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FTIR study of primate color visual pigments

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How do we distinguish colors? Humans possess three color pigments; red-, green-, and blue-sensitive proteins, which have maximum absorbance (λ_{max}) at 560, 530, and 420 nm, respectively, and contribute to normal human trichromatic vision (RGB). Each color pigments consists of a different opsin protein bound to a common chromophore molecule, 11-*cis*-retinal, whereas different chromophore-protein interactions allow preferential absorption of different colors. However, detailed experimental structural data to explain the molecular basis of spectral tuning of color pigments are lacking, mainly because of the difficulty in sample preparation. We thus started structural studies of primate color visual pigments using low-temperature Fourier-transform infrared (FTIR) spectroscopy, which needs only 0.3 mg protein for a single measurement. Here we report the first structural data of monkey red- (MR) and green- (MG) sensitive pigments, in which the information about the protein, retinal chromophore, and internal water molecules is contained. Molecular mechanism of color discrimination between red and green pigments will be discussed based on the structural data by FTIR spectroscopy.

Key words: color visual pigment, color tuning, FTIR, retinal, internal water molecule

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]. 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. Furthermore, in the case of bovine Rh, the structures of 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] have also been solved. 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 no color pigment has been crystallized thus far.

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 a difference of 30 nm in their λ_{max} values and their sequences differ by 15 amino acids [2]. Figure 1a illsuatrates the chromophore and surrounding 27 amino acids (within 5 Å) in bovine Rh. While monkey Rh (MRh) has identical amino acids, about half of them are replaced in MR and MG. 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 (Fig. 1b) [11]. Between MR and MG, three amino acids are different near the retinal chromophore, where O-H bearing residues are introduced in MR such as Ser, Tyr, and Thr (Fig. 1b). This is also the case in human green and red pigments. This strongly suggests that these hydroxyl groups are responsible for the different λ_{max} between green and red. This hypothesis was indeed confirmed by the previous site-directed mutagenesis studies [12–14].

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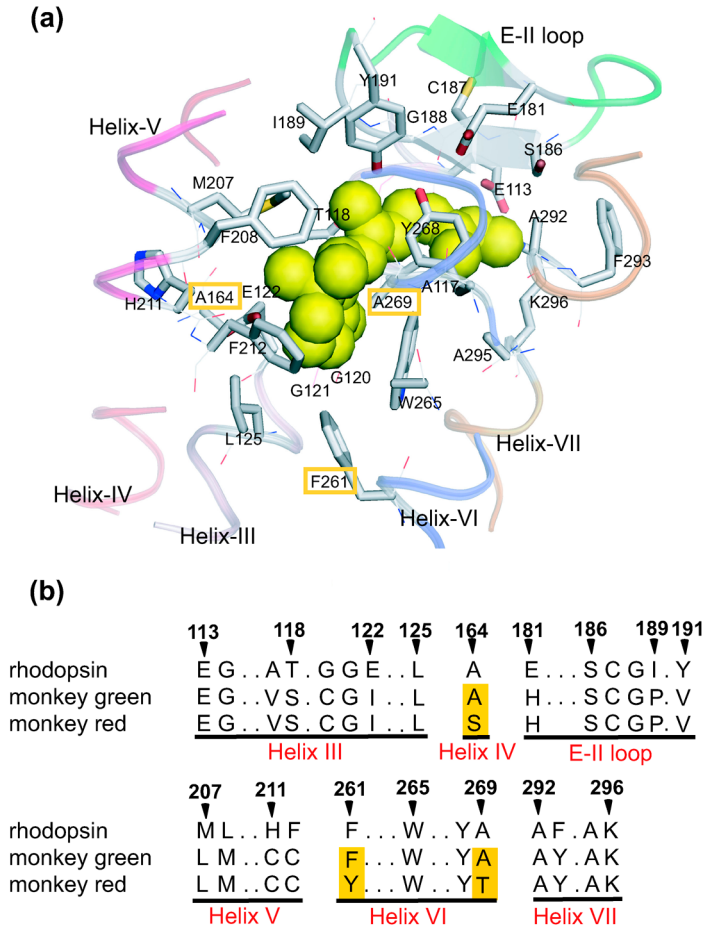

Figure 1 (a) X-ray crystal structure of the chromophore-binding site of bovine rhodopsin (1U19 [4]). The upper and lower regions correspond to the extracellular and cytoplasmic sides, respectively. The retinal chromophore is shown by yellow space-filling model. Side chains of the 27 amino acids within 5 Å from the retinal chromophore are shown by stick drawing. Ribbon drawings illustrate the secondary structures around the retinal. Corresponding amino acids in MR and MG are identical except for three 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 three amino acids that differ in MR and MG are highlighted orange. The residue numbers are based on the bovine Rh sequence. Reprinted with permission from ref. 34. Copyright 2010 Wiley-VCH Verlag.

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 protein-bound water molecules in proton-pumping microbial rhodopsins [15–17]. In fact, proton transfer via a transient linear water-molecule chain in a membrane protein, especially microbial rhodopsins, was discussed recently [18]. Protein-bound water molecules in Rh are experimentally monitored by X-ray crystallography [4,5], FTIR spectroscopy [19–21] and radiolytic labeling method [22,23]. Structural analysis of the protein-bound waters in our color visual pigments is thus intriguing, though indeed challenging.

The structural analysis on the green and red pigments was only reported by resonance Raman spectroscopy, in which the observed vibrational bands were very similar between human green and red pigments, indicating similar chromophore-protein interaction [24]. 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 [24], but no experimental confirmation has been obtained to date. In contrast, IR spectroscopy is able to provide vibrational signals not only from the chromophore, but also from protein and water molecules [19]. We previously reported difference FTIR spectra of the chicken red-sensitive pigment that was prepared from over 2000 chicken retinae [25], but identification of the vibrational bands of proteins is difficult for native proteins.

The first structural data of MR and MG

We expressed MR and MG in HEK293 cell lines for structural analysis using FTIR spectroscopy. As previously reported, 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 [19,26]. Thus, information

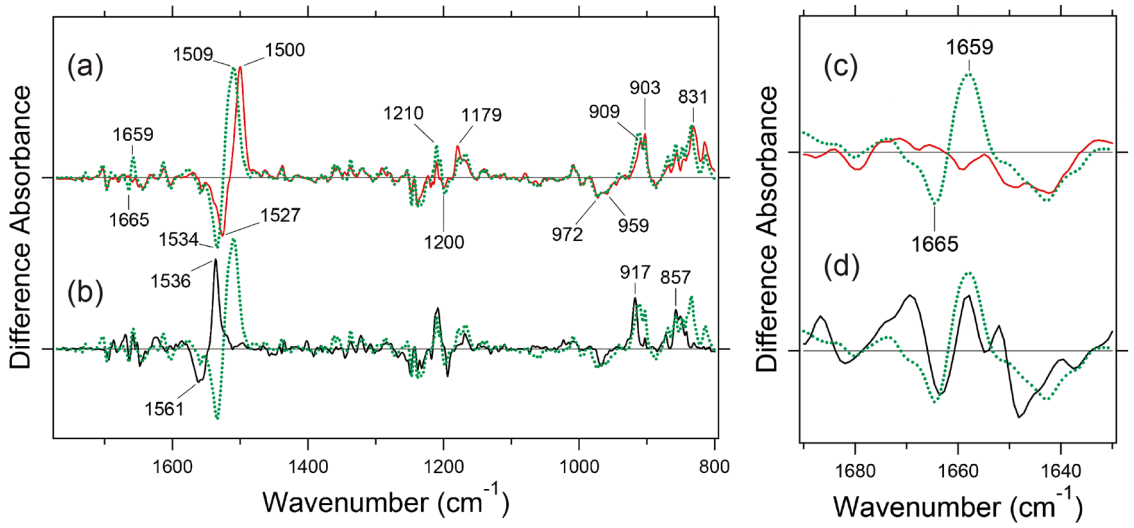

Figure 2 Light-induced difference FTIR spectra of MR (red curve), MG (green dotted curve) and MRh (black curve) in the 1,770–800 cm^{-1} (a, b) and 1,690–1,630 cm^{-1} (c) regions. Each pigment was irradiated with 543, 501, and 501 nm light (by use of an interference filter) for MR, MG, and MRh until photo-steady-state mixtures (batho intermediates) were produced. The samples were then irradiated for the reversion from batho intermediates to the original states with >660, >610, and >610 nm, respectively, and difference spectra were obtained between batho intermediates and original states. The spectra are measured at 77 K in D_2O. Each spectrum was taken with permission from ref. 34.

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 4,000–1,800 cm^{-1} that monitors X-H stretching vibrations. The measurements in D_2O further isolate H-D nonexchangeable and exchangeable vibrations at 4,000–2,700 and 2,700–1,800 cm^{-1}, respectively [20,26,27]. We isolated MR and MG from at least 3 L cultures of HEK293 cells, which were 40-times larger than those of MRh, because the expression level was much lower for MR and MG. Each protein expressed in HEK293 cell lines, was solubilized by a detergent, purified by antibody column, and reconstituted into L-α-phosphatidylcholine liposomes [28]. Since the sample amounts for FTIR spectroscopy were very limited, we were not able to optimize the preparation conditions, and we followed the methods applied for bovine Rh [29,30]. Nevertheless, we were able to obtain spectral data of MR and MG in the entire mid-IR region.

Figure 2 shows light-induced difference FTIR spectra measured at 77 K in D_2O. Formation of the batho intermediate is clear from the down-shifted ethylenic C=C stretches of the retinal chromophore at 1,561 (–)/1,536 (+), 1,534 (–)/1,509 (+), and 1,527 (–)/1,500 (+) cm^{-1} for MRh, MG, and MR, respectively, which correspond to the redshift in the visible region. The spectra of MR and MG are very similar (Fig. 2a), but it should be noted that these spectra are also similar to that of MRh (Fig. 2b). The reason probably comes from the fact that vibrational signals of the retinal chromophore dominate at 77 K in the spectral region shown in Figure 2, such as C=C stretch at 1,570–1,500 cm^{-1}, C-C stretches at 1,250–1,150 cm^{-1}, and hydrogen-out-of-plane vibrations at 1,000–800 cm^{-1}. The present observation that the vibrations due to the retinal chromophore were similar between MR and MG, is consistent with the previous resonance Raman results [24]. On the other hand, a clear spectral difference between MR and MG was seen in the amide-I region. MG possesses the bands at 1,665 (–)/1,659 (+) cm^{-1}, which are absent in MR (Fig. 2c). Since the frequency is characteristic of α_{II}-helix [31], retinal isomerization accompanies helical structural perturbation in MG, but not in MR. Notably, the spectral features between MG and MRh are slightly changed in the amide-I region, suggesting that 3 pigments possess their own structural elements, though they are provided the similar retinal binding pockets due to similar retinal signals in Figure 2a and b.

Although the difference FTIR spectra are similar for the 3 pigments in the 1,770–800 cm^{-1} region, the situation is entirely different in the X-D (Fig. 3a, b) and X-H (Fig. 3c, d) stretching regions. H-D exchangeable vibrations such as O-D and N-D stretches appear at 2,700–2,000 cm^{-1} in D_2O. The spectral features are identical between MR and MG, (Fig. 3a). In contrast, they are very different between MG and Rh (Fig. 3b). These facts indicate that hydrogen-bonding network involving water molecules is similar between MR and MG, but different in Rh.

Spectral comparison in the X-H stretch region (Fig. 3c, d) led to the same conclusion as for the X-D stretch (Fig. 3a, b) in view of the similarity between MR and MG, but not for Rh. Although MR and MG exhibit similar bands in the X-H stretch region, a negative band at 3,359 cm^{-1} in Figure 3c was only observed for MR, not for MG. A possible candidate for the 3,359 cm^{-1} band is one of the 3 amino acids possessing O-H groups (orange box in Fig. 1b). We previously identified O-H stretching frequencies of Thr at 3,500–3,300 cm^{-1}

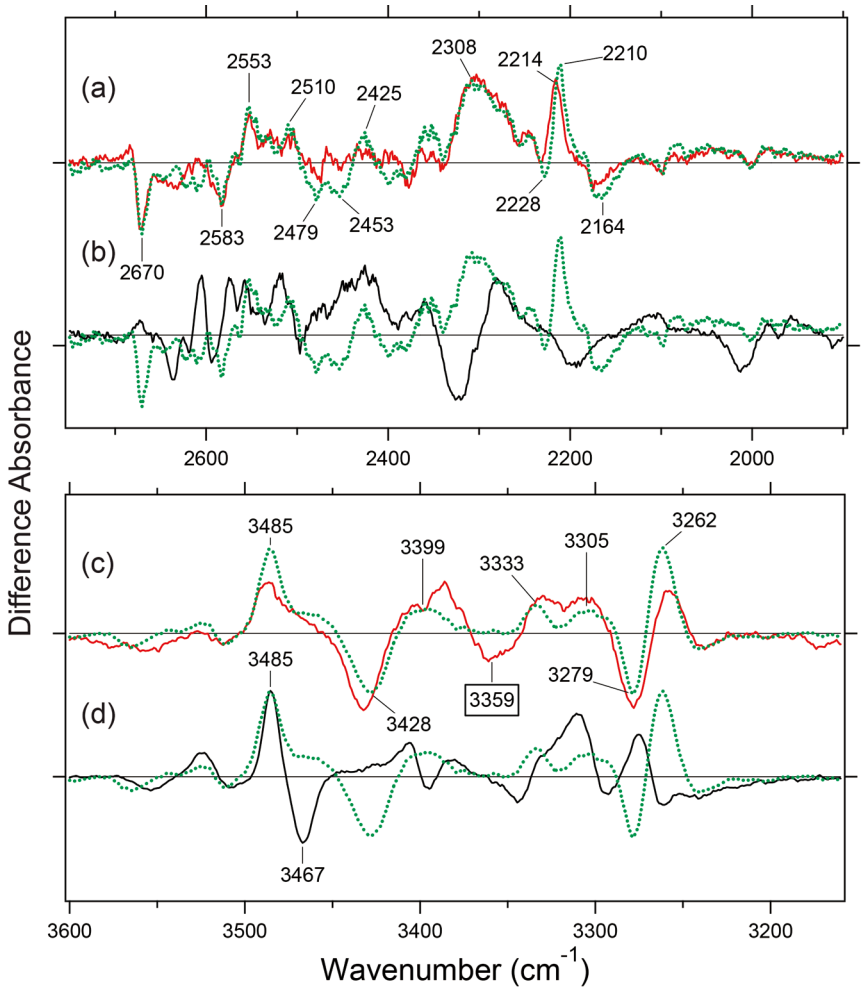

Figure 3 Light-induced difference FTIR spectra of MR (red curve), MG (green dotted curve) and MRh (black curve) in the 2,750-1,900 cm^{-1} (a, b) and 3,600-3,160 cm^{-1} (c, d) regions. The spectra are measured at 77 K in D_2O. The band highlighted at 3,359 cm^{-1} is unique to MR. Each spectrum was taken with permission from ref. 34, 35.

in bacteriorhodopsin [27] and *pharaonis* phoborhodopsin [32], and the frequency at 3,359 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.

Protein-bound water molecules in MR and MG

Successful detection of the accurate FTIR spectra in the X-D stretch region motivated us to identify protein-bound water molecules in MR and MG. How are water structures different between color pigments and Rh? The detailed spectral analysis of water can be performed for less-noisy spectra. By further improving of FTIR spectral accuracy, we could 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 corresponds to only the environment around the retinal.

Figure 4a and b show the X-D stretch region of MR and MG in D_2O (red curve) and $D_2{}^{18}O$ (blue curve), respectively, and the spectral changes at 2,700-2,450 cm^{-1} mostly originate from water stretching vibrations because of the downshift by isotope water.

From Figure 4a 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 Rh (Fig. 4c) [20]. While these peaks seem to be distributed randomly, the averaged frequencies are 2,579 and 2,591 cm^{-1} for MR and MG, respectively. Since MR and MG possess the λ_{max} at 560 and 530 nm, respectively, it is likely that the red-shifted pigments possess protein-bound water molecules at a lower frequency, i.e. under stronger H-bonds. Such correlation may be also applicable for Rh (λ_{max}: 500 nm), because the averaged frequency is 2,600 cm^{-1} [20]. It should be noted that the frequencies of internal water molecules are determined by their local environments, so

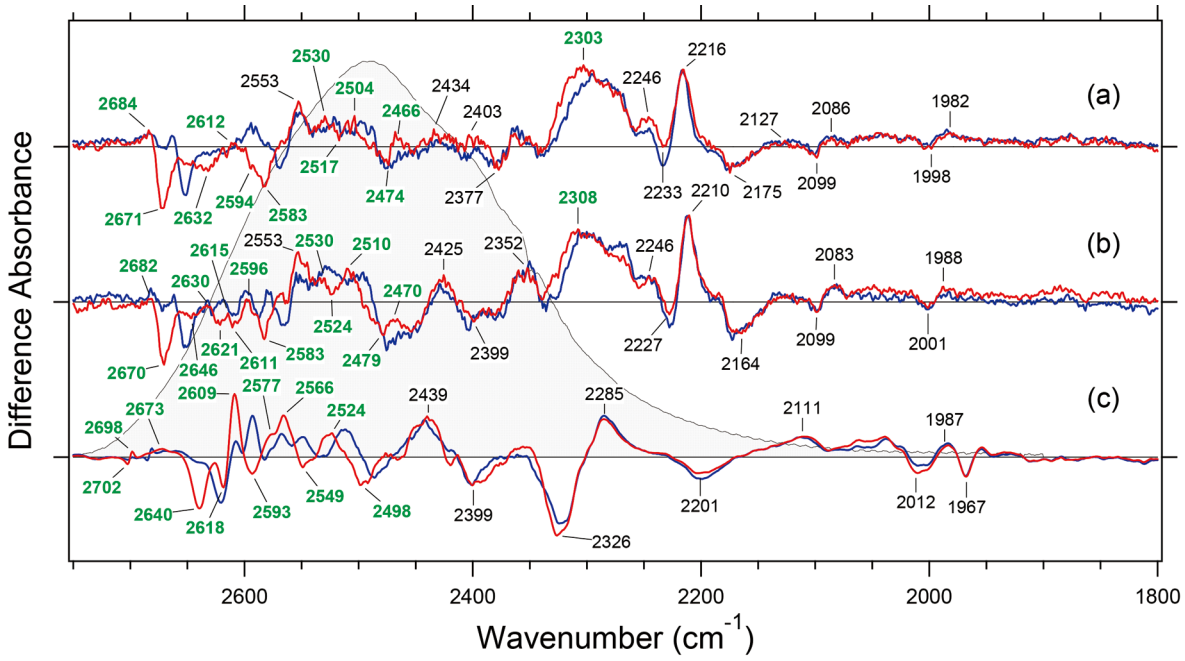

Figure 4 Light-induced difference FTIR spectra of MR (a), MG (b), and bovine Rh (c) in the 2,750–1,800 cm^{-1} region. The spectra are measured at 77 K in D_2O (red curve) and $D_2{}^{18}O$ (blue curve), respectively, and green labeled frequencies correspond to those identified as water stretching vibrations. The gray curve in the 2,700–2,000 cm^{-1} region represents O-D stretching vibrations of D_2O at room temperature. Each spectrum was taken with permission from ref. 35.

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-*trans* form after photoisomerization appear at 2,700–2,500 cm^{-1} in Rh (Fig. 4c). In contrast, the frequencies of the positive 2,303-cm^{-1} and 2,308-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. The frequencies are lower than that of pure deuterated water in the ice form (~2,400 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* [33]. Interestingly, MR, MG and HR all possess a Cl^--binding site near the retinal chromophore [11,33]. In the previous paper on HR, we discussed that Cl^- is weakly hydrated by internal waters, but photoisomerization perturbs the local structure of the anion biding site, resulting in the formation of a strong H-bond with the water [33]. This is also possibly the case with MR and MG.

Future Prospects

In this review, we showed the first FTIR spectral comparison of the red- and green-sensitive color visual pigments in the L group. The FTIR spectra of the color pigments were similar to those of Rh in the conventional 1,800–800 cm^{-1} region, whereas the spectra were entirely different in the X-D (2,700–2,000 cm^{-1}) and X-H (3,800–2,800 cm^{-1}) stretching regions. In addition, some spectral differences containing protein-bound water molecules between MR and MG were observed. Since X-H and X-D stretches are the direct probes of hydrogen-bonding environment, this study opens a new window in understanding the specific chromophore-protein interactions in color pigments. On the other hand, it should be noted that further FTIR analysis by use of mutant and/or isotope labeled proteins enable us to provide more detailed information about and discuss the basis of structure. Therefore, we have performed the measurement of some mutant proteins and isotope-labeled retinal protein, which can provide more detailed information about and discuss the basis of structure. Furthermore, we will go ahead with the structural analysis of monkey blue (MB) using FTIR. Molecular mechanism of our color discrimination can be understood from the structural basis in the end.

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