Raman Spectroscopy

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# Experimental Detection of the Intrinsic Difference in Raman Optical Activity of a Photoreceptor Protein under Preresonance and Resonance Conditions

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***Abstract:*** *Raman optical activity (ROA) is an advanced technique capable of detecting structural deformations of light-absorbing molecules embedded in chromophoric proteins. Resonance Raman (RR) spectroscopy is widely used to enhance the band intensities. However, theoretical work has predicted that under resonance conditions the ROA spectrum resembles the shape of the RR spectrum. Herein, we use photoactive yellow protein (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 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.*

Raman spectroscopy is widely used to better understand the physical basis of protein structure and function. In the case that molecules embedded in proteins exhibit an electronic transition, resonance Raman (RR) spectroscopy allows the enhancement of the vibrational modes of these chromophores with an excitation wavelength in resonance with this electronic transition. This greatly increases the amplitude of the detected Raman bands and depends strongly on the energy difference between the position of the electronic transition and the excitation wavelength.[1,2]

One advanced techniques in Raman spectroscopy is to measure light scattering using circularly polarized light. The difference in Raman scattered intensity between right ($I^R$) and left ($I^L$) circularly polarized incident light holds information on molecular chirality[3–6] and is called Raman optical activity (ROA). The sum of $I^R$ and $I^L$ corresponds to the Raman spectrum, and a dimensionless circular intensity difference (CID) is defined by Equation (1):

$$\Delta = \frac{I^R - I^L}{I^R + I^L} \quad (1)$$

Such ROA measurements are of particular interest for probing chromophoric proteins, which contain a light-absorbing cofactor at their functional active site.[7–9] Since Raman scattering is a relatively weak effect and the CID value is small ($\Delta < 10^{-3}$), resonance enhancement is of great importance in ROA studies. However, experimental[10–13] and theoretical[14,15] ROA studies under resonance conditions have so far been very limited. Importantly, theoretical work predicts that a resonance ROA (RROA) spectrum contains signals with the same relative intensities as those detected in the parent RR spectrum if a single excited electronic state is in resonance.[2,16] This theoretical work predicts that the sign of the RROA bands is opposite to that of the electronic circular dichroism (ECD) spectrum, and the CID value is minus one half of the ECD anisotropy ratio $g_{ECD}$ as shown in Equation (2):

$$\Delta = -\frac{1}{2} g_{ECD} = -\frac{1}{2}\frac{\Delta\varepsilon}{\varepsilon} \quad (2)$$

where $\varepsilon$ is the molar extinction coefficient and $\Delta\varepsilon$ ($=\varepsilon^L - \varepsilon^R$) corresponds to the ECD spectrum. In this description, the shape of the nonresonance ROA and RROA spectra of the same molecule are expected to differ greatly.

We recently applied near-infrared ROA with $\lambda = 785$ nm excitation to both bacteriorhodopsin[7] and photoactive yellow protein (PYP).[8,9] The ROA signals measured in these studies are dominated by vibrational modes originating from the *two* distinct chromophores of these two photoreceptors. We suggested that the ROA spectra of the chromophore are enhanced by a preresonance Raman effect.[7] Under the preresonance condition, the chromophore Raman intensities are moderately increased even if the excitation wavelength is lower in energy than the electronic absorption band of a sample.[17] Additionally, the shape of the ROA spectra, including the sign of the detected bands, is quite distinct from Raman spectra for these proteins.

In ROA studies on PYP,[8,9] the availability of its high-resolution crystal structures[18,19] allows quantum chemical calculations based on density functional theory (DFT) to simulate the ROA spectra. Such combined experimental and computational studies established the high sensitivity of the ROA spectra to out-of-plane deformations of the chromophore. These studies demonstrated that the near-infrared excitation allows accurate measurement of structurally infor-

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mative preresonance ROA spectra of a chromophore embedded in a protein.

Measurements under resonance conditions promise to allow more accurate measurements of the ROA signals of the chromophore in PYP by further increasing the amplitude of Raman scattering signals involved. We recently developed two ROA spectrometers with visible ($\lambda_{ex} = 532$ nm) and near-infrared ($\lambda_{ex} = 785$ nm) excitation.[7–9,20] In the case of the highly studied PYP from *Halorhodospira halophila* (Hh PYP), we were unable to measure the ROA spectrum using $\lambda_{ex} = 532$ nm excitation because of fluorescence from the sample, arising from direct excitation into the red edge of the absorbance band of Hh PYP. To avoid this complication, we measured ROA spectra for the PYP from *Salinibacter ruber* (Sr PYP). The absorption band of the chromophore $\pi \rightarrow \pi^*$ transition for Sr PYP (absorption maximum $\lambda_{max} =$ 432 nm) is 14 nm blue-shifted compared to that for Hh PYP ($\lambda_{max} = 446$ nm; Figure 1 a).[21,22] This modest blue-shift was sufficient to avoid fluorescence, and we were able to measure

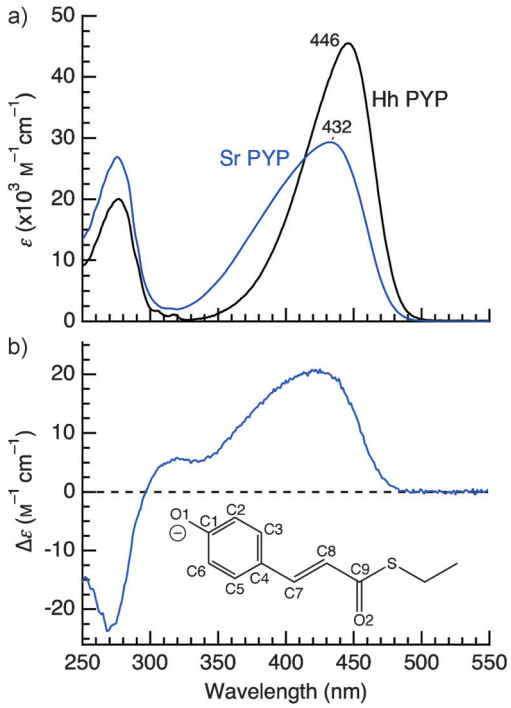


***Figure 1.*** a) Electronic UV/Vis absorption and b) circular dichroism spectra of Sr PYP in Tris-HCl buffer (10 mM, pH 7.5; blue traces). The absorption spectrum of Hh PYP is shown in (a; black trace) for comparison. Inset in (b): structure and atom numbering of the *p*CA chromophore.

its ROA spectra with both $\lambda_{ex} = 532$ and 785 nm excitation. We therefore proceeded to analyze these spectra and to compare their CID with the anisotropy ratio $g_{ECD}$ for the same protein.

The ECD spectrum of Sr PYP is shown in Figure 1 b. The strong ECD band around $\lambda = 430$ nm is due to the lowest $\pi \rightarrow \pi^*$ transition of the *p*-coumaric acid (*p*CA) chromophore. A moderate ECD band near $\lambda = 320$ nm can be assigned to the $n \rightarrow \pi^*$ transition of a carbonyl oxygen (O2; see inset in Figure 1 for numbering scheme).[23] The ECD anisotropy ratio for the $\lambda = 432$ nm band was estimated to be $g_{ECD} = 6.8 \times 10^{-4}$.

Figure 2 shows the Raman and ROA spectra of Sr PYP compared to those for Hh PYP. The upper three traces show the Raman spectra of a) Hh PYP and b) Sr PYP with $\lambda_{ex} =$ 785 nm excitation as well as the spectrum of c) Sr PYP with

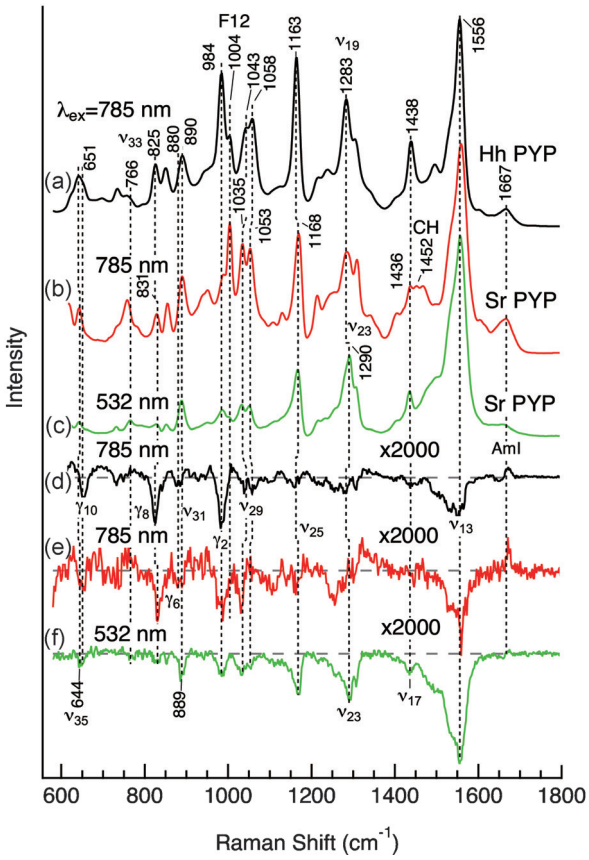


***Figure 2.*** Raman and ROA spectra of Sr PYP in Tris-HCl (10 mM, pH 7.5). The spectra for Hh PYP with excitation at $\lambda_{ex} = 785$ nm are also shown (a, d), which are adapted from Ref. [8]. The spectra for Sr PYP were obtained under the following conditions: b, e) 170 mW of $\lambda_{ex} = 785$ nm excitation light at the sample, 5.5 mM protein concentration, circa 63 h acquisition time; c, f) 630 mW of $\lambda_{ex} = 532$ nm excitation light at the sample, 2.1 mM protein concentration, circa 21 h acquisition time. The Raman spectra are normalized using the intense $\nu_{13}$ band at 1556 $cm^{-1}$, and the ROA spectra are magnified by a factor of 2000.

532 nm excitation. These spectra are normalized using the intense Raman band at 1556 $cm^{-1}$, which is due to a C=C stretching mode $\nu_{13}$ of the *p*CA chromophore.[8,24] Comparison of traces a and b reveals an overall spectral similarity between Hh PYP and Sr PYP. However, Sr PYP exhibits larger Raman intensities at 1667, circa 1450, and 1004 $cm^{-1}$, which are assigned to amide I (AmI) bands, CH deformation modes in the protein moiety, and phenylalanine F12 modes, respectively.[25,26] The greater band intensities for the protein Raman bands in Sr PYP imply smaller preresonance enhancement of the chromophore Raman bands for Sr PYP

compared to those for Hh PYP. This observation is consistent with a blue-shifted absorption maximum and smaller extinction coefficient for Sr PYP (see Figure 1 a). A different extent of preresonance enhancement also accounts for the spectral differences for the Raman spectra of Sr PYP between the different excitation wavelengths $\lambda_{ex} = 785$ nm (trace b) and 532 nm (trace c). Alteration of the excitation wavelength from $\lambda_{ex} = 785$ to 532 nm (trace b→c) distinctly diminished the relative intensities of the protein Raman bands mentioned above. This spectral change can be interpreted in terms of a larger preresonance effect for the chromophore Raman bands with the 532 nm excitation. Judging from the relative intensity ratio between the $\nu_{13}$ and AmI bands, the change in the excitation wavelength from $\lambda_{ex} = 785$ to 532 nm causes an approximate threefold increase in the Raman bands of the chromophore. In addition to the different degree of preresonance enhancement, the alteration in excitation wavelength also affects the Raman spectrum of the *p*CA chromophore. With $\lambda_{ex} = 785$ nm excitation, for example, the spectrum shows a band at 1283 $cm^{-1}$ ($\nu_{19}$), whereas the spectrum with 532 nm excitation exhibits a band at 1290 $cm^{-1}$ ($\nu_{23}$). A similar frequency difference was also detected for Hh PYP between 413.1 and 647.1 nm excitations.[24]

The lower part of Figure 2 displays the ROA spectra of Hh PYP and Sr PYP. Although there are several observable differences in the ROA spectra with $\lambda_{ex} = 785$ nm excitation between Hh PYP (trace d) and Sr PYP (trace e), their overall spectral features are similar. For example, both proteins show negative ROA bands around 1556 ($\nu_{13}$), 984 ($\gamma_2$), 825 ($\gamma_8$), and 650 $cm^{-1}$ ($\gamma_{10}$). Since the ROA spectra are sensitive to the out-of-plane distortion of the chromophore,[8,9] this result suggests that the distortions of the *p*CA chromophore are conserved between the two PYP proteins.

Figure 2 also examines the effects of different excitation wavelengths on the ROA spectra of Sr PYP. As a result of a larger preresonance enhancement, the signal-to-noise ratio of the ROA spectrum with an excitation wavelength of $\lambda_{ex} =$ 532 nm (trace f) is distinctly higher than that with excitation at 785 nm (trace e). Additionally, the alteration of excitation wavelength from $\lambda_{ex} = 785$ to 532 nm causes significant spectral changes in the ROA spectra. For instance, the negative ROA band around 1556 $cm^{-1}$ ($\nu_{13}$) changes its band shape, and its intensity increases significantly. Another notable effect is a decreased intensity of the signal attributable to $\gamma_8$ (831 $cm^{-1}$) with excitation at $\lambda_{ex} = 532$ nm. As noted above, the negative ROA band for $\gamma_8$ is one of characteristic features in the near-infrared ROA spectra of PYP. This band, however, shows a negligible intensity upon excitation at $\lambda_{ex} = 532$ nm.

Figure 3 shows the Raman and ROA spectra of Sr PYP with excitation at $\lambda_{ex} = 532$ nm, in which the ROA spectrum is multiplied by a factor of −3200. The data reveal that the Raman and the scaled ROA spectra are very similar each other, allowing us to draw four conclusions. First, all of the ROA bands have the same negative sign, except for the AmI band near 1667 $cm^{-1}$. Second, the negative sign of the chromophore ROA bands is opposite to that of the ECD spectrum. Third, the bands in the ROA spectrum have the same relative intensities compared to those within the parent

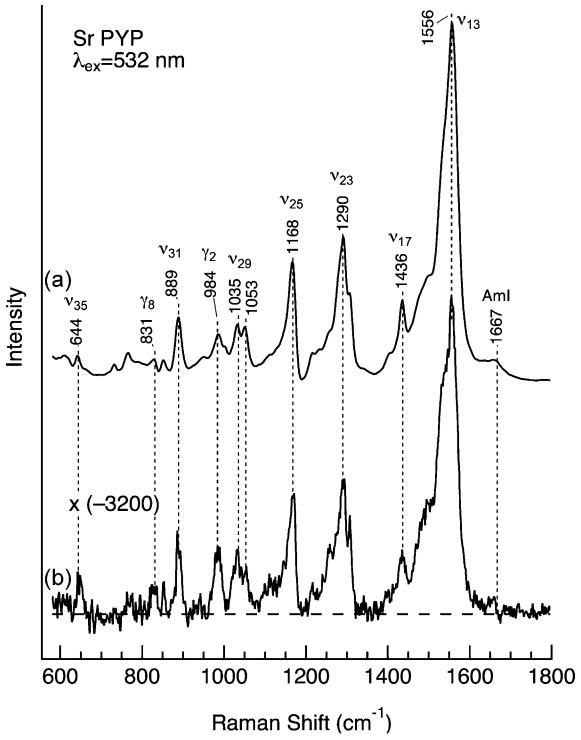


***Figure 3.*** The Raman and ROA spectra of Sr PYP in 10 mM Tris-HCl (10 mM, pH 7.5) with excitation at $\lambda_{ex} = 532$ nm. The ROA spectrum in (b) is magnified by a factor of −3200.

Raman spectrum with the exception of the AmI region. Finally, the CID value ($\Delta = -1/3200 = -3.1 \times 10^{-4}$) is close to minus one half the magnitude of the ECD anisotropy ratio ($g_{ECD} = 6.8 \times 10^{-4}$) for the $\pi \rightarrow \pi^*$ transition. These observations are fully consistent with the four theoretically predicted characteristic features for RROA.[2,14] Thus, the measured spectrum is a RROA spectrum of Sr PYP that is in resonance with the chromophore $\pi \rightarrow \pi^*$ transition around $\lambda = 434$ nm. While Sr PYP does not absorb light at $\lambda = 532$ nm (Figure 1 a), apparently the energy difference between the absorbance maximum and excitation wavelength is small enough for the spectrum to be dominated by the RROA effect. On the other hand, the absorption band of the peptide backbone ($\lambda_{max} \approx 220$ nm) is far from 532 nm, so that this excitation wavelength leads to nonresonance ROA spectra (that is, a positive/negative couplet) in the AmI region. Note that a small difference in band shape can be discerned for the intense $\nu_{13}$ bands between the Raman and ROA spectra (Figure 3). This indicates a minor deviation from a pure RROA spectrum.

We have reported the first investigation of the effects of excitation wavelength on the ROA spectra of a photoreceptor protein. As expected, the amplitudes of the Raman and ROA spectra of the *p*CA chromophore of Sr PYP are enhanced when the excitation wavelength is changed from $\lambda_{ex} = 785$ to 532 nm. This result indicates that the shorter excitation wavelength of 532 nm, which is closer to the electronic absorption band of the chromophore ($\lambda_{max} = 432$ nm), results in efficient enhancement of the chromophore Raman and ROA bands. For Sr PYP, we detected the RROA of the *p*CA

  

chromophore with an excitation wavelength of $\lambda_{ex} = 532$ nm. Since the relative intensities of the RROA bands are identical to those for the parent Raman spectrum, the additional structural information, such as out-of-plane distortions of the chromophore, present in the nonresonance ROA spectrum is lost. This implies that it is unfavorable to use an excitation wavelength that is too close to the edge of the absorption band. It is therefore important to choose a preresonance condition where RROA is not detected and the ROA bands from the chromophore can be preferentially measured with only very small contributions of the protein moiety. 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. We note the possibility that the ROA spectrum of PYP with an excitation wavelength of $\lambda_{ex} = 785$ nm contains a minor contribution from the RROA effect. Although we cannot completely exclude this possibility, a sharp difference in the ROA spectra between the two excitation wavelengths indicates that the contribution of the RROA effect is minor.

The present findings confirm the previously reported theoretical description of RROA[2,16] and will allow the selection of optimal experimental conditions for future ROA measurements on proteins molecules that contain cofactors at their active site. Structural distortions of such chromophores are considered to be important for biological function. For instance, the out-of-plane distortion of the chromophore is one of the key factors controlling its absorption spectra.[27,28] Furthermore, illumination produces primary high-energy intermediates with structurally perturbed chromophores,[29–32] which drive subsequent protein conformational changes.[31,33] Such structural distortions have proven difficult to measure experimentally. ROA spectroscopy will be useful to detect these functionally important structural distortions of chromophoric proteins.

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