Color Vision

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

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Humans have two kinds of vision: twilight vision mediated by rhodopsin in rod photoreceptor cells and color vision achieved by multiple color pigments in cone photoreceptor cells.[1] Humans have 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 red, green, blue (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.[3] On the molecular level, studying rhodopsin 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 rhodopsin, the structures have been further determined for photointermediates[6,7] and for 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 rhodopsin. 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 an approximately 30 nm difference in the λ_{max} value and have 15 amino acid sequence differences.[2] Figure 1a illustrates the chromophore and surrounding 27 amino acids (within 5 Å) in bovine rhodopsin. While monkey rhodopsin has identical amino acids, about half of them are replaced in monkey green and red pigments. E113 is the common counterion of the protonated Schiff base, but

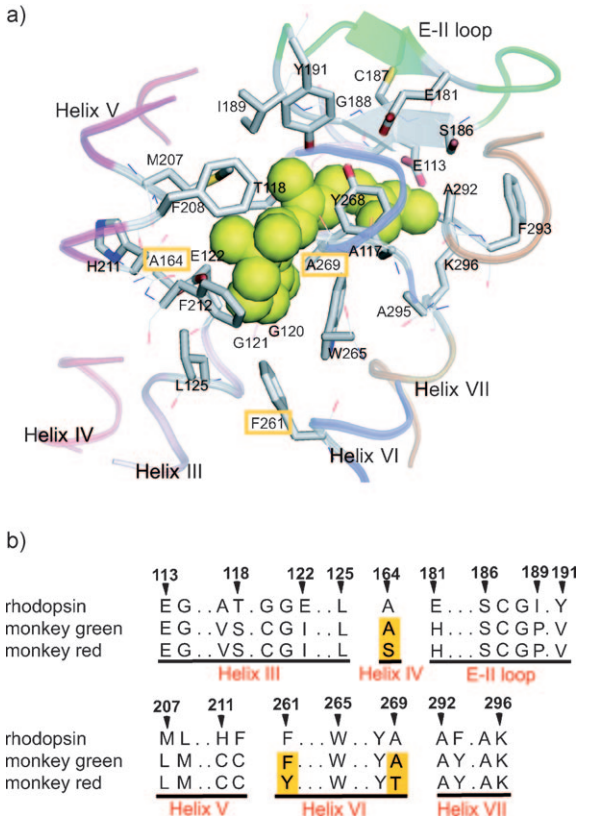

Figure 1. a) X-ray crystallographic structure of the chromophore-binding site of bovine rhodopsin (Protein Data Bank entry: 1U19[4]), which is viewed from the helix VI side. The upper and lower regions correspond to the extracellular and cytoplasmic sides, respectively. The retinal chromophore, which 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 monkey green and red pigments are identical except for three amino acids shown by orange boxes. b) Partial amino acid sequences of rhodopsin (bovine and monkey), monkey green, and monkey red. 27 amino acids within 5 Å from the retinal chromophore in bovine rhodopsin are shown. The amino acids are identical between bovine and monkey rhodopsins. The three amino acids that differ in monkey green and red are highlighted orange. The residue numbers are based on the bovine rhodopsin sequence.

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E181 is replaced by histidine that functions as a chloride binding site in the L group (Figure 1b).[9] Between monkey green and red, three amino acids are different near the retinal chromophore, where O–H bearing residues are introduced in monkey red such as Ser, Tyr, and Thr (Figure 1b). This is also the case in human green and red pigments. This strongly suggests that these hydroxy groups are responsible for the different λ_{max} between green and red. This hypothesis was indeed confirmed by the previous site-directed mutagenesis,[10–12] whereas distant hydroxy 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 rhodopsin 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, in which 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 to date. In contrast, IR spectroscopy is able to provide vibrational signals not only from the 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 over 2000 chicken retinae,[22] but identification of the vibrational bands of proteins is difficult for native proteins.

We thus attempted to express monkey green and red 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 indicate the changes in vibrational modes of the retinal chromophore and surrounding protein and water molecules.[21,23] Thus, information on local structural perturbation of the protein upon retinal photoisomerization can be obtained. In addition, information on hydrogen bonds can be obtained from the frequency region of 4000–1800 cm^{-1} that monitors X–H stretching vibrations. The measurements in D_2O identify H–D non-exchangeable and exchangeable vibrations at 4000–2700 and 2700–1800 cm^{-1}, respectively.[23–25] Herein, we report the FTIR spectral comparison of monkey green and red. We used cultures of monkey green and red that were 40-times larger than those of monkey rhodopsin, because the expression level was much lower for monkey green and red. Each protein expressed in HEK293 cell lines, was solubilized by a detergent, purified by antibody column, and reconstituted into L-α-phophatidylcholine liposomes. 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 rhodopsin.[26,27] Herein, the intensity of the FTIR signals for monkey red were smaller than those for monkey green, which yielded noisier spectra for X–H and X–D stretching frequencies. Nevertheless, we were able to obtain the difference spectra of monkey green and red in the entire mid-IR region.

Figure 2 shows light-induced difference FTIR spectra measured at 77 K in D_2O. 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 rhodopsin, green, and red, respectively, which correspond to the red-shift in the visible region. The spectra of monkey green and red are very similar (Figure 2a), but it should be noted that the spectra are also similar to that of monkey rhodopsin (Figure 2b). The reason is probably that vibrational signals of the retinal chromophore dominate in Figure 2, such as the 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 monkey green and red, the vibrations arising from the retinal chromophore were similar, and consistent with previous resonance Raman results.[20] On the other hand, a clear spectral difference was seen in the amide-I region. Monkey green has bands at 1665 (–)/1659 (+) cm^{-1}, which are absent in monkey red (shown in detail in Figure S1 of the Supporting Information). Since this frequency is characteristic of an α_{II} helix,[28] a retinal chromophore isomerization accompanies helical structural perturbation in monkey green, but not in monkey red.

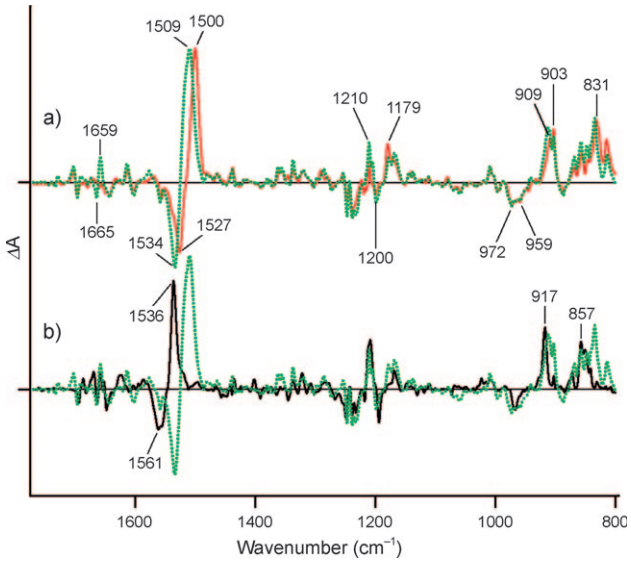

Figure 2. Light-induced difference FTIR spectra of monkey green (green dotted lines in (a) and (b)), monkey red (red line in (a)) and monkey rhodopsin (black line in (b)) in the 1770–800 cm^{-1} region. The spectra are measured at 77 K in D_2O. Positive and negative bands originate from the bathointermediate and unphotolyzed states, respectively. The spectra of monkey red, monkey 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.

Although the difference FTIR spectra are similar for the three pigments in the 1770–800 cm^{-1} region, the situation is entirely different in the X–D (Figure 3) and X–H (Figure 4) stretching regions. H–D exchange vibrations, such as O–D and N–D stretches, appear at 2700–2000 cm^{-1} in D_2O. The spectral features are identical in monkey green and red, though the spectrum of monkey red was noisier (Figure 3a).

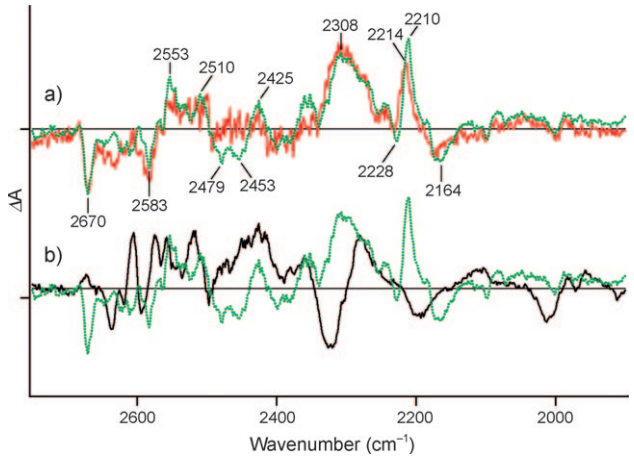

Figure 3. Light-induced difference FTIR spectra of monkey green (green dotted lines in (a) and (b)), monkey red (red line in (a)) and monkey rhodopsin (black line in (b)) in the 2750–1900 cm^{-1} region. The spectra are measured at 77 K in D_2O. The scaling factors are the same as in Figure 2. One division of the y axis corresponds to 0.000035 absorbance unit.

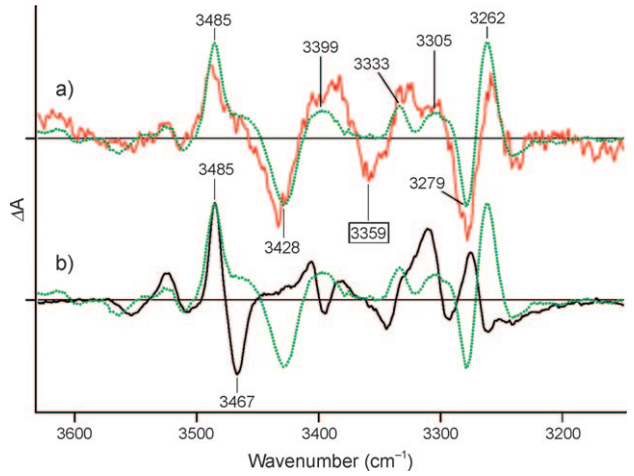

Figure 4. Light-induced difference FTIR spectra of monkey green (green dotted lines in (a) and (b)), monkey red (red line in (a)), and monkey rhodopsin (black line in (b)) in the 3630–3150 cm^{-1} region. The band highlighted at 3359 cm^{-1} is unique to monkey red. The spectra are measured at 77 K in D_2O. The scaling factors are the same as in Figure 2. One division of the y axis corresponds to 0.00005 absorbance unit.

In contrast, they are very different between monkey green and rhodopsin (Figure 3b). These facts indicate that the hydrogen-bonding network involving water molecules is similar in monkey green and red, but different in rhodopsin. One noticeable band is a sharp positive peak at 2210 and 2214 cm^{-1} in monkey green and red, respectively, which is absent in monkey rhodopsin. It may originate from 1) the N–D or O–D stretch of the protein, or 2) the 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 three hydroxy groups belong to the retinal binding pocket in monkey red, but not in green, their vibrational bands would only be expected in monkey red. Nevertheless, we did not observe bands specific to monkey red in the X–D stretch region.

Spectral comparison in the X–H stretch region (Figure 4) led to the same conclusion as for the X–D stretch region (Figure 3) in terms of the similarity between green and red, but not to rhodopsin. The sharp peaks at 3485 (+)/3467 (−) cm^{-1} in monkey rhodopsin (Figure 4b) are similar in frequency to those at 3487 (+)/3463 (−) cm^{-1} in bovine rhodopsin,[29] thus they probably originate from an O–H stretch of Thr118. Similarly, the peaks at 3485 (+)/3428 (−) cm^{-1} in monkey green and red are likely to originate from an O–H stretch of the corresponding Ser residue (Figure 1b). Although monkey green and red exhibit similar bands in the X–H stretch region, a negative band at 3359 cm^{-1} (highlighted in Figure 4a) was only observed for monkey red, not for monkey green. This band was noisy, but reproduced for three independent measurements. A possible candidate for the 3359 cm^{-1} band is one of the amino acids possessing O–H groups (orange box in Figure 1b). We previously identified O–H stretching frequencies of Thr at 3500–3300 cm^{-1} in bacteriorhodopsin[24] and *pharaonis* phoborhodopsin,[30] and the frequency at 3359 cm^{-1} suggests a strong hydrogen bond for an O–H stretch. Thus, the monkey-red-specific X–H stretch may be the key to understanding the unique chromophore–protein interaction in the red pigment. It should be noted that the position of the corresponding positive peak is not clear because of the overlap with noisy spectral features. Therefore, future FTIR analysis by use of mutant proteins will be needed to provide more detailed information.

In summary, we report the first FTIR spectral comparison of the green- and red-sensitive color visual pigments in the L group. We used a sample preparation procedure based on the HEK293 cell line. The FTIR spectra of the color pigments were similar to those of rhodopsin 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 monkey green and red 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.

Experimental Section

The cDNAs of monkey green, red, and rhodopsin were tagged by the Rho1D4 epitope sequence and introduced into expression vectors pcDLSR α296. They were expressed in the HEK293T cell line and regenerated with 11-*cis*-retinal as previously reported.[26,27,31] The reconstituted pigments were extracted with buffer A [2% (w/v) *n*-dodecyl-β-D-maltoside, 50 mM HEPES (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid), 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.5)] and purified by adsorption on an antibody-conjugated column and eluted with buffer B [0.06 mg mL^{-1} 1D4 peptide, 0.02% *n*-dodecyl-β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.5)]. For the FTIR analysis, solubilized samples were reconstituted into phosphatidylcholine liposomes with a 100-fold molar excess. Low-temperature FTIR spectroscopy was applied

to the films hydrated with D_2O at 77 K as described previously.[21–25] For the formation of bathointermediates, the samples were irradiated with 543, 501, and 501 nm light (by use of an interference filter) for monkey red, green, and rhodopsin, respectively. 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 monkey red, green, and rhodopsin, respectively.

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[1] Y. Shichida, H. Imai, *Cell. Mol. Life Sci.* **1998**, *54*, 1299 – 1315.
[2] D. D. Oprian, A. B. Asenjo, N. Lee, S. L. Pelletier, *Biochemistry* **1991**, *30*, 11367 – 11372.
[3] "Retinal binding proteins": H. Kandori in *cis-trans Isomerization in Biochemistry* (Ed.: C. Dugave), Wiley-VCH, Weinheim, **2006**, pp. 53 – 75.
[4] K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto, M. Miyano, *Science* **2000**, *289*, 739 – 745.
[5] M. Murakami, T. Kouyama, *Nature* **2008**, *453*, 363 – 367.
[6] H. Nakamichi, T. Okada, *Angew. Chem.* **2006**, *118*, 4376 – 4379; *Angew. Chem. Int. Ed.* **2006**, *45*, 4270 – 4273.
[7] H. Nakamichi, T. Okada, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12729 – 12734.
[8] P. Scheerer, J. H. Park, P. W. Hildebrand, Y. J. Kim, N. Krauß, H. W. Choe, K. P. Hofmann, O. P. Ernst, *Nature* **2008**, *455*, 497 – 502.
[9] Z. Wang, A. B. Asenjo, D. D. Oprian, *Biochemistry* **1993**, *32*, 2125 – 2130.
[10] R. Yokoyama, S. Yokoyama, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9315 – 9318.
[11] M. Neitz, J. Neitz, G. H. Jacobs, *Science* **1991**, *252*, 971 – 974.
[12] A. B. Asenjo, J. Rim, D. D. Oprian, *Neuron* **1994**, *12*, 1131 – 1138.
[13] P. B. Coto, A. Strambi, N. Ferre, M. Olivucci, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17154 – 17159.
[14] S. Sekharan, M. Sugihara, V. Buss, *Angew. Chem.* **2007**, *119*, 273 – 275; *Angew. Chem. Int. Ed.* **2007**, *46*, 269 – 271.
[15] K. Fujimoto, J. Hasegawa, H. Nakatsuji, *Chem. Phys. Lett.* **2008**, *462*, 318 – 320.
[16] A. Altun, S. Yokoyama, K. Morokuma, *J. Phys. Chem. B* **2008**, *112*, 6814 – 6827.
[17] G. Tomasello, G. Olaso-Gonzalez, P. Altoe, M. Stenta, L. Serrano-Andres, M. Merchan, G. Orlandi, A. Nottoni, M. Garavelli, *J. Am. Chem. Soc.* **2009**, *131*, 5172 – 5186.
[18] R. E. Stenkamp, S. Filipek, C. A. G. G. Driessen, D. C. Teller, K. Palczewski, *Biochim. Biophys. Acta Biomembr.* **2002**, *1565*, 168 – 182.
[19] R. J. Trabanino, N. Vaidehi, W. A. Goddard, *J. Phys. Chem. B* **2006**, *110*, 17230 – 17239.
[20] G. G. Kochendoerfer, Z. Wang, D. D. Oprian, R. A. Mathies, *Biochemistry* **1997**, *36*, 6577 – 6587.
[21] H. Kandori, A. Maeda, *Biochemistry* **1995**, *34*, 14220 – 14229.
[22] T. Hirano, H. Imai, H. Kandori, Y. Shichida, *Biochemistry* **2001**, *40*, 1385 – 1392.
[23] H. Kandori, N. Kinoshita, Y. Shichida, A. Maeda, *J. Phys. Chem. B* **1998**, *102*, 7899 – 7905.
[24] H. Kandori, N. Kinoshita, Y. Yamazaki, A. Maeda, Y. Shichida, R. Needleman, J. K. Lanyi, M. Bizounok, J. Herzfeld, J. Raap, J. Lugtenburg, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 4643 – 4648.
[25] Y. Furutani, Y. Shichida, H. Kandori, *Biochemistry* **2003**, *42*, 9619 – 9625.
[26] H. Imai, A. Terakita, Y. Shichida, *Methods Enzymol.* **2000**, *315*, 293 – 312.
[27] T. Nagata, A. Terakita, H. Kandori, D. Kojima, Y. Shichida, A. Maeda, *Biochemistry* **1998**, *37*, 17216 – 17222.
[28] S. Krimm, A. M. Dwivedi, *Science* **1982**, *216*, 407 – 409.
[29] T. Nagata, T. Oura, A. Terakita, H. Kandori, Y. Shichida, *J. Phys. Chem. A* **2002**, *106*, 1969 – 1975.
[30] Y. Sudo, Y. Furutani, K. Shimono, N. Kamo, H. Kandori, *Biochemistry* **2003**, *42*, 14166 – 14172.
[31] H. Imai, T. Hirano, H. Kandori, A. Terakita, Y. Shichida, *Biochemistry* **2001**, *40*, 2879 – 2886.

