Elucidation of the Active Site Structure in a Photoreceptor Protein by Raman Optical Activity

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Chapter 1

General Introduction

There are many types of chromoproteins that have a cofactor as an active site. For example, a photoreceptor protein uses a chromophore for its light absorption. The steric contribution of the surrounding protein moiety can cause structural distortions in the chromophore, and such effects have been considered to be crucial for biological function. We have applied Raman optical activity (ROA) to photoreceptor proteins to obtain structural information. ROA measures a small difference in the scattered intensity in right and left circularly polarized light. Although almost all ROA instrument has a 532 nm laser, its wavelength induces phosphorescence from most of photoreceptor. Thus, we made a ROA instrument with 785 nm excitation wavelength in order to avoid a generation of phosphorescence if we measure ROA spectra of many photoreceptor proteins. A photoactive yellow protein from *Halorhodospira halophira* (Hh PYP) is one of the photoreceptor proteins and used as the model system because of light molecular weight and small size of the chromophore.

![Figure 1. Raman optical activity based on incident circular polarization method.](image)

Our previous study shows that an ROA spectrum of Hh PYP consists of ROA bands attributed to vibrational mode of the chromophore and reflects the steric structure
of the chromophore. However, applications of ROA spectroscopy to reveal the steric structure of cofactor within other chromoproteins require enhancement of the ROA scattering intensity and acquisition structural information from the ROA spectrum.

In the Chapter 3, we use PYP from *Salinibacter ruber* (Sr PYP) to measure the first experimental data on the effect of changing the excitation wavelength on the ROA spectra of a protein. We observe a close similarity between the shape of the RR spectrum and the resonance ROA spectrum of Sr PYP. Furthermore, we experimentally verify the theoretical prediction concerning the ratio of the amplitudes of the ROA and Raman spectra. Our data demonstrate that selecting an appropriate excitation wavelength is a key factor for extracting structural information on a protein active site using ROA spectroscopy.

In the Chapter 4, $^{13}$C$_8$ labeled pCA is used to assign an intense signal at 826 cm$^{-1}$ in the ROA spectrum of PYP to a hydrogen out-of-plane (HOOP) vibration of the ethylenic moiety of the chromophore. Quantum chemical calculations based on density functional theory demonstrate that the sign of this ROA band reports the direction of the distortion in the dihedral angle about the C=C bond, while its amplitude is proportional to the dihedral angle. These results document the ability of ROA to quantify structural deformations of a cofactor molecule embedded in a protein moiety.

These results show that selecting an appropriate excitation wavelength and analysis by HOOP mode as spectroscopic ruler to obtain a steric structure of cofactor within chromoprotein.
Chapter 2

Experimental detection of the intrinsic difference in Raman optical activity of a photoreceptor protein under pre-resonance and resonance conditions

We have reported the first investigation of the effects of excitation wavelength on the ROA spectra of a photoreceptor protein. As we expected, the ROA spectrum of the pCA chromophore of *Salinibacter ruber* PYP (Sr PYP) is enhanced, when the excitation wavelength is changed from 785 to 532 nm (Figure 1). This result indicates that the shorter excitation wavelength of 532 nm, which is closer to the electronic absorption band of the chromophore ($\lambda_{\text{max}} = 432$ nm), is suitable to obtain an efficient enhancement for the chromophore Raman and ROA bands. For Sr PYP, we observed the RROA of the pCA chromophore with 532 nm excitation. Since the relative intensities of the RROA bands are identical to those for the parent Raman spectrum as shown in Figure 2, the structural information such as out-of-plane distortions of the chromophore present in the nonresonance ROA spectrum is partially lost. This observation implies that it is unfavorable to use an excitation wavelength that is too close to an edge of the absorption band. It is therefore important to choose a pre-resonance condition where RROA is not observed and the ROA bands from the chromophore with very small contributions of the protein moiety can be preferentially measured. Our experimental results demonstrate that under such conditions the Raman bands from the chromophore are enhanced without the loss of structural information from the ROA spectra. Here we should note a possibility that the RROA contribute to the ROA spectra of PYP with 785 nm excitation. Although we cannot completely exclude this possibility, a sharp difference in the ROA spectra between the two excitation wavelengths suggests a minor contribution of the RROA effect.
Figure 1. Raman and ROA spectra of Hh and Sr PYPs in Tris-HCl (10mM, pH 7.5)
Figure 2. Raman and ROA spectra of Sr PYP in 10 mM Tris-HCl, pH 7.5 with 532 nm excitation. The ROA spectrum (b) is magnified by a factor of $-3200$. 
Chapter 3

Spectroscopic Ruler for Measuring Active Site Distortions based on Raman Optical Activity of a Hydrogen Out-of-Plane Vibration

In the case of application of ROA to photoactive yellow protein (PYP), which contains \( p \)-coumaric acid (\( p \)CA) as a chromophore (Figure 1), the observed ROA spectra exhibit several intense bands below 1000 cm\(^{-1}\) that are mainly ascribed to hydrogen out-of-plane bending (HOOP) modes. Intensities of these ROA bands could significantly reflect the out-of-plane deformation of the chromophore. Here we use \( ^{13} \)C8-\( p \)CA to assign an intense ROA signal at 826 cm\(^{-1}\) to a HOOP mode \( \gamma_8 \) (Figure 2). To provide a quantitative test of the band assignments, we performed DFT calculations and examined the effects of the \( ^{13} \)C8 substitution of the \( p \)CA chromophore. We also performed systematic DFT calculations to explore what structural factors affect the ROA spectra, particularly the sign and intensity of HOOP bands.

Figure 3 shows the Raman and ROA spectra of PYP with 785 nm excitation. The figure also depicts the spectra for \( ^{13} \)C8-\( p \)CA PYP (red) as well as the \( ^{12} \)C/\( ^{13} \)C difference spectra (blue). These data reveal that four different ROA signals, including the prominent band at 826 cm\(^{-1}\) are clearly affected by the isotope editing, confirming the assignment of this band to \( \gamma_8 \).

To extract information on structural deformations of the \( p \)CA from the ROA spectra, we performed systematic DFT calculations. Figure 4 displays the effects of varying dihedral angle \( \tau(C4–C7–C8–C9) \) on the simulated Raman and ROA spectra. As can be seen in the figure, many of the ROA bands change its signs and/or intensities as a function of \( \tau(C4–C7–C8–C9) \). The \( \gamma_8 \) band is especially sensitive to this dihedral twist, and the sign of the ROA band reports the direction of the distortion in the dihedral angle.
about the C=C bond, while its amplitude is proportional to the dihedral angle. These results document the ability of ROA to quantify structural deformations of a cofactor molecule embedded in a protein moiety.

Figure 1. The structure and numbering of the pCA chromophore in PYP.

Figure 2. Atomic displacement vectors for the HOOP mode $\gamma_8$ of the pCA chromophore.
Figure 3 Raman and ROA spectra of the natural abundant (N.A.) (a, d) and the $^{13}$C-$^8$pCA PYP (b, e). The $^{12}$C/$^{13}$C difference spectra are also shown (c, f).
Figure 4. Simulated Raman and ROA spectra of the active site models for PYP.
Chapter 4

General Conclusions

In the first study, the present findings confirm the previously reported theoretical description of RROA and will allow the selection of optimal experimental conditions for future ROA measurements on proteins molecules that contain cofactors at their active site. In fact, structural distortions of a chromophore are considered to be important for a biological function. The second study shows that a HOOP ROA band works as a spectroscopic ruler for the our-of-plane distortion of the chromophore that is embedded in a protein environment. Since structural distortions of cofactors are considered to be important for many biological functions, ROA spectroscopy will be useful to detect this functionally important structural information. These results demonstrate that we need to select an appropriate excitation wavelength and analyze using HOOP mode as spectroscopic ruler in order to obtain a steric structure of cofactor within chromoprotein.
Chapter 5

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