Chemical Reactivity and Spectroscopy of the Thiol Ester−Linked p -Coumaric Acid Chromophore in the Photoactive Yellow Protein from Ectothiorhodospira halophila †

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Chemical Reactivity and Spectroscopy of the Thiol Ester-Linked *p*-Coumaric Acid Chromophore in the Photoactive Yellow Protein from *Ectothiorhodospira halophila*[†]

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ABSTRACT: We have recently identifed *p*-coumaric acid as the chromophore of the photoactive yellow protein (PYP) from the purple sulfur bacterium *Ectothiorhodospira halophila*, a blue-light photoreceptor with rhodopsin-like photochemistry [Hoff, W. D., Düx, P., Hård, K., Nugteren-Roodzant, I. M., Crielaard, W., Boelens, R., Kaptein, R., Van Beeumen, J., & Hellingwerf, K. J. (1994) *Biochemistry 33*, 13959−13962]. Here we report on the chemistry of the linkage of this new photoactive cofactor to apoPYP: (i) Analysis of chromophore−peptide conjugates of PYP by high-resolution mass spectrometry unambiguously shows that the *p*-coumaric acid molecule is bound to Cys 69 via a thiol ester bond. The PYP chromophore is the first cofactor known to be stably thiol ester-linked to its apoprotein. (ii) The chemical reactivity of this thiol ester bond with respect to dithiothreitol, performic acid, and high pH is similar to that of disulfide bridges. These treatments result in the cleavage of the thiol ester bond, concomitant with strong shifts in the UV/vis absorbance band of the chromophore. (iii) The spectral properties of the PYP chromophore under different conditions are related to the structural integrity of the protein, the presence of the thiol ester bond, and the ionization state of the phenolic proton of the chromophore. These results are important for the general problem of spectral tuning in photoreceptor proteins.

The photoactive yellow protein (PYP)[1] from *Ectothiorhodospira halophila* (Meyer, 1985) is the best-studied example of a new family of photoreceptor proteins (Meyer et al., 1990; Hoff et al., 1994b). It is the first photosensory protein from a eubacterium that has been characterized in detail. PYP probably functions as the photoreceptor for a new type of negative phototaxis response (Sprenger et al., 1993). Evidence has been obtained that large protein conformational changes occur during the photocycle of PYP (Meyer et al., 1989; Hoff et al., 1995; M. E. Van Brederode and W. D. Hoff, unpublished results) and it has been proposed that these conformational changes result in the formation of a signaling state of the protein [see Meyer et al. (1989) and Sprenger et al. (1993)]. The crystal structure of PYP at 2.4-Å resolution has been reported to show that the protein has a β-clam structure (McRee et al., 1989), previously only found in eukaryotic proteins (Flower et al., 1993). This model was refined assuming retinal to be the chromophore in PYP (McRee et al., 1989). However, it has recently been reported that this crystal structure is erroneous (Baca et al., 1994); it has been redetermined and refined to 1.4 Å, showing that PYP has an α/β fold (Borgstahl et al., 1995). This is in line with early circular dichroism spectra of PYP indicating 19% α-helix (Meyer et al., 1987; W. D. Hoff et al., unpublished results).

The photochemical characteristics of PYP have been studied in detail and show clear resemblances to those of the (archaebacterial) rhodopsins (Meyer et al., 1987, 1991; Stavenga et al., 1991; Hoff et al., 1992, 1994c; Miller et al., 1993). By absorbance of a photon, PYP is excited into a photocycle that involves a red-shifted and a blue-shifted intermediate (Meyer et al., 1987; Hoff et al., 1994c), very similar to what has been described for the sensory rhodopsins (Spudich & Bogomolni, 1988). However, we have recently determined the PYP cofactor to be a *p*-coumaric acid molecule [Hoff et al., 1994a; confirmed in Baca et al. (1994)] linked to Cys 69 [see Van Beeumen et al. (1993)], presumably via a thiol ester bond. Therefore, it is very different from the Schiff base-bound retinal molecule present in the rhodopsins. PYP is the first protein in which a *p*-coumaric acid chromophore has been found. The molecule was previously only known from secondary plant metabolism (Goodwin & Mercer, 1983). This result leads to a number of new questions: (i) What is the primary photochemical process occurring in the PYP chromophore? (ii) How does the PYP chromophore interact with the protein? (iii) What

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[1] Abbreviations: PYP, photoactive yellow protein; MS, mass spectrometry; ES, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; FAB, fast-atom bombardment; CID, collision-induced dissociation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; bR, bacteriorhodopsin; SR-II, sensory rhodopsin II; pCA, *p*-coumaric acid.

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are the spectral and chemical properties of this new cofactor and its linkage to the apoprotein? (iv) What is the biosynthesis route of the cofactor in *E. halophila*?

It has been proposed that the photochemical basis of the PYP photocycle is the *cis*−*trans* isomerization of the *trans* vinyl bond in the chromophore (Hoff et al., 1994a). Since the photochemistry of the rhodopsins has a similar basis, this resolves the paradox of how PYP and the rhodopsins can be very different structurally but very similar with respect to their photochemical characteristics.

The absorbance maximum of intact PYP lies at 446 nm (Meyer, 1985), while free *p*-coumaric acid absorbs maximally at 284 nm. A similarly large red shift of the chromophore absorbance maximum after binding to the apoprotein is observed in several proteins, which is essential for their biological function. However, the exact physical basis of this red shift, i.e., of the spectral tuning of chromophore absorbance by proteins in general, is not well understood. In the case of PYP the protonation state of the phenolic hydroxyl group of the chromophore has been proposed to be important (Baca et al., 1994).

In Hoff et al. (1994a) and Baca et al. (1994), the free PYP chromophore was identified after disruption of the apoprotein−chromophore linkage; it was inferred that this chromophore is bound via a thiol ester bond. Here we present data that unambiguously identify the chromophore−apoprotein linkage as a thiol ester and report on the chemical and spectral properties of this new photoactive cofactor and its linkage to the protein.

MATERIALS AND METHODS

Proteolysis of PYP. Intact PYP was isolated from *Ectothiorhodospira halophila* according to the procedure described by Meyer (1985), with modifications as described in Hoff et al. (1992) from cells grown as described in Hoff et al. (1994c). Aliquots of 1−2 ml of protein were used in proteolytic digestion experiments with two different proteases, during which absorbance spectra of the samples were recorded. First, 350 μg of intact PYP was digested with 20 μg of pepsin at pH 2.5 for 10 h. In a second proteolytic digestion, 11 mg of PYP in 5 mM Tris-HCl of pH 8 was incubated with 28 μg of proteinase K, which was added in three consecutive steps to ensure complete degradation of PYP. For the third proteolytic treatment performed in this study, 0.6 mg of native PYP in 10 mM Tris-HCl was incubated with 10 μg of pepsin for 2 h at pH 2.6. Next, the pH was brought to 7.5 with NaOH and 20 μg of proteinase K was added. After 70 min the pH was brought to 3 with HCl and the samples were frozen at −20 °C.

HPLC Isolation of Chromophore−Peptide Conjugates. Chromopeptides were isolated from proteolytic digests of PYP by reverse-phase HPLC as described in Van Beeumen et al. (1993). Chromatography was monitored at 220 nm to detect peptides and at 330 nm to identify fractions containing the PYP chromophore. A 1000 S diode-array detector (Applied Biosystems, Foster City, CA) was used to obtain full UV/vis absorbance spectra of the chromopeptides.

Electrospray and Matrix-Assisted Laser Desorption Ionization−Mass Spectrometry. Chromopeptides were analyzed by MALDI-MS, using a VG Tofspec time-of-flight mass spectrometer (VG Analytical, Wytenshawe, U.K.). One microliter of the HPLC fractions was mixed with 1 μL of a 50 mM solution of α-cyano-γ-hydroxycinnamic acid in acetonitrile/water/trifluoroacetic acid (50:49:1 v/v/v) and spotted on the target. Spectra obtained from typically 10−20 shots of the N_2 laser (337 nm) were averaged to obtain the final spectrum. The acceleration voltage was set at 20 kV. The mass spectrometer was calibrated using a mixture of gramicidin S and bovine insulin (Sigma). In the case of the consecutive digestion of PYP with pepsin and proteinase K, chromopeptides were analyzed by electrospray MS. Five microliters of the collected HPLC fractions were mixed with 5 μL of 50% acetonitrile/1% formic acid in water and injected into the electrospray source of a VG Bio-Q mass spectrometer (VG Biotech, Altrincham, U.K.). The capillary voltage was set at 4 kV, and the cone voltage at 40 V. Scans of 12 s, covering the mass range from 300 to 1500 Da, were accumulated during 2.5 min. The mass spectrometer was calibrated with horse myoglobin. In order to investigate the ability of DTT to break thiol esters, several CoA-SH esters were incubated overnight with 100 mM DTT at pH 7.8. The reaction mixture was separated on a self-made capillary column packed with reverse-phase Alltima C_{18} (Alltech, Deerfield, IL) and on-line detected by electrospray MS. The LC-MS configuration and conditions were similar to those described in Klarskov et al. (1994).

High-Resolution Mass Spectrometry. Fast-atom bombardment (FAB) mass spectrometry (MS) was carried out in MS-I of a JEOL JMS SX/SX102A (JEOL Ltd., Akishima, Japan) tandem mass spectrometer ($B_1E_1B_2E_2$ configuration) at an accelerating voltage of 10 kV and a mass resolution of 2000, coupled to a JEOL MS-HP7000 data system. The FABMS spectra were obtained by loading the samples in a matrix solution (glycerol, thioglycerol, and nitrobenzyl alcohol) onto a stainless steel probe and bombarded with xenon atoms with an energy of 3 keV. During the high-resolution FABMS measurements, a resolving power of 10 000 (10% valley definition) was used. Cesium iodide and glycerol were used to calibrate the mass spectrometer.

Unimolecular and collision-induced dissociation (CID) of the ions, selected by MS-I, took place in the field-free region after E_1, that is, the third field-free region, thus operating both MS-I and MS-II as double-focusing instruments. Helium or argon collision gases were introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 50%. The MS-I was operated at a resolution adjusted such that only the ^{12}C species of the ion to be analyzed was transmitted. The MS-II was operated at a resolution of 2000. The daughter-ion spectra in the first field-free region of MS-I, that is, in front of B_1, were obtained by CID of parent ions where the magnetic field (B_1) and the electric field (E_1) were changed, keeping the ratio B_1/E_1 constant.

Disruption of the Chromophore−ApoPYP Linkage. Treatment of PYP with performic acid was performed as described previously (Sanger, 1949), with minor changes. Since addition of H_2O_2 to formic acid, in order to initiate formation of performic acid, causes changes in the UV region of the spectrum, this mixture was allowed to react until its absorbance spectrum had become constant after approximately 20 min. Below 260 nm its absorbance was very strong; this background was subtracted from all following spectra. Ten microliters of a solution of PYP, containing approximately 160 pmol of protein, was then added to the mixture of 485 μL of formic acid and 5 μL of H_2O_2 (30%

w/v). Absorbance spectra were recorded after increasing time intervals with an Aminco DW-2000 spectrophotometer.

The effect of DTT on PYP was determined as follows. The (apo)protein was separated from the chromophore in SDS−polyacrylamide gels prepared according to Laemmli (1970). Prior to electrophoresis, the protein was incubated for 1 h at 37 °C in sample buffer, either with or without 0.1 M dithiothreitol (DTT). The gels were layered with 15 μL of a 66 μM solution of PYP, with an absorbance of 3.0 at 446 nm (i.e., approximately 14 μg, or 1 nmol), to which an equal volume of sample buffer had been added. The absorbance spectrum of the protein in the gel after electrophoresis was examined by placing the gel, clamped between two UV-transparent plates, in an Aminco DW-2000 spectrophotometer. The gel was either examined in electrophoresis buffer, immediately after electrophoresis, or in a buffer containing 20% (v/v) methanol plus 10% (v/v) acetic acid (i.e., after fixation). The correct position of the gel in the measuring beam of the spectrophotometer was determined by moving the gel manually, while measuring the absorbance at 280 nm. As a positive control, the spectrum of a partially purified cytochrome from *E. halophila* (Meyer et al., 1985), after electrophoresis in the same gel, was recorded. The sharp absorbance peak at 408 nm showed that the procedure used yields proper spectra of proteins in SDS gels after electrophoresis.

Absorption Spectroscopy. The effects of proteolysis and other treatments of PYP on its absorbance spectrum were measured with an Aminco DW-2000 spectrophotometer (SLM Instruments).

RESULTS AND DISCUSSION

Isolation of Small, Intact Chromophore−Peptide Conjugates. Since a number of lines of evidence suggested that the chromophore of PYP is (or is related to) retinal, experiments were performed aimed at the extraction of this chromophore in an organic solvent, using procedures developed for bacteriorhodopsin (Oesterhelt et al., 1973, 1974; Becher & Cassim, 1977). However, incubation of PYP at a range of pH values (between 3 and 10, adjusted with either HCl or acetic acid) with or without up to 200 mM hydroxylamine, either in the dark or in the light, or with SDS (in order to denature the protein) did **not** result in the release of any extractable material absorbing in the 250−500-nm range. As a positive control, bacteriorhodopsin was either treated with hydroxylamine or acetic acid, resulting in the release of the chromophore from the protein, as was concluded from the absorbance spectra of petroleum ether extracts (data not shown). This indicates that the chromophore in PYP behaves significantly different from Schiff base-bound retinal, as it is present in the rhodopsins: apparently it is not possible to detach the chromophore of PYP from the apoprotein under circumstances that promote the disruption of a Schiff base linkage. This conclusion was confirmed by the observation that addition of SDS caused PYP to denature and to lose its yellow color: the absorbance maximum shifted from 446 nm to approximately 350 nm. After subsequent extraction of most of the SDS with petroleum ether, the colorless protein in the water phase regained its initial yellow color (data not shown). Apparently, even SDS-induced denaturation of PYP does not lead to detachment of the chromophore and is readily reversible.

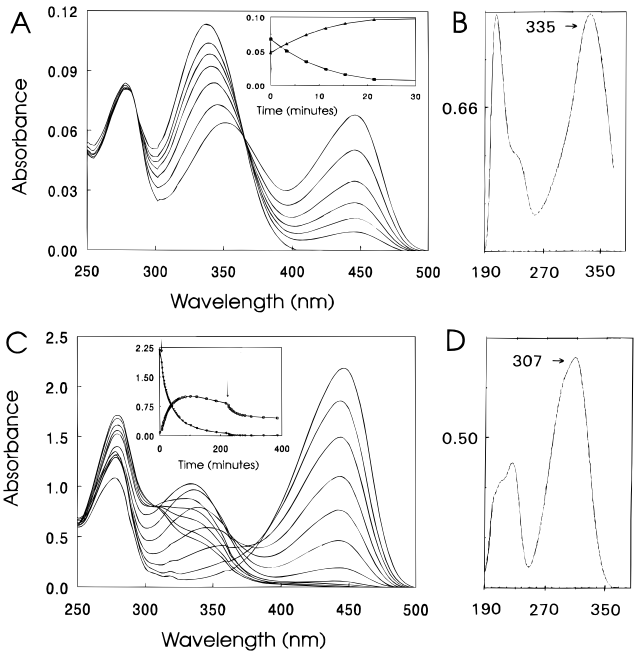

FIGURE 1: Effects of proteolysis with pepsin or proteinase K on the absorption spectrum of PYP from *Ectothiorhodospira halophila*. The changes in the absorbance spectrum of PYP during digestion with pepsin at pH 2.5 (A) and with proteinase K at pH 8.0 (C) were determined. The insets show the kinetics of the absorbance changes at 446 and 340 nm after the addition of protease; the second arrow in the inset in panel C indicates the addition of NaOH to stimulate proteinase K. The absorbance spectra of HPLC-purified chromopeptides obtained after these proteolytic treatments are shown in panels B and D, respectively.

Reversibility of denaturation of PYP, induced by either low pH, heat, or the addition of urea, has already been reported (Meyer, 1985; Meyer et al., 1987).

We then used a different strategy to obtain information about the PYP chromophore, in which native PYP was proteolytically digested in order to obtain small chromopeptides with an intact chromophore−apoPYP linkage. First, we extensively incubated intact PYP with pepsin at pH 2.5. At this low pH, PYP is present in two species which are in equilibrium (Meyer et al., 1987): the native species, absorbing at 446 nm, and the acid-denatured form, absorbing at 352 nm (W. D. Hoff and I. H. M. Van Stokkum, unpublished results). This proteolytic digestion led to the disappearance of the absorbance bands at 446 and 352 nm and the appearance of an absorbance band at 335 nm in an isosbestic transition (Figure 1A). The ϵ_{max} of this absorbance band is approximately 60% that of native PYP at 446 nm. We used this fraction in an HPLC isolation procedure [see Van Beeumen et al. (1993)] which yielded two major chromopeptide fractions, both with absorbance maxima at 210 and 335 nm (Figure 1B). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry of these fractions indicated that they contained the peptides N-61−E-74 and N-61−F-75 plus the chromophore of 147 Da (Table 1).

Since these peptides were rather large, complicating their analysis by high-resolution mass spectrometry, we decided to use the more aspecific proteinase K for a second digestion experiment. Unexpectedly, incubation of PYP at pH 8 resulted in a biphasic bleaching of the native 446-nm absorbance band from 446 nm via 340 nm to a 305-nm shoulder [Figure 1C; also see Van Beeumen et al. (1993)]. By HPLC we isolated a chromophore-containing fraction

Table 1: Properties of the Chromophore-Containing HPLC Fractions Obtained from PYP after Different Proteolytic Treatments

digestion	λ_{max}[a]	MW	structure
pepsin	335	1603/1749	pCA[b] + N-61→E-74/ N-61→F-75
proteinase K[c]	307	nd[d]	free *p*-coumaric acid[e]
pepsin/ proteinase K	335/307[f]	925/535[f]	pCA + F-63→C-69/ V-66→C-69

[a] Absorbance maximum (nanometers). [b] pCA = *p*-coumaric acid, thiol ester linked to Cys 69 in the indicated peptide. [c] Note that in Van Beeumen et al. (1993) a PYP sample that was digested less extensively with proteinase K showed two classes of HPLC fractions: some with an absorbance maximum at 307 nm and some at 355 nm. [d] Not determined. The presence of contaminants prevented us from obtaining reliable mass spectra of this fraction. [e] Determined by NMR measurements [see Hoff et al. (1994a)]. [f] Minor amounts.

from this mixture, which had absorbance maxima at 225 and 307 nm (Figure 1D). This experiment was repeated using 11 mg of pure PYP. NMR measurements of the resulting chromophore-containing fraction in D_2O indicated that in this procedure the bond between the chromophore and the apoprotein was broken [Table 1; see Hoff et al. (1994a)], explaining the difference in absorption spectra between this fraction and the pepsin-generated chromopeptide.

In an attempt to obtain *small* peptides to which the chromophore was still attached, we first digested PYP with pepsin at pH 2.5 and then for a shorter period with proteinase K at pH 7.5. HPLC analysis of this sample yielded a number of chromophore-containing fractions absorbing at 335 nm and only a small amount of material with an absorbance maximum at 307 nm (data not shown), indicating that indeed the protein−chromophore linkage was still intact in most peptides containing Cys 69. Two of these fractions could be identified as the chromophore linked to the tetrapeptide V-66−C-69 and the heptapeptide F-63−C-69, respectively, on the basis of electrospray ionization mass spectra (Table 1).

Mass Spectrometric Analysis of the Structure of the Chromophore and the Nature of Its Linkage to apoPYP. We further investigated the HPLC-purified chromophore-containing heptapeptide obtained after the combined pepsin/proteinase K digestion of PYP by high-resolution mass spectrometry. The full-scan FAB mass spectrum of this heptapeptide is presented in Figure 2A. The peaks corresponding to the protonated, $[M + H]^+$, and sodium-cationized, $[M + Na]^+$, molecules are the most predominant nonmatrix ion signals in the spectrum and are easily identified.

The abundances of the fragment ions are relatively low, and many of them are observed to be approximately the same as the abundance of the matrix ions. Thus it would be difficult to unambiguously obtain the amino acid sequence of this peptide on the basis of the FAB mass spectrum as shown in Figure 2A alone. Therefore, accurate measurements of the $[M + H]^+$, $[M + Na]^+$, and the related fragment ions were performed, the results being summarized in Table 2. As can be seen from this table, the determined masses are in good agreement with the corresponding calculated masses. Therefore, in this experiment we have detected the **intact** protein−chromophore conjugate containing *p*-coumaric acid in a thiol ester linkage to Cys 69.

To circumvent the problems of infrequent fragmentation and interference of matrix ions, we turned to the application

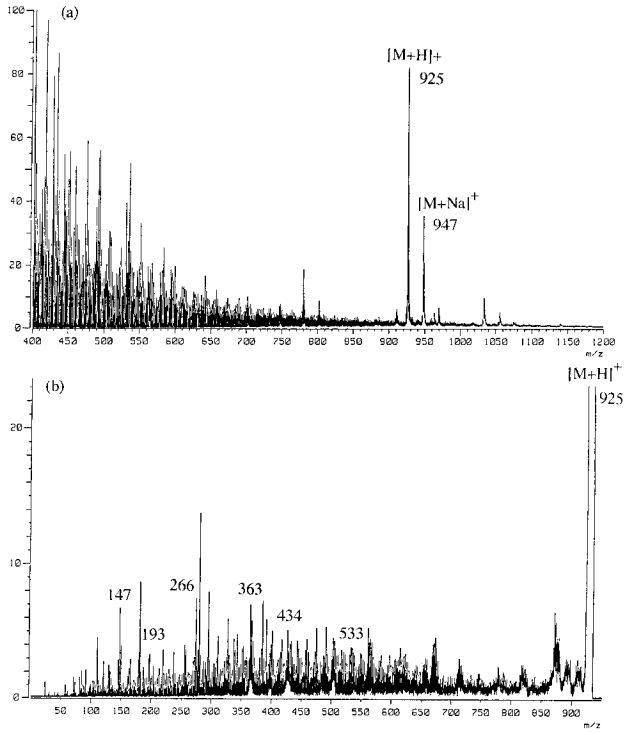

FIGURE 2: FABMS spectroscopy of intact PYP chromopeptides. The positive-ion FAB mass spectrum of the heptapeptide obtained after pepsin/proteinase K digestion of PYP (a) and positive-ion FAB MS/MS mass spectrum of the protonated molecule of the heptapeptide (b) are shown.

of tandem mass spectrometry (MS/MS) and collisional activation of the protonated molecule of the heptapeptide, as can be seen in Figure 2B. In addition to the presence of *m*/*z* 147 in the full-scan positive-ion FAB spectrum (not shown) and in the positive-ion FABMS/MS spectrum of the protonated molecule, corresponding with an elemental composition of $C_9H_7O_2$, we compared the MS/MS spectrum of the $[M-17]^+$ ion of commercially available *p*-coumaric acid (Figure 3A) with the MS/MS spectrum of the ion with *m*/*z* 147 generated from the positive-ion FAB mass spectrum from the heptapeptide (Figure 3B) to assign the structure of the chromophore. As can be seen from Figure 3, both FABMS/MS spectra are in excellent agreement with each other. These data unambiguously confirmed the identity of the chromophore as *p*-coumaric acid and its linkage to Cys 69 as a thiol ester.

Chemical and Spectral Properties of the Thiol Ester-Linked PYP Chromophore. To study the properties of the unique PYP chromophore and its linkage to the apoprotein, we investigated the spectral effects of different chemical treatments of PYP. In Figure 4A the spectral effects of incubation at high pH on PYP are shown. For pH > ca. 11.7, the native absorbance band (λ_{max} = 446 nm) shifts to 410 nm. The increase in absorbance at 250 and 295 nm occurring upon increasing the pH (see Figure 4A) is caused by Tyr deprotonation [see Mihalyi (1968)]. The shift from 446 to 410 nm occurs on a rather short time scale; it is complete within 1 min, i.e., the time needed for the spectrophotometer used in this experiment to obtain a spectrum after addition of NaOH. This process is reversible, since reneutralization of the sample leads to the re-formation of the native absorbance band at 446 nm (data not shown). However, this peak is somewhat reduced in intensity, indicating that in addition to the reversible 446 to 410 nm

Table 2: High-Resolution FAB Mass Spectrometric Data on the Heptapeptide−Chromophore Conjugate Obtained after the Combined Pepsin/Proteinase K Digestion of PYP[a]

key ions	elemental composition	calculated mass	measured mass	difference (ppm)
X[b]	$C_9H_7O_2$	147.0447	147.0446	0.6
X-CO	C_8H_7O	119.0497	119.0487	8.3
X + -S-CH_2	$C_{10}H_9O_2S$	193.0323	193.0315	4.3
Cys-X	$C_{12}H_{12}NO_4S$	266.0487	266.0457	11.0
-Pro-Cys-X	$C_{17}H_{19}N_2O_5S$	363.1015	363.1086	19.2
-Ala-Pro-Cys-X	$C_{20}H_{24}N_3O_6S$	434.1386	434.1399	3.0
-Val-Ala-Pro-Cys-X	$C_{25}H_{33}N_4O_7S$	533.2070	533.2036	6.4
-Asp-Val-Ala-Pro-Cys-X	$C_{29}H_{38}N_5O_{10}S$	648.2339	648.2351	1.8
-Lys-Asp-Val-Ala-Pro-Cys-X	$C_{35}H_{50}N_7O_{11}S$	776.3289	not detected	
(H)Phe-Lys-Asp-Val-Ala-Pro-Cys-X + H	$C_{44}H_{61}N_8O_{12}S$	925.4130	925.4203	7.9
(H)Phe-Lys-Asp-Val-Ala-Pro-Cys-X + Na	$C_{44}H_{60}N_8O_{12}SNa$	947.3949	947.3962	1.4

[a] The calculated exact masses for key ions generated upon positive ion FABMS from the heptapeptide−chromophore conjugate (Figures 2 and 3) are shown together with their measured masses and elemental composition. [b] X denotes the PYP chromphore.

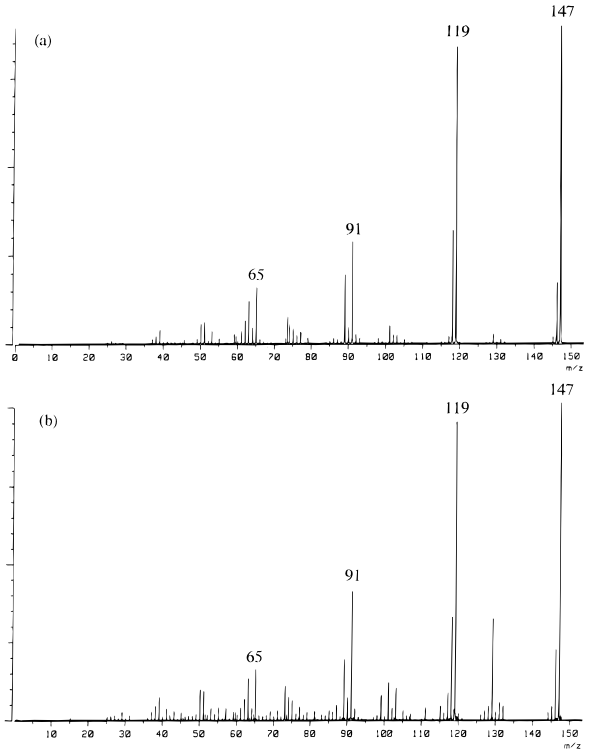

FIGURE 3: MS/MS spectroscopic identification of *p*-coumaric acid in a PYP chromopeptide. The positive-ion FAB MS/MS mass spectra of the $[M - 17]^+$ ion of commercially available *p*-coumaric acid (a) and the *m*/*z* 147 ion generated upon FABMS from the heptapeptide−chromophore complex (b) are shown.

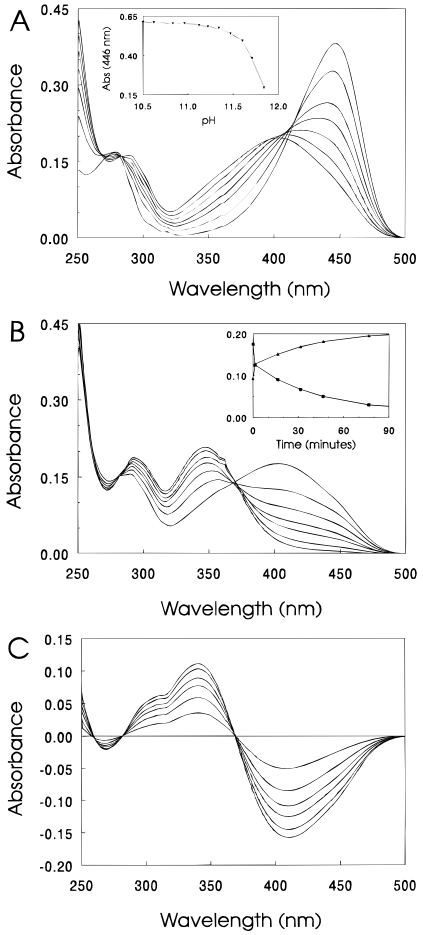

FIGURE 4: Effect of high pH on the absorbance spectrum of native PYP. (A) The absorbance spectrum of PYP from *E. halophila* was determined at pH values between 7.0 and 12.5 by titration with NaOH. The inset shows the pH dependence of the absorbance at 446 nm. (B) The sample was left at pH 12, and at different time intervals absorbance spectra were recorded. The inset shows the kinetics of the absorbance changes at 410 and 335 nm. (C) The difference spectra associated with the transition shown in panel B were calculated.

shift a second, irreversible process occurs. This process can also be observed when PYP is incubated at pH 12 for longer periods. In this case the newly formed 410-nm absorbance band is lost with the concomitant formation of an absorbance band near 340 nm in an isosbestic transition (Figure 4B,C). When, upon completion of the formation of this near-UV-absorbing product, the pH is returned to neutral, a reversible shift (with a p*K* of approximately 9.75) of the absorbance band to approximately 308 nm (shoulder) is observed (data not shown).

Also after proteolytic degradation of PYP with pepsin at pH 2.5, a drastic pH increase has pronounced effects: the absorbance band at 335 nm resulting after the proteolysis (see Figure 1A) shifts to 410 nm upon the addition of NaOH. Subsequently, this absorbance band decays in a way that is very similar to that observed for the native protein (data not shown).

Treatment of PYP with DTT results in the release of the chromophore from the protein, as was determined with electrospray ionization mass spectrometry (Van Beeumen et al., 1993). On this basis we initially proposed that the PYP chromophore is bound to the apoprotein via a disulfide bridge. However, subsequent experiments indicated that it is more likely that the chromophore is bound to PYP via a thiol ester (Hoff et al., 1994a; Baca et al., 1994). This point

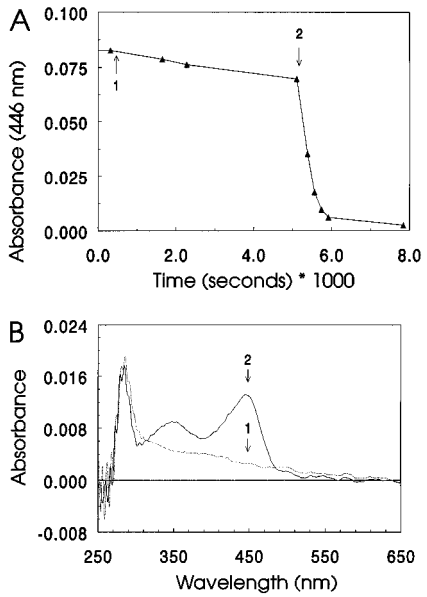

FIGURE 5: Bleaching of native PYP from *E. halophila* by DTT. (A) The effect of addition of 1.5 mM (trace 1) and 100 mM (trace 2) DTT on the absorbance of PYP from *E. halophila*, incubated at pH 10.0, was determined spectrophotometrically by monitoring the absorbance at 446 nm, the absorbance maximum of PYP. (B) The absorbance spectrum of PYP from *E. halophila* after incubation in sample buffer (with or without DTT), followed by SDS−PAGE, was determined. PYP from *E. halophila* was incubated in sample buffer with (trace 1) or without (trace 2) 0.1 M DTT for 1 h at 37 °C and subjected to SDS−PAGE. Subsequently, the gel was placed in a spectrophotometer to determine the UV/vis absorbance spectrum of the PYP samples in gel. The spectra shown have not been scaled and were smoothed to reduce scatter. The smoothing had no effect on the position or height of the peaks.

is definitely proven by the results reported here. Apparently, DTT can break not only disulfide bridges but also thiol ester bonds. We have studied this process in more detail. The effect of incubation of intact PYP at pH 10 with different concentrations of DTT was examined spectroscopically. Treatment of the protein with DTT caused the absorbance band at 446 nm to bleach (Figure 5A). However, since rather high concentrations of DTT were necessary to achieve this bleaching, the very strong absorbance band of DTT in the UV region precluded the determination of the absorbance spectrum of the bleached product. These experiments were further complicated by the spontaneous oxidation of DTT in air. Therefore, on the basis of this experiment only, it cannot be excluded that DTT causes bleaching of the chromophore to the UV region of the spectrum, in a form in which it might still be attached to the apoprotein.

In order to confirm that the chromophoric absorbance is released from the protein by treatment with DTT, both unbleached and DTT-bleached samples of PYP were subjected to SDS−PAGE. If the apoprotein−chromophore linkage is disrupted by the treatment with DTT, the chromophore should be separated from the protein after electrophoresis. That this indeed is the case can be concluded from Figure 5B, which shows the absorbance spectra of PYP in the polyacrylamide gel, determined immediately after electrophoresis. When no DTT was present in the sample buffer, the protein showed three absorbance peaks: one at 285 nm, caused by aromatic amino acids in the protein, and two caused by the chromophore. The first of these, at 350 nm, indicates that the protein is partly denatured (Meyer, 1985; Meyer et al., 1987) and the second, at 446 nm, shows the the remaining part of PYP in the sample is undenatured even after incubation with SDS and subsequent electrophoresis. This is in agreement with the SDS-induced denaturation experiment described above: even when SDS is bound to PYP, a considerable fraction of the protein shows an absorbance band at 446 nm, indicative of a physiologically intact protein conformation. The shape of the 446- and 350-nm absorbance bands is indistinguishable from those presented in the spectra of the native and low-pH bleached forms of PYP (Meyer, 1985).

When DTT was present in the sample buffer, however, all chromophore-derived absorbance disappeared: exclusively a 280-nm peak, of unchanged intensity, was observed in this sample (Figure 5B). Apparently, DTT treatment caused the release of the chromophore (and loss of this small molecule during electrophoresis), by disruption of the linkage between the chromophore and Cys 69.

When upon electrophoresis the acrylamide gel was stored in a buffer containing 20% methanol plus 10% acetic acid, identical results were obtained, except that in the sample not treated with DTT, all the protein was present in the denatured form. This resulted in a higher peak at 350 nm and no peak at 446 nm (data not shown).

In Van Beeumen et al. (1993) it was originally shown that the PYP chromophore is released from the holoprotein by treatment with DTT. Now knowing that a thiol ester-linked *p*-coumaric acid molecule forms the PYP chromophore, we investigated whether other thiol ester bonds also are cleavable by DTT. The model compound acetyl coenzyme A was therefore incubated with DTT and the reaction mixture was analyzed by LC-ESMS. A single peak corresponding to the mass of coenzyme A (767.5 Da) was detected, indicating the disruption of the thiol ester linkage (data not shown).

An alternative and chemically distinct method described for the disruption of disulfide bridges in proteins is treatment with performic acid (Sanger, 1949). We studied the effects of this reagent on PYP to investigate its reactivity toward the unusual thiol ester bond in this photoreceptor. In contrast to DTT, performic acid is transparent in the UV region of the spectrum, allowing UV/vis spectra of the detached chromophore to be recorded. Incubation of PYP with formic acid plus H_2O_2 caused marked changes in the absorbance spectrum of the protein (Figure 6A). The chromophore-derived absorbance maximum of the protein in pure formic acid is 337 nm, indicating that the protein is fully denatured; this absorbance spectrum is stable in time. Incubation in a mixture of formic acid plus H_2O_2 induced a shift of the absorbance maximum from 337 nm to approximately 314 nm. This spectroscopic transition is isosbestic at 322 nm and has maximal absorbance changes at 300 and 347 nm (Figure 6B). The absorbance difference spectrum shows fine structure near 300 nm with a spacing of 8 nm: shoulders are visible at 292 and 308 nm. HPLC analysis of a sample obtained by extraction of this sample with organic solvents showed the presence of a compound with an absorbance spectrum identical to that of *p*-coumaric acid, with absorbance maxima at 224 and 284 nm (data not shown).

CONCLUSION

PYP from *Ectothiorhodospira halophila* is the best-studied representative of a protein family [see Hoff et al. (1994b)] that forms a new class of photoreceptors. In a short time, it

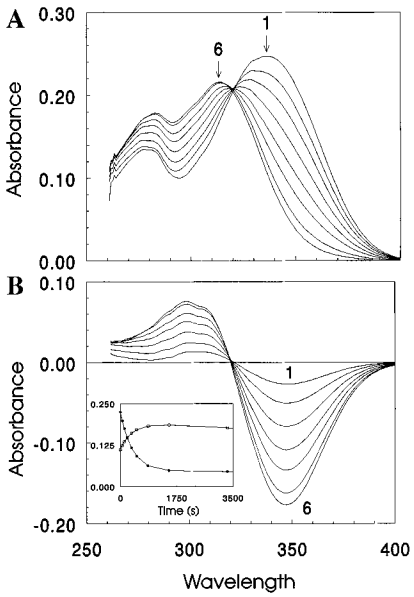

FIGURE 6: Absorbance changes in PYP induced by performic acid. (A) PYP was added to a mixture of formic acid and H_2O_2 (spectrum 1) and was incubated at room temperature. After increasing time intervals spectra were recorded, showing a shift in the absorbance maximum of the chromophore from 337 nm (spectrum 1) to 314 nm (spectrum 6). (B) Difference absorbance spectra, calculated from panel A of this figure. The inset shows the time course of the spectral transition at 300 and 347 nm.

has become a well-studied photoreceptor, and since only a very small number of photoactive proteins are known, it will be interesting to further compare the properties of PYP with the rhodopsins and phytochromes. In all three classes of photoreceptors, *cis*−*trans* photoisomerization of the covalently bound chromophore probably takes place and protein conformational changes have been implicated to play an essential role. In PYP, a thiol ester-bound *p*-coumaric acid molecule forms the photoactive cofactor. Here we have studied the chemical and spectroscopic properties of the new PYP chromophore and its unusual cofactor−apoprotein linkage.

Three different methods leading to the disruption of the apoPYP−chromophore thiol ester linkage were examined by UV/vis absorbance spectroscopy: (i) incubation at alkaline conditions, (ii) treatment with DTT, and (iii) treatment with performic acid. From the results shown in Figure 4B it can be concluded that at high pH the deprotonated chromophore is liberated from PYP by a nucleophilic attack of OH^- on the thiol ester, resulting in an absorbance shift from 410 to 340 nm. Also, the slow bleaching process observed during proteinase K digestion of PYP at pH 8.0 (Figure 1D) is caused by thiol ester hydrolysis. The rate of thiol ester hydrolysis is strongly pH-dependent and is expected to occur at a significant rate above pH 8 (Liu, 1977). Disruption of the thiol ester bond by DTT was also observed (Figure 5). Hydrolysis of this bond by performic acid (at low pH) yields doubly protonated *p*-coumaric acid with an absorbance band at approximately 310 nm (Figure 6A). Both DTT and performic acid are classical reactants for the disruption of disulfide bridges (Cleland, 1964; Sanger, 1949). Our results show that these reactants also disrupt thiol ester linkages. Alkaline lysis not only is effective for thiol esters (Figure 4B; Fox & Whitesell, 1994) but also has been reported to be effective for disulfide bridges (Donovan & White, 1971). Apparently, the chemistry of these two bonds in proteins is quite similar.

The absorbance band of the *p*-coumaric acid molecule is strongly affected by pH, the thiol ester linkage, and by interactions with the polypeptide chain of PYP. The linkage of *p*-coumaric acid to the protein via a thiol ester linkage leads to a red shift from 284 to 335 nm. Deprotonation of this molecule then results in a further red shift to 410 nm. Deprotonation of *p*-coumaric acid is also crucial in determining the position of the absorbance maximum of intact PYP. In the ground state of PYP the chromophore occurs in its deprotonated form, as proposed by Baca et al. (1994), confirmed by crystallographic data (Borgstahl et al., 1995), and proven by resonance Raman measurements (Kim et al., 1995). The final red shift from 410 to 446 nm remains unexplained. The strong blue shift during the PYP photocycle probably is the result of the transient reprotonation of the chromophore [see also Meyer et al. (1993)].

PYP is a powerful model system to study spectral tuning effects in detail, since for a number of reasons, PYP is amenable to detailed biochemical and biophysical studies: (i) its high-resolution 3D structure is available, (ii) it has a simple, nondegenerate chromophore, and (iii) it displays a well-studied photocycle. Due to its excellent experimental accessibility, PYP is the most promising candidate to obtain information on the function of photoactive proteins with atomic resolution.

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