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Complete Chemical Structure of Photoactive Yellow Protein: Novel Thioester-Linked 4-Hydroxycinnamyl Chromophore and Photocycle Chemistry[†,#]

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ABSTRACT: The unique ability of photoactive proteins to capture and use energy from a photon of light depends on the chromophore, its linkage to the protein, and the surrounding protein environment. To understand the molecular mechanisms by which a chromophore and protein interact to undergo a light cycle, we are studying photoactive yellow protein (PYP), a 14-kDa water-soluble photoreceptor from *Ectothiorhodospira halophila* with a photocycle similar to that of sensory rhodopsin. Here, we report the cloning and sequencing of the *pyp* gene and the chemical identification of both the chromophore and its covalent linkage to the protein. Elemental composition data from high-resolution mass spectrometry of a proteolytically derived chromopeptide, pH titrations and UV−visible spectroscopy of the protein-bound and chemically released chromophore, and fragmentation mass spectrometry of the liberated chromophore amide were combined with results from the 1.4-Å-resolution protein crystal structure to identify the chromophore in PYP as a 4-hydroxycinnamyl group covalently bound to the sole cysteine residue via a thioester linkage. While 4-hydroxycinnamate is a metabolic product of the phenylpropanoid pathway and a key molecule in plant stress response, this is the first report of covalent modification of a protein by this group. In the dark (yellow) state of PYP, the protein stabilizes the chromophore as the deprotonated phenolate anion. By combining our biochemical characterization of the chromophore with other published observations, we propose a chemical basis for the photocycle: following the initial absorption of a photon, the photocycle of PYP involves protonation of the chromophore to a neutral phenol form corresponding to the observed photobleached intermediate.

Photoactive yellow protein (PYP)[1] isolated from *Ectothiorhodospira halophila* is a small soluble protein of 14 kDa with a distinct bright yellow color, resulting from an absorbance maximum at 446 nm (Meyer, 1985). The action spectrum of the repellent response to blue light in *E.*

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[1] Abbreviations: PYP, photoactive yellow protein; *pyp*, DNA encoding PYP; PCR, polymerase chain reaction; ORF, open reading frame; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; GuHCl, guanidinium chloride; MS, mass spectrometry; UV, ultraviolet; LC, liquid chromatography; HPLC, high-performance LC; FAB, fast atom bombardment; CID, collision-induced dissociation.

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halophila matches the absorption spectrum of PYP (Sprenger et al., 1993), implicating PYP as the photoreceptor for the negative phototactic response. PYP undergoes a photocycle (Miller et al., 1993; Meyer et al., 1991, 1989, 1987) similar to that of the membrane-bound proteins bacteriorhodopsin, halorhodopsin, and the two sensory rhodopsins from the unrelated halophilic archaebacterium *Halobacterium halobium*. In the photocycle of PYP, the protein is excited to an intermediate I_1 with a red-shifted absorbance ($\lambda_{max} = 495$ nm) in less than 10 ns. The second intermediate (I_2, $k \sim 10^4$ s^{-1}) is bleached to 340 nm. The photocycle is completed by a slower return to the dark state ($k \sim 2$ s^{-1}) (Meyer et al., 1987). The absorbance maximum of the PYP holoprotein at 446 nm is shifted to about 340 nm upon denaturation (Meyer et al., 1987) or at pH below 2.7 (Meyer, 1985). The PYP photocycle involves the net uptake of one proton during formation of the fully bleached intermediate, followed by an equivalent proton release upon return of PYP to the ground state (Meyer et al., 1993). The unknown chromophore is covalently linked to Cys69 and has a mass of 147 Da (Van Beeumen et al., 1993). The unbleached ground-state structure of PYP has been determined to 1.4-Å resolution (G. E. O. Borgstahl, D. R. Williams, and E. D. Getzoff, manuscript in preparation), revising the initial chain tracing (McRee et al., 1989). PYP has an α/β fold consisting of an N-terminal 28-residue helical lariat structure and a central 45-residue, chromophore-bound helical loop section located on opposite sides of a six-stranded β-sheet (G. E. O. Borgstahl, D. R. Williams, and E. D. Getzoff, manuscript in preparation).

Here, we report the cloning and DNA sequencing of the complete *pyp* gene. Furthermore, through combined analysis of the properties of both the protein-bound and chemically released chromophore, the high-resolution mass determination of a proteolytically derived chromopeptide fragment, and the 1.4-Å resolution electron density image, we have identified the chromophore in PYP as a 4-hydroxycinnamyl group covalently bound to Cys69 via a thioester linkage.

METHODS

Protein Production and Purification. E. halophila strain BN9626, kindly provided by T. E. Meyer, was grown in 15-L carboys as described previously (Meyer, 1985) and yielded about 4−5 g of cells (wet weight)/L. In a Waring blender at low speed, 180−200 g of cell pellets was homogenized in Tris/EDTA buffer [10 mM Tris buffer, pH 7.5, 2 mM ethylenediaminetetraacetic acid (EDTA)] plus 1.0 mM phenylmethanesulfonyl fluoride. The homogenized cells were then passed twice through a French pressure cell at 18 000 psi. The cell debris was removed by centrifugation at 16 000*g* for 5−6 h and supernatant dialyzed into Tris/EDTA buffer. This step was repeated on the pellet after sonication in Tris/EDTA buffer to extract additional PYP from the cell debris. Following dialysis, the combined lysate was loaded onto a DE-52 anion-exchange column (Whatman) and washed with Tris/EDTA buffer until the absorbance of the eluant at 280 nm was negligible. Then, yellow fractions containing PYP were eluted with 80 mM NaCl and 50 mM Tris, pH 7.5, and further purified with a 75% ammonium sulfate precipitation. After dialysis of the supernatant into Tris/EDTA buffer, PYP was concentrated over a YM-10 Amicon membrane to ≤50 mL, loaded onto an HQ-poros anion-exchange column (Perseptive Biosystems), and eluted at 25−50 mM NaCl with a NaCl gradient in Tris/EDTA buffer. All visibly yellow fractions were then dialyzed into Bis-Tris buffer, pH 6.5, and further purified by chromatofocusing with Polybuffer 74 (diluted 1:10) at pH 3.8 using a mono P column (Pharmacia). The PYP was then run on a G-75 sizing column (Pharmacia) to remove the ampholytes, dialyzed into 20 mM sodium phosphate buffer or Hepes buffer, pH 7.0, and concentrated to ≈20 mg/mL.

Cloning the pyp Gene. Two degenerate oligonucleotides were designed to amplify the PYP coding region from *E. halophila* chromosomal DNA by the polymerase chain reaction (PCR) using Taq polymerase (Innis et al., 1990). The oligonucleotide sequences were chosen by back-translation from the PYP amino acid sequence (Van Beeumen et al., 1993) with the codon usage patterns for highly expressed genes in enteric bacteria. Two regions with minimal genetic code degeneracy were selected. The 16-fold degenerate primer 5′ATACCATGGARGAYATYGARAACAC3′ (where R = purine and Y = pyrimidine) included a *Nco*I restriction site and codons corresponding to PYP amino acid residues 9−14. The 32-fold degenerate primer 5′AATGTCGACTANGCYTTYTTCATRTG3′ (where N = purine or pyrimidine) included a *Sal*I site and a stop codon as well as the complement of the sequence coding for residues 108−112. Amplification was achieved by 30−35 cycles of PCR with denaturation at 94 °C for 1 min, annealing at 37 °C for 2 min, and extension at 72 °C for 3 min. The main PCR product corresponded to the expected size for the *pyp* gene between the codons for amino acids 9 and 112. The 315 bp between the *Nco*I and *Sal*I sites of the PCR product were cloned between the corresponding sites of the pPHSOD1LacI (Getzoff et al., 1992) for production of a probe to be used in identifying the *pyp* gene from a partial genomic library and between the same sites of pBluescriptII SK$^-$ (Stratagene) for sequencing. Additional PCR amplifications and recloning of the 315-bp fragment were done using the high-fidelity Vent polymerase (New England Biolabs).

E. halophila genomic DNA was prepared according to the miniprep method of Ausubel et al. (1992). Preliminary Southern blotting experiments performed on complete restriction enzyme digestions of *E. halophila* chromosomal DNA revealed that the smallest piece of DNA hybridizing with the cloned PCR product was a 2.5-kb *Pst*I fragment. Consequently, DNA fragments resulting from *Pst*I digestion of the *E. halophila* genomic DNA were fractionated by size with a sucrose gradient, and those containing fragments of approximately 2.5 kb of DNA were purified and ligated into pBluescriptII KS$^+$. The library of these plasmids was transformed into *Escherichia coli* MC1061 and screened by colony hybridization (Grunstein & Hogness, 1975) using the cloned PCR product as probe.

DNA Sequencing. Both the 315-bp PCR clones (resulting from amplifications with Taq and Vent polymerase) and a 1540-bp segment of the 2.5-kb genomic clone containing the *pyp* gene were sequenced on an Applied Biosystems automatic sequencer. Due to the GC-rich nature of the sample, the cycle sequencing reaction protocol recommended by the manufacturer (Applied Biosystems) was modified by adding 2.5% Triton X-100 "hydrogenated" (Calbiochem) and

4 additional units of AmpliTaq (Perkin-Elmer) and by raising the denaturation temperature to 98 °C. Additional internal sequencing primers were synthesized to allow complete sequencing of both DNA strands up to nucleotide 1094. Beyond this point, sequence data were acquired by sequencing only the coding strand with a minimum of two independent sequencing reactions. The sequence data were analyzed using the GCG (Genetics Computer Group) and Intelligenetics Suite computer software packages.

Mass Spectrometry. All mass spectrometry (MS) experiments, with the exception of high-resolution spectra, were performed on a Sciex API-III triple quadrupole ion-spray instrument. Sample introduction was usually by direct infusion into the spectrometer using a syringe pump (Harvard Applications) operating at 5 μL/min. For liquid chromatography/mass spectrometry (LC/MS) experiments, an Applied Biosystems 140B dual syringe pump HPLC was used in conjunction with an Applied Biosystems aquapore C8 reverse-phase column (2.1 × 100 mm). Linear acetonitrile gradients in 0.1% aqueous TFA were used at a flow rate of 150 μL/min, and approximately one-third the column eluant was directed into the mass spectrometer. For tandem mass spectrometry (MS/MS) experiments, collision-induced dissociation (CID) of the parent precursor ion was effected by collision with argon gas. The collision gas thickness was $\sim 8 \times 10^{14}$ atoms/cm^2, with the collision energy (quadrupole-2 rod offset voltage) set at −50 V. High-resolution mass spectra were obtained on a VG ZAB-2VSE mass spectrometer using a fast atom bombardment (FAB) ionization source and 3-nitrobenzyl alcohol as the sample matrix. Bradykinin was used as an internal mass calibrant (monoisotopic mass: 1059.5614) in determining the high-resolution mass of the chromopeptide fragment.

Proteolytic Digestion and Peptide Sequencing. PYP (130 μM solution in 10 mM ammonium bicarbonate, pH 7) was digested with 6 μM trypsin (Sigma) for 7 h at 37 °C. The digestion mixture was analyzed by mass spectrometry using both direct infusion and LC/MS. PYP was also digested with *Staphyloccocus aureus* V8 protease (Boehringer Mannheim) using the same conditions as for trypsin, except in 100 mM sodium phosphate, pH 7.8. The V8 protease digestion mixture was analyzed by LC/MS.

PYP (5.44 μg in 4 μL of H_2O) was digested with the arginine-specific protease clostripain (Sigma, 2.5 μg or 0.25 unit in 16 μL of 10 mM Tris, pH 7.5, 2 mM DTT, and 1 mM $CaCl_2$, activated for 2.5 h in this buffer) for 2 h at 25 °C. The proteolytic fragment containing residues 53−124 was then isolated from the digestion mixture by PAGE on a 20% acrylamide gel blotted onto a ProBlot membrane in 10 mM CAPS, pH 10, 10% methanol, 5% SDS, and 5 mM DTT and N-terminally sequenced with an Applied Biosystems peptide sequencer.

Cleavage and Purification of Chromophore. Removal of the chromophore was done by two methods. In the first method, a 14 μM solution of PYP was successively dialyzed overnight at 4 °C in the presence of 1, 14, and 140 mM hydroxylamine adjusted to pH 7.0 with NaOH. In the second approach, the chromophore was released at room temperature by dilution of PYP into 4.5 M GuHCl and 0.5 M ammonium chloride, pH 10. Progress of the cleavage reaction at room temperature was monitored by observing the change in chromophore absorbance from 398 to 348 nm with a Hewlett-Packard 8542A diode array spectrophotometer. Following cleavage, the reaction mixture was acidified with an equal volume of glacial acetic acid, and the liberated chromophore was purified by reverse-phase HPLC on a C4 column (Vydac, 1.0 × 25 cm) using linear gradients of acetonitrile in 0.1% aqueous TFA. UV monitoring at 214 and 310 nm was used to distinguish between peaks containing the liberated chromophore and the apoprotein.

Titration Experiments. Spectrophotometric titrations were performed on protein-bound and chemically liberated chromophore and on 4-hydroxycinnamic acid. Unfolded holoprotein was titrated by diluting solutions of native protein 1:100 into 4 M GuHCl solutions buffered between pH 3 and pH 10.5 at 0.5 pH unit intervals. Titrations of chemically liberated chromophore and 4-hydroxycinnamic acid (Aldrich) were performed by diluting an aqueous solution of chromophore 1:100 into 33 mM solutions of Tris/Bis-Tris/CAPS (1:1:1) buffered between pH 8.25 and pH 9.75 at 0.25 pH unit intervals.

Crystallographic Refinement. The atomic structure of PYP, including all 125 amino acid residues, the 4-hydroxycinnamyl chromophore in thioester linkage with Cys 69, and 94 well-defined water molecules, has been determined and refined to 1.4-Å resolution (G. E. O. Borgstahl, D. R. Williams, and E. D. Getzoff, manuscript in preparation). The diffraction data with a signal-to-noise ratio ($I/\sigma I$) greater than 3 are 90% complete from 20- to 1.5-Å resolution, and more than 50% complete for the highest (1.4−1.5-Å) resolution data. The chromophore model was built into a 1.4-Å resolution $F_o - F_c$ electron density map with phases calculated from the protein atoms only. Following crystallographic refinement with X-PLOR (Brünger et al., 1987), the crystallographic residual error (R-factor) between these data from 20- to 1.4-Å resolution and the complete model is 18.6%, and the root-mean-square deviations from ideality of bond lengths and angles are 0.012 Å and 1.57°, respectively, and there are no deviant main-chain torsion angles on the Ramachandran plot.

RESULTS

Cloning and Sequencing. The 315-bp PCR product including amino acids 9−112 of PYP was amplified from *E. halophila* chromosomal DNA and used to identify a 2.5-kb *Pst*I clone containing the *pyp* gene from a partial genomic library. The *pyp* probe hybridized to only one DNA band in Southern blots of genomic DNA digested with a variety of restriction enzymes, suggesting that *pyp* occurs as a single-copy gene in the *E. halophila* chromosome.

A 1540-bp segment of the 2.5-kb *Pst*I genomic clone was sequenced and analyzed for all possible open reading frames (ORF) on each strand (Figure 1). The G+C composition of this sequence is 67%, which is in the range expected for the genus (Stacey et al., 1989), and the codon usage shows a strong preference for codons ending with G (33%) or C (61%). The ORF encoding PYP was found between two other ORF's encoding putative unknown proteins (Figure 1). The *pyp* ORF (bases 769−1143) is preceded by a potential ribosome binding site very similar to the canonical Shine-Dalgarno sequence, and it is followed by a pair of nearly perfect inverted repeats (bases 1157−1170 and 1174−1187) that could have a regulatory role at the transcriptional level. The partial translation product of the first ORF (bases 1−660) showed significant homology with *E. coli* D-amino acid

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   1 CTGCAGTTCACCGCGGCCCTGGCGCAGCGACTCACCGCCGAAGGCGCCGAACTGTGGCTCGACACTACGGCACGCAGGCTGCTCGGCGGC   90
     L  Q  F  T  A  A  L  A  Q  R  L  T  A  E  G  A  E  L  W  L  D  T  T  A  R  R  L  L  G  G
  91 CCAGCGGGTGTGGAGGGCGTCGAGACCAGCCGCGGGATCATCGACGCGGACGCCGTAGTTGTCGCGGCGGGCAGTTACAGCCCGGCCCTG  180
     P  A  G  V  E  G  V  E  T  S  R  G  I  I  D  A  D  A  V  V  V  A  A  G  S  Y  S  P  A  L
 181 ACCCGCAGGTTCGGTCTGCGCCTGCCCATCGAACCCGTCAAGGGGTATTCGGTGAGCATCCCGCTGCACGGGATAGAGGGCGCACCCGGG  270
     T  R  R  F  G  L  R  L  P  I  E  P  V  K  G  Y  S  V  S  I  P  L  H  G  I  E  G  A  P  G
 271 ATGTCCATCATCGACGACGCCCGCAAGGTGGTCGTTACCCGACTGGGTGAGCAGCTGCGTATCGCCGGCAAGGCGGAGATCACCGGCTTC  360
     M  S  I  I  D  D  A  R  K  V  V  V  T  R  L  G  E  Q  L  R  I  A  G  K  A  E  I  T  G  F
 361 GATCTCAGGCTCGACGAGCGGCGCTGGCGAGCCGTCCGCGAGCAGGGTCTGAGCCGCTTCCCGCGCCTGGCCGAACAGCTGGCGGACGCC  450
     D  L  R  L  D  E  R  R  W  R  A  V  R  E  Q  G  L  S  R  F  P  R  L  A  E  Q  L  A  D  A
 451 CCGAGCCAACCCTGGGCCGGCCTGCGCCCGATGACCTGCGACGGCCCCCCGATCCTGGGTCCGACCCCGATCTCCGGCCTGCACCTGGCC  540
     P  S  Q  P  W  A  G  L  R  P  M  T  C  D  G  P  P  I  L  G  P  T  P  I  S  G  L  H  L  A
 541 ACGGGCGTCGGCCACCTGGGCTGGACGTTCGCCGCCGGCAGCGCGCGCCTGGTCGCCGACCAGCTGGAGGAGCGCACCTGCGACCTCGAT  630
     T  G  V  G  H  L  G  W  T  F  A  A  G  S  A  R  L  V  A  D  Q  L  E  E  R  T  C  D  L  D
 631 CCCGCCCCGTACTCGCTCCAACGCTACGGATGAACAAAGGGACGGTTCTTGTTATACAAAGGCTTTACATCTAGGCTGGAGTCTCGAGAG  720
     P  A  P  Y  S  L  Q  R  Y  G  *
 721 TGAGCAAGGCTCACCACGAAGCCCCCGGTCCATGAAAGGAGTATCACGATGGAACACGTAGCCTTCGGTAGCGAGGACATCGAGAACACC  810
                                        M  E  H  V  A  F  G  S  E  D  I  E  N  T
 811 CTCGCCAAGATGGACGACGGCCAGCTCGACGGCCTGGCCTTCGGCGCCATCCAGCTCGACGGCGACGGCAACATCCTTCAGTACAACGCC  900
     L  A  K  M  D  D  G  Q  L  D  G  L  A  F  G  A  I  Q  L  D  G  D  G  N  I  L  Q  Y  N  A
 901 GCGGAGGGCGACATCACCGGCCGCGACCCGAAGCAGGTCATCGGCAAGAACTTCTTCAAGGACGTGGCCCCGTGCACTGACAGCCCGGAG  990
     A  E  G  D  I  T  G  R  D  P  K  Q  V  I  G  K  N  F  F  K  D  V  A  P  C  T  D  S  P  E
 991 TTCTACGGCAAGTTCAAGGAAGGGGTGGCCTCGGGCAACCTGAACACGATGTTCGAGTACACCTTCGATTACCAAATGACGCCCACGAAG 1080
     F  Y  G  K  F  K  E  G  V  A  S  G  N  L  N  T  M  F  E  Y  T  F  D  Y  Q  M  T  P  T  K
1081 GTGAAGGTGCACATGAAGAAGGCCCTCTCCGGCGACAGCTACTGGGTCTTCGTCAAGCGCGTCTAGACCAGACCCCGCTGCCCGTGCCCG 1170
     V  K  V  H  M  K  K  A  L  S  G  D  S  Y  W  V  F  V  K  R  V  *
1171 TCCCGGGGCGCGGGCAGCCGCGAAAGGACCGACCGATGCAAGGGCTGAATGCCGATGAAGTGCTGCGGTTGCTGCGCAGCCTGATCCCCGG 1260
                                        M  Q  G  L  N  A  D  E  V  L  R  L  L  R  S  L  I  P  G
1261 TGAGCTGGCCACCGGCCGCGGTCAACGGGGCGACCCGCCCGAAGCGCAGGACCTGTGCGCCGATAGCCGCCTGGATGCCGAACCAATCCG 1350
      E  L  A  T  G  R  G  Q  R  G  D  P  P  E  A  Q  D  L  C  A  D  S  R  L  D  A  E  P  I  R
1351 GGCGGACTCCCTGGATCGACTCAATCTAGCCAGCGCGCTCAACCGCTTCTTCCGCCTGCATGAGACCGGTGTGGAGGACCGTCTGTTGGC 1440
      A  D  S  L  D  R  L  N  L  A  S  A  L  N  R  F  F  R  L  H  E  T  G  V  E  D  R  L  L  A
1441 CGTGCGCCGGATCGGCGACATGGCCGAACTCATCGCCGACGCCAGTCAGCACACCAGCGGCCTGACCTTCTCGACCTCGGGCAGCACGGG 1530
      V  R  R  I  G  D  M  A  E  L  I  A  D  A  S  Q  H  T  S  G  L  T  F  S  T  S  G  S  T  G
1531 CACCCCGCAG 1540
      T  P  Q
```

FIGURE 1: Nucleotide sequence of the *pyp* gene and flanking sequences. Numbering corresponds to nucleotides. For three open reading frames (ORF), the nucleotide sequence is translated into one-letter amino acid code below the first nucleotide of each codon. An asterisk indicates stop codons. The first ORF includes nucleotides 1−660, the second ORF (encoding PYP) includes nucleotides 769−1143, and the third ORF includes nucleotides 1205−1540. Putative ribosome binding sites are indicated with double underline, and the pair of inverted repeats of a potential hairpin loop of possible regulatory function are indicated with a single underline.

dehydrogenase (a 47-kDa flavoprotein) with 37% identity and 61% similarity for a 218 amino acid overlap. Interestingly, a 47-kDa flavoprotein has been isolated from *E. halophila* (Meyer, 1985). A third partial ORF (bases 1205−1540), located downstream from *pyp*, may also encode a protein since it is preceded by a potential ribosome binding site, but the sequence is not significantly homologous to any genes or proteins in GenBank 83.0 or EMBL 38.0 databases.

Translation of the nucleotide sequence of the *pyp* ORF matched the PYP peptide sequence (Van Beeumen et al., 1993) except for one putative amino acid difference (Glu56 → Gln56). Our DNA sequence data were obtained from three independent clones: one Taq PCR product, a Vent PCR product, and the 2.5-kb genomic clone. In all three clones, sequencing of both DNA strands revealed the presence of the CAG codon normally coding for glutamine in position 56 of PYP. In order to resolve the apparent difference between the peptide sequence and the DNA sequence, we also sequenced the amino terminus of the PYP proteolytic fragment containing residues 53−124, generated by clostripain digestion. The protein sample was treated to minimize possible deamidation, and the amino-terminal sequence 53-DPKQ... identified residue 56 as Gln. Further confirmation as to the identity of residue 56 came from MS of the tryptic digest of PYP, revealing a proteolytic fragment of mass 543.7 ± 0.4 Da which corresponds to the fragment 56-QVIGK-60 (calculated average mass: 543.7 Da) and is therefore consistent with Gln at residue 56.

Improved PYP Purification. Modifications of the PYP purification protocol improved both protein yield and purity. To increase the efficiency of cell lysis, the resuspended cell pellets were homogenized in a Waring blender and then put through the French press twice. To extract all PYP from the cells, centrifugation and dialysis steps were repeated on the sonicated cell debris pellet. The 75% ammonium sulfate precipitation, additional anion-exchange chromatography, and chromatofocusing steps were added to improve purity. The yield was 66 μg of PYP/g of cells. The ratio of the 280: 446 nm absorbances averaged 0.5, equivalent to the purity obtained in the best fractions of the original purification procedure, and has been as low as 0.4. By ion-spray MS, the measured mass of purified PYP is 14 020 ± 4 Da (compared to a calculated average mass for apoprotein of 13 874 Da), indicating a 1:1 ratio of protein to chromophore (see below). PYP elutes at pH 4.2−4.1 from the chromatofocusing column, consistent with its previously measured p*I* of 4.3 (McRee et al., 1986). PYP purified by using the chromatofocusing protocol routinely provides clean, single crystals that do not twin.

Chromophore Chemistry. We measured the UV−visible absorption spectra of denatured PYP as a function of pH. From acidic to slightly alkaline pH the chromophore maximally absorbed at 340 nm, but at more alkaline pH, the absorption shifted to 398 nm (Table 1, lines 3 and 4). The pK_a of this transition is 9.0 ± 0.5.

Table 1: Acid–Base Absorption Maxima of PYP Chromophore

protein-bound or cleaved chromophore	pH	λ_{max}	reference
1. native protein	7	446	Meyer, 1985
2. native protein	2	345	Meyer, 1985
3. denaturated protein	10	398	this work
4. denaturated protein	7	≈340	Meyer, 1987
5. NH_3 cleaved	13	≈340	this work
6. NH_3 cleaved	2	300	this work

The mass of the chromophore was determined by ion-spray MS for both the whole protein and V8 protease-cleaved chromopeptide 66-VAPCTDSPE-74 (data not shown). The chromophore mass was derived from the difference (146 Da) between the experimentally measured mass and the mass calculated from the protein sequence alone. Taking into account that the calculated masses of apoprotein and peptide included the sulfhydryl proton of free cysteine and that this proton must be absent in the chromophore-bound polypeptides, the mass of the chromophore is 147 Da.

Chemical Cleavage of the Chromophore from the Protein. Hydroxylamine treatment of PYP cleaved the chromophore linkage to produce the apoprotein, as detected by MS analysis, but also caused other side reactions. Cleavage by hydroxylamine suggested a thioester linkage (Weimbs & Stoffel, 1992), rather than a disulfide or thioether linkage, to Cys69. To test this, PYP was treated with ammonia under alkaline conditions that were expected to result in thioester, but not disulfide, cleavage to the free thiol and the amide derivative of the chromophore (Connors & Bender, 1961; Jencks, 1969). If, in fact, the chromophore were linked via a thioester, then the predicted mass of the liberated chromophore amide would be 163, as calculated on the basis of the masses of acyl chromophore as attached to PYP (147 Da) and a terminal NH_2 group (16 Da).

Treatment of PYP holoprotein with pH 10 ammonium buffer in GuHCl resulted in a spectrophotometrically observed shift in chromophore absorption maxima from 398 to 348 nm, displaying an isosbestic point at 362 nm. Given that the pH remained constant at 10, this change in λ_{max} was interpreted as chemical modification of the chromophore. The reaction was deemed complete in approximately 4 h, as judged from the change in absorption maxima. Analysis of the reaction mixture by HPLC at dual wavelengths of 214 and 350 nm showed a small early-eluting peak with absorbance at 350 nm which presumably contained the liberated chromophore as an amide derivative. The late-eluting protein peaks absorbed at 214 nm but not at 350 nm, indicating that the chromophore was detached from the protein (Figure 2A). Apoprotein was, however, identified among the later eluting broad protein peaks. The early-eluting peak could not be analyzed by ion-spray MS due to intense low-mass noise caused by salt contamination.

Characterization of the Liberated Chromophore. To confirm the presence of the liberated chromophore, the early-eluting peak was characterized by LC/MS and UV spectroscopy. The mass of the purified amide form of the chromophore as determined by LC/MS (which allowed the subtraction of background ions) was 163, precisely as predicted for aminolysis of a chromophore–protein thioester bond by ammonia (Figure 2C). There was insufficient chromophore amide sample to allow high-resolution FAB-MS analysis. UV monitoring of the LC effluent confirmed

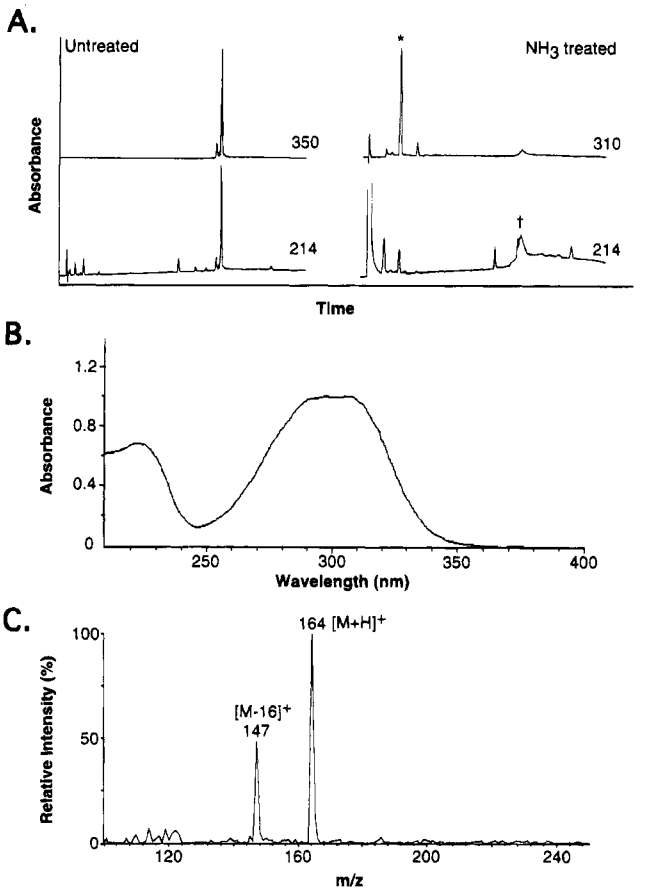

FIGURE 2: Chemical cleavage and characterization of chromophore. (A) HPLC profiles of PYP before and after chemical release of chromophore. Peaks were eluted from a C4 reverse-phase column using a 1%/min acetonitrile gradient in 0.1% aqueous TFA. Run times were 60 min. Simultaneous 214- and 350-nm monitoring clearly distinguishes the chromophore-bearing protein from other minor contaminants. Absorbances are not to the same scale, and each has been adjusted to adequately show the peaks of interest. Left pair of chromatograms show elution of native PYP. Right pair of chromatograms correspond to separation of PYP following aminolytic cleavage of chromophore by aqueous ammonium chloride, pH 10. Elution of the cleaved chromophore fragment (*) could be selectively identified by monitoring the column eluent at 310 nm, and this peak was collected for further study. Apoprotein (†) elutes within a broad peak and is detectable at 214 nm. (B) UV absorption spectra of cleaved chromophore, as purified by HPLC. Sample solvent was 25% acetonitrile in 0.1% aqueous TFA (pH 2). Absorption maxima occur at 224 nm and a broad peak centered at 300 nm. The ratio of 224:300-nm absorbance was 0.7. (C) MS analysis of liberated chromophore. HPLC-purified material was analyzed by LC/MS, and the mass spectrum shown corresponds to elution of the 310-nm absorbing species. The spectrum has been corrected for background ions by subtraction of the average background spectrum. As predicted for cleavage of a thioester protein–chromophore linkage by ammonia, the predominant *m/z* 164 peak corresponds with the protonated chromophore cleavage product ($[M + H]^+$). The ion at *m/z* 147 is presumably a fragment ion resulting from loss of ammonia (NH_3) from the protonated parent ion at 164 (also see Figure 3).

that the elution time of the mass 163 species matched that of the peak absorbing at 310 nm. The absorption spectrum of free chromophore in 25% acetonitrile and 0.1% TFA (pH 2) displayed maxima at 224 nm and at a broad peak centered at 300 nm (Figure 2B). While the extinction coefficients could not be determined due to unknown concentration, the ratio of the 224:300 nm absorbances was approximately 0.7:1. The UV absorbance peak of the liberated chromophore shifted from 300 to ≈340 nm under alkaline conditions

(Table 1, lines 6 and 5). The pK_a of 9.0 ± 0.3 for this transition matched that seen for the 340/398-nm transition for the chromophore bound to denatured PYP. The absorbance of the chromophore at a relatively long wavelength for its low molecular weight suggests not only a conjugated system of unsaturated bonds but also aromatic character. The alkaline pK_a of both free and bound chromophores is most consistent with the titration of a phenolic hydroxyl, though other possibilities are an aromatic amine or aromatic nitrogen heterocycle. However, the pK_a of simple aromatic amines and aromatic nitrogen heterocycles is typically much lower (pK_as of aniline and pyridine are ≈5).

High-Resolution Mass Spectrometry. In order to determine the elemental composition of the chromophore, we measured the high-resolution mass (1063.427 ± 0.015 Da) of the V8 protease derived chromopeptide, 66-VAPCTDSPE-74, by FAB-MS. Given the chromophore−protein linkage to be a thioester, the mass of the liberated chromophore amide was therefore derived as 163.074 ± 0.015 Da. Limiting the elements to C, H, N, O, and S, consideration of all potential elemental compositions of the form $C_aH_bN_cO_dS_e$ of mass 163.074 ± 0.015 Da generates over 30 possibilities. However, all but two of these candidates can be eliminated by imposing three constraints. First, the cleaved chromophore should contain at least one oxygen atom (in the primary amide bond). Second, the UV absorption properties of cleaved chromophore indicate aromatic or conjugated double bonds; accordingly, the minimum index of hydrogen deficiency [as calculated from the molecular formula (Silverstein et al., 1981)] should be at least 3. Third, the nitrogen rule (Silverstein et al., 1981) requires that the molecular formula of cleaved chromophore have an odd number of nitrogen atoms due to its odd mass (163). Two candidates for the chromophore amide satisfied all of these restrictions: $C_8H_9N_3O$ or $C_9H_9NO_2$. An elemental composition of $C_8H_7N_2O$ for the acyl chromophore would be consistent with an aromatic amine or nitrogen heterocycle, while $C_9H_7O_2$ could represent a phenol.

Mass Spectrometric Fragmentation. Fragmentation patterns for the amide derivative of the free chromophore, as liberated by alkaline ammonia, were analyzed in a MS/MS experiment. The molecular ion at *m/z* 164 ($[M + H]^+$) was fragmented by collision with argon gas to generate a well-defined set of daughter ions at *m/z* 147, 119, 91, and 65, along with minor ions at 103, 77, and 44 (Figure 3). Assuming a sequential loss of fragments to generate the observed ions, this CID spectrum indicates consecutive losses of *m/z* 17 (164 → 147), 28 (147 → 119), 28 (119 → 91), and 26 (91 → 65). The 164 → 147 → 119 series is characteristic of a terminal primary amide and reflects loss of neutral NH_3 (164 → 147) followed by C=O (147 → 119) or, alternatively, scission of the charged $[O{=}C{=}NH_2]^+$ fragment leading to the minor ion at 44. The consecutive fragment ions at *m/z* 91 (tropylium), 77 (phenyl), and 65 (cyclopentadienyl) all suggest the presence of a single phenyl ring in the chromophore. Loss of second mass 28 fragment (119 → 91) suggested loss of an additional neutral C=O, which would be characteristic of a phenol (Davis & Frearson, 1987). Other less commonly observed mass 28 fragments are N_2, C_2H_4, and HC=NH. A nitrogen-containing chromophore is harder to reconcile with the observed data. Upon fragmentation, both aromatic amines and nitrogen heterocycles characteristically lose HCN (27), not HC=NH (28).

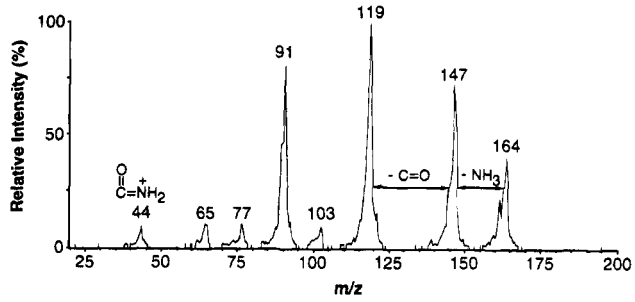

FIGURE 3: MS/MS fragmentation of cleaved chromophore. The protonated molecular ion at *m/z* 164 was fragmented by collision with neutral argon gas. The CID spectra obtained show sequential losses of mass 17 (147), 28 (119), 28 (91), and 14 (77) or 26 (65). The presence of a primary amide is confirmed by characteristic losses from the parent ion of 17 (NH_3) followed by 28 (C=O) or, alternatively, loss of the charged $[O{=}C{=}NH_2]^+$ fragment (*m/z* 44). Consecutive ions at 91, 77, and 65 suggest the presence of a phenyl ring, while the loss of a second mass 28 fragment is consistent with a phenol substructure.

Furthermore, the fragment ions at *m/z* 91 and 77 do not support the presence of either annular nitrogen (aromatic heterocycle) or nitrogen external to an aromatic ring (aromatic amine). Loss of the mass 26 fragment (91 → 65) is undoubtedly due to ejection of the neutral HC≡CH, a feature commonly observed in the fragmentation of aromatic hydrocarbons.

MS fragmentation therefore provided additional proof that the chemically cleaved chromophore contained a primary amide, as predicted from aminolysis of a protein chromophore thioester bond by ammonia. In conjunction with the titration data, it provided strong evidence that the chromophore was phenolic, in which case the molecular formula of chemically cleaved chromophore amide can only be $C_9H_9NO_2$.

Crystallographic Chromophore Structure. The 1.4-Å-resolution "omit" electron density map (Figure 4) made with phases calculated from the PYP protein atomic model without the chromophore (G. E. O. Borgstahl, D. R. Williams, and E. D. Getzoff, manuscript in preparation) clearly defined the positions and bonding of the heavy (non-hydrogen) atoms in the chromophore. This map revealed the chromophore to be composed of a six-membered ring, with a single heavy atom substituent at position 1 of the ring and four heavy atoms in a connecting linker between position 4 of the ring and the sulfur of Cys69. All heavy atoms of the chromophore lay approximately in a single plane, indicating an aromatic ring conjugated with the linker. Higher electron density (10σ contours, Figure 4) for the ring substituent (O1) and the branch from the connecting linker (O2) indicated N or O heteroatoms. Atom O2 is the carbonyl oxygen in the thioester bond, providing additional confirmation as to the nature of the chromophore−protein linkage. When the heteroatom electron density values were compared to those of the protein main chain, the electron density for atom O1 matched that of the carbonyl oxygen better than that of the nitrogen (Figure 4). Thus, the electron density maps supported the elemental formula of the acyl chromophore of $C_9H_7O_2$ corresponding to a 4-hydroxycinnamyl moiety (Figure 4).

The PYP protein atomic model including the 4-hydroxycinnamyl chromophore was refined against 1.4-Å diffraction data. The resulting stereochemistry (bond lengths are

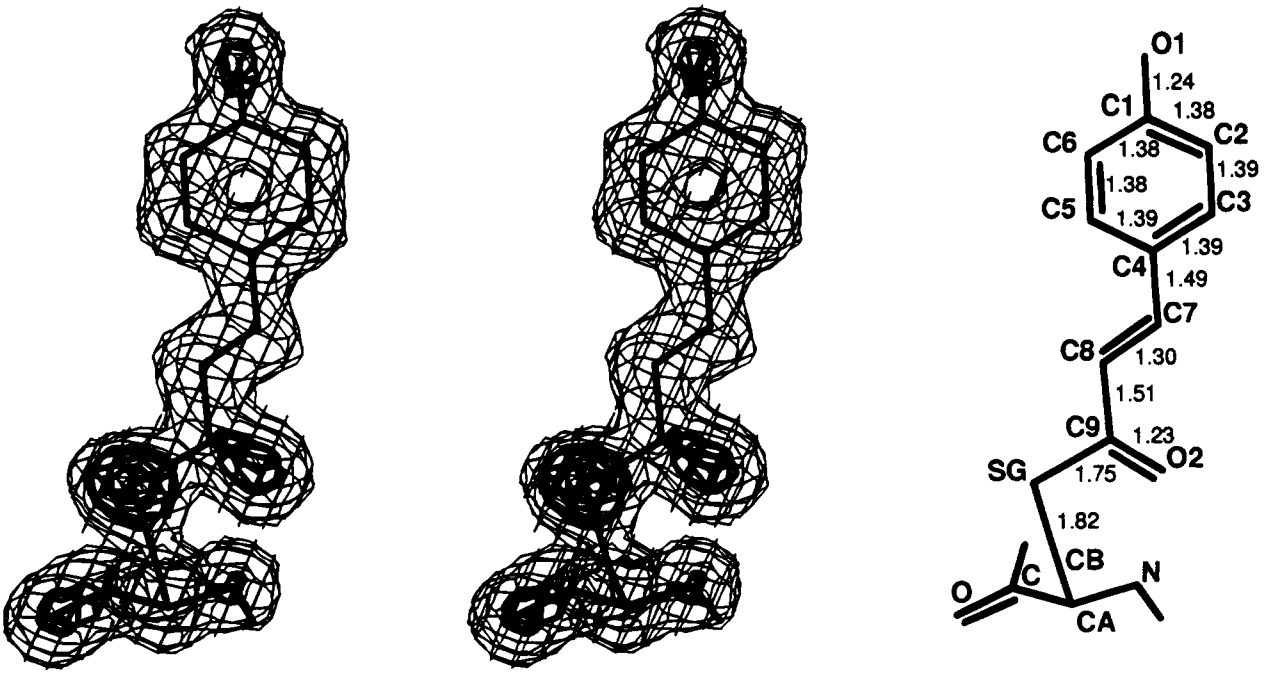

FIGURE 4: 1.4-Å electron density image and atomic model of the PYP chromophore. The stereo diagram to the left shows the PYP chromophore model with its omit $F_o - F_c$ electron density (thin lines 3σ and thick lines 10σ). The chromophore was excluded from the phase calculation to avoid model bias. The higher contour (thick) lines at the thioester sulfur, the thioester carbonyl oxygen, and the cinnamyl hydroxyl indicate the presence of atoms with more electrons than carbon (thin contour lines). Atom designations and refined bond lengths are indicated on the stick diagram to the right.

indicated in Figure 4) confirm that O2 is a carbonyl oxygen and that there is a double bond between C7 and C8, thus extending conjugation from the aromatic ring through to the thioester bond. The refinement of the C1−O1 bond length was given special consideration. When the structure was refined with a 1.376-Å restraint [typically used for Tyr hydroxyl (Brünger et al., 1987)], the refined bond length converged to 1.345 Å. Setting the restraint to 1.310 Å [value from potassium 4-(4-nitrophenyl)phenolate small molecule structure (Haase et al., 1991)] resulted in a value of 1.292 Å. Finally, a restraint of 1.249 Å [value typically used for Asp or Glu carboxylate (Brünger et al., 1987)] was used and resulted in a value of 1.240 Å. This short C1−O1 bond length indicates a double bond and thereby a deprotonated state for O1 in the context of the folded protein.

Comparison of the Cleaved Chromophore to 4-Hydroxycinnamic Acid. Proof of the proposed chromophore structure was provided by comparing the properties of the chemically cleaved chromophore with commercially available 4-hydroxycinnamic acid. This compound differs from the liberated chromophore in that it is the free acid, while the latter is a primary amide (Figure 5A). The UV absorption spectrum and MS/MS fragmentation pattern of 4-hydroxycinnamic acid support the proposed chemical structure of the cleaved chromophore as 4-hydroxycinnamide (Figure 5B,C). Both compounds exhibit absorption maxima at about 225 and 300 nm with a similar 225:300-nm absorbance ratio. Fragmentation of the molecular ion of 4-hydroxycinnamic acid (Figure 5C) generated the same set of major daughter ions as observed for the cleaved chromophore (Figure 3). This indicates these two compounds are composed of the same structural elements. The pK of the phenolic hydroxyl in 4-hydroxycinnamic acid is 9.5 ± 0.3 (for transition between 310 and 340 nm), a value similar to that obtained for the cleaved chromophore (9.0 ± 0.3). The 0.5 increase in pK for 4-hydroxycinnamic acid relative to the 4-hydroxycinnamide chromophore is not surprising, given that deprotonation of the phenolic hydroxyl is somewhat disfavored by the negatively charged carboxylate group.

DISCUSSION

The first step in photosensing is the capture of energy from a photon. Establishing the chemical nature of a chromophore, its covalent linkage to the protein, and the protein's exact primary structure is of fundamental importance in defining the mechanism of protein photosensing. Here we have cloned and sequenced the DNA encoding PYP (Figure 1). The *pyp* gene is the first protein-coding gene ever sequenced from a member of the *Ectothiorhodospira* genus, the single genus of the *Ectothiorhodospiraceae* family of purple sulfur bacteria (Stacey et al., 1989). The *Ectothiorhodospiraceae* family is smaller and less well characterized than the other purple sulfur bacterial family, *Chromatiaceae*. Genetic studies have been recently undertaken for some species of the *Chromatiaceae*, especially for *chromatium vinosum*; whereas in the *Ectothiorhodospiraceae*, only ribosomal RNA genes have been sequenced so far. The translated amino acid sequence from the *pyp* gene matches the previously published protein sequence (Van Beeumen et al., 1993) with one exception: Gln56 replaces Glu56. The DNA and revised protein sequences for PYP (Figure 1) not only establish the protein's primary structure but also open the door to future mutagenesis studies.

In PYP, the covalent linkage of the chromophore to Cys69 is cleaved by dithiothreitol, suggesting a disulfide or thioester but not a thioether bond (Van Beeumen et al., 1993). On the basis of the inability of 25% aqueous TFA to cleave chromophore from PYP or its chromopeptides, these researchers further proposed that the chromophore is attached through a disulfide rather than a thioester bond and noted

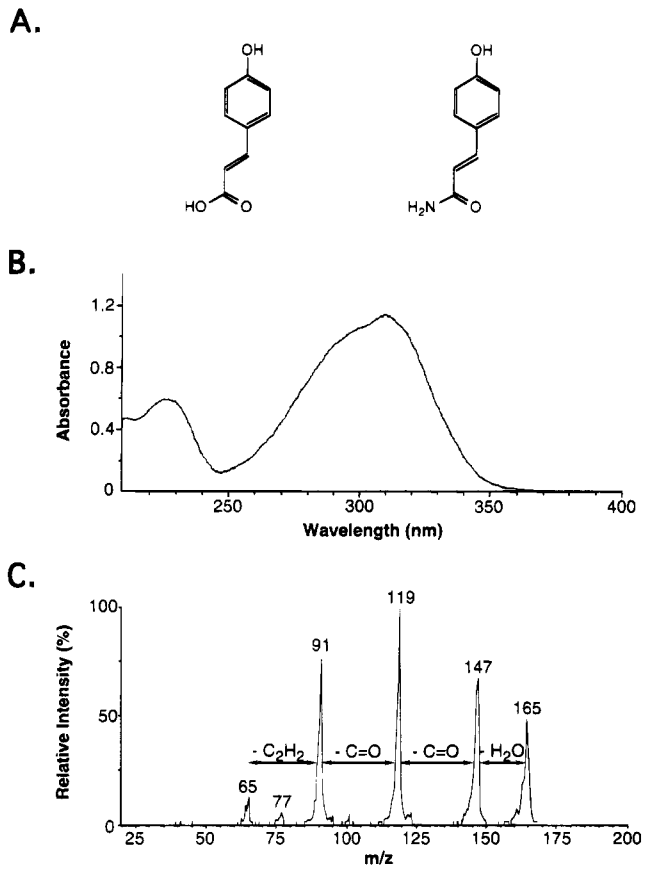

FIGURE 5: Chemical structure and spectroscopic properties of 4-hydroxycinnamic acid. (A) Chemical structures of 4-hydroxycinnamic acid (left) and the chemically cleaved chromophore 4-hydroxycinnamide (right). (B) UV absorption spectra of 4-hydroxycinnamic acid. Sample solvent was 25% acetonitrile in 0.1% aqueous TFA (pH 2). Absorption maxima occur at 228 and 310 nm and the 228:310 nm absorbance ratio is 0.5. The absorption properties of 4-hydroxycinnamic acid are thus very similar to those of the cleaved chromophore (cf. Figure 2). (C) MS/MS fragmentation of 4-hydroxycinnamic acid. The protonated molecular ion at *m/z* 165 was fragmented by collision with neutral argon gas. Mass and identity of sequential fragment losses from the parent ion were 18 (H_2O), 28 (C=O), 28 (C=O), and 14 (CH_2) or 26 (HC≡CH). These losses resulted in a set of daughter ions (147, 119, 91, 77, and 65) essentially identical to that obtained for the cleaved chromophore, both in mass and relative intensity (cf. Figure 3).

the rarity (or total absence) of known disulfide-linked protein cofactors in nature. However, given the low rate of thioester hydrolysis by aqueous acid (Jencks, 1969), the chemical nature of the chromophore−protein bond warranted reinvestigation. Here, we demonstrate that the chromophore is released both by hydroxylamine cleavage, as also previously attempted by Meyer et al. (1987) and McRee et al. (1986), and by alkaline aminolysis to liberate the amide derivative of the chromophore (Figure 2A). Both these methods are known to cleave Cys thioester bonds while leaving disulfide bonds intact (Connors & Bender, 1961; Jencks, 1969; Weimbs & Stoffel, 1992).

The elemental composition of the acyl chromophore was limited to $C_8H_7N_2O$ or $C_9H_7O_2$ by the high-resolution mass of the proteolytic chromopeptide fragment 66-VAPCTDSPE-74. The extinction coefficient (45.5 mM^{-1} cm^{-1}) (Meyer et al., 1989) and absorption maxima (Table 1) indicate an aromatic or highly conjugated chromophore. Combining the elemental composition data with the high-resolution electron density map of the protein-bound chromophore (Figure 4), the MS fragmentation pattern (Figure 3), and pK_a (9.0 ± 0.3) of the chromophore amide uniquely identifies the PYP chromophore as the 4-hydroxycinnamyl group. Comparisons of the UV absorption, pH titration, and MS fragmentation data for the chemically cleaved chromophore (Figure 3) and 4-hydroxycinnamic acid (Figure 5) confirmed this identification.

4-Hydroxycinnamic acid (also known as *p*-coumaric acid) is an intermediate in the phenylpropanoid pathway in plants and is metabolically derived from both phenylalanine and tyrosine. Interestingly, the incorporation of 4-hydroxycinnamic acid into flavonoids, lignins, and other phenolic compounds proceeds via a thioester-linked 4-hydroxycinnamyl-coenzyme A intermediate. If the 4-hydroxycinnamyl group is similarly activated in *E. halophila* as a coenzyme A adduct, then co- or posttranslational attachment to PYP could occur via transthioesterification, catalyzed by an as yet undiscovered enzyme. Regardless of the mechanism of incorporation, this is the first report of covalent modification of a protein by 4-hydroxycinnamic acid and also, to the best of our knowledge, the first identification of this metabolite in the Proteobacteria. In plants, 4-hydroxycinnamic acid is a key molecule involved in the stress response pathway: wound healing, pathogen infection, or exposure to UV. It is interesting to note that 4-hydroxycinnamate is evolutionarily conserved in a bacterium where it plays a role in the negative phototactic response to blue light (Sprenger et al., 1993).

Spectroscopic and crystallographic data suggest that PYP's chromophore in the dark state is stabilized within the folded protein as a phenolate anion. Three factors contribute to the yellow color of PYP: conjugation, deprotonation, and burial of the chromophore in a hydrophobic protein environment. The contribution of each of these factors to the absorption maximum of the chromophore can be demonstrated from the absorption data. Increased conjugation from covalent attachment to the protein increases the absorption maximum by 40 or 58 nm, depending on the protonation state (Table 1, lines 4−6 or 3−5). Deprotonation further red-shifts the absorption maximum by another 58 nm (Table 1, lines 3 and 4). Placing the chromophore within the folded protein changes the absorption maximum by either 48 or 106 nm, depending on whether the chromophore is buried in the deprotonated or protonated form (Table 1, lines 1−3 or 1−4). The smaller change of 48 nm is more plausible since placing conjugated organic compounds in hydrophobic solvents is expected to increase the absorption maximum by only 20 nm or less (Parikh, 1974), indicating that the chromophore is in an anionic form in the dark state of PYP. Finally, in the 1.4-Å resolution PYP crystal structure (G. E. O. Borgstahl, D. R. Williams, and E. D. Getzoff, manuscript in preparation), the C1−O1 bond length refines to that of a double, rather than a single, bond (Figure 4) and further indicates that the chromophore is in an anionic form in the dark state of PYP.

During the photocycle, PYP takes up a single proton upon bleaching and subsequently releases it upon return to the dark state (Meyer et al., 1993). When the protein is denatured at neutral pH, the covalently bound chromophore is protonated, as shown by pH titrations (Table 1, lines 3 and 4). The 340-nm absorption maximum characteristic of the protonated chromophore in the denatured protein has a striking resemblance to that of the bleached intermediate (I_2) of the PYP

photocycle (Meyer et al., 1987). Likewise, very acidic pH titration of the native protein bleaches the absorption maximum of the chromophore (Table 1, line 2), indicating that the dark state chromophore is not only deprotonated but also buried and that the protonation at pH 2.7 is probably correlated to solvent exposure of the chromophore. The anionic form of the chromophore in the dark state and the 340-nm absorbance common to both the bleached PYP intermediate and the protonated protein-bound chromophore suggest that the PYP light cycle occurs through the protonation and deprotonation of the chromophore.

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