

Resonance Raman Evidence That the Thioester-Linked 4-Hydroxycinnamyl Chromophore of Photoactive Yellow Protein Is Deprotonated[†]

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ABSTRACT: Resonance Raman spectra of the ground state of photoactive yellow protein (PYP), a photoactive pigment found in *Ectothiorhodospira halophila*, have been obtained with excitation at 413.1 nm using a microspinning sample cell. The resonance Raman spectra of the thioester-linked 4-hydroxycinnamyl chromophore in the protein are compared with the preresonance Raman spectra of the 4-hydroxycinnamyl phenyl thioester and 4-hydroxycinnamic acid model compounds at various pH values. Bands at 1568, 1542, 1500, 1434, and 1166 cm⁻¹ in the Raman spectrum of the anionic form of the 4-hydroxycinnamyl phenyl thioester are shown to be characteristic for the deprotonation of the chromophore. The observation of bands in PYP exhibiting very similar frequency and intensity patterns provides strong evidence that the chromophore in PYP is stabilized as a phenolate anion at pH 7.4, in support of conclusions from crystallographic studies. Furthermore, the insensitivity of the PYP Raman spectrum to placement of the protein in D₂O buffer is consistent with the absence of the exchangeable phenolic proton on the cinnamyl chromophore. These results establish the feasibility of elucidating the molecular mechanism of light-to-information transduction by this new photosensory pigment with resonance Raman spectroscopy.

Photoactive yellow protein (PYP),¹ a water-soluble yellow-colored protein with a visible absorption maximum at 446 nm, is found in several halophilic phototrophic bacteria including *Ectothiorhodospira halophila* (Meyer, 1985; Meyer et al., 1990; Hoff et al., 1994c). *E. halophila* exhibits a negative phototactic reaction or repellent response to physiological intensities of blue light (Sprenger et al., 1993). The wavelength dependence of this photoresponse matches the absorption spectrum of PYP, leading to the hypothesis that PYP functions as the photoreceptor in *E. halophila* (Sprenger et al., 1993). Absorption of a photon by PYP initiates a cyclic photochemical reaction with at least two photointermediates (Meyer et al., 1987; Hoff et al., 1994a). The photocycle of PYP is similar to those of membrane-bound rhodopsins isolated from *Halobacterium salinarum* (Meyer et al., 1987; Hoff et al., 1994a). However, the protein structure of PYP is very different from that of bacteriorhodopsin (McRee et al., 1989; Van Beeumen et al., 1993); PYP has an α/β -fold characterized by a central, twisted, six-stranded, antiparallel β -sheet flanked on both sides by loops and helices (Borgstahl et al., 1995), while bacteriorhodopsin contains seven closely packed α -helices (Henderson et al., 1990). In addition, while the chromophore of PYP is a 4-hydroxycinnamyl group covalently bound to a cysteine via a thioester linkage (Hoff et al., 1994b; Baca et al., 1994), all rhodopsins contain a Schiff base-linked retinal chro-

mophore. PYP therefore exemplifies a new class of photoreceptor proteins whose protein structure is now well known (Hoff et al., 1994b; Baca et al., 1994; Borgstahl et al., 1995). However, little is known about the structure of the chromophore and especially the light-induced structural changes in the chromoprotein that lead to the signaling state of this photoreceptor.

We report here the first resonance Raman vibrational spectra of PYP. Resonance Raman spectroscopy provides a sensitive and selective probe for monitoring the structure and structural changes of chromophores embedded in proteins [for example, see Mathies et al. (1987)]. Interestingly, X-ray crystallographic data suggest that the chromophore in the ground state of PYP is stabilized within the folded protein as a phenolate anion (Baca et al., 1994; Borgstahl et al., 1995). This suggestion encouraged us to examine the ionization state of the chromophore using resonance Raman spectroscopy. To interpret the basic vibrational characteristics of the PYP chromophore, preresonance Raman spectra of the 4-hydroxycinnamyl phenyl thioester and 4-hydroxycinnamic acid model compounds were also obtained at various pH values. Comparison of PYP and model compound Raman data provides strong evidence that the chromophore in native PYP is deprotonated at the phenolic hydroxyl group at pH 7.4 and also gives new insight into how the chromophore interacts with the encompassing protein. This work establishes the feasibility of studying the various PYP photointermediates to elucidate its mechanism of phototransduction.

MATERIALS AND METHODS

Photoactive yellow protein from *E. halophila* was isolated by a modification of Meyer's method (1985) as described by Hoff et al. (1992). Typically, 200 μ g of freeze-dried

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¹ Abbreviations: PYP, photoactive yellow protein; HCPT, 4-hydroxycinnamyl phenyl thioester.

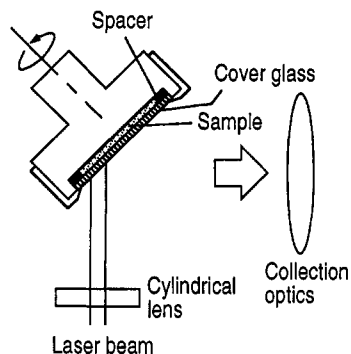


FIGURE 1: Microspinning sample cell for resonance Raman spectroscopy. Typically, $\sim 80 \mu\text{L}$ of aqueous protein solution was secured into a shallow circular chamber (18 mm diameter) in a stainless steel block that was formed by clamping a thin circular cover glass (18 mm diameter, $150 \mu\text{m}$ thick) on a $360 \mu\text{m}$ thick Teflon spacer using a threaded ring. The resonance Raman scattering was collected at about 90° to the incident laser beam.

protein was dissolved in $180 \mu\text{L}$ of 10 mM Tris-HCl (pH 7.4) buffer, and then the solution was centrifuged at ~ 4000 rpm in a microcentrifuge tube for 1 min to remove insoluble particles.

The model compound, 4-hydroxycinnamyl phenyl thioester (HCPT), was prepared by the reaction of thiophenol with acyloxypyridinium salt formed from free 4-hydroxycinnamic acid and 2-fluoro-1-methylpyridinium *p*-toluenesulfonate, as described previously (Duran et al., 1987). The final purification of the model compound was performed with a Rainin HPLC, which was equipped with a C18 reverse phase column (25 mm \times 500 mm) run with a linear gradient from 60% acetonitrile in water to 100% acetonitrile over 40 min at 4.0 mL min^{-1} . The eluted solution was then vacuum evaporated. The purity was checked with EI-MS (m/e 256, 147, 119, 109, 91, 65), elemental analysis (Calcd for $\text{C}_{15}\text{H}_{12}\text{O}_2\text{S}$: C, 70.28; H, 4.72. Found: C, 70.02; H, 4.93) and UV-vis absorption ($\lambda_{\text{max}} = 337 \text{ nm}$ in MeOH).

A microspinning sampling system, consisting of a thin ($150\text{-}\mu\text{m}$) circular cover glass (18 mm diameter) and a $360\text{-}\mu\text{m}$ Teflon spacer, was developed for resonance Raman studies on PYP. The sample spinning system, which is shown in Figure 1, allows us to record resonance Raman spectra from as little as $80 \mu\text{L}$ of colored-protein solution at any rotation speed from 1 to 30 Hz. This minimized the contamination of spectra with scattering from photoproducts that might build up using stationary cell formats. For preresonance Raman spectra, a rectangular Suprasil cell was used in the 90° geometry. The Raman spectra, excited by the 413.1- and 647.1-nm lines from a Spectra Physics 2025-11 Kr⁺ laser and by the 488.0-nm line of a Spectra Physics 2020 Ar⁺ laser, were measured using a modified Spex 1400 polychromator (Mathies and Yu, 1978) equipped with a Princeton Instruments Model LN-CCD with a UV-coated EEV Model 88130 (298×1152 pixel) array. The spectral slit width was 10, 8, and 6 cm^{-1} for 413.1-, 488.0-, and 647.1-nm excitations, respectively. Typical data acquisition times were 45 and 10 min for the protein and model compounds, respectively. The data were collected with the use of CSMA software (Princeton Instruments) on a PC and processed by Igor Pro version 2.03 (WaveMetrics) on a Macintosh Quadra 700. The spectra were calibrated by comparison to the Raman bands of cyclohexanone for 647.1- and 488.0-nm excitation and to those of 25% acetone/water for 413.1-nm excitation.

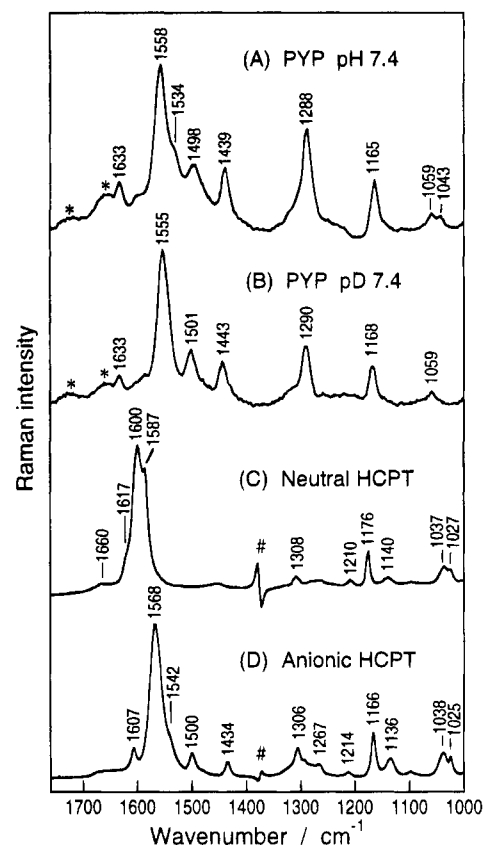


FIGURE 2: Comparison of the resonance Raman spectrum of PYP with the preresonance Raman spectra of the 4-hydroxycinnamyl phenyl thioester model compound in the $1750\text{--}1000 \text{ cm}^{-1}$ region: (A) $80 \mu\text{M}$ PYP in Tris buffer, pH 7.4, excited with 30 mW at 413.1 nm; (B) $80 \mu\text{M}$ PYP in Tris buffer, pD 7.4, excited with 30 mW at 413.1 nm; (C) 1 mM 4-hydroxycinnamyl phenyl thioester in a 50% $\text{CH}_3\text{CN}/0.1 \text{ M}$ phosphate buffer, pH 6.0 solution (solvent subtracted), excited with 300 mW at 488.0 nm; (D) 1 mM 4-hydroxycinnamyl phenyl thioester in a 50% $\text{CH}_3\text{CN}/0.1 \text{ M}$ borate buffer, pH 9.5 solution (solvent subtracted), excited with 200 mW at 647.1 nm. The bands marked with an asterisk are due to photointermediates of PYP. The derivative feature, seen near 1380 cm^{-1} in C and D is due to an imperfect subtraction of the CH_3CN solvent band.

Normal coordinate analysis was performed using the semiempirical PM3 Hamiltonian running under HyperChem version 4.0 (Hypercube Inc.) on a NEC Image P90 with Intel 90-MHz Pentium microprocessor.

RESULTS AND DISCUSSION

The 413.1-nm excited resonance Raman spectra of PYP in H_2O and D_2O in the $1750\text{--}1000 \text{ cm}^{-1}$ region are compared in Figure 2 with the 488.0-nm excited preresonance Raman spectrum of the neutral form of 4-hydroxycinnamyl phenyl thioester (HCPT) at pH 6.0 and further with the 647.1-nm excited preresonance Raman spectrum of the anionic form of HCPT at pH 9.5. At a glance, the resonance Raman spectrum of PYP in H_2O in Figure 2A is more similar to the Raman spectrum of the anionic form of HCPT in Figure 2D than to the spectrum of its neutral form in Figure 2C. Specifically, the bands at 1558, 1534, 1498, 1439, 1288, and 1165 cm^{-1} in Figure 2A exhibit a similar intensity and frequency pattern to those at 1568, 1542, 1500, 1434, 1306, and 1166 cm^{-1} in Figure 2D.

Before examining the details of the protein spectra, the effect of deprotonation on the molecular vibrations of HCPT

must be examined to determine the proper chemical structure to be discussed. Upon deprotonation, the most intense doublet band centered at $1600/1587\text{ cm}^{-1}$ in Figure 2C downshifts to $1568/1542\text{ cm}^{-1}$. The two bands at 1600 and 1587 cm^{-1} are analogous to the Y8a and Y8b modes of tyrosine, respectively [for review, see Harada and Takeuchi (1986)]. The deprotonation of tyrosine to form tyrosinate is known to shift the Y8a and Y8b modes from 1617 to 1603 cm^{-1} and from 1601 to 1558 cm^{-1} , respectively. The latter large 43-cm^{-1} downshift is partially due to the involvement of phenolic hydrogen motions in the corresponding mode of tyrosine (Harada & Takeuchi, 1986). Thus, it is reasonable that the bands at 1600 and 1587 cm^{-1} for the neutral form of HCPT would be very sensitive to deprotonation. Second, in the spectrum of the anionic form, two distinctive bands show up at 1500 and 1434 cm^{-1} , that can be ascribed to the Y19a and Y19b modes of tyrosine, respectively. Third, the band at 1176 cm^{-1} in Figure 2C downshifts by 10 cm^{-1} to 1166 cm^{-1} in Figure 2D. Thus, there are a variety of distinctive marker bands for the deprotonation of the PYP chromophore.

The above three spectral characteristics accompanying deprotonation are also confirmed in the protein spectrum in Figure 2A. In particular, the low frequencies of the $1558/1534\text{-cm}^{-1}$ doublet, the presence of the characteristic modes at 1498 and 1439 cm^{-1} , and the excellent frequency correspondence of the 1165-cm^{-1} mode all argue that the chromophore in the ground state of PYP is deprotonated. Thus, the resonance Raman spectrum of PYP at pH 7.4 can be understood in terms of the deprotonation of the 4-hydroxycinnamyl chromophore at the hydroxyl group.

An additional line of evidence that the chromophore in PYP is deprotonated is obtained by examining spectra in D_2O . Solvent replacement by D_2O does not cause any significant change in the spectrum of the protein, as shown in panels A and B of Figure 2. There is only a small difference in peak position and relative intensity between the protein spectra in H_2O and D_2O . For example, the prominent bands at 1558 , 1498 , 1439 , and 1165 cm^{-1} in H_2O shift by only $3\text{--}4\text{ cm}^{-1}$, and the band near 1290 cm^{-1} is somewhat weaker in D_2O than in H_2O . The slight spectral differences between H_2O and D_2O might suggest that some perturbation is imposed on the deprotonated chromophore, e.g., by hydrogen-bonding donors in the protein and/or internal water molecules vibrationally coupled to the chromophore. It is important to note that the spectrum of neutral HCPT is sensitive to D_2O exchange as expected, while that of anionic HCPT is not (data not shown).

Two broad bands are seen near 1670 and 1720 cm^{-1} in the protein spectrum of Figure 2A. The intensity of these bands increases when the spinning speed of the Raman sample cell is slowed from 20 to 1 Hz (data not shown). Upon speeding up the rotation rate, the intensity of both bands immediately drop, demonstrating that they are due to transient intermediates rather than permanent photodegradation. A more detailed characterization of these intermediate spectra and comparison with model compounds will be presented elsewhere.

There are two other interesting features to be noted in the resonance Raman spectrum of PYP. First, there is an intense band at 1288 cm^{-1} that is probably due to stretching vibrations delocalized over the 4-hydroxycinnamyl skeleton, mainly ascribable to tyrosinate C—O and C—C stretching

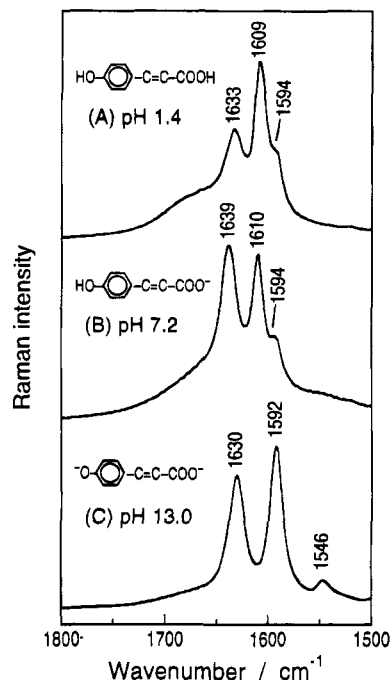


FIGURE 3: Raman spectra of 5 mM 4-hydroxycinnamic acid in the $1500\text{--}1800\text{ cm}^{-1}$ region at various pH values: (A) pH 1.4; (B) pH 7.2; (C) pH 13.0. The laser power at 488.0 nm was 200 mW .

of the acryloyl fragment —C=C—C(—S—)O . As shown in the model compound spectra in panels C and D of Figure 2, this band is seen near 1307 cm^{-1} ; no substantial change in the peak position is induced upon deprotonation, but the relative intensity of the band is considerably enhanced. The difference in wavenumber seen between PYP and 4-hydroxycinnamyl phenyl thioester might be associated with the conformation around the C—C single bond of the —C=C—C(—S—)O fragment of the chromophore. An atomic model (Baca et al., 1994; Borgstahl et al., 1995), where the positions and bonding of the heavy (non-hydrogen) atoms in the 4-hydroxycinnamyl chromophore were crystallographically determined based on 1.4-\AA diffraction data of PYP, provides insight into the conformation around the C—C single bond. The model clearly indicates that the chromophore takes up an *s-cis* conformation in the binding site of the protein. Although the conformation around the C—C single bond of the 4-hydroxycinnamyl phenyl thioester model compound in solution is not known, it is reasonable to assume that the model compound is found in the *s-trans* conformation. This suggests in turn that the wavenumber difference seen for the band near 1300 cm^{-1} between PYP and 4-hydroxycinnamyl phenyl thioester is due to alteration in the torsion angle about the C—C single bond for an *s-cis* form. Normal mode calculations on the thioester chromophore support the idea that the C—C mode frequencies are sensitive to conformational changes. The above results suggest that the band near 1300 cm^{-1} could be an important clue to determine the conformation of the chromophore in other photoinduced intermediates involved in the PYP photocycle.

The second feature to be noted is that a distinctive band is observed at 1633 cm^{-1} in the protein spectrum of Figure 2A but the corresponding band is seen at 1607 cm^{-1} in the HCPT spectrum of Figure 2D. Figure 3 shows that there are at least three bands near 1600 cm^{-1} for the 4-hydroxycinnamic acid model compound in the neutral and monoanionic forms. Further, there are at least two bands at 1630

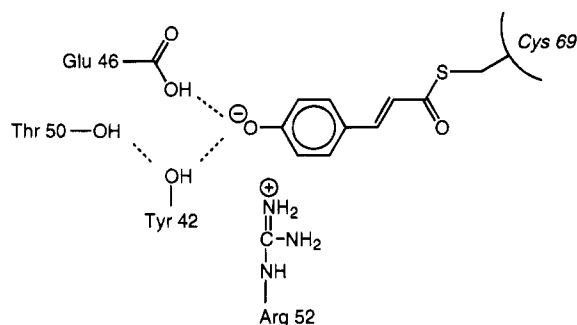


FIGURE 4: Schematic of the binding pocket for the thioester-linked 4-hydroxycinnamyl chromophore in PYP.

and 1592 cm^{-1} in this region for 4-hydroxycinnamic acid in the dianionic form. Based on analogy with tyrosine (Harada & Takeuchi, 1986) and also with the HCPT results discussed above, the bands near 1610 and 1594 cm^{-1} seen in Figure 3, panels A and B, are assigned to vibrational modes analogous to Y8a and Y8b of tyrosine, respectively; the bands at 1592 and 1546 cm^{-1} in Figure 3C can be assigned to vibrational modes equivalent to Y8a and Y8b of tyrosinate, respectively. An important common feature for the above three forms of 4-hydroxycinnamic acid is that a distinctive band is seen in the $1630\text{--}1640\text{ cm}^{-1}$ range. A semiempirical calculation for 4-hydroxycinnamic acid using PM3 indicates that a stretching vibration of the central $\text{C}=\text{C}$ double bond makes a significant contribution to the 1635 cm^{-1} mode. Recently, D'Ordine et al. (1994) found drastic changes in the Raman features for cinnamyl-coenzyme A, when the ligand binds to crotonase. The downshift of the acryloyl $\text{C}=\text{C}$ stretching vibration to 1563 cm^{-1} was explained by an electronic rearrangement of the π -electrons mainly in the acryloyl portion of the cinnamyl chromophore upon binding to crotonase. There is, however, no evidence that a similar effect is occurring on the acryloyl portion of the chromophore in PYP, since the $\text{C}=\text{C}$ double bond stretching vibration of the chromophore is seen at 1633 cm^{-1} in the encompassing protein, close to the frequency observed in the model compounds. The appearance of this band at 1633 cm^{-1} in the protein spectrum of Figure 2A is quite interesting, because it provides a way to probe the structure of the central $\text{C}=\text{C}$ double bond in the ground state and in the various photointermediates. Further experimental and theoretical work on the model compounds closer to the exact structure of the chromophore in PYP is in progress.

An important outcome of the present work is that comparative Raman studies on PYP in H_2O and D_2O and on its model compounds provide the unequivocal conclusion that the 4-hydroxycinnamyl chromophore in PYP at pH 7.4 is deprotonated in the ground state. The full crystallographic atomic model of PYP proposes that the deprotonated chromophore is stabilized by hydrogen bonds from the side chains of Tyr42 and Glu46 to the phenolic oxygen atom and by electrostatic complementarity with the positively charged Arg52. These three amino acids are interlocked by Thr50 through a network of hydrogen bonds (Borgstahl et al., 1995). All structural information available now on PYP, including this Raman study, leads to a model of the binding pocket of PYP in the ground state, as shown in Figure 4. The 4-hydroxycinnamyl chromophore is deprotonated to form a phenolate anion. The three-center hydrogen-bonding interactions with the side chains of Tyr42 and Glu46, in concert with the positively charged side chain of Arg52, are

presumably used to stabilize the phenolate anion, resulting in a lowering the pK_a of the PYP chromophore by 2 or more units. It is estimated that the pK_a of the chromophore in the unfolded holoprotein is 9.0 ± 0.5 (Baca et al., 1994). Resonance Raman studies on photointermediates of PYP are in progress and will provide dynamical information on the structure of the chromophore and its interactions with apoprotein during its progression through the photocycle. In particular, it will be important and interesting to determine if light absorption produces changes in $\text{C}=\text{C}$ configuration, $\text{C}-\text{C}$ conformation, and/or protonation state.

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