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**DOI**

[10.1074/jbc.271.50.31949](https://doi.org/10.1074/jbc.271.50.31949)

**Publication date**

1996

**Document Version**

Final published version

**Published in**

The Journal of Biological Chemistry

[Link to publication](#)

**Citation for published version (APA):**

Kroon, A. R., Hoff, W. D., Fennema, H. P. M., Gijzen, J., Koomen, G. J., Verhoeven, J. W., Crielaard, W., & Hellingwerf, K. J. (1996). Spectral Tuning, Fluorescence, and Photoactivity in Hybrids of Photoactive Yellow Protein, Reconstituted with Native or Modified Chromophores. *The Journal of Biological Chemistry*, 271(50), 31949-31956. <https://doi.org/10.1074/jbc.271.50.31949>

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# Spectral Tuning, Fluorescence, and Photoactivity in Hybrids of Photoactive Yellow Protein, Reconstituted with Native or Modified Chromophores\*

(Received for publication, July 9, 1996, and in revised form, September 3, 1996)

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Photoactive yellow proteins (PYPs) constitute a new class of eubacterial photoreceptors, containing a deprotonated thiol ester-linked 4-hydroxycinnamic acid chromophore. Interactions with the protein dramatically change the (photo)chemical properties of this cofactor. Here we describe the reconstitution of apoPYP with anhydrides of various chromophore analogues. The resulting hybrid PYPs, their acid-denatured states, and corresponding model compounds were characterized with respect to their absorption spectrum, pK for chromophore deprotonation, fluorescence quantum yield, and Stokes shift. Three factors contributing to the tuning of the absorption of the hybrid PYPs were quantified: (i) thiol ester bond formation, (ii) chromophore deprotonation, and (iii) specific chromophore-protein interactions. Analogues lacking the 4-hydroxy substituent lack both contributions (chromophore deprotonation and specific chromophore-protein interactions), confirming the importance of this substituent in optical tuning of PYP. Hydroxy and methoxy substituents in the 3- and/or 5-position do not disrupt strong interactions with the protein but increase their pK for protonation and the fluorescence quantum yield. Both deprotonation and binding to apoPYP strongly decrease the Stokes shift of chromophore fluorescence. Therefore, coupling of the chromophore to the apoprotein not only reduces the energy gap between its ground and excited state but also the extent of reorganization between these two states. Two of the PYP hybrids show photoactivity comparable with native PYP, although with retarded recovery of the initial state.

Many proteins bind cofactors to extend the physicochemical range of photoactivity, offered by their amino acid side chains. In photobiology these cofactors play a central role since they are responsible for both light absorption and its conversion into a biologically relevant response. In many cases, the altered physicochemical characteristics of the chromophores enable the protein-chromophore complex to optimally perform its bio-

logical function. This phenomenon, called spectral tuning, has been studied extensively in rhodopsins, all members of the family of 7-transmembrane  $\alpha$ -helical proteins. Here we have used the photoactive yellow protein (PYP)<sup>1</sup> as a model system to study such protein-cofactor interactions.

PYP is a water-soluble protein, which was first isolated from the halophilic purple phototrophic eubacterium *Ectothiorhodospira halophila* (1). Upon excitation, the protein enters a cyclic chain of dark reactions, *i.e.* a photocycle, resembling the one observed in the sensory rhodopsins from archaeobacteria (2). The first intermediate in this photocycle (red-shifted intermediate of PYP) is red-shifted to 465 nm. Subsequently, a blue-shifted intermediate (pB<sub>355</sub>) develops on a sub-millisecond time scale. The photocycle is completed by the reformation of the initial state of the protein (pG), in about 1 s (3).

*E. halophila* displays negative phototaxis to blue light. PYP has been implicated to function as the photosensor in this response, since its absorption spectrum matches its wavelength dependence (4). Although PYP resembles the sensory rhodopsins both functionally and photochemically, its chromophore is not retinal but a novel type of chromophore, 4-hydroxycinnamic acid (5, 6), linked to Cys-69 via a thiol ester bond (7). Therefore, PYP represents a unique type of photoreceptor. PYP homologues have been detected in several eubacteria (8–11). Thus, it has been proposed to refer to this novel family of blue-light photoreceptors as Xanthopsins (11, 12).

The photochemical basis of the photocycle of PYP has recently been shown to reside in the photoisomerization of the vinyl double bond in the chromophore, from *trans* to *cis* (13). The quantum yield of this process was initially reported to be 0.64 (14); however, when measurements were extended to a large range of laser pulse energies, a value of 0.35 was obtained (15). Besides photoactivity, PYP shows weak fluorescence too. The quantum yield of pG is  $\sim 2 \times 10^{-3}$ , with an emission maximum at 495 nm (16, 17).

During progression through its photocycle PYP undergoes a large conformational change, which exposes hydrophobic sites to the solvent (14), due to a partial unfolding of the protein upon the formation of pB (18). The altered protein conformation of pB is thought to initiate signal transduction, ultimately affecting the flagellar rotation and thus leading to phototaxis

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<sup>1</sup> The abbreviations used are: PYP, photoactive yellow protein; HAP, histidine-tagged apoPYP overproduced in *E. coli*; pCA, 4-hydroxycinnamic acid (*p*-hydroxycinnamic acid); GFP, green fluorescent protein; GdnHCl, guanidinium hydrochloride; pG, Initial or groundstate of PYP and its hybrids; pB, blue-shifted intermediate of PYP; pB<sub>dark</sub>, Low pH-induced blue-shifted intermediate of PYP.

(19). A decrease in pH spontaneously transforms PYP to a blue-shifted state, resembling pB (1).<sup>2</sup>

Free pCA in aqueous solvents and at neutral pH absorbs maximally at 284 nm (20); however, within the chromophore-binding pocket in the apoprotein, the absorption of the chromophore is strongly red-shifted (to 446 nm). Three contributions to this shift have been identified (6, 7, 19): (i) formation of the thiol ester bond between pCA and Cys-69, (ii) deprotonation of the chromophore (21), and (iii) specific protein-chromophore interactions.

The structure of PYP has recently been re-determined by x-ray crystallography at 1.4-Å resolution (22). The protein has an unusual  $\alpha/\beta$  fold, which resembles eukaryotic proteins involved in signal transduction. PYP is the first photosensory protein for which the crystal structure has been resolved, and this provides the possibility to study its photochemistry and function with atomic resolution (23).

The limited availability of PYP has been a bottleneck in further biophysical and biochemical characterizations. However, recently we have overproduced a histidine-tagged version of apoPYP (HAP) in *Escherichia coli* (11). In addition, the *in vitro* reconstitution of apoPYP (obtained after removal of the chromophore from PYP isolated from *E. halophila*) with the anhydride of pCA has been reported (24).

Here we report the *in vitro* reconstitution of heterologously produced HAP with its natural chromophore and a number of chromophore analogues. We refer to these proteins as hybrid PYPs in view of the fact that we combined the wild type apoprotein *in vitro*, with its natural chromophore and with several modified chromophores. Two classes of chromophore analogues have been investigated. Class A chromophores possess (an) additional ring substituent(s) (3-hydroxy, 3-methoxy, and 3,5-dimethoxy groups) that allows the analysis of the effect of a disturbance of the fit of the chromophore into its binding pocket. In class B chromophores the phenolic hydroxy group has been replaced by a 4-amino, a 4-methoxy, or a 4-dimethylamino substituent. Hybrids based on this class can thus be used to examine the role of the phenolic deprotonation. Here we present an analysis of these PYP hybrids with respect to their absorption and fluorescence characteristics in the pG and pB<sub>dark</sub> state.

#### MATERIALS AND METHODS

**Synthesis of Cinnamic Acid Anhydrides**—To reconstitute HAP, anhydrides of I–VII (Fig. 2) were prepared from the free acids. To accomplish this, 1 mmol of each carboxylic acid was stirred overnight with 1.2 equivalent of dicyclohexylcarbodiimide in dry *N,N*-dimethylformamide. Dicyclohexyl urea precipitated from the solution as the reaction proceeded. After completion of the reaction, the suspension was centrifuged in an Eppendorf centrifuge (2 min at 14,000 rpm), and the clear supernatant was used for reconstitution experiments. Anhydrides were stored at 77 K to prevent decomposition.

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was obtained from Acros Organics (Pittsburgh, PA). 4-Coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), and caffeic acid (3, 4-dihydroxycinnamic acid) were obtained from Sigma. 4-Aminocinnamic acid, 4-dimethylaminocinnamic acid, and 4-methoxycinnamic acid were obtained from Aldrich. All were obtained as the *trans*-isomer.

4-Methoxycinnamic acid anhydride was obtained as a by-product of the coupling reaction between 4-methoxycinnamic acid and 4-nitrothiophenol. This latter anhydride has been obtained exclusively in crystalline form.

**Synthesis of Butyl Thiol Ester Model Compounds**—We used the butyl thiol esters of I–IV as model compounds for the characterization of the absorption and fluorescence of hybrids I–IV. These model compounds were synthesized according to Ref. 25. Each carboxylic acid (1 mmol)

was dissolved in dry *N,N*-dimethylformamide and ~20 mg of dimethylaminopyridine, and 4 equivalents of isobutyl thiol was added, except for the synthesis of the 4-hydroxycinnamic acid thiol ester, for which tertiary butyl thiol was used. Subsequently, 1.2 equivalents of dicyclohexylcarbodiimide was added. The reaction was carried out overnight, while slowly stirring at room temperature. The precipitated dicyclohexyl urea was filtered off repeatedly, and following concentration *in vacuo*, the filtrate was taken up in CH<sub>2</sub>Cl<sub>2</sub>. It was subsequently extracted two times with 0.5 M HCl, two times with a saturated NaHCO<sub>3</sub> solution, and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel (0.035–0.07 mm), using a petroleum ether (60–80 °C), ethyl acetate mixture (2/1, v/v). The purity of each product was checked by IR, NMR, and mass spectrometry.

**Production of HAP**—The polyhistidine-tagged apoPYP (HAP) was expressed in *E. coli* M15, containing the plasmid pHisP (11). HAP production was induced during exponential growth in Luria Bertani medium in the presence of ampicillin (100 mg/liter), via addition of 0.2 mM isopropylthiogalactoside.

**Purification and Reconstitution of HAP into PYP Hybrids**—Two protocols were developed to obtain reconstituted PYP and its hybrids from HAP. Both protocols were carried out at room temperature.

To investigate the kinetics of the reaction between HAP and the anhydride of pCA, HAP was purified before its use as a substrate for the reconstitution reaction. *E. coli* M15/pHisP cells were harvested through centrifugation after 5 h of induction. The cell pellets were resuspended in 50 mM phosphate buffer (pH = 7.5), and cells were disrupted by sonication. The membrane and cytosolic fraction were separated by centrifugation 1 h at 200,000 × *g* in a Centricon T-1055 ultracentrifuge. The cytoplasmic fraction, containing HAP, was dialyzed 16 h against 50 mM phosphate buffer (pH = 7.5) with two changes. Next, the extract was mixed with Ni<sup>2+</sup>-nitrilotriacetic acid resin, incubated for 1 h under gentle agitation at room temperature, loaded in a column, and washed with 0.2 M phosphate, 0.1 M citrate buffer (pH = 7.2) until the absorption at 280 nm (*A*<sub>280</sub>) was constant. The absorbed proteins were eluted with the same buffer, using a linear pH gradient from pH = 7.2 to pH = 3.5. Fractions with the highest *A*<sub>280</sub> values were brought to neutral pH with sodium hydroxide, washed in an Amicon pressure filter concentrator, and stored at –20 °C or used immediately for reconstitution experiments. We observed that HAP has a tendency to precipitate more readily than holoPYP, especially at lower pH values.

Hybrids used for spectral characterization were isolated after reconstitution with the chromophore analogues listed in Fig. 2, because this considerably improved the yield in the purification procedure. Cell-free extract was prepared according to the following procedure. *E. coli* cells (from 20 liters of culture broth) were harvested by centrifugation and resuspended in 1.5 liters of 50 mM sodium phosphate buffer (pH = 7.2). The cells were prepared for lysis by 60 min incubation at 37 °C with 0.5 g of lysozyme (Sigma). Subsequently, the pH was increased to pH = 10 with 8 N NaOH, and cells were incubated for 5 min at this pH to reduce the viscosity of the lysate, after which the pH was re-adjusted to 7 with 37% (w/w) HCl. After removal of the debris by low speed centrifugation (30 min, 10,000 × *g*), the supernatant was brought to 50% ammonium sulfate saturation and subjected to high speed centrifugation (30 min, 40,000 × *g*) to clarify the extract. The clear supernatant was distributed over 7 fractions (~200 ml per fraction, each containing approximately 140 mg of HAP). These fractions were incubated for 60 min, each with an excess of one of the anhydrides of the chromophore analogues, under gentle mixing (2 mmol in total, *i.e.* a 200-fold molar excess, via two successive additions). The high concentration of proteins other than HAP, and of ammonium sulfate, in these samples did not significantly interfere with the rate or the final level of reconstitution with the anhydrides tested (data not shown). After reconstitution, the mixture was dialyzed overnight against 50 mM phosphate buffer (pH = 7.2) to remove excess anhydride and free acid and concentrated to a volume of 25 ml.

To purify the seven hybrid PYPs in parallel, we modified a rapid isolation procedure using Ni<sup>2+</sup>-nitrilotriacetic acid resin (27). Resin (0.3 ml bed volume) was incubated for 1 h with 1 ml of reconstituted extract, containing approximately 3 mg of hybrid PYP, under gentle agitation at room temperature. After binding, the resin was washed with 50 bed volumes 50 mM phosphate buffer (pH = 7.2). This was carried out by centrifuging each sample 12 times for 10 s in an Eppendorf centrifuge, followed by resuspension of the resin. The adsorbed proteins were eluted using 10 bed volumes 100 mM Na<sup>+</sup>-EDTA in 50 mM phosphate buffer (pH = 7.2). The eluates were washed with 25 mM phosphate buffer (pH = 7), concentrated to a volume of 1 ml (Centricon 10), and stored at –20 °C until further analysis. The purity of the samples was estimated by their purity index (pl) which has been defined as the ratio

<sup>2</sup> W. D. Hoff, I. H. M. Van Stokkum, J. Gural, M. E. Van Brederode, and K. J. Hellingwerf, submitted for publication.

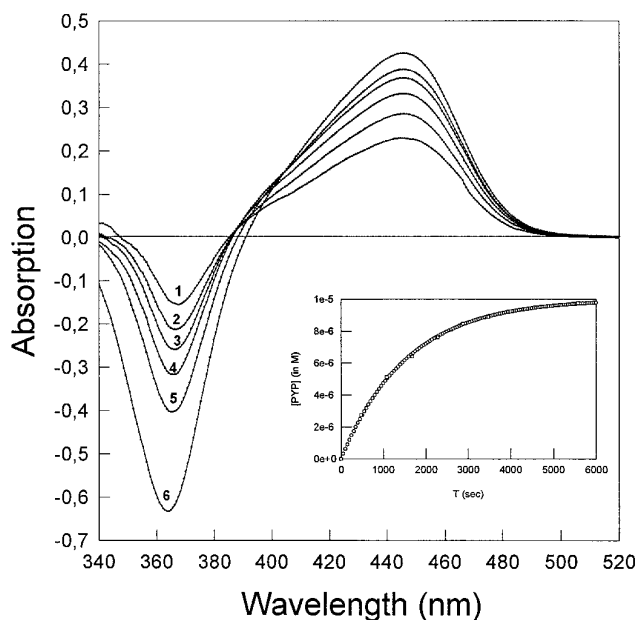


FIG. 1. Reconstitution of polyhistidine-tagged apoPYP (HAP), overproduced in *E. coli*. A time series of absorption difference spectra (after minus before mixing) of 4 mM 4-hydroxycinnamic anhydride with 10  $\mu$ M apoPYP at pH = 4.8 is shown. Spectra 1–6 correspond to 8, 18, 28, 38, 48, and 93 min, respectively. The inset shows a kinetic analysis of the reconstitution of HAP at pH = 4.8, as measured at 446 nm.

of its absorption at 280 and at 446 nm (1). The pI of the sample reconstituted with the native chromophore was 0.9 (see Fig. 3). Thus this sample is approximately 60% pure.

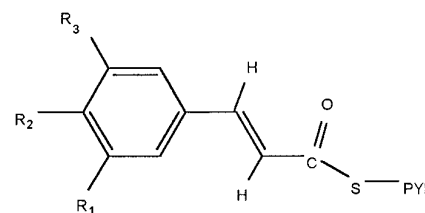
**Absorption and Fluorescence Spectroscopy**—To monitor the reconstitution reaction between HAP and the anhydride of pCA, quartz cuvettes with two compartments, separated by a transparent barrier, were used to measure sum spectra of the apoprotein and the anhydrides in solution, before and after mixing (Hellma Benelux B.V., Rijswijk, The Netherlands). Electronic absorption spectra were recorded in an SLM-Instruments Aminco DW2000 spectrophotometer with a resolution of approximately 1 nm. Fluorescence spectra were recorded in a SPEX-Fluorolog 2 spectrometer. Quantum yields were measured relative to quinine sulfate (dissolved in 1 M  $H_2SO_4$ ;  $\phi = 0.55$  (27)).

Photoactivity was analyzed by measuring the electronic absorption spectrum of the PYP hybrids in 50 mM sodium phosphate buffer, pH = 7 (or 50 mM glycine buffer, pH = 10, for hybrid IV), on a Hewlett-Packard 8453A diode array spectrometer, modified to allow illumination of the sample with a 200-watt high pressure mercury arc lamp, at right angles to the measuring beam, with a time resolution of approximately 0.1 s. All spectroscopy was carried out at room temperature.

**Miscellaneous**—HAP in complex samples was quantified with rocket immunoelectrophoresis (11). pK values of model compounds and hybrids were determined through manual fits of the data of spectrophotometric titrations, recorded with a Cary-3 UV/Vis spectrophotometer (Varian).

## RESULTS AND DISCUSSION

**Purification of Polyhistidine-tagged ApoPYP and Its Reconstitution with Various Chromophores**—HAP was isolated from the cytoplasmic fraction of recombinant *E. coli* by  $Ni^{2+}$ -affinity chromatography and reconstituted into holoPYP with the anhydride of pCA. This process was monitored by UV/Vis absorption spectroscopy after mixing 4 mM pCA anhydride with 10  $\mu$ M HAP (Fig. 1). At neutral pH values the rate of this transesterification reaction is beyond the time resolution of a conventional spectrophotometer, as was found for the reconstitution of apoPYP from *E. halophila* with this anhydride (24). However, lowering the pH to 4.8 slows down the rate of the reconstitution reaction (see Fig. 1), allowing its kinetic analysis. The increase in absorption at 446 nm indicates the formation of holoPYP, while the decrease in absorption at 365 nm is



No	Systematic name	R1	R2	R3
I	4-hydroxy cinnamic acid	H	OH	H
II	3,4-dihydroxy cinnamic acid	OH	OH	H
III	3-methoxy-4-hydroxy cinnamic acid	OCH <sub>3</sub>	OH	H
IV	3,5-dimethoxy-4-hydroxy cinnamic acid	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
V	4-Amino cinnamic acid	H	NH <sub>2</sub>	H
VI	4-Dimethyl amino cinnamic acid	H	N(CH <sub>3</sub> ) <sub>2</sub>	H
VII	4-Methoxy cinnamic acid	H	OCH <sub>3</sub>	H

FIG. 2. Structure of cinnamic acid and the substituents of the aromatic ring of the cinnamic acid derivatives that have been used for reconstitution of PYP hybrids.

caused by the decomposition of pCA anhydride. The reconstitution reaction is complicated by the fact that pCA anhydride not only reacts with HAP, to form holoPYP, but also with water, yielding pCA. This can account for the absence of an isosbestic point in the difference spectra, recorded during the reconstitution reaction (see Fig. 1). The rate of both reactions increases with increasing pH. For the reaction between the anhydride and HAP this can be explained by assuming that Cys-69, binding the chromophore, reacts with the anhydride in its ionized form, which is expected to be formed with a pK of approximately 10. The spontaneous hydrolysis of the anhydride in water presumably is base-catalyzed.

The progress of the reaction as monitored by the absorption changes at 446 nm can best be described as a second-order reaction, with a specific rate constant of the reaction between HAP and 4-hydroxycinnamic acid anhydride of  $0.16 \text{ M}^{-1}\text{s}^{-1}$  at pH = 4.8 (see Fig. 1, inset). More detailed investigations of this reaction requires stopped-flow analysis.

From the pI of the reconstituted protein, it can be concluded that the extent of reconstitution is more than 95% of the amount of HAP available (data not shown). The absorption spectra of native PYP, isolated from *E. halophila*, and of reconstituted HAP are indistinguishable, including the absorption maximum at 446 nm and a characteristic fine-structure at 318 nm. From this, it can be concluded that HAP is an excellent substrate for reconstitution. HAP is overproduced at a level of approximately 50 mg per liter of culture per OD unit at 660 nm (see Ref. 11) and therefore now available for biophysical and structural studies in large amounts. This also renders PYP accessible to studies by site-directed mutagenesis, which are in progress in our group, and it opens the way to reconstitute apoPYP with pCA analogues.

To assess the feasibility of this latter option, we synthesized the anhydrides of six pCA analogues (Fig. 2). In the first three analogues the aromatic ring of cinnamic acid carries one (II, III) or two (IV) additional substituents at the 3- and/or 5-position. In the remaining three analogues (V–VII) the phenolic hydroxy group is lacking, being substituted by an amino, a methoxy, and a dimethyl amino group, respectively. These chromophore analogues were chosen (i) to test the effect of perturbation of the chromophore binding pocket, by the added

substituents, on the absorption and fluorescence characteristics of PYP, and (ii) to investigate the proposed importance of the 4-OH group for spectral tuning of the chromophore. Like the reconstitution with the anhydride of pCA, the reaction of the other chromophore analogues with HAP at neutral pH also proceeds beyond the time resolution of the spectrophotometer used (data not shown). This indicates that even in IV, which carries two bulky methoxy substituents flanking the 4-hydroxy group, no compelling steric impediments exist to fitting the (*trans*)-cinnamic chromophore unit into its binding site in the apoprotein. This result shows that the reaction between apoPYP and anhydrides can be used as a general method to obtain hybrid PYPs.

In the study of photoactive proteins, the use of modified chromophores has proven to be a very powerful strategy. Besides the ones used in this study, several additional chromophore analogues may further help elucidating the mechanism of functioning of PYP. Some examples are as follows: (i) replacement of the vinyl bond by either a single or a triple bond, (ii) isotopically labeled derivatives, and (iii) chromophore analogues in which the vinyl bond is locked through a covalent bridge (compare Ref. 28).

**UV/Vis Absorption Characteristics of Hybrid PYPs**—To investigate the mechanism of spectral tuning in PYP, we aimed at the separation of the three factors involved in this process (see Introduction) in the seven hybrids and their quantification (in  $\text{cm}^{-1}$ ). In this report we will refer to the spectral shift induced by the formation of the thiol ester bond for the uncharged chromophore as  $\Delta\lambda_{\text{thiolest}}$ , to the shift caused by the deprotonation of the thiol ester-bound chromophore as  $\Delta\lambda_{\text{deprot}}$ , and to the shift caused by specific interactions between the chromophore and the protein as  $\Delta\lambda_{\text{protein}}$ . The sum of these three factors is referred to as  $\Delta\lambda_{\text{tot}}$ .

The  $\Delta\lambda_{\text{tot}}$  of the hybrids was determined by measuring the absorption spectra of these proteins, after their purification, and of the corresponding chromophore-derived model compounds (Fig. 3 and Table I). The values for  $\Delta\lambda_{\text{tot}}$  were calculated, using the absorption maxima observed for the hybrid PYPs and the free acids at pH = 7, with the exception of the PYP hybrid containing IV, which displayed maximal spectral tuning at pH values above 9 (see below).

The two classes of chromophores clearly lead to very different magnitudes of spectral tuning. For hybrids II to IV,  $\Delta\lambda_{\text{tot}}$  was approximately  $13,000 \text{ cm}^{-1}$ , essentially identical to the value observed in PYP, containing its native chromophore (Table II). However, for V to VII,  $\Delta\lambda_{\text{tot}}$  is reduced to approximately  $7,100 \text{ cm}^{-1}$ , confirming the importance of the 4-OH group in the process of the tuning of the chromophore absorption. In line with this, hybrids reconstituted from analogues lacking the 4-hydroxy group show pH-independent spectral characteristics in the pH range investigated (Table I). Nevertheless, also these latter hybrids (*i.e.* containing a class B chromophore), all display an absorption band of the aromatic amino acids of the apoprotein and a clearly discernible absorption band caused by the chromophore. The absorption maxima of this latter band range from 353 (V) to 436 nm (VI) in class B hybrids.

To quantify the contribution of  $\Delta\lambda_{\text{deprot}}$ , the absorption spectra of the hybrids, denatured by 4 M guanidinium HCl (GdnHCl) at pH = 7, where the chromophore is present in its neutral form, were compared with those recorded at pH = 11. At the latter pH, the chromophores containing an OH group are present in their anionic form (see below; note that denaturation of PYP at pH = 13 yielded almost identical absorption maxima) (Fig. 3). Comparison of the absorption maxima of the free acids and the GdnHCl-denatured hybrid PYPs at pH = 7 (and 3) allowed the calculation of  $\Delta\lambda_{\text{thiolest}}$ . For PYP and all hybrids,

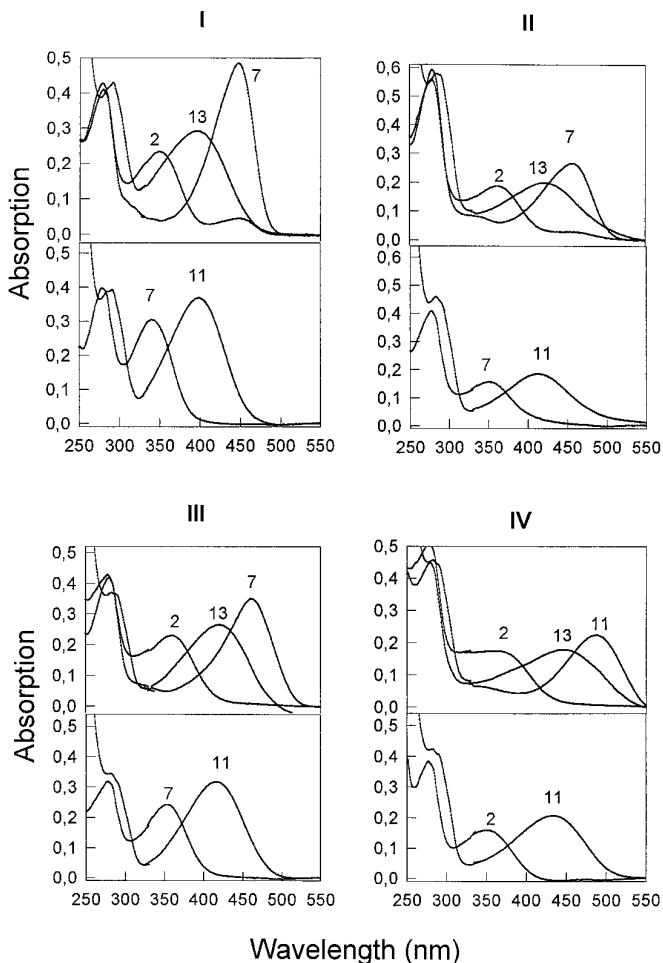


FIG. 3. Absorption spectra of PYP hybrids, reconstituted with modified chromophores I, II, III, or IV, at various pH values and in the presence and absence of 4 M guanidinium hydrochloride.

the  $\Delta\lambda_{\text{thiolest}}$  was approximately  $6,000 \text{ cm}^{-1}$ , whereas  $\Delta\lambda_{\text{deprot}}$  was around  $4,700 \text{ cm}^{-1}$  for I–IV and absent for V–VII (see Table I).

This analysis indicates (see Table II) that approximately 80% of  $\Delta\lambda_{\text{tot}}$  can be explained on the basis of two chemical modifications of the chromophore upon binding to the apoprotein, *i.e.* formation of the thiol ester bond and the deprotonation. This leaves approximately 20% of the shift to be caused by  $\Delta\lambda_{\text{protein}}$ . This effect is largest for the native chromophore (*i.e.*  $3,000 \text{ cm}^{-1}$ ; see Table I) but still significantly present in II–IV (approximately  $2,000 \text{ cm}^{-1}$ ). Apparently, the modifications in these latter three class A chromophores leave the specific protein-chromophore interactions, which give rise to  $\Delta\lambda_{\text{protein}}$ , largely intact. However, in the chromophores that lack the 4-OH group (V–VII), the effect of  $\Delta\lambda_{\text{protein}}$  is absent. Apparently, this phenolic hydroxy group does not only contribute to the spectral tuning by its deprotonation but is also essential for the interactions with the protein, leading to  $\Delta\lambda_{\text{protein}}$ .

Below pH = 3, PYP is converted to a blue-shifted state (1), which can be regarded as the acid-denatured state of PYP.<sup>2</sup> This state, called  $\text{pB}_{\text{dark}}$ , has an absorption spectrum, similar to the blue-shifted intermediate pB from the photocycle of PYP, but is slightly red-shifted with respect to the absorption expected (and observed) for thiol ester-linked pCA (19).<sup>2</sup> We have also examined the presence of a red-shift in this latter intermediate ( $\Delta\lambda_{\text{pB}_{\text{dark}}}$ ) in the hybrids studied here (Tables I and II) and found that for II–IV this parameter is approximately  $720 \text{ cm}^{-1}$ , slightly lower but similar to the value observed for native

TABLE I

Summary of spectroscopic data of cinnamic acid derivatives, their respective anhydrides, and the hybrid PYPs

The  $\lambda_{\max}$  of the free acid, the anhydride, and the PYP-linked chromophore, at various pH values and in the presence and absence of guanidinium hydrochloride, has been indicated. The asterisk indicates that this sample was measured at pH 11 rather than at pH 7. ND, not determined; GdnHCl, guanidinium hydrochloride.

Analogue	Hybrid PYP in buffer			Hybrid PYP in 4 M GdnHCl			Free acid pH = 7	Anhydride <sup>a</sup> pH = 7
	pH = 2	pH = 7	pH = 3	pH = 3	pH = 7	pH = 11		
	<i>nm</i>							
I	350	446	394	339	339	397	284	342
II	361	457	417	350	351	413	287	335
III	359	460	417	351	353	416	287	367
IV	357	488*	444	349	343	433	306	376
V	353	353	353	353	353	353	302	337
VI	436	436	436	436	436	436	324	439
VII	ND	355	ND	ND	ND	ND	272	355

<sup>a</sup> Determined from absorbance difference spectra.

TABLE II

Spectral characteristics of the various absorption bands of model compounds and hybrid PYPs ( $\lambda_{\max}$ ,  $\epsilon$ , and band width) and tuning parameters of hybrid PYPs

The extinction coefficients were calculated assuming 100% reconstitution and equivalent hybrid protein concentrations after normalization at 280 nm.  $\Delta\lambda$ , redshift caused by the thioester bond;  $\Delta\lambda_{\text{deprot.}}$ , redshift caused by the deprotonation of the chromophore,  $\Delta\lambda_{\text{protein}}$ , redshift caused by specific chromophore-protein interactions;  $\Delta\lambda$ , total redshift of coumaric acid and its derivatives upon binding to PYP;  $\Delta\lambda_{\text{pBdark}}$ , redshift of the chromophore in pB<sub>dark</sub> (i.e. difference in wavelength of maximal absorbance of PYP in the pB<sub>dark</sub> form, with and without guanidinium hydrochloride).

Hybrid	Absorption characteristics			Tuning parameters				
	$\lambda_{\max}$ <i>cm</i> <sup>-1</sup>	$\epsilon$ <i>M</i> <sup>-1</sup> <i>m</i> <sup>-1</sup>	fwhm <sup>a</sup> $\Delta$ <i>cm</i> <sup>-1</sup>	$\Delta\lambda$	$\Delta\lambda_{\text{deprot.}}$	$\Delta\lambda_{\text{protein}}$ $\Delta$ <i>cm</i> <sup>-1</sup>	$\Delta\lambda$	$\Delta\lambda_{\text{pBdark}}$
I	22,421	4.55 * 10 <sup>4</sup>	3,022	5,712	4,310	2,960	12,982	927
II	21,882	2 * 10 <sup>4</sup>	3,541	6,353	4,277	2,099	12,729	871
III	21,739	3 * 10 <sup>4</sup>	3,831	6,514	4,291	2,242	13,047	635
IV	20,491	2 * 10 <sup>4</sup>	3,508	3,225	6,060	2,031	11,316	642

<sup>a</sup> fwhm, full-width at half-maximum.

PYP (927 *cm*<sup>-1</sup>), whereas it is absent in V and VI. This indicates that (i) also in pB<sub>dark</sub> a protein-induced bathochromic shift occurs in I–IV and that (ii) like  $\Delta\lambda_{\text{protein}}$  this shift depends on the presence of the 4-OH group. Apparently, also in pB<sub>dark</sub>, the chromophore absorption is red-shifted by specific interactions with the protein. It is interesting to note that in hybrids II–IV, both  $\Delta\lambda_{\text{protein}}$  and  $\Delta\lambda_{\text{pBdark}}$  are reduced to approximately 75% of the value observed in native PYP.

In addition to  $\Delta\lambda_{\text{tot}}$ , we also examined the bandwidth and maximal extinction coefficient  $\epsilon$  of hybrids II–IV and compared it to native PYP (Table II). Assuming identical levels of reconstitution and purity for HAP reconstituted with I–IV,  $\epsilon$  is reduced from 45.5 to approximately 20–30 *mm*<sup>-1</sup>*cm*<sup>-1</sup> in II–IV (Table II). Thus, compared with PYP, the other hybrids have a lower  $\epsilon$ . However, since they also have a larger bandwidth at half height (see Table II), this probably only has a minor effect on the optical transition probability in hybrids II and III.

**Protein-induced Shifts in the pK for Chromophore Deprotonation**—As described above, deprotonation of the phenolic hydroxy group of I–IV leads to a significant bathochromic shift in the absorption spectrum. The pK for deprotonation of the thiol ester model compounds of I–IV is approximately 8.7 (Table III). Therefore, chromophore deprotonation at neutral pH can only occur if the protein environment lowers the pK for this process. For native PYP it has been reported that a  $\Delta\text{pK}$  occurs from 8.8 to 2.8.<sup>2</sup> It has been proposed that Glu-46 and Arg-52 are important factors in causing this  $\Delta\text{pK}$  (see below).

To further examine the effect of the chromophore modifications in II–IV, we measured the pK for chromophore deprotonation in these hybrids (Table III). It is clear that the pK in hybrids II and III is also strongly affected by the protein, be it somewhat less than in native PYP (shifting it to 3.8 and 3.5, respectively). However, in hybrid IV the pK of the chromophore is not at all affected by binding to the protein. This suggests that in this hybrid the phenolate anion at neutral pH is proto-

TABLE III

pK<sub>a</sub> values for the phenolic hydroxy group of the chromophore of hybrid PYPs and model thiol esters

For further details: see "Materials and Methods."

	Hybrid PYPs	Model thiol esters
I	2.8	8.8
II	3.8	8.4
III	3.5	8.7
IV	8.7	8.7

nated, possibly because steric hindrance by the two methoxy groups prevents Arg-52 from approaching the chromophore homologue sufficiently to allow it to charge-compensate a phenolate anion. It is interesting to note that hybrid IV is the only one in which the absorption maxima observed at pH = 13 and = 11 in GdnHCl are not indistinguishable. This supports the notion that the electrostatic interactions in this hybrid are significantly different from those in native PYP, apparently leading to an increased resistance of the protein toward alkaline denaturation. Since it has been proposed that this interaction is important for the stability of PYP, it will be of interest to investigate how the temperature stability of hybrids I–IV correlates with these pK values.

From Table II it can be seen that the  $\Delta\lambda_{\text{tot}}$  in hybrid IV (at high pH) is still quite significant and almost indistinguishable from the corresponding values of II and III, whereas its  $\Delta\text{pK}$  has been reduced to 0. Apparently, the  $\Delta\text{pK}$  and  $\Delta\lambda_{\text{tot}}$  are not directly correlated.

**Fluorescence of Hybrids I–IV**—To complement the data obtained by absorption spectroscopy described above, we further probed the transition between pG and its excited state pG\* by studying the fluorescence characteristics of hybrids I–IV. We focused on two parameters, the fluorescence quantum yield ( $\Phi_{\text{fl}}$ ) and the Stokes shift, as calculated from the difference

TABLE IV  
Fluorescence characteristics of PYP, three of its hybrids, and their corresponding model thiol esters

$\Phi$ , quantum yield of fluorescence, St., Stokes shift; difference in wavelength of maxima of fluorescence excitation and emission.

	Hybrid PYPs								Butyl thioesters							
	pH = 2				pH = 7 <sup>a</sup>				pH = 7				pH = 11			
	$\lambda_{\text{ex}}$	$\lambda_{\text{em}}$	St.	$\Phi$	$\lambda_{\text{ex}}$	$\lambda_{\text{em}}$	St.	$\Phi$	$\lambda_{\text{ex}}$	$\lambda_{\text{em}}$	St.	$\Phi$	$\lambda_{\text{ex}}$	$\lambda_{\text{em}}$	St.	$\Phi$
	nm	nm	cm <sup>-1</sup>	%	nm	nm	cm <sup>-1</sup>	%	nm	nm	cm <sup>-1</sup>	%	nm	nm	cm <sup>-1</sup>	%
I	360	439	4999	0.3	446	494	2173	0.2	340	460	7673	0.01	379	496	6224	0.1
II	360	458	5944	0.4	450	527	2761	1.9	349	489	8203	0.03	375	580	6667	0.4
III	358	471	6702	0.9	461	527	2717	5.4	344	490	8662	0.3	403	521	5620	0.2
IV	372	482	6135	0.7	485	548	2371	2.7	345	505	9184	0.1	425	544	5092	0.1

<sup>a</sup> Readings were taken at pH = 11 for hybrid IV.

between the maxima of chromophore fluorescence emission and excitation (Table IV). Comparison with Table I shows that a strict correlation exists between the position of the absorption maxima and the maxima of fluorescence excitation. The only exception is hybrid IV at low pH. This is related to the difficulty of accurate determination of the absorption maximum at this pH, which may be affected by light scattering. Also, increased absorption of the apoprotein, due to tyrosine deprotonation, may complicate this measurement. The quantum yield determined for I, *i.e.*  $2 \times 10^{-3}$ , agrees reasonably well with previously determined values (16, 17).

Examination of fluorescence emission and excitation spectra (as an example, the spectra of IV are displayed in Fig. 4, for both the pG and pB<sub>dark</sub> state) shows that the Stokes shift in hybrids II–IV is approximately 2600 cm<sup>-1</sup>, similar to the value of 2179 cm<sup>-1</sup> observed in I (Table IV). However, their  $\Phi_{\text{fl}}$  is significantly increased, up to  $5 \times 10^{-2}$  for II. To examine whether the origin of this increased  $\Phi_{\text{fl}}$  is intrinsic to the chromophore analogues used, or to protein-chromophore interactions, we also determined the fluorescence spectra and  $\Phi_{\text{fl}}$  for the thiol ester model compounds I–IV. At pH 11, the Stokes shift of these compounds (in their deprotonated form) is approximately 5900 cm<sup>-1</sup>, significantly larger than in the PYP hybrids. This may be caused by a significantly impaired flexibility of the chromophore in the apoprotein environment. However, the  $\Phi_{\text{fl}}$  of model compounds II–IV was approximately  $2 \times 10^{-3}$ , comparable with the low value observed in native pG. Apparently, interaction of chromophores II–IV with the apoprotein increases their  $\Phi_{\text{fl}}$  5–25-fold (Table IV). In parallel to the increased quantum yields, it was observed that also the fluorescence lifetime of II and III is strongly increased with respect to I. The multi-exponential nature of these lifetimes, however, complicates their straightforward analysis.

To further probe the protein-chromophore interactions in pB<sub>dark</sub>, alluded to above, we also determined the fluorescence characteristics of hybrids I–IV at pH 2 and compared these results with the corresponding protonated model compounds (at pH 7). This analysis shows that I–IV display fluorescence not only after excitation of their pG form but also of the pB<sub>dark</sub> state (Table IV). As for the pG state, it was found that the Stokes shifts of the chromophores in pB<sub>dark</sub> (approximately 5900 cm<sup>-1</sup>) are significantly smaller than those observed for the protonated model compounds, measured at pH 7 (approximately 8400 cm<sup>-1</sup>). For hybrid I the  $\Phi_{\text{fl}}$  is higher in pB<sub>dark</sub> than in pG, whereas for hybrids II–IV the opposite is observed; the fluorescence in II–IV is much less affected by the protein in its pB<sub>dark</sub> state than in its pG state. This suggests that in hybrids II–IV the protein-chromophore interactions in pB<sub>dark</sub> are less perturbed by the additional ring substituents than those in pG.

**Spectral Tuning of PYP in Its pG State**—To find possible mechanisms involved in  $\Delta\lambda_{\text{protein}}$ , it is useful to compare spectral tuning in PYP and in the rhodopsins, since its physical basis has been studied extensively in the latter proteins. Two

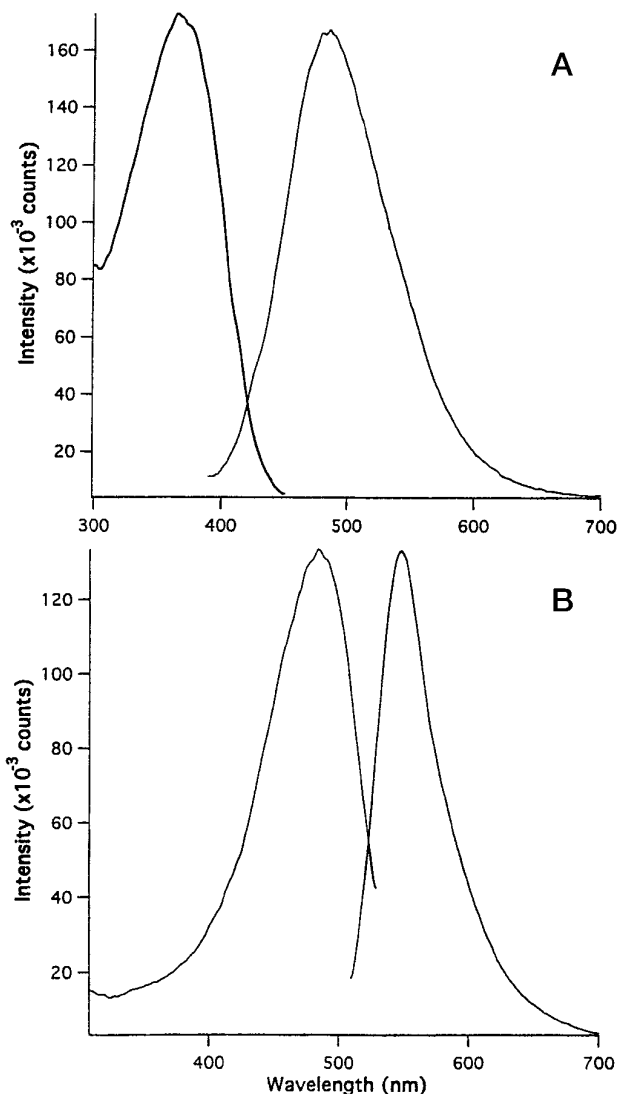


FIG. 4. Fluorescence excitation and emission spectra of hybrid IV in the pB<sub>dark</sub> (A) and pG (B) form (*i.e.* at pH = 2 and pH = 7), respectively.

chemical modifications occur upon binding of retinal to opsins, both of which are analogous to those occurring in PYP: (i) the formation of a Schiff base between a Lys residue and retinal, and (ii) the protonation of this Schiff base, aided by a strong protein-induced increase in its pK. These two processes shift  $\lambda_{\text{max}}$  from 370 to 440 nm but are usually not considered to be part of the opsin shift. Specific protein-chromophore interactions lead to a further bathochromic shift from 440 to 568 nm (called opsin shift (29)) for the light-adapted state of bacteriorhodopsin. This shift of 5,100 cm<sup>-1</sup> corresponds to the  $\Delta\lambda_{\text{protein}}$

of approximately  $3,000\text{ cm}^{-1}$  in hybrid I. Although the opsin shift in bacteriorhodopsin is still only partially understood, two contributing factors involved are generally accepted (30–32). First, the protonated Schiff base is weakly stabilized (the so-called external point charge model (29)) by a complex counterion, involving the charges of Asp-85, Asp-212, and Arg-82. Second, the protein forces the retinal ring into the 6-*s-trans* conformation, thus leading to a co-planarization of the  $\beta$ -ionone ring and the polyene chain of retinal, thereby extending the conjugated system (30). Recently, a third factor has been identified, the stabilization of the excited state of retinal by polar or polarizable side chains in the retinal binding pocket (31).

Since the negative charge on the deprotonated PYP chromophore(s) is buried within the protein (22), the presence of a counterion for this charge at a relatively large distance can be proposed to explain the  $\Delta\lambda_{\text{protein}}$ . Arg-52 is a likely candidate to contribute to this function. Our analysis indicates that the 4-OH group is essential for  $\Delta\lambda_{\text{protein}}$ . Second, the counterpart of the 6-*s-trans* conformation in retinal bound to native bacteriorhodopsin is formed by the conformation around the C–C single bond of the  $-\text{C}=\text{C}-\text{C}(-\text{S})=\text{O}$  fragment. Both the 1.4-Å x-ray data (22) and resonance Raman data (21) indicate that this single bond is in the *s-cis* conformation. Since it seems reasonable to expect that for model compounds in solution this bond is in the *s-trans* conformation, this factor may affect the absorption spectrum of the chromophore. However, at this point it is difficult to quantitate this effect.

A third possibility that we have considered is protein-induced torsional strain on the *trans* C=C bond in the chromophore. Such strain would destabilize the ground state and would stabilize the excited state, since this latter state is expected to have an energy minimum at a double bond angle of  $135^\circ$ . Initial results of essential dynamics calculations<sup>3</sup> suggest that such strain indeed is present in the pG state. This proposal implies that the degree of torsional strain imposed on the chromophore is decreased by the additional ring substituents in II–IV. Four residues in the pCA binding site of PYP are of primary importance: (i) Arg-52, which has already been discussed, and (ii) the hydrogen bonding network between the phenolate anion and Glu-46, Tyr-42, and Thr-50. These interactions can be expected to reduce the mobility of the chromophore, which may be a prerequisite for the application of torsional stress on the C=C bond. In this way, this proposal can explain the absence of a  $\Delta\lambda_{\text{protein}}$  in V–VII.

Binding of a chromophore to apoPYP leads to a decrease in the magnitude of its Stokes shift by more than a factor of 2. It is interesting to note that PYP displays both the strongest decrease in Stokes shift and the largest  $\Delta\lambda_{\text{protein}}$ . This decrease in Stokes shift is not easily compatible with the notion of torsional stress; however, torsional stress would tend to decrease the difference between the ground state and the first excited state.

**Photoactivity of the Hybrid PYPs**—The typical photobleaching and dark recovery of native PYP (and of I) is readily observable in hybrids II and III but not in IV. The rate of the dark recovery reaction, however, was slightly (in III) and even strongly (in II) decreased. For the other hybrids (in particular IV) transient kinetic analyses will have to be applied, to determine whether or not a short-lived intermediate exists.

**Comparison of PYP Hybrids with Green Fluorescent Protein (GFP)**—An interesting comparison can be made between the highly fluorescent hybrids (III and IV; see Table IV) and GFP, based on their mutual similarity and differences. At neutral

pH, for instance, IV and GFP display two absorption bands (for the latter at approximately 395 and 475 nm). However, whereas in IV these two forms are due to the titration of the phenolate anion, in GFP they appear to be due to isomerization (33), although some interconversion of the absorption bands at 395 and 475 nm occurs, in the pH range in which GFP can be titrated, without interference by rapid denaturation (34). Surprisingly, fluorescence emission of GFP from both its absorption bands (data not shown) gives rise only to a single emission band (at 508 nm), with a only minor red shoulder. This is in striking contrast to PYP, in which both protonation states of the apoprotein-bound chromophore give rise to a fluorescent protein, with slightly higher fluorescence quantum yield at neutral pH but with clearly separated emission bands for the two states. This can be concluded from comparisons of the fluorescence of pG and pB<sub>dark</sub>. It is not known what the effect is of isomerization of the chromophore of PYP on the fluorescence emission. The transient character of pB complicates its fluorescence characterization. In view of the intriguing fluorescence characteristics of GFP, it will be of great interest to characterize the isomerization state of the chromophore of GFP with IR spectroscopy, for example, as a function of irradiation dose and pH. The fluorescence characteristics of GFP may be due to emission from a deprotonated chromophore.

The increase in  $\Phi_{\text{fl}}$  of hybrids II and III and their photochemical activity provides new approaches for future work. First, time-resolved fluorescence spectroscopy can be employed as a new and powerful tool to investigate the primary photochemistry of PYP. Second, the photochemical properties of PYP can be manipulated by developing different chromophore analogues, strongly enhancing the scope for application of PYP hybrids in practical applications, for example, like in optical data storage. In addition, the biophysical basis for the distribution of the quantum yield of each of the three parallel reaction pathways, available to the excited state of pG (*i.e.* photochemistry, fluorescence, and radiationless decay (see Ref. 15), becomes accessible to experiments. This may lead to better insight in the way nature tunes chromophores, to function optimally as photosensory light absorber (PYP) or bioluminescent light emitter (GFP).

**Concluding Remarks**—In this study we present a general method for the reconstitution of PYP with various chromophores, based on the ability to reconstitute apoPYP with 4-hydroxycinnamic acid anhydride (24) and the heterologous overexpression of HAP (11). The N-terminal histidine tag of the overproduced protein allows for its efficient purification and if necessary can be specifically removed via enterokinase digestion of the isolated protein. This makes PYP available in large amounts and amenable to site-directed mutagenesis and labeling with NMR-visible isotopes (*e.g.* with  $^{15}\text{N}$  and  $^{13}\text{C}$ ).

Here we have characterized seven PYP hybrids and have found that the protein dramatically changes chemical and physical properties of the various chromophores. Hybrids II–IV, which have additional ring substituents, still show a strong chromophore-protein interaction, as is apparent from the change in (i) wavelength of maximal absorption of the chromophore, (ii) pK for deprotonation, (iii) fluorescence quantum yield, and (iv) Stokes shift. For all hybrids studied here, a quantitative description of the absorption changes between the free acid and the chromophore bound to the native protein was obtained, in which the formation of the thiol ester accounts for a red-shift of  $\sim 6000\text{ cm}^{-1}$  ( $\Delta\lambda_{\text{thiolest}}$ ), chromophore deprotonation accounts for  $\sim 4700\text{ cm}^{-1}$  ( $\Delta\lambda_{\text{deprot}}$ ), and further protein-chromophore interactions for  $\sim 2300\text{ cm}^{-1}$  ( $\Delta\lambda_{\text{protein}}$ ). Our data are consistent with the presence of torsional stress on the vinyl double bond of the chromophore, but also the conformation of

<sup>3</sup> D. M. F. Van Aalten, W. D. Hoff, J. B. C. Findlay, and K. J. Hellingwerf, submitted for publication.



the single bond in the  $-C=C-C(-S-)=O$  fragment may be of importance for  $\Delta\lambda_{\text{protein}}$ .

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