Evidence for a Protonated and *cis* Configuration Chromophore in the Photobleached Intermediate of Photoactive Yellow Protein

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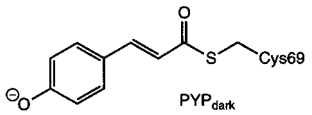
The photoactive yellow protein (PYP) from phototrophic bacterium *Ectothiorhodospira halophila* is a small water-soluble photoreceptor protein in which a 4-hydroxycinnamyl chromophore is covalently linked to Cys69 through a thioester bond.[1] In a dark state (PYP_{dark}), the chromophore is stabilized in the *trans*



configuration and its phenolic hydroxyl group is deprotonated.[2] Upon photoelectronic excitation, PYP enters a photocycle that shows several spectrally distinct intermediate states.[3] The longest lived photobleached intermediate PYP_M is presumed to be the signaling state of PYP.[4] Previous UV−vis[1b] and infrared[5] studies have suggested that the chromophore becomes protonated in PYP_M. In addition, a *cis* configuration chromophore has been also suggested.[6] In fact, time-resolved X-ray crystallography[4] has found a *cis*-chromophore for PYP_M, although a recent NMR study shows that the protein structure of PYP_M in solution differs significantly from the crystalline state.[7] Here we report the first resonance Raman (RR) investigation of PYP_M. We have performed an isotopic labeling experiment and calculation using density functional theory (DFT) and present evidence for a protonated and *cis* configuration chromophore for PYP_M in solution.

Figure 1 depicts the RR spectra[8] of wild-type PYP_{dark}, PYP_M, and acid-induced bleached state, $PYP_{M,dark}$. The RR spectrum of PYP_{dark} (λ_{max} = 446 nm) excited at 406.7 nm is in agreement with that reported previously.[2a] This species displays intense

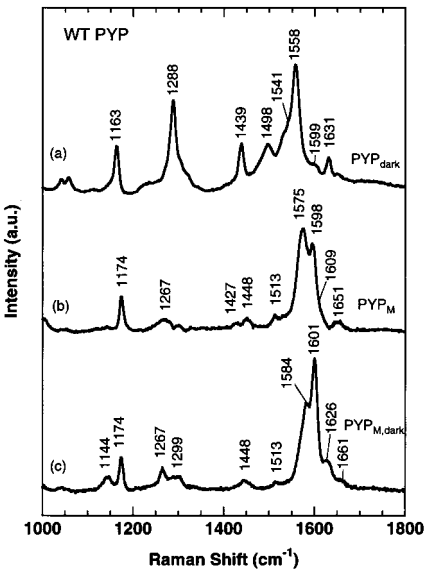


Figure 1. RR Spectra of wild-type PYP. Trace a: the spectrum of PYP_{dark} in 10 mM Tris/HCl buffer at pH 7.4 with excitation at 406.7 nm (0.7 mW). Trace b: The difference spectrum between spectra with and without the 441.6 nm pump beam (13 mW). The sample was dissolved in 10 mM citrate/20 mM phosphate buffer at pH 5.0 and excited at 325.0 nm (2.6 mW). Trace c: The spectrum of $PYP_{M,dark}$ in 20 mM citrate/HCl buffer at pH 2.0 with excitation at 325.0 nm (2.6 mW).

Raman bands at 1163, 1288, and 1558 cm^{-1} as well as some bands with moderate intensities at 1439, 1498, and 1631 cm^{-1}. We measured the RR spectrum of PYP_M by probing at 325.0 nm and pumping at 441.6 nm to accumulate the photointermediate state. Because the excitation wavelength of 325.0 nm is only on resonance for PYP_M (λ_{max} = 355 nm),[3a] the difference spectrum between spectra with and without the 441.6 nm light corresponds approximately to the RR spectrum of PYP_M. Figure 1 illustrates the dramatic changes in the spectrum upon the photoconversion of PYP_{dark} to PYP_M, especially in the following three alterations. (i) The most remarkable difference is a ∼30 cm^{-1} upshift of the main band at 1558 cm^{-1} (a doublet appeared at 1575 and 1598 cm^{-1}). (ii) The band at 1163 cm^{-1} for PYP_{dark} also upshifts by 11 cm^{-1} to 1174 cm^{-1}. (iii) Most of the Raman bands of PYP_M below 1520 cm^{-1} are significantly diminished in intensity compared to those for PYP_{dark}. Kim et al.[2a] measured the Raman spectrum of 4-hydroxycinnamyl phenyl thioester as a model for the PYP chromophore. They showed that a protonation of the hydroxyl group leads to upshifts of the main band near 1570 cm^{-1} and the band at 1166 cm^{-1} by ∼30 and ∼10 cm^{-1}, respectively. In addition, the intensities of the bands below 1520 cm^{-1} were significantly reduced upon protonation. These effects on protonation of the model compound are essentially identical to those seen in Figure 1, demonstrating that the chromophre in PYP_M is protonated.

To discuss *cis*/*trans* isomerization of the chromophore, we next compare the RR spectra of PYP_M and $PYP_{M,dark}$. Upon lowering

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(8) RR spectra were obtained on a Spex 500M spectrometer equipped with a liquid nitrogen-cooled CCD detector (Instrument S.A., Inc.). Rayleigh scattering was rejected with a prefilter, Triax190 spectrometer (Instrument S.A., Inc.). Samples were excited with either the 406.7 or 325.0 nm line available from BeamLok 2065 krypton ion (Spectra-Physics Lasers, Inc.) or IK5651R-G helium−cadmium (Kimmon Electric Co., Ltd.) lasers, respectively. For the measurement of PYP_M, the 441.6 nm line of the helium−cadmium laser was used to illuminate the sample. All spectra were taken at room temperature. The measurements were made on samples contained in a quartz spinning cell. Preparation of wild-type PYP has been described.[9] ^{13}C-labeled PYP was prepared by reconstitution of apoPYP protein with 4-hydroxycinnamic anhydride whose carbonyl carbon atom was labeled with ^{13}C.

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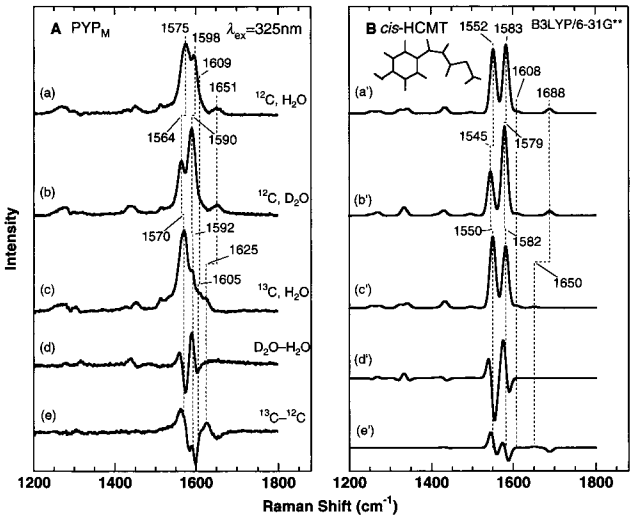

Figure 2. Experimentally obtained resonance Raman and simulated Raman spectra of PYP_M. (A) For trace b, the sample was dissolved in a buffered 90% D_2O/10% H_2O solution. Other experimental conditions are the same as those in Figure 1. (B) Gaussian band shapes with a 10 cm^{-1} width are used.

Table 1. Selected Vibrational Frequencies (cm^{-1}) and Absorption Maxima (nm) of PYP_M and $PYP_{M,dark}$

PYP_M	$PYP_{M,dark}$	*cis*-HCMT	*trans*-HCMT	
ν_{exp}[a]	ν_{exp}[a]	ν_{cal}[b]	ν_{cal}[b]	assignment
1575 ± 0.1	1584 ± 0.4	1552	1569	ν_{C-C} (ring) + $\nu_{C=C}$
1598 ± 0.2	1601 ± 0.1	1583	1590	ν_{C-C} (ring) + $\nu_{C=C}$
1609 ± 2.1	1626 ± 0.4	1608	1616	ν_{C-C} (ring) + $\nu_{C=C}$
1651 ± 0.4	1661 ± 0.8	1688	1696	$\nu_{C=O}$
λ_{exp}[a]	λ_{exp}[a]	λ_{cal}[b]	λ_{cal}[b]	
355[c]	345[d]	310	295	

[a] Experimentally observed values. The uncertainties for the ν_{exp} are the standard deviations calculated from the fitting analysis. [b] Theoretically calculated values. For the geometry optimization the starting geometry was taken from the crystal structure of PYP_M[4] or PYP_{dark},[2b] and the planar structure was obtained. [c] Reference 3a. [d] Reference 10.

the pH below 3, PYP_{dark} is reversibly converted into a stable bleached state, $PYP_{M,dark}$ (λ_{max} = 345 nm), which presumably contains a *trans*-chromophore with a protonated phenolic oxygen.[10] In fact, Figure 1 shows that overall spectral features for PYP_M and $PYP_{M,dark}$ are similar, indicating that the chromophore in $PYP_{M,dark}$ is also protonated. However, an inspection of traces b and c reveals a shift ($3-17$ cm^{-1}) of the main Raman bands near 1600 cm^{-1}. To interpret this difference, we have examined by both an isotopic labeling experiment and a DFT calculation.[11] Figure 2A shows the RR spectra of PYP_M in buffered H_2O and D_2O solutions, as well as the spectrum of the ^{13}C=O labeled protein. The figure also illustrates the H/D and $^{13}C/^{12}C$ difference spectra. Figure 2B displays the calculated spectra of *cis*-4-hydroxycinnamyl methyl thioester (HCMT), which is a model of the PYP_M chromophore. Clearly overall agreements between the experimental and calculated spectra are achieved satisfactorily,[13] indicating a protonated *cis*-chromophore for PYP_M. We also calculated the vibrational frequencies of a protonated *trans*-HCMT as summarized in Table 1. We have found that the central C=C stretching vibration contributes to the intense Raman bands at $1575-1625$ cm^{-1} (see the figure in the Supporting Information) and the *cis*/*trans* isomerization reproduces the observed differences ($\sim$10 cm^{-1}) for these bands between PYP_M and $PYP_{M,dark}$. This is reasonable because a *trans*-isomer tends to exhibit a higher C=C stretching frequency than a *cis*-isomer by about 10 cm^{-1}.[14] Furthermore, semiempirical ZINDO[11] calculations for *cis*- and *trans*-HCMT explain the observed difference in the absorption maximum between PYP_M and $PYP_{M,dark}$ (Table 1). These results strongly suggest that the chromophore of PYP_M in solution is in *cis* configuration, whereas $PYP_{M,dark}$ is characterized by a *trans*-chromophore.

Another significant observation in Figure 2 is a large $^{12}C/^{13}C$ isotope shift (-26 cm^{-1}) of the band at 1651 cm^{-1}. We assign the band to the carbonyl C=O stretching vibration, since the frequency is especially sensitive to the isotopic substitution of the carbonyl carbon atom. The assignment is further confirmed by the DFT calculation as shown in Figure 2B, where the $\nu_{C=O}$ (1688 cm^{-1}) shifts downward by 38 cm^{-1}. It is noteworthy that the bandwidth of the $\nu_{C=O}$ for PYP_M ($\sim$16 cm^{-1}) is more than twice as large as the corresponding one at 1631 cm^{-1} for PYP_{dark} ($\sim$7 cm^{-1}).[15] This broadness in PYP_M could reflect disordered protein structures[7] with different hydrogen bonding strengths.[16]

In conclusion, the results reported here establish that the phenolic oxygen of the chromophore in PYP_M is protonated. We further present evidence that suggests a *cis* configuration chromophore for PYP_M in solution. These results are in line with the currently proposed photocycle mechanism of PYP. We have also successfully assigned some important structural marker bands, such as the $\nu_{C=O}$ of the chromophore. With this information in hand, RR spectroscopy provides a unique approach for studying protein dynamic processes in PYP. Further studies using time-resolved RR as well as complete vibrational assignments including the low-frequency region are currently in progress.

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Supporting Information Available: A figure showing the atomic displacements of selected normal modes of 4-hydroxycinnamyl methyl thioester (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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