-minus-dark difference FTIR spectra in the 3600-3150 cm\(^{-1}\) region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D\(_2\)O. One division of the y-axis corresponds to 0.0003 absorbance unit.
Figure 4-5  Light-minus-dark difference FTIR spectra in the 2700-2440 cm\(^{-1}\) region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D\(_2\)O. Green labeled frequencies correspond to those identified as water stretching vibrations by measuring spectra in D\(_2\)\(^{18}\)O (data not shown). Box labeled frequencies represent the common originated from water molecules. One division of the y-axis corresponds to 0.00015 absorbance unit.
Figure 4-6 Light-minus-dark difference FTIR spectra in the 2390-2100 cm⁻¹ region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D₂O. Green labeled frequencies correspond to those identified as water stretching vibrations by measuring spectra in D₂¹⁸O (data not shown). One division of the y-axis corresponds to 0.00012 absorbance unit.
mutated pigments are nearly identical to that of MR-wt except for MR-Y277F. The water signals of MR-S180A and MR-T285A are similar to MR-wt. On the other hand, the water signal of Y277F is similar to that of MG-wt, not MR-wt, specially at 2630-2600 cm\(^{-1}\). The bands at 2622 (+)/2616 (-), 2612 (+)/2605 (-) cm\(^{-1}\) correspond to the 2630 (+)/2621 (-), 2615 (+)/2611 (-) cm\(^{-1}\) for MG-wt. This result suggests that the water bands around 2600 cm\(^{-1}\) for MR-Y277F and MG-wt are located in the vicinity of amino acid at the position of 277. From the crystal structure of bovine Rh (27), there are four water molecules near F261 (corresponding position of 277 for color pigments. Interestingly, NPxxY motifs that are highly conserved in GPCRs family is situated near these water clusters. MG-wt specific two pair bands at 2630 (+)/2621 (-), 2615 (+)/2611 (-) cm\(^{-1}\) slightly exhibit shift to 2635 (+)/2626 (-), 2612 (+)/2608 (-) cm\(^{-1}\) for MG-N318Q, the mutant of one of the NPxxY motifs. Thus, these results strongly imply that retinal photoisomerization causes the local structural perturbation of internal water molecules extended to the NPxxY motifs.

O-D stretches of water molecules located at 2700-2450 cm\(^{-1}\) correspond to weakly and/or moderate H-bonded strength. As reported previously, strongly H-bonded water molecules located at <2400 cm\(^{-1}\) are absent for bovine Rh (22). In contrast, they were observed for the unphotolyzed state of MR and MG, but a water band appears at ~2300 cm\(^{-1}\) in MR and MG after retinal photoisomerization (14). From our previous data, we infer that strongly H-bonded water molecule constitutes the Cl\(^{-}\)-binding site, which is the same as for a light-driven Cl\(^{-}\) pump HR (31). Binding of Cl\(^{-}\) causes a red shift in the absorption maximum among L-group (long-wavelength-sensitive visual pigments). However, the band frequencies of strongly H-bonded water molecule are 2303 and 2308 cm\(^{-1}\) for MR and MG, respectively, so that Cl\(^{-}\)-binding conditions are similar between MR and MG. Therefore, Cl\(^{-}\)-binding would not be involved in color tuning mechanism between MR and MG. In fact, the spectra for MR mutants provide the same frequency of MR-wt (2303 cm\(^{-1}\); (Figure 4-6)). The frequency of MG-N318Q is coincident with that of
MG-wt. In the crystal structure of bovine Rh (27), Glu181 which corresponds to His197, one of the amino acids of constitution for Cl'-binding site is placed at the opposite side of mutated position.

By using preferential illumination wavelengths at 77 K, we can selectively accumulate the 9-cis form (21, 32), and we already established the experimental conditions for MR and MG in the previous study (14). So, we can estimate the location of the water molecules in detail by comparing the measurements of the 9-cis form. Figure 4-7 shows the corresponding difference FTIR spectra of MR-wt, MR-S180A, MR-Y277F, MR-T285A, MG-wt, and MG-N318Q, respectively, in the 2700-2450 cm\(^{-1}\) region, where the positive and negative sides are all-trans and 9-cis forms, respectively. The band features of water molecules at <2550 cm\(^{-1}\), moderate H-bonded region, for each pigments are similar, which is also the case for all-trans and 11-cis forms (Figure 4-5). Nevertheless, the water signal behaviors in the 2640-2595 cm\(^{-1}\) region are entirely different. Both MR-Y277F and MR-T285A have new water O-D stretches at 2633 (+), 2605 (-), 2599 (+) or 2633 (+), 2613 (-), and 2585 cm\(^{-1}\), which appear in MG-wt and MG-N318Q. Interestingly, water signal of MR-Y277F is MG-wt like for both all-trans minus 11-cis and all-trans minus 9-cis forms, whereas that of MR-T285A is only MG-wt like for all-trans minus 9-cis forms. As reported previously, the difference signals of water in bovine Rh are identical between all-trans minus 11-cis, and all-trans minus 9-cis forms, but MR and MG possess water vibrations differing between the 11-cis and 9-cis forms at 2710-2530 cm\(^{-1}\) (14). According to the X-ray crystal structures of bovine Rh (27) and 9-cis Rh (33), internal water molecules are absent along the retinal polyene chain (particularly C11 and C9 position), which is consistent with our previous FTIR data (14). It is thus suggested that MR and MG possess specific internal water molecules near C11 and C9, which is more influenced by mutation of T285. According to the Rh structure, T285 (A269 in bovine Rh) is 7.7 Å and 9.4 Å from the C9 and C11 atom of the retinal chromophore, while the position of Y277 (F261 in bovine Rh) is far distant (9.4 Å and 11.1 Å,
Figure 4-7  Light-minus-dark difference FTIR spectra in the 2700-2440 cm\(^{-1}\) region for the 9-cis form of MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D\(_2\)O. Green labeled frequencies correspond to those identified as water stretching vibrations by measuring spectra in D\(_2\)\(^{18}\)O (data not shown). Box labeled frequencies represent the common originated from water molecules. One division of the y-axis corresponds to 0.00015 absorbance unit.
respectively). This structural information is consistent with the FTIR results.

**Effect of the Mutations on the Protonated Schiff Base (PSB)** It is well known that the strength of interaction between protonated Schiff base of retinal chromophore (PSB) and its counterion is one of the key factors for determinant of absorption maxima among pigments. However, Prof. Mathies group reported the resonance Raman study of human color visual pigments, and concluded that (i) the structure of retinal chromophore is similar, and (ii) the Schiff base hydrogen-bond is similar between red and green pigments (12). They evaluated the strength of the Schiff base hydrogen-bond using C=NH and C=ND stretching vibrations. To monitor the H-bond strength of the Schiff base, difference C=NH and C=ND is a smart method, but so complicated and not direct measure of the H-bonding strength. On the other hand, N-H (N-D) stretching vibration is a direct probe of hydrogen bonding strength, where frequency is lowered if hydrogen bond is stronger (Kandori et al. 2002). In fact, we observed a peak pair at 2250-2200 cm\(^{-1}\) for each pigment (Figure 4-8a), which did not exhibit isotope shift for \(^{18}\)O water (14). The frequencies are shifted among each pigment, which are determined by Gaussian curve fitting.

Figure 4-8b shows absorption spectra of MR-wt, MR-S180A, MR-Y277F, MR-285A, and MG-wt, possessing the \(\lambda_{\text{max}}\) at 560, 556, 550, 547, and 530 nm, respectively. Figure 4-8c shows the plot of the absorption maxima in energy and the frequency of the bands. It is clearly shown that the X-D stretching frequency shows linear correlation with \(\lambda_{\text{max}}\) of the pigments. The strong candidate is the N-D stretch of the retinal Schiff base. In the case of E. coli and *Halobacterium salinarum* cells, the Schiff base nitrogen can be labeled by adding \(^{15}\)N-lysine into culture media (31, 34-39). In contrast, isotope label of proteins in HEK293 cells is not well established. Therefore, in the present study, in order to assign the Schiff base band in the FTIR spectra of pigments, I used a different way. I attempted to assign by use of C\(_{15}\)-2H (D)-substituted retinal derivative (Figure 4-9), by which I expected the isotope effect on the Schiff base N-D stretch. Figure 4-10a shows the result of unlabeled and
Figure 4-8  
A. Light-minus-dark difference FTIR spectra in the 2250-2190 cm\(^{-1}\) region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D\(_2\)O. The fitted curves (red) using the Gaussian functions. One division of the y-axis corresponds to 0.00012 absorbance unit.

B. UV-vis absorption spectra of the wild-type and mutant proteins after purification. One division of the y-axis corresponds to 0.02 absorbance unit. The results are taken from Figure 4-2b.

C. Correlation between \(\lambda_{\text{max}}\) in visible and the X-D bond frequencies for the unphotolyzed 11-cis from (negative peaks in a-f).
Figure 4-9  Upper; Structural formula for retinal protonated Schiff base (PSB) in the 11-cis form in D$_2$O and synthetic $^{15}$D-labeled. “R” represents the protein moiety including the lysine residue (Lys296 in bovine Rh; Lys312 in color pigments) that forms a covalent bond with the chromophore. Bottom; Confirmation of HPLC chromatographs of the single 11-cis-retinal with $^{15}$D-labeled.
Figure 4-10  A. Light-minus-dark difference FTIR spectra of unlabeled (black line) and 15D-labeled (red line) MG-wt in the 2250-1950 cm⁻¹ region. One division of the y-axis corresponds to 0.00002 absorbance unit. B. Light-minus-dark difference FTIR spectra in the 2140-2055 cm⁻¹ region for the MR-wt (a), MR-S180A (b), MR-Y277F (c), MR-T285A (d), MG-wt (e), and MG-N318Q (f), which are measured at 77 K in D₂O. The fitted curves (red) using the Gaussian functions. One division of the y-axis corresponds to 0.00003 absorbance unit. C. Correlation between λ_max in visible and the N-D bond frequencies of Schiff base for the unphotolyzed 11-cis from (negative peaks in a-f).
C\textsubscript{15}-deuterated MG-wt. Unexpectedly, the pair bands at 2228 (-)/2210 (+) cm\textsuperscript{-1} do not exhibit the isotope shift, while the clear isotope shift is observed for the bands at 2099 (-)/2083 (+) cm\textsuperscript{-1}. Therefore, the Schiff base N-D stretch is located at about 2100 cm\textsuperscript{-1}, not at about 2220 cm\textsuperscript{-1}(Figure 4-10b). Then I attempted to the N-D stretch by Gaussian curve fitting. Figure 4-8c plots the N-D stretch of the Schiff base versus \(\lambda_{\text{max}}\), but there was no clear correlation. I concluded that the hydrogen-bonding strength of the retinal Schiff base is identical (<2 cm\textsuperscript{-1} in N-D stretch) between MG and MR. This is consistent with previous investigated Raman data (12). Then, a remaining question is the origin of the pair bands at 2250-2200 cm\textsuperscript{-1}. It is clear that they come from protein, not retinal chromophore, but the frequency is correlated with color.

**Discussion**

In the present study, I measured the difference FTIR spectra of mutant pigments, where MR becomes MG-like. According to the previous our FTIR data of MR-wt and MG-wt, there were at least three different IR signals between MR and MG; 1) MG specific amide-I bands at 1665 (-)/1659 (+) cm\textsuperscript{-1} which were absent in MR (13), 2) MR specific X-H stretch at 3359 cm\textsuperscript{-1} which was absent in MG (13), and 3) protein-bound water molecules, in particular appeared at 2650-2600 cm\textsuperscript{-1} region (moderate H-bonded water molecules). Unexpectedly, mutant MR did not show the MG-specific amide-I bands, while X-H stretch still remained.

Although structural difference between MR and MG is not originated from three amino acids, I found that N318 in the NPxxY motifs is crucial for structural difference. Amide-I bands at 1665 (-)/1659 (+) cm\textsuperscript{-1} of MG-N318Q (Figure 4-3f) was decreased compared with MG-wt (Figure 4-3e), suggesting helical structural perturbation. D99 (D83 in bovine Rh) may be involved in the network, and previous paper suggested that the C=O stretches of D83 connected with internal water molecule and peptide C=O of G120 (40, 41). Therefore, internal water molecules would be involved in helical structural change between MR and MG.
Figure 4-11  Structure of the retinal-binding pocket and interhical cavity to cytoplasmic side including NPxxY motif in bovine rh (31), which is viewed from the helix V side. The upper and lower regions correspond to the extracellular and cytoplasmic sides, respectively. Six helices (helix I, II, III, IV, VI, and VII) are illustrated by cartoon drawing, and the retinal chromophore and side chain of several important amino acid residues are shown by stick drawing. Residues that are different between green and red pigments are drawn in violet, and that constitute the interhical cavity are drawn in green. Water molecules correspond to cyan spheres.
The present FTIR spectra of mutated pigments showed that the replacement of amino acids at the position of 277 and 318 caused change of the water bands frequency at 2630-2600 cm\(^{-1}\) region, strongly suggesting that water hydrogen bonding change upon photoisomerization in both MR and MG takes place toward the cytoplasmic side of retinal. According to crystal structure of bovine Rh, the position close to NPxxY motif is more hydrophilic environment than \(\beta\)-ionone ring side of retinal, where three different amino acids between MR and MG present. Previous studies (42) have shown that 9 protein-bound water signals for each negative and positive side are detected by low-temperature FTIR measurements of squid Rh. More numerous waters are identified in the crystal structure of squid Rh (43), where 9 water molecules fill the cavity in the cytoplasmic side. According to this structure, these water molecules connect between retinal binding pocket and the NPxxY motifs (43).

From the analysis of 9-cis form, I concluded that protein-bound water molecule locates near the position of 285. Here I propose the structural model based on the crystal structure of bovine Rh in Figure 4-11, which illustrates how hydrogen bonding change included water upon retinal photoisomerization propagates toward NPxxY motif in cytoplasmic side. It is conceivable that the alteration of hydrogen bonding network.

**Hydrogen bonding network included water plays a role in signal transduction**

As well-known, there are several different molecular properties between Rh and color visual pigments. Previous studies clearly showed that color visual pigments exhibit faster regeneration from 11-cis-retinal and opsin and faster formation and decay of physiologically active meta-II intermediate than does Rh (44). In addition, the faster regeneration and the faster decay of the active state in color visual pigments might correlate with the faster recovery after photoresponse (45) and the lesser activation of signal transduction cascade (46, 47), respectively. Moreover, it was reported that single amino acid at position 122 plays a crucial role for determinant of molecular properties between Rh and color visual pigments (48).
pigments, amino acid at position 122 correspond to Glutamate and Isoluecine, respectively. From the crystal structure of Rh, E122 locates in the vicinity of β-ionone ring of retinal, and can be formed the hydrogen bond with peptide backbone C=O of H211 presented near E122. Thus, the absence of E122 in color visual pigments causes the formation of hydrogen bonding network system different from that in Rh and results in faster regeneration and the faster decay of the active state of meta-II. Water-containing H-bond network may play an important role to distinguish between Rh and color visual pigments.

**How color regulation accommodates between red and green sensitive pigments?** This work assigned N-D stretching vibration of retinal Schiff base by using isotope label of retinal (¹⁵D), which led to no correlation between the assigned N-D stretching vibration and λ<sub>max</sub> (Figure 4-10c). If so, the present systematic mutagenesis FTIR study also steered a traditional attitude that the dominant physical mechanism responsible for the color discrimination between red and green is the interaction of dipolar amino acids. However, I would like to emphasize that there is more interesting question remained in this study. I observed clear correlation between the pair bands at 2250-2200 cm⁻¹ for each pigments and λ<sub>max</sub>. What is the origin of the pair bands? What is the meaning of linear correlation between X-D stretching vibration and λ<sub>max</sub>? This vibration originates from protein, not retinal because of insensitive isotope shift, and the amino acid may be a good monitor of color tuning, or it may even actively participate color tuning. Judging from the structure of Rh, N-D stretch of W265 (W281 for MR and MG) may be a candidate for the X-D stretching vibration.
REFERENCES


Chapter 5
An FTIR Study of the Photoreaction of Bovine Rhodopsin in the Presence of Hydroxylamine

Introduction

Rh is one of G protein-coupled receptors (GPCR), which has diverged into a photoreceptive protein in retinal visual cells (1-4). 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 GPCR. The 11-cis-retinal is bound to the Lys-296 side chain in the transmembrane helix 7 through a protonated Schiff base linkage. Absorption of a photon by the chromophore causes isomerization to the all-trans form, followed by conformational changes of protein (5). Several intermediate states in the bleaching process have been identified, such as photorhodopsin, bathorhodopsin (Batho), lumirhodopsin (Lumi), metarhodopsin-I (Meta-I), and metarhodopsin-II (Meta-II) (6). Meta-II catalyzes the GDP-GTP exchange reaction in the trimeric G protein transducin (1, 3, 4).

Rh is the best studied GPCR, and one of the current foci of research is the structural analysis of Meta-I and Meta-II states, because it is directly related to the activation mechanism. Nevertheless, high resolution structures have never been reported for Meta-I and Meta-II, possibly because of the large structural changes induced in the dark state of Rh by light (2). Light-induced difference Fourier-transform infrared (FTIR) spectroscopy is a powerful method to study structure-function relationships in Rh (7, 8). The Meta-I minus Rh and Meta-II minus Rh spectra have been measured (7, 8), which led to ample structural information with atomic details, including a proton transfer from the Schiff base to Glu113 in Meta-II (9, 10), unique peptide backbone alteration in Meta-I and Meta-II (7, 8), and water structural changes (11, 12). Structural alteration accompanying the complex formation with transducin has been identified as well (13-16). In addition, an
interesting observation was reported on opsin, the protein moiety without retinal, by Vogel and Siebert (17). G-protein activation by opsin is low at physiological pH, but, using FTIR, they clearly showed that opsin exists in two pH-dependent conformations with a pKa of the transition at 4.1, where the low pH opsin conformation is similar to that of Meta-II. This observation is highly consistent with the X-ray crystallographic structure of opsin at acidic pH, that showed partially open structure at the cytoplasmic side (18). Possible crystallization of an opsin forming a complex with the C-terminal peptide of the α-subunit of transducin (19) further suggests that the structure of opsin at low pH mimics that of the active state of Rh.

In the FTIR study of opsin by Vogel and Siebert, hydroxylamine was used to extract the retinal chromophore from opsin (17). Hydroxylamine has been a useful reagent in the structure-function study of Rh, because it interacts with the retinal Schiff base and forms a retinal oxime. Since oxime has its absorption maximum at about 360-370 nm, Rh loses its color upon formation of retinal oxime. The Schiff base is not reactive to hydroxylamine in the dark, which is consistent with the well-protected retinal binding site (20). In contrast, upon illumination, all-trans retinal oxime forms easily, resulting in the loss of color. This suggests that activation of Rh creates a specific reaction channel for hydroxylamine, or loosens the chromophore binding pocket. The reaction mechanism of hydroxylamine with the photoactivated Rh is thus intriguing, though the structural information has been scarce even after the report by Vogel and Siebert (17).

In this paper, I studied hydroxylamine effects on the photoactivated Rh by FTIR spectroscopy. The sample preparation was our first challenge. In the FTIR spectroscopy of Rh, we normally use hydrated films, which is advantageous for monitoring light-induced structural changes of Rh in atomic details. In fact, we can observe hydrogen-bonding alteration of even a single water molecule (21). In contrast, since molecular diffusion is significantly restricted in hydrated films, we could not observe the complex formation between bovine Rh and transducin (13).
Therefore, we previously conducted FTIR measurements in highly concentrated suspension of the mixture (13, 14). The situation turned out to be different for the interaction of Rh and hydroxylamine. The present study clearly shows that hydrated films can be used for the interaction between protein (Rh) and small molecules (hydroxylamine). I studied the hydroxylamine effect on Meta-I and Meta-II states at their characteristic temperatures, 240 and 280 K, respectively, under the physiological pH (~7). In addition, time-dependent formation of retinal oxime was monitored at 265 K by UV/visible and FTIR spectroscopy. Molecular mechanism of the interaction between hydroxylamine and photoactivated Rh is discussed based on the present FTIR observations.

**Materials and Methods**

Frozen bovine retinas were purchased from J. A. & W. L. Lawson Co. (Lincoln, NE). Crude rod outer segments (ROS) were isolated from the retinas in ROS buffer (10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM MgCl₂, 0.1 mM PMSF, 1 mg/L aprotinin 1 mg/L leupeptin, 1 mM DTT, pH 7.3) by a sucrose flotation method as described previously (12, 22). A discontinuous sucrose gradient was used to purify ROS (12, 22). ROS were then washed five times with distilled water by centrifugation at 80,000 g. The ROS suspension was diluted with 2 mM phosphate buffer (pH 7.0), and its final concentration was adjusted to 3 mg/ml (A₅₀₀nm = 3 OD). For the UV/visible spectroscopy of the sample in buffer solution, the ROS suspension was further diluted in 2 mM phosphate buffer (pH 7.0) (A₅₀₀nm = 0.8 OD), and the sample in the absence and presence of 1 M hydroxylamine was illuminated at 265 or 280 K.

For the measurements of the sample in hydrated films, a 50 μL aliquot of ROS suspension in the absence and presence of 100 mM hydroxylamine was deposited on a BaF₂ window with a diameter of 18 mm. The dry film was then well rehydrated, and mounted in an Oxford DN-1704 cryostat. The experimental set-up was the same as described previously (12, 22). The cryostat was equipped with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. The
FTIR spectra were recorded in a Bio-Rad FTS40 spectrometer at 2 cm\(^{-1}\) resolution and constructed from 128 interferograms. The sample was illuminated at 240, 265, or 280 K. In the present measurements, the sample was illuminated by >560 nm light for 1 minute, where the light source for the illumination was a 1-kW halogen-tungsten lamp in a slide projector.

**Results**

**Photoreaction in the Presence and Absence of Hydroxylamine at 240 K.** The black curve in Figure 5-1 shows light-induced spectral change of bovine Rh in the UV/visible region at 240 K. This is characteristic of the Meta-I minus Rh spectrum, possessing positive and negative peaks at 459 and 535 nm, respectively. For the measurement, native ROS membranes in 2 mM phosphate buffer (pH 7) were first dried and then rehydrated on an IR window. Red curve in Figure 5-1 represents light-induced spectral change of bovine Rh in the presence of hydroxylamine at 240 K, where hydrated film sample was prepared from native ROS membranes containing 100 mM hydroxylamine in 2 mM phosphate buffer (pH 7). Identical spectra indicate formation of Meta-I even in the presence of hydroxylamine at 240 K. This fact may suggest that hydroxylamine does not react with the Schiff base of Meta-I, similar to the Schiff base of Rh. It should however be noted that another interpretation is possible: namely, diffusion is prohibited at 240 K, so that hydroxylamine cannot reach the Schiff base of Meta-I.

Figure 5-2 compares the difference FTIR spectra corresponding to those of Figure 5-1, where black dotted and red curves represent the measurements in the absence and presence of hydroxylamine, respectively. Both spectra are essentially similar, indicating that Meta-I specific structural changes occur in the presence of hydroxylamine, as can be judged from the bands at 1736 (-), 1727 (-), 1701 (+), 1551 (-), 1538 (+), 1238 (-), 1215 (-), 1198 (+), 971 (-), and 950 (+) cm\(^{-1}\). The spectral features observed in D\(_2\)O are also characteristic of the Meta-I minus Rh spectrum (data not shown).
Figure 5-1 Light-minus-dark difference UV/visible spectra of bovine Rh at 240 K. A hydrated film sample of native ROS membranes prepared at pH 7 was illuminated at >560 nm. Red and black spectra represent the measurements in the presence and absence of hydroxylamine, respectively. One division of the y-axis corresponds to 0.05 absorbance units.
Figure 5-2  Light-minus-dark difference FTIR spectra of bovine Rh at 240 K in the 1800-800 cm\(^{-1}\) region. A hydrated film sample of native ROS membranes prepared at pH 7 was illuminated at >560 nm. Red and black dotted spectra represent the measurements in the presence and absence of hydroxylamine, respectively. One division of the y-axis corresponds to 0.002 absorbance units.
Although typical Meta-I minus Rh spectra were obtained in the presence of hydroxylamine, some deviations were observed from the spectra obtained in its absence, suggesting that hydroxylamine influences structural changes upon formation of Meta-I. In fact, the two spectra in Figure 5-2 are somewhat different in the range of 1700-1500 cm\(^{-1}\). The influence of hydroxylamine was more clearly observed in the frequency region of X-H and X-D stretching vibrations in D\(_2\)O. Figures 5-3a and b compare the Meta-I minus Rh spectra in the X-D stretching region between the presence and absence of hydroxylamine. The bands at 2633 (-) and 2617 (+) cm\(^{-1}\) in the absence of hydroxylamine (black dotted curve in Figure 5-3a) presumably originate from O-D stretching vibrations of water, as we previously identified the bands at 2629 (-) and 2616 (+) cm\(^{-1}\) in the Meta-I minus Rh spectrum in D\(_2\)O to be water O-D stretches by use of D\(_2\)\(^{18}\)O (12). Interestingly, the spectral shape at these frequencies is considerably different in the presence of hydroxylamine (red curve in Figure 5-3a), where two negative peaks appear at 2642 and 2609 cm\(^{-1}\). This suggests that water-containing hydrogen-bonding network is altered in the presence of hydroxylamine.

Figure 5-3b shows the frequency region of strongly hydrogen-bonded X-D stretching vibrations, and we previously suggested that the bands at 2262 (+), 2012 (-) and 1966 (-) cm\(^{-1}\) in the Meta-I minus Rh spectrum are ascribable to the N-D stretching vibrations of the Schiff base (12). While the negative bands at 2010 and 1965 cm\(^{-1}\) were unchanged by the presence of hydroxylamine, clear spectral change was seen for the positive band (Figure 5-3b). The frequency in the presence of hydroxylamine (2294 cm\(^{-1}\)) is up-shifted by about 30 cm\(^{-1}\) from that in the absence (2262 cm\(^{-1}\)). These results indicate that hydrogen-bonding strength of the Schiff base is identical for Rh regardless of the presence of hydroxylamine, but the hydrogen bond is weakened for Meta-I in the presence of hydroxylamine.

Together with the results of Figure 5-3a, the effect of hydroxylamine on the hydrogen-bonding network in the Schiff base region can be summarized as follows.
Figure 5-3  Light-minus-dark difference FTIR spectra of bovine Rh at 240 K in the X-D (a and b) and X-H (c) stretching region in D₂O. A hydrated film sample of native ROS membranes prepared at pH 7 was illuminated at >560 nm. Red and black dotted spectra represent the measurements in the presence and absence of hydroxylamine, respectively. One division of the y-axis corresponds to 0.0005 (a), 0.0002 (b), and 0.001 (c) absorbance units.
In the dark state of Rh, hydrogen bonding of water molecules is changed by the presence of hydroxylamine, but it does not affect the hydrogen bond of the Schiff base. This may be the reason why hydroxylamine does not react with the Schiff base in Rh. On the other hand, hydrogen bonds of water molecules and the Schiff base are affected in Meta-I by the presence of hydroxylamine, indicating that the hydrogen-bonding network in the Schiff base region is perturbed in Meta-I.

Figure 5-3c shows the Meta-I minus Rh spectra in the X-H stretching region, where H-D unexchangeable O-H or N-H stretching vibrations appear. A negative band at 3475 cm\(^{-1}\) possibly originates from the O-H stretch of Thr118, consistent with a sharp negative peak appearing at 3463 cm\(^{-1}\) in the Batho minus Rh spectrum (23). From the frequency, the bands at 3301 (+) and 3283 (-) cm\(^{-1}\) are ascribable to amide-A vibrations of \(\alpha\)-helix, N-H stretches of the peptide backbone. Higher frequency shift upon Meta-I formation indicates weakened hydrogen bond of the peptide in \(\alpha\)-helical regions, and this spectral feature correlates with that for amide-I vibrations, C=O stretches of the peptide backbone, which appeared at 1664 (+) and 1656 (-) cm\(^{-1}\) (Figure 5-2). Unlike the data in Figures 5-3a and b, the Meta-I minus Rh spectra are similar between the presence and absence of hydroxylamine, except for a broad positive band at 3460-3420 cm\(^{-1}\). These results suggest that structural changes in the H-D unexchangeable region are not affected by hydroxylamine. Thus, hydroxylamine specifically influences hydrogen-bonding network near the Schiff base region in Rh and Meta-I.

**Photoreaction in the Presence and Absence of Hydroxylamine at 280 K.** I next examined the effect of hydroxylamine on Meta-II. Black and red curves in Figure 5-4a correspond to the light-induced UV/visible spectral change of bovine Rh in buffer solution and hydrated film samples (pH 7), respectively, in the absence of hydroxylamine at 280 K. The positive peaks at 386 and 378 nm are characteristic of the formation of Meta-II. The difference spectra differ in the range of 400-500 nm, where black curve shows more positive contributions. This fact suggests that in
Figure 5-4  Light-minus-dark difference UV/visible spectra of bovine Rh in buffer solution (black curve) and in hydrated films (red curve) at 280 K. Native ROS membranes are in buffer solution containing 2 mM phosphate buffer (pH 7.0), which was prepared in the absence and presence of 1 M hydroxylamine. A hydrated film sample of native ROS membranes was prepared from 2 mM phosphate buffer (pH 7.0) in the absence and presence of 100 mM hydroxylamine. (a) Black and red spectra represent the measurements in buffer solution and in hydrated films, respectively, in the absence of hydroxylamine. (b) Black and red spectra represent the measurements in buffer solution and in hydrated films, respectively, in the presence of hydroxylamine. One division of the y-axis corresponds to 0.2 absorbance units.
Solution there are photoproducts absorbing at these wavelengths, such as Meta-I and/or Meta-III. In contrast, the difference spectrum of hydrated film looks like the pure difference between Meta-II and Rh. Black and red curves in Figure 5-4b correspond to the light-induced spectral change of bovine Rh in buffer solution and hydrated film samples, respectively, in the presence of hydroxylamine at 280 K (pH 7). The positive peaks, blue-shifted compared to those from Figure 5-4a, are consistent with formation of retinal oxime. It is thus obvious that hydroxylamine can react with the Schiff base even in the hydrated films, forming a retinal oxime.

I next compared the difference FTIR spectra under the illumination conditions identical to those of the red curves in Figure 5-4. Black dotted and red curves in Figure 5-5a represent the measurements in the absence and presence of hydroxylamine, respectively. As can be clearly seen, the spectra significantly differ from each other. Black dotted curve looks like a typical Meta-II minus rhodopsin spectrum, as obvious from the bands at 1768 (-), 1748 (+), 1727 (-), 1713 (+), 1665 (-), and 1644 (+) cm^{-1} (7, 8). On the other hand, the positive part of the red curve probably originates from the retinal oxime and opsin.

Spectral changes in the range of protonated carboxylic acids vibrations have been extensively studied for the Meta-II minus Rh spectrum, and the bands at 1768 (-), 1727 (-), and 1713 (+) cm^{-1} were identified as the COOH stretches of Asp83 in Rh, Glu122 in Rh, and Glu113 in Meta-II, respectively (9, 10). These peak frequencies were unchanged in the presence of hydroxylamine, but remarkably reduced (red curve in Figure 5-5b). It should be noted that the two spectra in Figure 5-4 are normalized arbitrarily at 280 K, but the analysis at 265 K strongly (see below) suggests that the numerical comparison here is reasonable. Signal reduction may partially originate from similar structures of the product and Rh, or involvement of additional changes of protonated carboxylic acids and lipids. Among these vibrations, the positive band at 1713 cm^{-1} should be particularly noted. Previous site-directed mutagenesis studies reported that this band originates from protonation of Glu113 in Meta-II (9, 10),
Figure 5-5  Light-minus-dark difference FTIR spectra of bovine Rh at 280 K in the 1800-800 (a) and 1800-1500 (b) cm$^{-1}$ region. A hydrated film sample of native ROS membranes prepared at pH 7 was illuminated at >560 nm. Black dotted and red spectra represent the measurements in the absence and presence of hydroxylamine, respectively. One division of the y-axis corresponds to 0.005 absorbance units.
Figure 5-6  (a) Upper panel; Black dotted and red spectra represent the measurements of bovine Rh in the absence and presence of hydroxylamine, respectively, at 280 K, which is reproduced from Figure 5-5b. Lower panel; Double difference spectrum between black dotted and red spectra (red minus black dotted lines). Since black and red lines correspond to the (Meta-II) minus (Rh), and (Retinal Oxime and Opsin) minus (Rh and Hydroxylamine), the blue spectrum corresponds to (Retinal Oxime and Opsin) minus (Meta-II and Hydroxylamine). (b) Upper panel; Black dotted and red spectra represent the measurements of bovine Rh just after and 10 min after the illumination, respectively, in the presence of hydroxylamine at 265 K, which is reproduced from Figure 5-8b. Lower panel; Double difference spectrum between black dotted and red spectra (red minus black dotted lines). The similarity of the blue spectra between a and b suggests the same origin.
suggesting that Meta-II formation is accompanied by a proton transfer from the Schiff base to the counterion, Glu113. In Meta-II, the neutral pair of the Schiff base and Glu113 appears to be energetically more favorable than an ion pair (protonated Schiff base and negatively charged Glu113). The positive peak at 1713 cm\(^{-1}\) is preserved in the presence of hydroxylamine (red curve in Figure 5-5), whereas its intensity is less than half of that in the absence of hydroxylamine. This suggests that Glu113 is not fully protonated in the retinal oxime and opsin. In other words, pKa of Glu113 is elevated upon Meta-II formation, but is lowered by the formation of retinal oxime.

Amide-I vibrations in the presence of hydroxylamine were largely different from those of Meta-II. Unlike the up-shifted amide-I vibration of \(\alpha\)-helix in Meta-I (1664 (+)/1656 (-) cm\(^{-1}\); Figure 5-2), a down-shifted amide-I vibration of \(\alpha\)-helix is characteristic of Meta-II (1665 (-)/1644 (+) cm\(^{-1}\); black dotted curve in Figure 5-5). This difference is probably coupled to the different global conformational changes in Meta-I and Meta-II. In the presence of hydroxylamine, the bands at 1665 (-)/1644 (+) cm\(^{-1}\) are significantly reduced, and instead, a new peak appears at 1639 (-) cm\(^{-1}\). It should be noted that the frequency at 1639 (-) cm\(^{-1}\) is the typical frequency of \(\beta\)-sheet. Rh is mainly comprised of \(\alpha\)-helices and loops, and the only \(\beta\)-sheet structure can be seen in the extracellular region, where the 3rd and 4th transmembrane helices are connected by an antiparallel \(\beta\)-sheet composed of Arg177-Glu181 and Ser186-Asp190 (20). Interestingly, the antiparallel \(\beta\)-sheet constitutes the retinal binding pocket by covering the chromophore from the extracellular bulk. Present results suggest that the \(\beta\)-sheet structure is disrupted by the formation of retinal oxime. It should be noted that the antiparallel \(\beta\)-sheet exhibits additional amide-I vibration at about 1685 cm\(^{-1}\) (24), which is not obvious in Figure 5-5b probably because of strong positive band at 1686 cm\(^{-1}\). Nevertheless, double difference in Figure 5-6a shows a spectral contribution at 1687 cm\(^{-1}\) together with that at about 1640 cm\(^{-1}\) (peak at 1642 cm\(^{-1}\)). This fact also supports the structural disruption of the antiparallel \(\beta\)-sheet by the formation of retinal oxime. From the FTIR spectra (Figures 5-5 and 5-6),
disruption of the β-sheet structure is much smaller in the formation of Meta-II. I expected that the influence of hydroxylamine can be observed more clearly in the higher frequency region, such as that of X-H stretches, but unfortunately, such spectra could not obtained with sufficient quality.

The effect of hydroxylamine on Meta-II can be summarized as follows. Upon formation of Meta-II, Glu113 is protonated by the Schiff base proton. The protonation of Glu113 is partially preserved upon the formation of retinal oxime (ret-C15=N-OH), whereas the antiparallel β-sheet constituting the retinal binding pocket at the extracellular surface is structurally disrupted. Interestingly, the previous FTIR spectra in the presence of hydroxylamine at pH 4 were very similar to those of Meta-II, with no reduction of the positive 1713-cm\(^{-1}\) band, and without the negative 1639-cm\(^{-1}\) band (17). This is consistent with the idea that deprotonation of Glu113 and disruption of the β-sheet are coupled. It is likely that protonation of Glu113 stabilizes the β-sheet structure and vice versa at the extracellular surface.

**Time-Dependent Spectral Changes in the Presence and Absence of Hydroxylamine at 265 K.** So far, I have studied the influence of hydroxylamine upon formation of Meta-I and Meta-II at the selected constant characteristic temperatures, 240 and 280 K, respectively. At these temperatures, the only products in the absence of hydroxylamine were Meta-I and Meta-II, and the influence of hydroxylamine was directly monitored. No reaction took place with Meta-I (240 K), but hydrogen-bonding network was considerably perturbed (Figures 5-1-5-3). At 280 K, retinal oxime was formed, presumably through the reaction of hydroxylamine with Meta-II, and protein structural changes different from those occurring upon formation of Meta-II were observed (Figures 5-4 and 5-5). These results apparently indicate that hydroxylamine can react only with Meta-II, but not with Meta-I. However, the sample is frozen at 240 K, the temperature at which diffusion hardly takes place, and the lack of hydroxylamine reactivity may originate from this frozen environment. Since Meta-I and Meta-II are in equilibrium, it may be difficult to conclude if hydroxylamine
can react with Meta-I or not. Thus, I studied the effect of hydroxylamine at the temperature higher than 240 K, and observed time-dependent formation of retinal oxime at 265 K.

Figure 5-7 shows light-induced spectral changes of bovine Rh in the UV/visible region at 265 K. Curves in Figure 5-7a and b correspond to the difference spectra of the ROS sample in buffer solution (pH 7) from 0 min (orange) to 10 min (blue) after the illumination. In the absence of hydroxylamine (Figure 5-7a), Meta-II is formed instantaneously, as shown by the peaks at 386 (+) and 521 (-) nm, and the spectrum does not change with time. The negative peak (521 nm) is more red shifted than that at 280 K (511 nm; Figure 5-4a), suggesting the presence of Meta-I as an additional product at 265 K. Previous study reported more numerous formation of Meta-I under these experimental conditions (25). While the reason is unclear, the present results were reproduced for both buffer solution and hydrated films. In the presence of hydroxylamine, two positive peaks were observed, at 366 nm and at about 460 nm, just after the illumination (orange curve in Figure 5-7b), which probably originate from retinal oxime and Meta-I, respectively. Interestingly, the population of Meta-I is higher in the presence of hydroxylamine (orange curve in Figure 5-7b) than in the absence (orange curve in Figure 5-7a), under the identical pH and temperature conditions, suggesting that hydroxylamine somehow alters the equilibria (structural information will be shown below). As the time goes, the positive peak at about 460 nm gradually decreases and, concomitantly, the peak at 366 nm increases, possessing an isosbestic point at 403 nm. This observation can be interpreted as the conversion of Meta-I into retinal oxime, possibly through Meta-II. After 10 min, Rh was almost entirely converted into retinal oxime (blue curve in Figure 5-7b).

Essentially similar properties were observed for the hydrated films. Curves in Figure 5-7c and d correspond to the difference spectra of the ROS sample in hydrated films (pH 7) from 0 min (orange) to 10 min (blue) after the illumination. In the absence of hydroxylamine, Meta-II is formed instantaneously, as shown by the peaks
Figure 5-7  Light-minus-dark difference UV/visible spectra of bovine Rh in buffer solution (a and b) and in hydrated films (c and d) at 265 K. Native ROS membranes are in buffer solution containing 2 mM phosphate buffer (pH 7.0), which was prepared in the absence (a) and presence (b) of 1 M hydroxylamine. A hydrated film sample of native ROS membranes was prepared from 2 mM phosphate buffer (pH 7.0) in the absence (c) and presence (d) of 100 mM hydroxylamine. Orange, red, light pink, pink, and blue spectra correspond to 0, 2, 4, 6, and 10 min time points after the illumination, respectively. One division of the y-axis corresponds to 0.5 (a) and 0.12 (b) absorbance units.
at 389 (+) and 521 (-) nm, where Meta-I is also formed to some extent. In the presence of hydroxylamine, two positive peaks were observed, at 376 nm and at about 460 nm, just after the illumination (orange curve in Figure 5-7d). While the latter originates from Meta-I, as in solution, the former presumably originates from the mixture of Meta-II and retinal oxime, unlike in solution. I suggest that since hydroxylamine diffusion is significantly restricted in hydrated films, Meta-II could be observed. In fact, as the time goes, the peak at 376 nm is blue-shifted without exhibiting an isosbestic point, as the positive peak at about 460 nm gradually decreases. This process can be interpreted in terms of the conversion of the Meta-I/Meta-II equilibrium mixture into retinal oxime. The initial observation of Meta-II in the presence of hydroxylamine also suggests that retinal oxime is formed by the interaction of hydroxylamine with Meta-II, not Meta-I. After 10 min, Rh was almost entirely converted into retinal oxime (blue curve in Figure 5-7d).

Next, I measured difference FTIR spectra under the illumination conditions identical to those of the curves in Figure 5-7c and d. The spectra obtained in the absence of hydroxylamine (265 K) exhibited no time-dependent changes, and the spectral features looked similar to those of Meta-II (data not shown). Black dotted and red curves in Figure 5-8 represent the measurements just after and 10 min after illumination in the presence of hydroxylamine, respectively (see Figure 5-6b for the double difference spectrum). As is clearly shown, both spectra differ significantly from each other. Black dotted curve is highly similar to the typical Meta-II minus Rh spectrum, as indicated by the bands at 1768 (-), 1748 (+), 1727 (-), 1710 (+), 1656 (-), and 1644 (+) cm⁻¹ (7, 8). Interestingly, the formation of Meta-I, observed by the visible spectroscopy (the orange curve in Figure 5-7d), was not apparent from the FTIR spectra. The spectrum (black dotted lines in Figure 5-8) is similar to that of Meta-II (black dotted lines in Figure 5-5), which strongly suggests that protein structure of the product is Meta-II like. It appears that hydroxylamine affects the equilibrium of the Schiff base protonation in the Meta-II like conformation.
Figure 5-8  Light-minus-dark difference FTIR spectra of bovine Rh in the presence of hydroxylamine at 265 K in the 1800-800 (a) and 1800-1500 (b) cm\(^{-1}\) region. A hydrated film sample of native ROS membranes prepared at pH 7 was illuminated at >560 nm. Black dotted and red spectra represent the measurements just after and 10 min after the illumination, respectively. One division of the y-axis corresponds to 0.002 absorbance units.
The difference FTIR spectra changed with the time, and that at 10 min after the illumination (the red curve in Figure 5-8) is very similar to the red curve in Figure 5-5, indicating the formation of retinal oxime and opsin. Thus, similarity of the spectra at 280 K (red curve in Figure 5-5) and 265 K (red curve in Figure 5-8) further confirmed the conversion of Rh into retinal oxime and opsin. It should be noted that the scaling in the spectral comparison between the presence and absence of hydroxylamine in Figure 5-5 is arbitrary, but the two spectra in Figure 5-8 can be quantitatively compared, because of the time-dependent spectral changes.

**Discussion**

In a typical application of FTIR spectroscopy to Rh, we use hydrated films (21). This method is highly advantageous for monitoring the light-induced structural changes of Rh. In fact, we can observe hydrogen-bonding alteration of even a single water molecule (21). In contrast, hydrated films may be disadvantageous for monitoring protein-protein interactions, because molecular diffusion is significantly restricted. Previously, when we could not observe the complex formation between bovine Rh and G-protein transducin in hydrated films, we applied FTIR measurements to the highly concentrated solution of the mixture (13, 14). The present study clearly shows that hydrated films can be used for studying the interaction between protein (Rh) and small molecules (hydroxylamine). This allows the detailed structural analysis of the activation of Rh in the presence of such molecule, not only in the conventional low frequency region (1800-800 \text{ cm}^{-1}), but also in the X-H and X-D stretching vibrational regions (4000-1800 \text{ cm}^{-1}), the latter of which is highly advantageous for monitoring hydrogen-bonding alterations (5, 26). Indeed, using this system, I could observe structural perturbation of water stretching vibration by hydroxylamine at 240 K. Below, I discuss the influence of hydroxylamine on the photoactivation processes of bovine Rh.

**Influence of Hydroxylamine on Meta-I.** Identical light-induced UV/visible spectra obtained in the absence and presence of hydroxylamine at 240 K (Figure 5-1) show
that hydroxylamine does not react with the Schiff base of Meta-I, which is also the case in unphotolyzed Rh. This is possibly explained by completely frozen environment at such low temperature, where diffusion is prohibited. Nevertheless, the observation of Meta-II in hydrated films at 265 K (Figure 5-7d) suggests that hydroxylamine only reacts with Meta-II, not Meta-I. Relaxed structure around the retinal Schiff base is necessary for the formation of retinal oxime.

Despite the fact that no reaction takes place between Meta-I and hydroxylamine at 240 K, the present FTIR spectroscopy shows that hydroxylamine influences structural changes upon formation of Meta-I. The influence of hydroxylamine was clearly observed in the frequency regions of X-H and X-D stretching vibrations in D$_2$O, suggesting that hydroxylamine affects water-containing hydrogen-bonding network (Figure 5-3). From the spectral analysis of Rh mutants and their batho intermediate at 77 K, we previously identified that water O-H stretches at 3564 and 3538 cm$^{-1}$ originate from the water molecules near Gly120 and Glu113, respectively (27, 28). They further correspond to 2640 and 2618 cm$^{-1}$ bands as the water O-D stretches, respectively (12). The water O-D stretch at 2633 cm$^{-1}$ in the Meta-I minus Rh spectrum in D$_2$O (Figure 5-3a) presumably corresponds to the water molecules in the Schiff base region, and the influence of hydroxylamine on the water stretching vibration is reasonable.

Unlike water vibrations, hydrogen-bonding strength of the Schiff base is identical for the unphotolyzed Rh in the absence and presence of hydroxylamine (Figure 5-3b). In contrast, the hydrogen bond of the Schiff base is weakened for Meta-I in the presence of hydroxylamine (Figure 5-3b). Hydroxylamine does not react with the Schiff base in Rh and Meta-I, but it is likely that the Schiff base environment of Meta-I prepares the reaction accompanying its decay. On the other hand, structural changes in the H-D unexchangeable region are not affected by hydroxylamine (Figure 5-3c). Thus, hydroxylamine specifically influences hydrogen-bonding network near the Schiff base region in Rh and Meta-I.
**Influence of Hydroxylamine on Meta-II.** The present study showed that hydroxylamine can react with the Schiff base even in the hydrated films, forming a retinal oxime (Figures 5-4 and 5-6). Spectral changes in protonated carboxylic acids vibrations in the Meta-II minus Rh spectrum were unchanged in frequency, but reduced considerably in their amplitudes. Reduction of the intensity of Glu113 band suggests that Glu113 is not fully protonated after the formation of retinal oxime and opsin. While the pKa of Glu113 is elevated upon Meta-II formation, it is lowered by the formation of retinal oxime.

Amide-I vibrations in the presence of hydroxylamine were largely different from those of Meta-II. In the presence of hydroxylamine, the bands at 1665 (-)/1644 (+) cm\(^{-1}\) are significantly reduced, and instead, a new peak appears at 1639 (-) cm\(^{-1}\). This characteristic frequency of \(\beta\)-sheet suggests the structural disruption in the extracellular region by hydroxylamine, where the 3rd and 4th transmembrane helices are connected by an antiparallel \(\beta\)-sheet composed of Arg177-Glu181 and Ser186-Asp190. It should be noted that the Meta-II specific bands at 1635 (+)/1621 (-) cm\(^{-1}\) (Figure 5-5a), which are much smaller in Meta-I, can be also interpreted as the structural perturbation of \(\beta\)-sheet, because characteristic amide-I frequency of \(\beta\)-sheet is 1640-1610 cm\(^{-1}\). This suggests that the antiparallel \(\beta\)-sheet forming the retinal binding pocket (Arg177-Glu181 and Ser186-Asp190) does not change in Meta-I, but does in Meta-II. Observation of the \(\beta\)-sheet characteristic band at 2366 (-) cm\(^{-1}\) (amide-A, N-D stretching vibration, of peptide amide in D\(_2\)O) in the Meta-II minus Rh spectrum, but not in the Meta-I minus Rh spectrum, further supports this interpretation (12). Altered \(\beta\)-sheet structure in Meta-II is further perturbed by hydroxylamine. Since the frequency at 1639 cm\(^{-1}\) corresponds to weaker hydrogen bond in \(\beta\)-sheet than that at 1621 cm\(^{-1}\), the region of the structural changes is different in \(\beta\)-sheet of the retinal binding pocket between Meta-II and opsin (with retinal oxime).

Amide-I vibrations in the presence of hydroxylamine were fundamentally different from those of Meta-II at neutral pH (Figures 5-5 and 5-7), but were earlier shown to be
very similar at pH 4 (17). The observed changes were (i) reduction of the bands characteristic of α-helices (at 1665 (-)/1644 (+) cm⁻¹), and (ii) appearance of the band characteristic of β-sheet (at 1639 (-) cm⁻¹). This suggests that pH-dependent structural alteration of opsin (and retinal oxime) accompanies such secondary structural changes. The β-sheet structure constituting the retinal binding pocket is largely disrupted at neutral pH, but considerably restored at low pH. In parallel, Meta-II like helical structure appears to be restored at low pH. Recent X-ray crystallographic structures of free opsin (18) and opsin complexed with the C-terminal peptide of α-subunit of transducin (19) at low pH suggest that the pH-dependent structural transition is also coupled to the changes at the cytoplasmic surface region.

Implication of the Hydroxylamine Reaction into Visual Pigments in the Dark. The present study revealed that hydrolysis of the Schiff base in Meta-II by hydroxylamine accompanies disruption of the β-sheet constituting the retinal binding pocket. This is consistent with the X-ray structure of bovine Rh, because the retinal binding site is highly protected from aqueous solution in the dark (20). On the other hand, if the counterion Glu113 is neutralized, the hydroxylamine can react to the Schiff base even in the dark (29). The mechanism must be closely related to the lowered pKa of the Schiff base from >12 (wild type) to about 6 (the mutants) (30). Hydrogen-bonding network in the Schiff base region plays an important role in stabilizing the structure, which prevents from the attack of hydroxylamine. It is however noted that the structure of the retinal binding site is never static, but dynamic. Recently, we have observed a slow but measurable hydrogen/deuterium exchange (HDX) of the O-H group of Thr118, which is highly isolated from the aqueous phase and in steric contact with the buried retinal chromophore (31). This observation implies that a protein structural fluctuation drives the retinal binding pocket transiently open, and such motion well explains the mechanism of dark noise in vertebrate visual cells. Protein structural fluctuation could take place more frequently in the Glu113 mutants than in the wild type.
Similar discussion is possible for color visual pigments. Unlike Rh, hydroxylamine can react to the Schiff base of color visual pigments such as iodopsin even in the dark (32). It is also established that cone photoreceptors display 103-104 higher rates of dark noise than rod photoreceptors (33-35). I predict that the structural fluctuation takes place more frequently in color pigments than in Rh (31), where stability of the β-sheet plays a crucial role. Although the structural information of color visual pigments has been little, we recently succeeded in measuring accurate light-induced FTIR spectra of MG and MR (36). This opens a new window in understanding the structural basis of color visual pigments, including the retinal binding pocket.
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Chapter 6

Conclusion and Perspectives

In this thesis, I addressed questions related to our visual pigments. In particular, I challenged structural analysis of primate color pigments, which has been never tried. Fortunately, I was able to report the first structural study. The results in each chapter are summarized as follows.

In chapter 2, I attempted to express MG and MR in HEK293 cell lines for structural analysis using FTIR spectroscopy, and succeeded in measuring the first FTIR spectra of MG and MR (1). The obtained FTIR spectra of the color pigments were similar to those of Rh in the conventional 1800-800 cm\(^{-1}\) region, whereas the spectra were entirely different in the X-D (2700-2000 cm\(^{-1}\)) and X-H (3800-2800 cm\(^{-1}\)) stretching regions. In addition, some spectral differences between MG and MR were observed. Since X-H and X-D stretches are the direct probes of hydrogen-bonding environment, the present FTIR study opened a new window to understanding of the specific chromophore-protein interaction in color pigments.

In chapter 3, based on FTIR spectra, I identified protein-bound water molecules of MG and MR (2). In the case of MR, I observed 6 O-D stretches for the original 11-cis form, on the other hand, 7 O-D stretches were observed for the original 11-cis form in the case of MG, suggesting the presence of 3-8 water molecules near the retinal chromophore. Although there are no strongly H-bonded water molecules in MR and MG, a water band appears at ~2300 cm\(^{-1}\) in MR and MG after retinal isomerization, which is not the case in Rh. It was unexpected that I observed correlation between the averaged frequencies of water and color of visual pigments. The averaged frequencies of water for MR, MG and bovine Rh are 2579, 2591 and 2600 cm\(^{-1}\), respectively. Therefore, red-shifted pigments possess protein-bound water molecules at low frequency. Although protein-bound water molecules are probably distributed randomly, this correlation between the averaged water frequency and \(\lambda_{\text{max}}\) may suggest that water molecules participate in color tuning, whose mechanism is
entirely unknown.

In chapter 4, I investigated low-temperature FTIR spectroscopy of mutants to elucidate the key residues causing different vibrational bands between MG and MR, and the location and/or distribution of internal water molecules (3). According to the results, Y277F mutant causing 10-nm blue-shift for MR shows similar hydrogen bonding alteration upon photoisomerization to MG-wt, suggesting that the amino acid at the position of 277 plays a crucial role for regulation of different hydrogen bonding network included water between MG and MR. In addition, I succeeded in assignment of the retinal Schiff base mode by use of isotope label of retinal (C\textsubscript{15}-D), which was similar between MG and MR. On the other hand, I found a vibration of amino acid, that has a positive correlation with $\lambda_{\text{max}}$, suggesting that the amino acid may be a monitor of color tuning, or it may even actively participate in color tuning.

In chapter 5, I showed that hydrated films can be used for the interaction between protein (Rh) and small molecules (hydroxylamine). I studied the hydroxylamine effect on Meta-I and Meta-II states at their characteristic temperatures, 240 and 280 K, respectively, under the physiological pH (~7) (4). Time-dependent formation of retinal oxime was monitored at 265 K by UV/visible and FTIR spectroscopy. As the results, I elucidated that hydroxylamine does not react with the Schiff base of Meta-I, which is also the case in unphotolyzed Rh, in contrast, hydroxylamine only reacts with Meta-II, suggesting that relaxed structure around the retinal Schiff base is necessary for the formation of retinal oxime.

I have the following research plan in near future. First, as shown in chapter 4, I would like to assign the X-D stretching vibration band. From the present structural study for color pigments, I confirmed that dipolar residues near the $\beta$-ionone ring are the color determinant between green and red sensitive pigments. On the other hand, I found an X-D stretch of protein correlating with $\lambda_{\text{max}}$. Since the mechanism is interesting, I'd like to assign this amino acid using mutant proteins. Second, I would like to achieve structural analysis of monkey blue-sensitive visual pigment (MB) and
reveal the structural factor of seeing blue. The most mysterious question for color tuning study is that Rh and color-pigments both contain a common chromophore molecule, 11-cis-retinal, whereas different chromophore-protein interactions allow preferential absorption of different colors. Thus, it is necessary to do structural analysis of MB to finish my journey of the world of color vision.

In far future, I would like to try to investigate the structural basis for not only visual pigments but also several types of GPCR. As is clearly understood from the Novel Prize for Chemistry in 2012, which was rewarded for the structural study of adrenergic receptor (5), remarkable advances in our knowledge of GPCR structure have been recently achieved by X-ray crystallography. This has led to insights into the conformational changes during GPCR activation and has provided opportunities for virtual screening of molecular libraries and fragment-like ligands. In this thesis (in my Ph. D course), I have achieved the structural study of color pigments which is one of GPCRs family. Therefore, in future, I like to apply spectroscopic methods to other GPCR proteins like taste, adenosine, adrenergic receptors and elucidate the interaction between receptors and ligands on the basis of structure.
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