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## Accelerated Publications

# Thiol Ester-Linked *p*-Coumaric Acid as a New Photoactive Prosthetic Group in a Protein with Rhodopsin-Like Photochemistry†

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ABSTRACT: A number of Eubacteria contain a photoactive yellow protein which has a photosensory function in negative phototaxis. It has been proposed that the cofactor responsible for the intense yellow color of this protein is retinal [McRee, D. E., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6533−6537]. This would make it the first eubacterial rhodopsin. Here we report the chemical structure of this chromophoric group to be *p*-coumaric acid, which is covalently bound to a unique cysteine in the apoprotein via a thiol ester bond, and thus not retinal. This makes PYP the first example of a protein containing *p*-coumaric acid, a metabolite previously found only in plants, as a prosthetic group and establishes the photoactive yellow proteins as a new type of photochemically active receptor molecule.

The photoactive yellow proteins (PYP) constitute a homologous group of proteins found in many Eubacteria (Meyer, 1985; Meyer et al., 1990; Hoff et al., 1994a). The structural and photochemical characteristics of the PYP isolated from *Ectothiorhodospira halophila* have been studied in some detail. Since PYP was isolated in 1985, a number of proposals concerning the chemical structure of the cofactor responsible for the yellow color of the protein have been advanced (Meyer, 1985; McRee at al, 1989; Van Beeumen et al., 1993), but the true nature of this chromophore remained unclear. The amino acid sequence (Van Beeumen et al, 1993) and crystal structure (McRee et al., 1989) of PYP at 2.4-Å resolution have been reported and show that the protein is composed of two perpendicular plates of $\beta$-sheet, forming a $\beta$-clam structure very similar to the fold of a number of eukaryotic proteins (Flower et al., 1993), without PYP sharing obvious sequence similarity with these proteins. *E. halophila* displays a negative phototaxis response toward physiological intensities of blue light (Sprenger et al., 1993). Evidence has been presented that the photocycle that PYP performs after absorption of a photon, in which the primary photoproduct recovers to the ground state on a subsecond time scale via two photocycle intermediates (Meyer et al., 1987; Hoff et al., 1994b), initiates this response. The photochemical characteristics of PYP (Meyer

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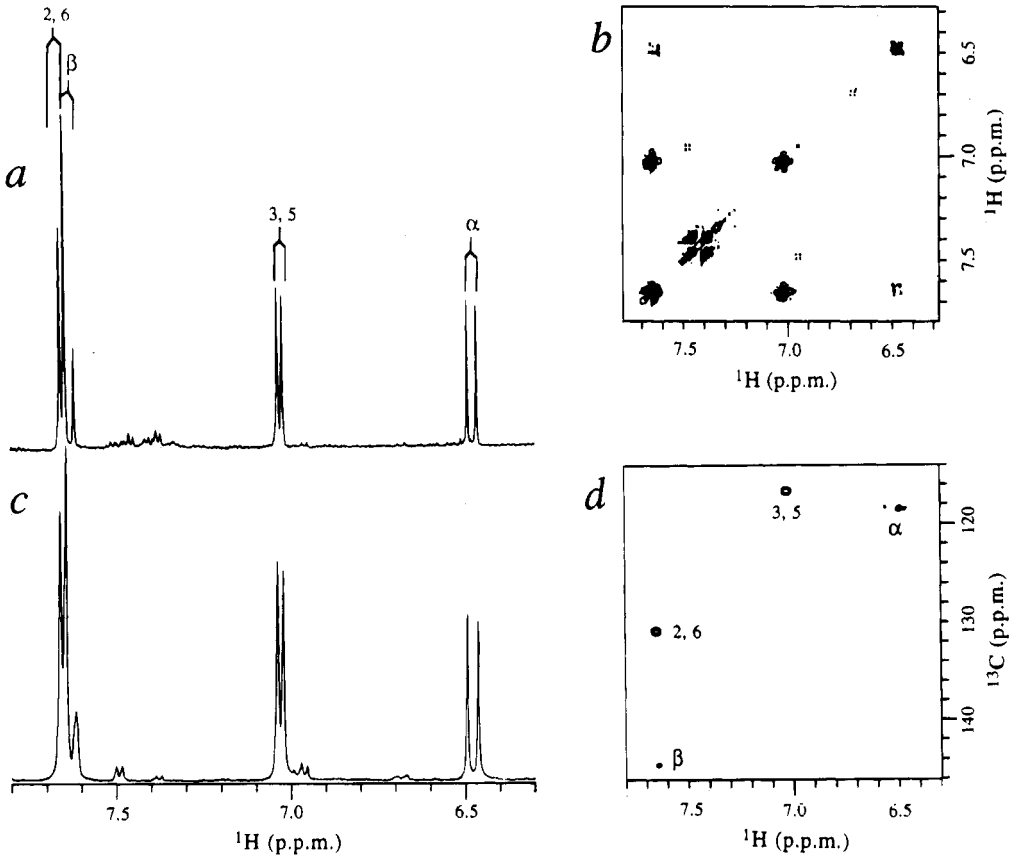


FIGURE 1: NMR spectra of the chromophore from PYP (a, b, d) and of pure *p*-coumaric acid (c). One-dimensional $^1$H NMR spectra of the chromophore (a) and *p*-coumaric acid (c) together with contour plots of a 2D TOCSY (Bax & Davis, 1985) spectrum (b) and a natural abundance $^1$H–$^{13}$C correlated spectrum (HSQC; Bodenhausen & Ruben, 1980) (d) of the chromophore are shown. The signals are marked according to the numbering shown in Figure 2. Only the spectral regions including the signals from the chromophore, respectively, *p*-coumaric acid, are shown.

et al., 1987, 1991; Hoff et al., 1992, 1994b; Miller et al., 1993) are strikingly similar to those of the archaebacterial sensory rhodopsins (Meyer et al., 1987; Stavenga et al., 1991; Spudich & Bogomolni, 1988). This suggested that, like the rhodopsins, PYP contains a retinal molecule bound to a lysine residue via a Schiff base, as was subsequently deduced from X-ray crystallography data (McRee et al., 1989). We have recently reported, however, that the chromophore in PYP is bound to Cys 69 and is strikingly different from retinal (Van Beeumen et al., 1993). These results lead to a very interesting photophysical paradox: how can the photochemical characteristics of PYP be so similar to those of the rhodopsins while the structure of its apoprotein and chromophore are completely different? To provide a basis to resolve this question, we have examined the chemical structure of the cofactor that is responsible for the photochemical activity of PYP.

## EXPERIMENTAL PROCEDURES

*Isolation of PYP Chromophore–Peptide Conjugates.* PYP was isolated from *E. halophila* SL1 as described before (Meyer, 1985; Hoff et al., 1992) and was subjected to a 40-h incubation with proteinase K at pH 8.0. This complex peptide mixture was subjected to reverse-phase high-pressure liquid chromatography (HPLC) as described (Van Beeumen et al., 1993).

*NMR Spectroscopy.* HPLC fractions containing the chromophore were examined by NMR spectroscopy in $D_2O$ at 311 K and pH 4.6. The 1D $^1$H NMR spectra of the isolated chromophore and of *p*-coumaric acid were recorded at 600 and 500 MHz, respectively. A 2D TOCSY spectrum of the chromophore was obtained at 500 MHz with a mixing time of 60 ms, using a spectral width of 5 kHz and acquiring 512 free induction decays of 64 scans each. A HSQC spectrum of the chromophore was acquired at 600 MHz with a spectral width of 21 kHz in $t_1$ and 8 kHz in $t_2$. A total of 512 free induction decays of 160 scans each were acquired.

*Capillary Electrophoresis.* The chromatography was performed in 28 mM valeric acid (pH 8.2) in an Applied Biosystems 270A-HT capillary electrophoresis system using vacuum injection of the samples (Karger et al., 1989).

*Materials.* The proteinase K used for the proteolytic digestion of PYP was from Boehringer Mannheim, and the *p*-coumaric acid used as a standard was obtained from Sigma.

## RESULTS

*Isolation and NMR Analysis of the PYP Chromophore.* We subjected PYP to proteolytic digestion in order to obtain small peptide–chromophore conjugates after high-pressure liquid chromatography (HPLC) purification. NMR analysis (Figure 1a) of an HPLC fraction with an absorbance maximum at 307 nm—indicating the presence of the chromophore—did not reveal any amino acids to be present in the sample. Apparently the chromophore–apoprotein linkage had been broken during the proteolytic digestion. Therefore, the NMR spectra shown in Figure 1 originate from the free chromophore and suggest that *p*-coumaric acid [*trans*-β-(4-hydroxyphenyl)acrylic acid] is the chromophore

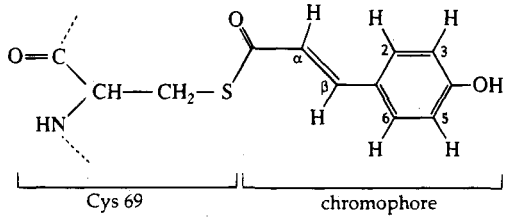


FIGURE 2: Chemical structure of the PYP chromophore, linked to Cys 69 of the apoprotein via a thiol ester bond.

in PYP. This can firmly be concluded from the identical $^1H$ and $^{13}C$ chemical shifts and $^1H-^1H$ scalar coupling patterns displayed by the PYP chromophore and authentic *p*-coumaric acid (Figure 1). $^1H$ NMR measurements in DMSO-$d_6$ of the chromophore and *p*-coumaric acid in both cases showed an additional peak at 9.95 ppm (data not shown). This signal can be assigned to the *para*-hydroxyl proton of the ring and is not present in 1D NMR spectra of pure cinnamic acid in DMSO-$d_6$. Independent evidence for the identity of the PYP chromophore is provided by the UV absorbance spectrum of the chromophore, which is indistinguishable from that of *p*-coumaric acid.

*The Apoprotein–Chromophore Linkage.* Previously, we have proposed that the chromophore of PYP is linked to the apoprotein via a disulfide bridge, on the basis of its sensitivity to dithiothreitol (DTT), a reagent that reduces disulfide bonds (Van Beeumen et al., 1993). However, because the chromophore of PYP has now been identified as *p*-coumaric acid, it is evident that in the native protein it must be bound to Cys 69 via a thiol ester bond (Figure 2). Using the thiol ester in acetyl-coenzyme A as a model, we showed by electrospray ionization mass spectrometry (ESIMS) that this bond is indeed cleaved by DTT (data not shown). ESIMS measurements of PYP chromopeptides obtained after a modified proteolysis procedure with both pepsin and proteinase K—in which the thiol ester bond remained intact—confirmed the expected mass of the thiol ester-linked chromophore as 147.15 ± 0.20. High-resolution mass spectrometry using fast atom bombardment (FAB) ionization unambiguously confirmed the structure proposed in Figure 2, with a precision of 7 ppm (W. D. Hoff et al., unpublished results).

Since the thiol ester bond between the apoprotein and the chromophore is expected to be hydrolyzable at high pH (Fox & Whitesell, 1994), we examined the effect of the addition of NaOH on the absorbance spectrum of native PYP. Upon incubation at pH values above 11.5, dramatic changes occur in the spectroscopic properties of PYP: the native absorbance band is bleached in a biphasic process, resulting in a species absorbing near 340 nm (data not shown). After neutralization, this sample was examined by capillary electrophoresis in order to confirm the disruption of the thiol ester bond. As can be concluded from Figure 3b, a molecule which behaves very similar to pure *p*-coumaric acid (Figure 3a) is liberated from the protein. Simultaneous electrophoresis of the protein sample and pure *p*-coumaric acid showed a single, sharp elution peak of additive area, confirming that the liberated PYP chromophore and *p*-coumaric acid are also indistinguishable with this high-resolution separation technique (Figure 3c).

## DISCUSSION

The molecular weight, the UV absorbance and NMR spectra, and the chromatographic behavior of the prosthetic

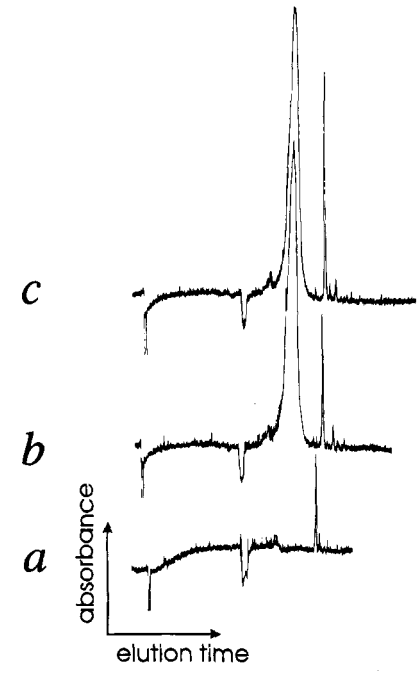


FIGURE 3: Capillary electrophoretic identification of the PYP chromophore. Capillary electrophoresis was performed with 2.5 μM *p*-coumaric acid (a) and with PYP that had been incubated at high pH (b). The elution was monitored at 284 nm. The horizontal arrow corresponds to 4 min. The chromophore released from the protein is chromatographically identical to *p*-coumaric acid as can be conclude from the experiment shown in spectrum c, where 2.5 μM of pure *p*-coumaric acid was added to the PYP sample investigated in spectrum b: only a single, sharp elution peak was observed, with an area that was the sum of the areas of the chromophore peaks in spectra a and b. The areas were 65514, 121270, and 186784 in spectra a, b, and c, respectively.

group of PYP described above unambiguously identify it as *p*-coumaric acid which is linked to Cys 69 via a thiol ester. To our knowledge, this is the first example of a thiol ester-bound photoactive cofactor. In plants *p*-coumaric acid is a secondary metabolite synthesized from tyrosine, but the occurrence of this molecule in Eubacteria has not yet been reported (Goodwin & Mercer, 1983). PYP is the first protein reported to contain *p*-coumaric acid as a prosthetic group.

In PYP, light absorption causes the protein to enter a photocycle in which the absorbance maximum of the native protein (446 nm, $\epsilon_{446}$ = 45 500 $M^{-1}$ $cm^{-1}$; Meyer et al., 1987) is shifted to 465 nm within a nanosecond. On a submillisecond time scale this photocycle intermediate is converted to a state absorbing at 355 nm, which recovers to the ground state on a subsecond time scale (Meyer et al., 1987; Hoff et al., 1994b). We propose that the photochemical basis of this process is the *cis–trans* isomerization of the vinyl *trans* double bond in the chromophore. The NMR spectra of the PYP chromophore (see Figure 1) display a large scalar coupling of 16 Hz between the two vinyl protons, which indicates a *trans* configuration. This coupling is also observed in NMR spectra of intact PYP, suggesting a *trans* configuration of the double bond of the chromophore in the ground state of PYP (data not shown). The initiation of the photocycle therefore probably involves the photochemical conversion of this bond to the *cis* form.

Since the absorbance changes of the rhodopsins are also initiated by chromophore photoisomerization, this provides a rationale for the close photochemical similarities between the rhodopsins and PYP despite the lack of structural

similarity between the two types of protein. The chemical structure of the PYP chromophore reported here establishes the PYP protein family as a new class of photoreceptors, which may have biotechnological applications as an alternative to bacteriorhodopsin (Birge, 1992). The recently published chromophore of the green fluorescent protein from the jellyfish *Aequorea victoria* (Cody et al., 1993; Chalfie et al., 1994) shows some resemblance to that of the PYP chromophore reported here. However, in the former case the chromophore is not photochemically active and originates from an intrapeptide cyclization process of the residues Ser-dehydroTyr-Gly resulting in a second cyclic structure bound to the vinyl group.

Due to the small size of both its protein and chromophore part, PYP is exceptionally well suited for biophysical studies and opens new possibilities for obtaining information with atomic resolution on processes occurring during photoreception.

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