BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Protein-Bound Water Molecules in Primate Red- and Green-Sensitive Visual Pigments

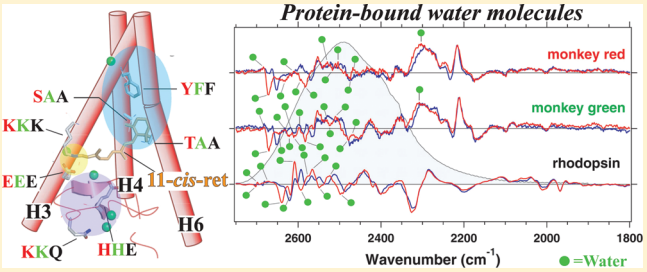
Kota Katayama,[†] Yuji Furutani,[†,§] Hiroo Imai,[‡] and Hideki Kandori*[,†]

[†]Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

[‡]Primate Research Institute, Kyoto University, Inuyama 484-8506, Japan

Supporting Information

ABSTRACT: Protein-bound water molecules play crucial roles in the structure and function of proteins. The functional role of water molecules has been discussed for rhodopsin, the light sensor for twilight vision, on the basis of X-ray crystallography, Fourier transform infrared (FTIR) spectroscopy, and a radiolytic labeling method, but nothing is known about the protein-bound waters in our color visual pigments. Here we apply low-temperature FTIR spectroscopy to monkey red (MR)- and green (MG)-sensitive color pigments at 77 K and successfully identify water vibrations using D_2O and $D_2{}^{18}O$ in the whole midinfrared region. The observed water vibrations are 6−8 for MR and MG, indicating that several water molecules are present near the retinal chromophore and change their hydrogen bonds upon retinal photoisomerization. In this sense, color visual pigments possess protein-bound water molecules essentially similar to those of rhodopsin. The absence of strongly hydrogen-bonded water molecules (O−D stretch at <2400 cm^{-1}) is common between rhodopsin and color pigments, which greatly contrasts with the case of proton-pumping microbial rhodopsins. On the other hand, two important differences are observed in water signal between rhodopsin and color pigments. First, the water vibrations are identical between the 11-*cis* and 9-*cis* forms of rhodopsin, but different vibrational bands are observed at >2550 cm^{-1} for both MR and MG. Second, strongly hydrogen-bonded water molecules (2303 cm^{-1} for MR and 2308 cm^{-1} for MG) are observed for the all-*trans* form after retinal photoisomerization, which is not the case for rhodopsin. These specific features of MR and MG can be explained by the presence of water molecules in the Cl^--biding site, which are located near positions C11 and C9 of the retinal chromophore. The averaged frequencies of the observed water O−D stretching vibrations for MR and MG are lower as the λ_{max} is red-shifted, suggesting that water molecules are involved in the color tuning of our vision.



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. 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 in a complex with the C-terminal 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 the 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 an ∼30 nm difference in the λ_{max} and have 15 amino acid sequence differences.[2] Previous site-directed mutagenesis studies revealed that three amino acids are mainly responsible for discrimination between green and red color (Figure 1).[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. The 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. The functionally important role of protein-bound water molecules has been extensively studied for microbial rhodopsins.[14−17] Water molecules bound to Rh are experimentally monitored by X-ray crystallography,[4,5] Fourier transform infrared (FTIR) spectroscopy,[18−20] and a radiolytic

Received: November 6, 2011
Revised: December 29, 2011
Published: January 19, 2012

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

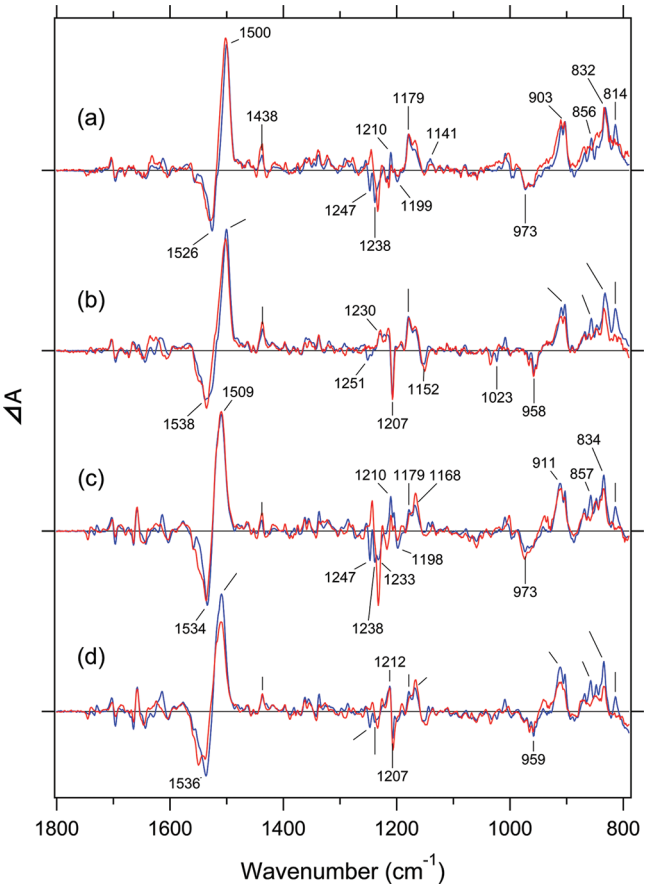


Figure 1. Light-minus-dark difference FTIR spectra of MR (a and b) and MG (c and d) in the 1800−800 cm^{-1} region, which are measured at 77 K in H_2O (red line) and D_2O (blue line). Positive bands originate from the all-*trans* form (bathointermediate, Batho), while negative bands originate from the 11-*cis* (a and c) or 9-*cis* (b and d) form. One division of the *y*-axis corresponds to 0.0024 absorbance unit.

labeling method.[21,22] Structural analysis of the protein-bound waters in our color visual pigments is thus intriguing, though indeed challenging.

We recently conducted the first structural analysis of monkey red (MR) and green (MG) using low-temperature FTIR spectroscopy.[23] The obtained FTIR spectra of MR and MG in D_2O 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 in the 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,[23] indicating a structural difference on 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 D_2O are similar between MR and MG, the reported spectra contained considerable noise, especially for MR.[23] The detailed spectral analysis of water should be performed for less noisy spectra. In this paper, on the basis of the highly accurate FTIR spectra, we 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. The water signals differ not only from those of Rh but also between MR and MG.

To identify the position of water molecules, we normally use mutant proteins. In fact, a previous comprehensive FTIR study of various mutants revealed the position of protein-bound water molecules in bacteriorhodopsin (BR), a light-driven proton pump.[14,24] 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 of protein, which corresponds to 300 culture plates of HEK293 cells). Instead, here we estimated the location of the water molecules using the measurements of the 9-*cis* form, which can be photochemically formed at 77 K. The role of protein-bound water molecules is discussed on the basis of our FTIR observations.

MATERIALS AND METHODS

Sample Preparation. The cDNAs of monkey red (MR) and monkey green (MG) were tagged by the Rho1D4 epitope sequence and introduced into expression vector pcDLSRα296. They were expressed in the HEK293T cell line and regenerated with 11-*cis*-retinal as previously reported.[23,25−27] The reconstituted pigments were extracted with buffer A [2% (w/v) *n*-dodecyl β-D-maltoside, 50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.5)], 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 $MgCl_2$ (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)].

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).[23] 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, the spectral change in MR was smaller than in MG in the previous paper, though the expression levels were similar (0.3 mg of protein from 300 culture plates of HEK293 cells).[23] This fact suggests considerable bleaching of MR during sample preparation, which yields FTIR spectra of MR that are noisier than those of MG. Therefore, in this study, we used night-vision goggles (NOG-7-2, Night Optics) for the sample preparation of MR.

FTIR Spectroscopy. A 30 μL aliquot of the pigment suspension, which contains 0.3 mg of 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 had been evacuated with 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 ~10 min after hydration.

The experimental setup was the same as that described previously,[18,19,23,24] 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 batho intermediates, the samples were irradiated with 543 and 501

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

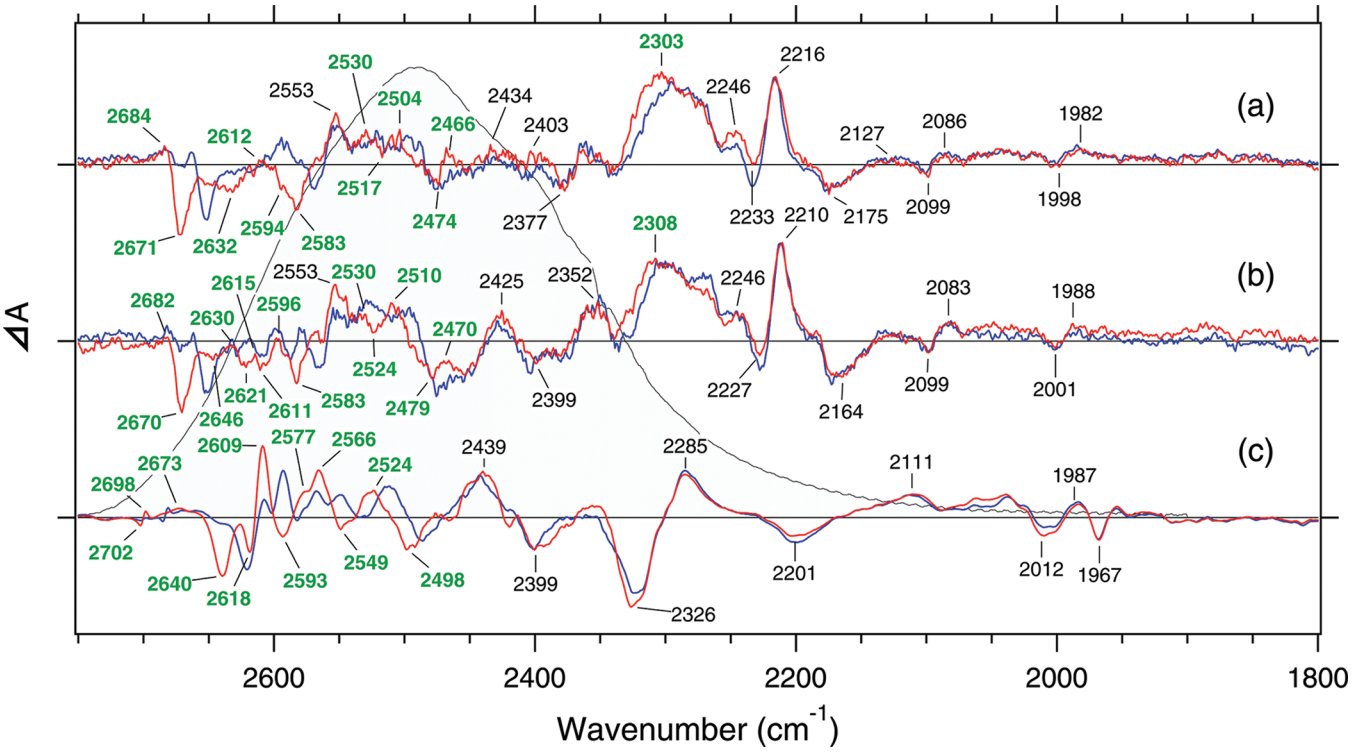


Figure 2. Light-minus-dark difference FTIR spectra of MR (a), MG (b), and bovine Rh (c), with 11-*cis*-retinal as the chromophore, in the 2750−1800 cm^{-1} region measured at 77 K. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively, and green labeled frequencies correspond to those identified as water stretching vibrations. The results for bovine Rh are from ref 19. The grayish curve in the 2700−2000 cm^{-1} region represents O−D stretching vibrations of D_2O 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.

nm light (by use of an interference filter) for MR and MG, respectively, at 77 K.[23] For the reversion from batho intermediates to the original states, the samples were irradiated with >660, and >610 nm light. On the other hand, for the reversion from batho intermediates to the iso (9-*cis*-retinal) states, the samples were irradiated with >560 and >530 nm light. For each measurement, 64 recordings were averaged, yielding the difference FTIR spectra between the all-*trans* and 11-*cis* forms and between the all-*trans* and 9-*cis* forms. The reproducibility of the difference FTIR spectra was confirmed by different sample preparations. Linear dichroism experiments revealed a random orientation of the pigment molecules in the film, so we have not applied polarized FTIR measurements.

RESULTS

Difference FTIR Spectra of the 11-*cis* and 9-*cis* Forms of MR and MG in the Low-Frequency Region. Figure 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 and b) and MG (c and d) in H_2O (red curves) are measured for the first time, while blue spectra in panels a and c of Figure 1 (hydrated with D_2O) reproduced those reported previously.[23] In the difference spectra, the negative signal originates from 11-*cis* or 9-*cis* forms and the positive signal from the batho intermediate (Batho) possessing all-*trans*-retinal. Formation of the Batho state is clearly seen by the strong HOOP vibrations of the retinal chromophore at 900−800 cm^{-1}. In addition, the lowered C=C stretching band of retinal is characteristic of formation of the Batho state, as seen for the pair bands at 1526 (−)/1500 (+) cm^{-1}, 1538 (−)/1500 (+) cm^{-1}, 1534 (−)/1509 (+) cm^{-1}, and 1536 (−)/1509 (+) cm^{-1} in panels a−d of Figure 1, respectively. The negative 1207 cm^{-1} band in panels b and d of Figure 1 can be assigned for the C−C stretching vibration of the retinal chromophore characteristic of the 9-*cis* form.[28]

We 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 S1a of the Supporting Information) and MG (blue curve in Figure S1c) in $D_2{}^{18}O$ are identical to those in D_2O (red curves in Figure S1) in the 1800−800 cm^{-1} region. In fact, double-difference spectra of MR (Figure S1b) and MG (Figure S1d) are coincident with the baseline. Identical spectra between D_2O and $D_2{}^{18}O$ indicate that water vibrations cannot be observed at 1800−800 cm^{-1}. It should be noted that the O−D bending vibration of water appears at ∼1200 cm^{-1}. This implies that the bending vibration is less sensitive to H-bonding alteration between the 11-*cis* and all-*trans* states, presumably because of the small atomic motion of oxygen, which is a great contrast to the case for O−D stretching vibrations as shown below.

Identification of the Water Signal in MR and MG. Red curves in panels a and b of Figure 2 show the light-minus-dark difference FTIR spectra of MR and MG (11-*cis* form) in D_2O, 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 they are entirely different from that of Rh (red curve in Figure 2c).[23] Nevertheless, these measurements with the improved signal:noise ratio provide a clear difference even between MR and MG. Blue curves in

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

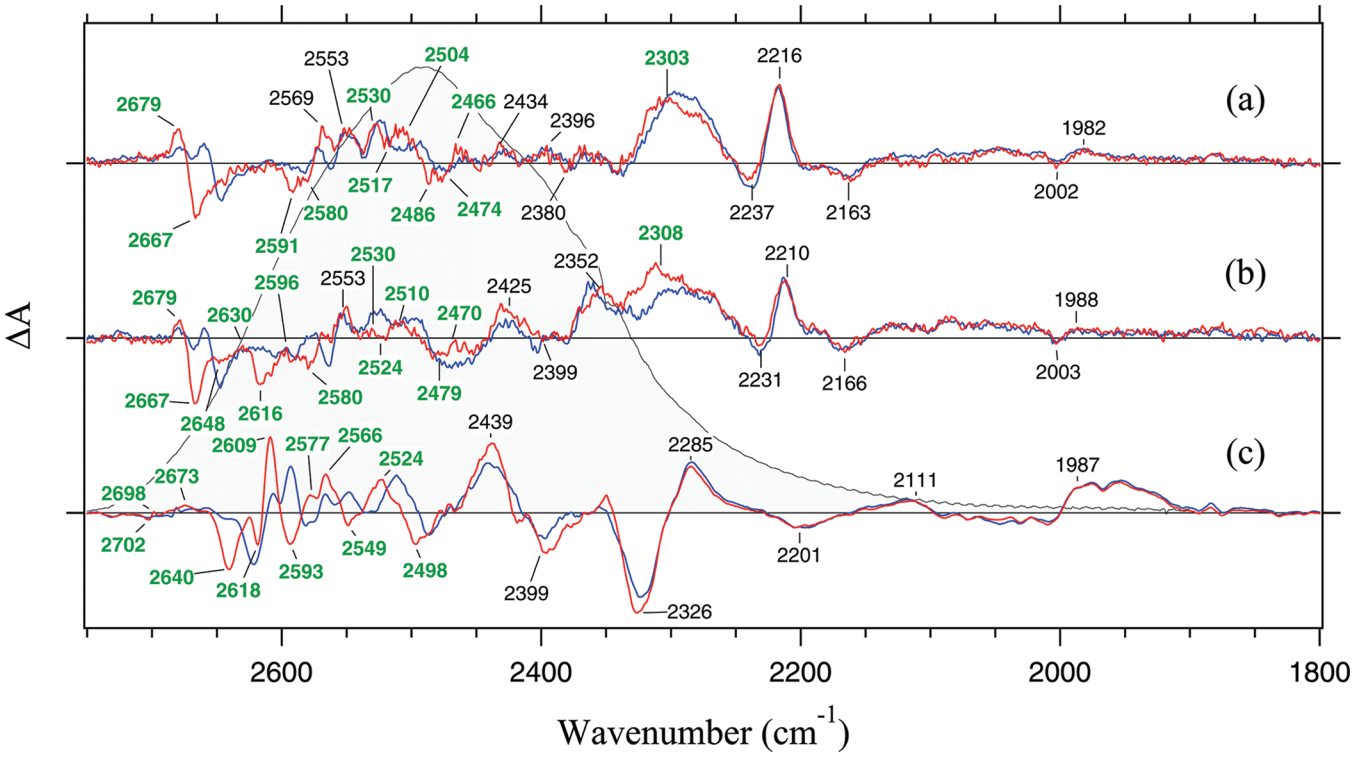


Figure 3. Light-minus-dark difference FTIR spectra for the 9-*cis* form of MR (a), MG (b), and bovine Rh (c) in the 2750−1800 cm^{-1} region measured at 77 K. Red and blue lines represent the spectra in D_2O and $D_2{}^{18}O$, respectively, and green labeled frequencies correspond to those identified as water stretching vibrations. The results for bovine Rh are from ref 19. The grayish curve in the 2700−2000 cm^{-1} region represents O−D stretching vibrations of D_2O 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 spectrum between the all-*trans* and 9-*cis* forms of MR (a), whereas a positive band appears at 2612 cm^{-1} in the difference spectrum between the all-*trans* and 11-*cis* forms of MR (Figure 2a). We thus find that all-*trans* and 9-*cis* forms of MR possess a water band at 2612 cm^{-1}.

Figure 2 correspond to the measurements in $D_2{}^{18}O$, 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_2O 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 $D_2{}^{18}O$ (Figure 2a). Similarly, the spectrum of MG in D_2O 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 $D_2{}^{18}O$ (Figure 2b). 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 panels a and b of Figure 2, MR shows six positive and six negative water bands, while MG shows eight positive and seven negative water bands. The numbers are comparable or slightly greater than those of bovine Rh (Figure 2c).[19] Negative bands for the 11-*cis* form appear at 2700−2450 cm^{-1} for 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. Because MR and MG possess λ_{max} values of 560 and 530 nm, respectively, it is likely that the red-shifted pigments possess protein-bound water molecules at low frequencies, i.e., under strong H-bonds. Such correlation may be also applicable for bovine Rh (λ_{max} = 500 nm), because the averaged frequency is 2600 cm^{-1}.[19] 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 the dielectric environment of the chromophore binding site in MR, MG, and Rh.

Positive bands for the all-*trans* form after photoisomerization appear at 2700−2500 cm^{-1} in bovine Rh (Figure 2c). In contrast, the frequencies of the positive 2303 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. Because MR and MG do not have strongly H-bonded waters for the unphotolyzed state, photoisomerization yields a spectral downshift of the 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 a similar observation only for halorhodopsin (HR) from *Natronomonas pharaonis*,[29] 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.[29,30] In the previous paper on HR, we discussed Cl^- being weakly hydrated by internal waters, but photoisomerization perturbs the local structure of the biding site, resulting in a strong H-bond of the water.[29] This is possibly the case in MR and MG.

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

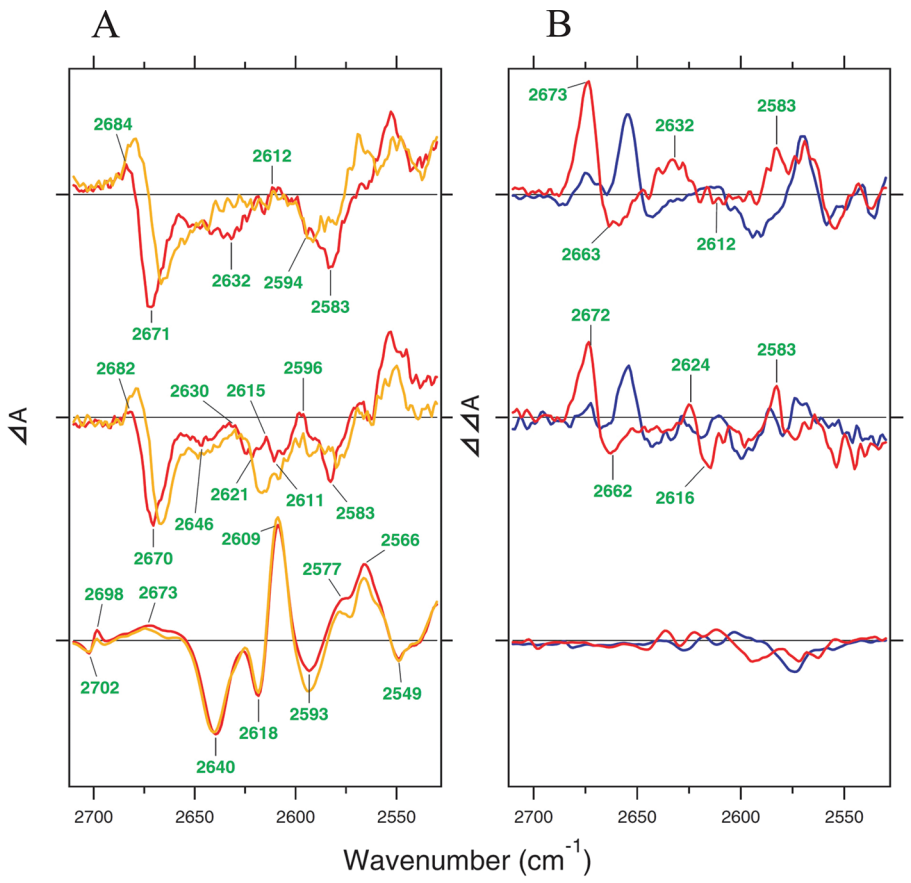


Figure 4. (A) Light-minus-dark difference FTIR spectra of MR (top), MG (middle), and bovine Rh (bottom) in the 2710−2530 cm^{-1} region measured at 77 K. Red and orange lines represent the Batho-minus-11-*cis* form and Batho-minus-9-*cis* form spectra in D_2O, respectively. Green labeled frequencies correspond to those identified as water stretching vibrations for the Batho-minus-11-*cis* form spectra in Figure 2. (B) Double-difference FTIR spectra of panel A, 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 D_2O and $D_2{}^{18}O$, respectively. One division of the *y*-axis corresponds to 0.00011 absorbance unit.

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,[18,31] and we established the experimental conditions for MR and MG in this study. Panels a and b of Figure 3 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, the difference signals of water are identical for bovine Rh, between Batho and the 11-*cis* form (Figure 2c), and between Batho and the 9-*cis* form (Figure 3c).[19] This is not the case for MR and MG, especially at >2500 cm^{-1}.

Figure 4A compares the all-*trans* minus 11-*cis* (red curves) and all-*trans* minus 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 4B represent the double-difference spectra of Figure 4A, which correspond to the 11-*cis* minus 9-*cis* spectra. The spectrum in the bottom portion of Figure 4B coincides with the baseline, indicating no difference between 11-*cis* and 9-*cis* forms of bovine Rh. On the other hand, the top and middle portions of Figure 4B 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

This 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 5. In the case of MR, we observed six O−D stretches for the original 11-*cis* form, while six and seven O−D stretches were identified for the all-*trans* and 9-*cis* forms, respectively. In the case of MG, seven O−D stretches were observed for the original 11-*cis* form, while eight and six O−D stretches were identified for the all-*trans* and 9-*cis* forms, respectively. With regard to the slight discrepancy in the number of water stretching vibrations, one observed band may be composed of multiple O−D stretches. In summary, the number of observed water vibrations is 6−8 for MR and MG, suggesting the presence of three to eight water molecules near the retinal chromophore. In the case of bovine Rh, we reported six O−D stretches for all 11-*cis*, all-*trans*, and 9-*cis* forms.[19] Thus, color visual pigments possess essentially similar protein-bound water molecules, though the numbers may be slightly greater than those of Rh.

For bovine Rh, we reported the lack of water bands under strong H-bonding conditions,[19] which is entirely different from the case for microbial rhodopsins. Our comprehensive FTIR analysis of microbial rhodopsins revealed that proton-pumping

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

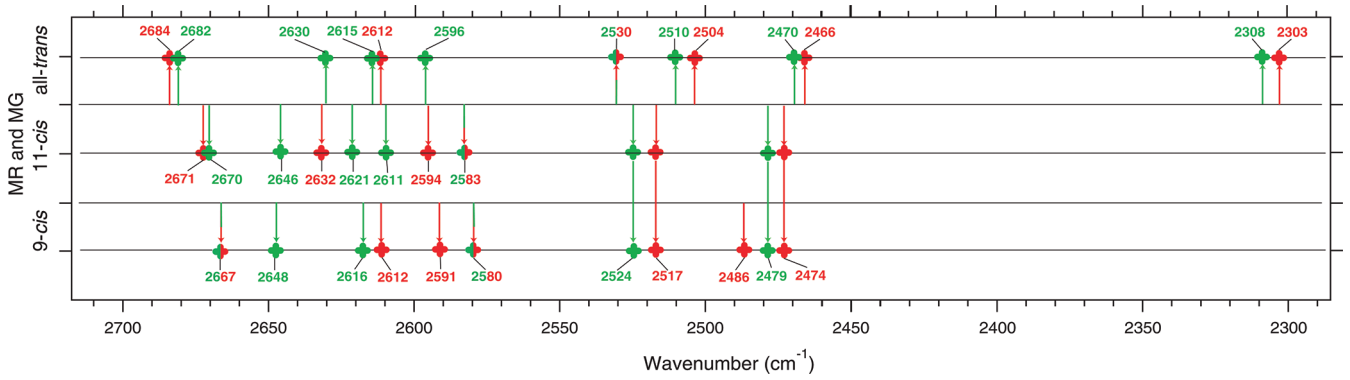


Figure 5. Observed O−D stretching vibrations of water molecules for MR (red) and MG (green). Top, middle, and bottom panels represent the results for all-*trans*, 11-*cis*, and 9-*cis* forms, respectively. Identical frequencies of water between MR and MG are represented as a unique marker, which illustrates the symbols colored half red and half green.

rhodopsins possess strongly H-bonded water molecules, whose O−D stretch is located at <2400 cm^{-1}.[3,16] 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 located at the pentagonal cluster.[24] 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.[16,32] Therefore, strongly H-bonded water molecules are energetically important for proton-pumping rhodopsins. In contrast, the absence of such water molecules under strong H-bonding conditions is common among Rh and color pigments.

Figure 6 illustrates the structure of bovine Rh,[4] which includes three important regions of color pigments: (i) the Schiff base moiety (region I), (ii) the Cl^--binding site (region II), and (iii) the 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 an H-bond with Glu113 in bovine Rh. A 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.[19] Similar results for MR and MG in this 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 2). This indicates that photoisomerization yields a spectral downshift of the water stretching vibration at >150 cm^{-1} for MR and MG. Among many studies of visual and microbial rhodopsins, we made a similar observation only for a light-driven Cl^- pump HR.[29] 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 that bisects the D−O−D angle on a straight line joining an oxygen atom and Cl^-.[33] Hydrogen bonds of water are weak in this configuration. On the other hand, photoisomerization in protein enforces the structural perturbation at the Cl^--binding site, causing the O−D group to be located on a straight line between the oxygen and Cl^-, by which a strong H-bond is formed in the K state for HR or Batho for MR or MG.

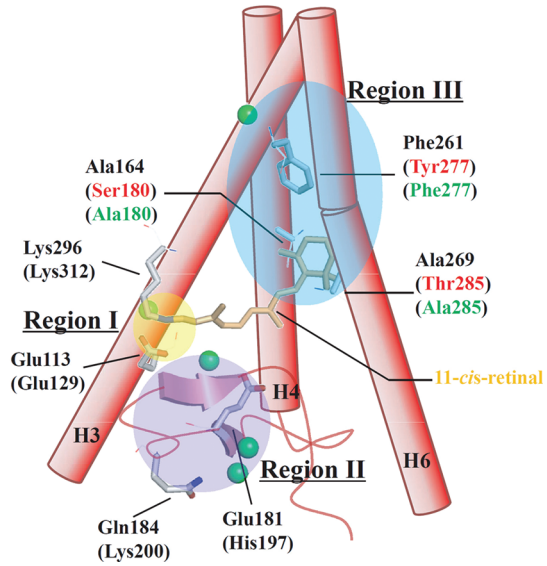


Figure 6. X-ray crystallographic structure of the chromophore-binding site of bovine Rh (Protein Data Bank entry 1U19),[40] which is viewed from the helix VII side. The top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. Three helices (helices 3, 4, and 6) are illustrated by a cylindrical drawing, and the retinal chromophore and side chains of several important amino acids are shown as stick drawings. Numbers of amino acids originate from bovine Rh and MR or MG (in parentheses). Three important regions are highlighted: (i) the Schiff base moiety (region I, yellow circle), (ii) the Cl^--binding site (region II, purple circle), and (iii) the β-ionone ring moiety (region III, blue oval). According to the structure of bovine Rh, regions I−III contain one water, three waters, and one water, respectively.

Figure 6 shows that His and Lys residues in region II constitute the Cl^--biding site in both MR and MG.[30] In the structure of bovine Rh, Glu181 is 4.8 Å from 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 is largely modified from Rh, and its location is probably along the polyene chain. It is likely that the retinal moiety near C11 and the β-sheet region, including His197 and Lys200, constitute the Cl^--binding site (region II) in MR and MG. We infer that the water vibration at ~2300 cm^{-1} is the result of retinal photoisomerization in the restricted protein environment.

dx.doi.org/10.1021/bi201676y | *Biochemistry* 2012, 51, 1126−1133

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 conditions. With regard to the counterpart of the strongly H-bonded water (~2300 cm^{-1}) in MR and MG, the results of Figure 4 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 4). The straightforward interpretation is that MR and MG possess internal water molecules near C11 and C9. Interestingly, X-ray structures of bovine Rh[4] and 9-*cis* Rh[34] showed no water molecules near C11 and C9 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 6). Thus, the results in Figure 4 suggest that MR and MG possess internal water molecules near C11 and C9. According to the X-ray structures of bovine Rh[4] and 9-*cis* Rh,[34] the nearest atoms of the retinal chromophore from Glu181 [Cl^- binding His in MR and MG (Figure 6)] are C11 (4.8 Å) and C12 (4.9 Å), respectively. A previous FTIR study of chicken red-sensitive pigment suggests the Cl^--binding site is near C14 of the retinal chromophore.[35] Therefore, it is likely that the Cl^--binding site is located along the polyene chain, which is hydrated by internal water molecules in MR and MG, but not in Rh. The fact that there are more water molecules in MR and MG than in Rh may originate from the water molecules in this region (region II).

Region III in Figure 6 contains the three amino acids differing between MR and MG, which are thought 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 6). What 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 5), suggesting that the water molecules are distant from C9 and C11 in MR and MG. Such water molecules may be located in region III, though we cannot reach a conclusion at present. Further experiments using mutant proteins are needed for clarification.

Finally, we did not expect to observe a 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 frequencies 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 λ_{max} may suggest that water molecules participate in color tuning, whose mechanism is entirely unknown. A color tuning mechanism has been theoretically investigated on the basis of homology modeling of Rh,[36−39] whereas the role of internal water molecules has never been taken into account. This observation of protein-bound water molecules in our color visual pigments thus stimulates further experimental and theoretical studies, leading to an improved understanding of the color tuning mechanism in our vision.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectral comparison between D_2O and $D_2{}^{18}O$ in the 1800−800 cm^{-1} region. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan. Phone and fax: 81-52-735-5207. E-mail: kandori@nitech.ac.jp.

Present Address

§Department of Life and Coordination-Complex Molecular Science, Institute for Molecular Science, 38 Nishigo-Naka, Myodaiji, Okazaki 444−8585, Japan.

Funding

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to H.K. (20108014 and 22247024), Y.F. (21023014 and 21026016), and H.I. (21370109), the global COE program for biodiversity and cooperative research program of PRI, the Research Foundation for Opto-Science and Technology, and the Takeda Foundation for Science to H.I. and Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists to K.K.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. S. Koike, A. Onishi, Y. Shichida, and R. S. Molday for providing 293T cell lines, the expression vector, and 1D4 antibodies.

■ ABBREVIATIONS

Rh, rhodopsin; MR, monkey red; MG, monkey green; FTIR, Fourier transform infrared; BR, bacteriorhodopsin; HOOP, hydrogen out-of-plane; H-bond, hydrogen bond; HR, halorhodopsin.

■ REFERENCES

(1) Shichida, Y., and Imai, H. (1998) Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci. 54*, 1299−1315.

(2) Oprian, D. D., Asenjo, A. B., Lee, N., and Pelletier, S. L. (1991) Design, chemical synthesis, and expression of genes for the three human color vision pigments. *Biochemistry 30*, 11367−11372.

(3) Kandori, H. (2011) Protein-Controlled Ultrafast Photoisomerization in Rhodopsin and Bacteriorhodopsin. In *Supramolecular Photochemistry: Controlling Photochemical Processes* (Ramamurthy, V., and Inoue, Y., Eds.) pp 571−596, John Wiley & Sons, Inc., Hoboken, NJ.

(4) Palczewski, K., Kumasaka, K., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science 289*, 739−745.

(5) Murakami, M., and Kouyama, T. (2008) Crystal structure of squid rhodopsin. *Nature 453*, 363−367.

(6) Nakamichi, H., and Okada, T. (2006) Crystallographic analysis of primary visual photochemistry. *Angew. Chem., Int. Ed. 45*, 1−5.

(7) Nakamichi, H., and Okada, T. (2006) Local peptide movement in the photoreaction intermediate of rhodopsin. *Proc. Natl. Acad. Sci. U.S.A. 103*, 12729−12734.

(8) Choe, H. W., Kim, Y. J., Park, J. H., Morizumi, T., Pai, E. F., Krauss, N., Hofmann, K. P., Scheerer, P., and Ernst, O. P. (2011) Crystal structure of metarhodopsin II. *Nature 471*, 651−655.
(9) Standfuss, J., Edwards, P. C., D'Antona, A., Fransen, M., Xie, G., Oprian, D. D., and Schertler, G. F. (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature 471*, 656−660.
(10) Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H. W., Hofmann, K. P., and Ernst, O. P. (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature 455*, 497−502.
(11) Yokoyama, R., and Yokoyama, S. (1990) Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. *Proc. Natl. Acad. Sci. U.S.A. 87*, 9315−9318.
(12) Neitz, M., Neitz, J., and Jacobs, G. H. (1991) Spectral tuning of pigments underlying red-green color vision. *Science 252*, 971−974.
(13) Asenjo, A. B., Rim, J., and Oprian, D. D. (1994) Molecular determinants of human red/green color discrimination. *Neuron 12*, 1131−1138.
(14) Kandori, H. (2000) Role of internal water molecules in bacteriorhodopsin. *Biochim. Biophys. Acta 1460*, 177−191.
(15) Garczarek, F., and Gerwert, K. (2006) Functional waters in intraprotein proton transfer monitored by FTIR difference spectroscopy. *Nature 439*, 109−112.
(16) Kandori, H. (2010) Hydrogen bonds of protein-bound water molecules in rhodopsins. In *Hydrogen bonding and transfer in the excited state* (Han, K.-L., and Zhao, G.-J., Eds.) pp 377−391, John-Wiley & Sons Ltd., West Sussex, U.K.
(17) Freier, E., Wolf, S., and Gerwert, K. (2011) Proton transfer via a transient linear water-molecule chain in a membrane protein. *Proc. Natl. Acad. Sci. U.S.A. 108*, 11435−11439.
(18) Kandori, H., and Maeda, A. (1995) FTIR spectroscopy reveals microscopic changes of the protein around the rhodopsin chromophore upon photoisomerization. *Biochemistry 34*, 14220−14229.
(19) Furutani, Y., Shichida, Y., and Kandori, H. (2003) Structural changes of water molecules during the photoactivation processes in bovine rhodopsin. *Biochemistry 42*, 9619−9625.
(20) Ota, T., Furutani, Y., Terakita, A., Shichida, Y., and Kandori, H. (2006) Structural changes in the Schiff base region of squid rhodopsin upon photoisomerization studied by low-temperature FTIR spectroscopy. *Biochemistry 45*, 2845−2851.
(21) Angel, T. E., Chance, M. R., and Palczewski, K. (2009) Conserved waters mediate structural and functional activation of family A (rhodopsin-like) G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A. 106*, 8555−8560.
(22) Angel, T. E., Gupta, S., Jastrzebska, B., Palczewski, K., and Chance, M. R. (2009) Structural water define a functional channel mediating activation of the GPCR, rhodopsin. *Proc. Natl. Acad. Sci. U.S.A. 106*, 14367−14372.
(23) Katayama, K., Furutani, Y., Imai, H., and Kandori, H. (2010) An FTIR study of monkey green- and red-sensitive visual pigments. *Angew. Chem., Int. Ed. 49*, 891−894.
(24) Shibata, M., and Kandori, H. (2005) FTIR studies of internal water molecules in the Schiff base region of bacteriorhodopsin. *Biochemistry 44*, 7406−7413.
(25) Imai, H., Terakita, A., and Shichida, Y. (2000) Analysis of amino acid residues in rhodopsin and cone visual pigments that determine their molecular properties. *Methods Enzymol. 315*, 293−312.
(26) Nagata, T., Terakita, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1998) The hydrogen bonding network of water molecules and the peptide backbone in the region connecting Asp83, Gly120 and Glu113 in bovine rhodopsin. *Biochemistry 37*, 17216−17222.
(27) Imai, H., Hirano, T., Kandori, H., Terakita, A., and Shichida, Y. (2001) Difference in molecular structure of rod and cone visual pigments studied by Fourier transform infrared spectroscopy. *Biochemistry 40*, 2879−2886.
(28) Siebert, F., Mantele, W., and Gerwert, K. (1983) Fourier-transform infrared spectroscopy applied to rhodopsin. The problem of the protonation state of the retinylidene Schiff base re-investigated. *Eur. J. Biochem. 136*, 119−127.
(29) Shibata, M., Muneda, N., Sasaki, T., Shimono, K., Kamo, N., Demura, M., and Kandori, H. (2005) Hydrogen-bonding alterations of the protonated Schiff base and water molecule in the chloride pump of *Natronobacterium pharaonis*. *Biochemistry 44*, 12279−12286.
(30) Wang, Z., Asenjo, A. B., and Oprian, D. D. (1993) Identification of the Cl^--binding site in the human red and green color vision pigments. *Biochemistry 32*, 2125−2130.
(31) Hirano, T., Fujioka, N., Imai, H., Kandori, H., Wada, A., Ito, M., and Shichida, Y. (2006) Assignment of the vibrational modes of the chromophore of Iodopsin and Bathoiodopsin: Low-temperature Fourier transform infrared spectroscopy of ^{13}C- and ^{2}H-labeled Iodopsins. *Biochemistry 45*, 1285−1294.
(32) Rozenberg, M., Loewennschuss, A., and Marcus, Y. (2000) An empirical correlation between stretching vibration redshift and hydration bond length. *Phys. Chem. Chem. Phys. 2*, 2699−2702.
(33) Ohtomo, N., Arakawa, K., Takeuchi, M., and Ohtaki, H. (1981) Neutron diffraction study of aqueous hydrochloric and hydrobromic acid solutions. *Bull. Chem. Soc. Jpn. 54*, 1314−1319.
(34) Nakamichi, H., Buss, V., and Okada, T. (2007) Photoisomerization mechanism of rhodopsin and 9-cis-rhodopsin revealed by X-ray crystallography. *Biophys. J. 92*, 106−108.
(35) Hirano, T., Imai, H., Kandori, H., and Shichida, Y. (2001) Chloride effect on Iodopsin studied by low-temperature visible and infrared spectroscopies. *Biochemistry 40*, 1385−1392.
(36) Stenkamp, R. E., Filipek, S., Driessen, C. A. G. G., Teller, D. C., and Palczewski, K. (2002) Crystal structure of rhodopsin: A template for cone visual pigments and other G protein-coupled receptors. *Biochim. Biophys. Acta 1565*, 168−182.
(37) Trabanino, R. J., Vaidehi, N., and Goddard, W. A. (2006) Exploring the molecular mechanism for color distinction in humans. *J. Phys. Chem. B 110*, 17230−17239.
(38) Fujimoto, K., Hasegawa, J., and Nakatsuji, H. (2008) Origin of color tuning in human red, green, and blue cone pigments: SAC-CI and QM/MM study. *Chem. Phys. Lett. 462*, 318−320.
(39) Tomasello, G., Olaso-González, G., Altoè, P., Stenta, M., Serrano-Andrés, L., Merchán, M., Orlandi, G., Bottoni, A., and Garavelli, M. (2009) Electrostatic control of the photoisomerization efficiency and optical properties in visual pigments: On the role of conterion quenching. *J. Am. Chem. Soc. 131*, 5172−5186.
(40) Okada, T., Sugihara, M., Bondar, A.-N., Elstner, M., Entel, P., and Buss, V. (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure. *J. Mol. Biol. 342*, 571−583.