

Structure of the photoactive yellow protein reconstituted with caffeic acid at 1.16 Å resolution

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A structural study is described of the photoactive yellow protein (PYP) reconstituted with the chromophore derivative 3,4-dihydroxycinnamic acid. The crystal structure of PYP reconstituted with this chromophore at 1.16 Å resolution is reported in space group $P6_5$. This is the first high-resolution structure of a photoreceptor containing a modified chromophore. The introduction of an extra hydroxyl group in the native chromophore (*i.e.* *p*-coumaric acid) appears to perturb the structure of the hybrid yellow protein only slightly. The chromophore is bound by the protein in two different conformations, separated by a rotation of 180° of the catechol ring. In combination with available spectroscopic data, it is concluded that the caffeic acid chromophore binds to the protein in a strained conformation, which leads to a faster ejection from the chromophore-binding pocket upon pB formation.

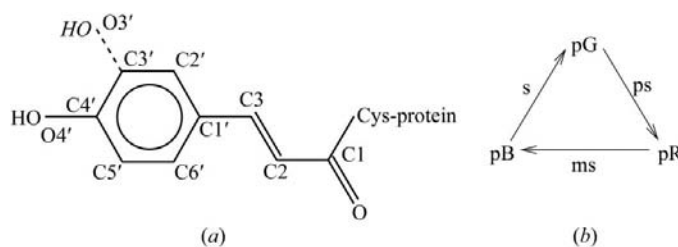
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1. Introduction

The photoactive yellow protein (PYP) is a water-soluble photoreceptor protein first isolated from *Ectothiorhodospira halophila* (Meyer, 1985; Meyer *et al.*, 1987). The protein appears to be involved in a phototactic response of this bacterium to blue light (Sprenger *et al.*, 1993). It binds a chromophore, *p*-coumaric acid, which gives the protein its characteristic yellow color, *via* a thiol-ester linkage to its unique cysteine (Baca *et al.*, 1994; Hoff, Düx *et al.*, 1994) (Fig. 1). PYP can be activated with light to go through a photocycle consisting of three main intermediates (Fig. 1). The ground state (pG) has a UV/Vis absorbance maximum at 446 nm. After absorption of a blue photon, the protein returns from the primary excited state into a transient ground state on

**Figure 1**

Photocycle and PYP structure. (a) Chemical structure of the native PYP chromophore and 3,4-dihydroxycinnamic acid (dotted line and hydroxyl group in italics). 'Cys' denotes Cys69 of the protein, to which the chromophore is bound. (b) The three-state photocycle is depicted with the names and conversion rates of the various intermediates.

the picosecond timescale (Baltuska *et al.*, 1997; Chaugenet *et al.*, 1998; Chosrowjan *et al.*, 1997; Meyer *et al.*, 1991) and then into a second strongly red-shifted intermediate (Ujj *et al.*, 1998). A more moderately red-shifted intermediate (pR), absorbing maximally at 465 nm, is formed on the nanosecond timescale (Hoff, van Stokkum *et al.*, 1994) (Fig. 1). The pR intermediate spontaneously converts into a blue-shifted intermediate (pB; absorbing maximally at 355 nm) on the sub-millisecond timescale (Hoff, van Stokkum *et al.*, 1994; Meyer *et al.*, 1987) (Fig. 1). The pB intermediate subsequently relaxes back to pG on a sub-second timescale (Hoff *et al.*, 1992; Hoff, van Stokkum *et al.*, 1994; Meyer *et al.*, 1987, 1991) or more rapidly, in a light-dependent reaction (Miller *et al.*, 1998). These transitions are accompanied by a complex double-bond isomerization of the *p*-coumaryl chromophore of PYP (Genick, Borgstahl *et al.*, 1997; Genick *et al.*, 1998; Kort *et al.*, 1996; Perman *et al.*, 1998; Xie *et al.*, 1996).

The structure of the ground state of PYP (*i.e.* pG) has been solved previously to 1.4 Å and subsequently to 0.82 Å resolution (Borgstahl *et al.*, 1995; Genick *et al.*, 1998), revealing the intimate contacts that the chromophore makes with specific residues lining a pocket inside the protein. Subsequent studies using Laue diffraction and cryo-crystallography have given hints as to what happens during and after chromophore isomerization (Genick, Borgstahl *et al.*, 1997; Genick *et al.*, 1998; Perman *et al.*, 1998). First, light absorption induces a rotation of the carbonyl group of the chromophore around its long axis, which is suggested to occur in a concerted motion with *trans* to *cis* isomerization of the vinyl bond of the chromophore. At low temperature (<150 K), this isomerization appears to be blocked about halfway (Genick *et al.*, 1998). In the later stages of the photocycle, the phenolic hydroxyl group of the chromophore is exposed to solvent, probably owing to a rotation around the C—S single bond of the chromophore (Genick, Borgstahl *et al.*, 1997). These studies have shown that the conformational changes in PYP are mainly limited to the chromophore and its surroundings. Interestingly, for PYP in aqueous solution formation of the blue-shifted intermediate pB is paralleled by a major conformational transition that carries typical features of a (partial) protein unfolding event, as reported by UV/Vis and FTIR spectroscopy, mass spectrometry and ¹H-NMR analyses (Hoff *et al.*, 1999; Rubenstenn *et al.*, 1998; van Brederode *et al.*, 1996; Xie *et al.*, 2001). These observations are more in line with a signaling function for PYP, which should ultimately result in a negative phototactic response of the bacterium. Molecular-dynamics simulations have identified regions in the PYP protein quite separated from the chromophore that may fluctuate in concert with it (van Aalten *et al.*, 1998, 2000).

In addition to crystallographic studies, attempts have been made to gain further insight into the structural and dynamic photocycle events in PYP by engineering the protein through site-directed mutagenesis (Devanathan *et al.*, 1998; Genick, Devanathan *et al.*, 1997; van Aalten *et al.*, 2002) or its chromophore through reconstitution studies (Cordfunke *et al.*, 1998; Devanathan *et al.*, 1997; Kroon *et al.*, 1996). Chromophore engineering in particular has attractive features, as a

Table 1

Details of data collection and refinement.

Values in parentheses are for the highest resolution shell. All measured data in the range 8.0–1.16 Å were included in the refinement. *R* factors were calculated with no σ -cutoff on the data unless stated otherwise. Each step represents several rounds of model building, during which occasional disordered stretches of residues were omitted (see §2).

Space group	<i>P6₅</i>
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 40.58, <i>c</i> = 117.80
Resolution range (Å)	15–1.16 (1.20–1.16)
No. of observed reflections	333681
No. of unique reflections	36609 (3352)
Redundancy	9.1
$\langle I/\sigma(I) \rangle$	15.7 (2.7)
Completeness (%)	96.7 (89.4)
<i>R_{merge}</i>	0.040 (0.546)
<i>R</i> , <i>R_{free}</i> (no σ cutoff)	0.162, 0.203
<i>R</i> , <i>R_{free}</i> (4 σ cutoff)	0.147, 0.188
No. of atoms	971; 135 waters
No. of <i>SHELX</i> parameters	9856
R.m.s.d. from bond ideality (Å)	0.009
R.m.s.d. from angle ideality (°)	1.12
R.m.s.d. on <i>B</i> factors of bonded atoms (Å ²)	3.7
Average isotropic <i>B</i> (Å ²)	20.24

wide range of small substitutions can be designed with the aim of studying their effects on PYP dynamics while not disturbing the structure of the protein. One promising chromophore derivative in this respect is 3,4-dihydroxycinnamic acid (caffeic acid). Static and transient UV/Vis spectroscopy of this PYP hybrid suggests that its structure is not disturbed, yet its photocycle kinetics are significantly affected (Devanathan *et al.*, 1997; Kroon *et al.*, 1996). Hence, caffeic acid is a suitably engineered chromophore to study the effects of the chromophore on PYP structure and dynamics, with the aim of further delineating the functional transitions in the photocycle. Here, we describe the structure of PYP reconstituted with 3,4-dihydroxycinnamic acid refined to 1.16 Å resolution. We compare this structure with the available spectroscopic data and propose that although the structure is not significantly affected, the 3,4-dihydroxy cinnamic acid chromophore does introduce strain in the PYP structure.

2. Materials and methods

2.1. Purification, reconstitution and crystallization

Apo-PYP was purified as described previously (Kroon *et al.*, 1996). Briefly, apo-PYP was expressed in *Escherichia coli* M15/pHis. Lysed cells were dialysed against 50 mM phosphate buffer pH 7. The resulting apo-PYP-containing solution was used for reconstitution with a chromophore (Kroon *et al.*, 1996). *p*-Coumaric acid (4-hydroxycinnamic acid) (Fig. 1a) was used to yield native PYP (WTPYP). Reconstitution with caffeic acid (3,4-dihydroxycinnamic acid) leads to the formation of the hybrid CAFPYP. After reconstitution, further purification was achieved with Ni²⁺-affinity chromatography using the N-terminal polyhistidine tag. This tag was subsequently removed by enterokinase treatment followed by ion-exchange chromatography.

CAFPYP did not crystallize under the conditions reported previously for crystallization of native PYP (Borgstahl *et al.*, 1995; McRee *et al.*, 1986, 1989), but crystals were obtained using a recently described protocol (van Aalten *et al.*, 2000).

Freeze-dried CAFYP was dissolved in 50 mM phosphate buffer pH 7, passed over a gel-filtration column and concentrated to 25 mg ml⁻¹. Crystals were obtained by vapor-diffusion experiments in which 1 µl of protein solution was mixed with an equal volume of well solution containing PEG 2K monomethyl-ether and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5. Crystals appeared within 3 d and grew as hexagonal rods.

2.2. Data collection, structure solution and refinement

Diffraction data were collected on beamline X26C at the National Synchrotron Light Source, Brookhaven National Laboratory at a wavelength of 1.08 Å using a 300 mm MAR Research image-plate detector. Data were collected at 100 K by flash-freezing crystals in an Oxford Cryosystems Cryostream. Diffraction data were integrated and scaled with the *HKL* package (Otwinowski & Minor, 1997). The crystal belonged to the hexagonal space group *P*6₅, with unit-cell parameters $a = b = 40.58$, $c = 117.80$ Å and one molecule in the asymmetric unit. Further details of the data-collection statistics are presented in Table 1.

The CAFYP structure was solved by molecular replacement using *AMoRe* (Navaza, 1994) with PYP previously crystallized in space group *P*6₃ (PDB entry 2phy; Borgstahl *et al.*, 1995) as a search model, excluding the chromophore. Using 8.0–4.0 Å data a single solution was obtained, which after rigid-body refinement in *AMoRe* gave an *R* factor of 0.341 (correlation coefficient 0.70). Initial refinement was performed with *CNS* (Brünger *et al.*, 1998) with iterative model building using *O* (Jones *et al.*, 1991). After a few cycles of model building and placement of some well defined water molecules (4σ peaks in an $F_o - F_c$ electron-density map and at least one hydrogen bond to the protein), the caffeic acid chromophore could be clearly seen in the $F_o - F_c$ maps (Fig. 2*a*) and was built into the model. Further rounds of model building and refinement allowed placement of additional water molecules and assignment of some alternate side-chain conformations. Subsequent refinement was performed with *SHELX97* (Sheldrick & Schneider, 1997). After introduction of anisotropic *B* factors, further alternate

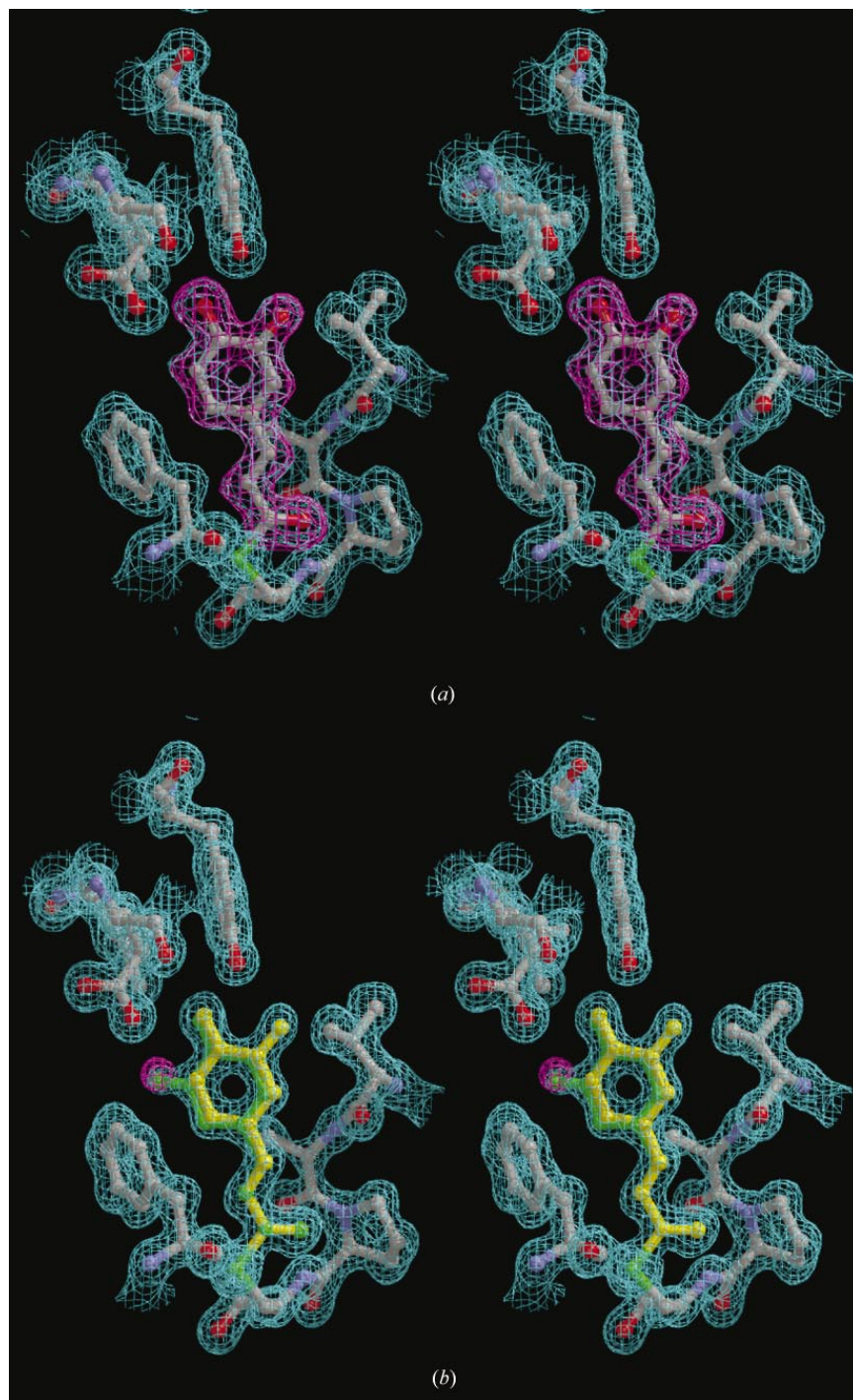


Figure 2

Stereoviews of the electron density around the chromophore. $F_o - F_c$ maps just before including the chromophore are shown in magenta. $2F_o - F_c$ maps after building in the chromophore and subsequent refinement are shown in cyan. (a) CAFYP (15–1.39 Å *CNS* maps), (b) CAFYP (15–1.16 Å *SHELX* maps). σ levels were 1.0 and 2.5 for the $2F_o - F_c$ and $F_o - F_c$ *CNS* maps, respectively, in (a) and 1.75 and 2.75 for the $2F_o - F_c$ and $F_o - F_c$ *SHELX* maps, respectively, in (b). In (b) the two alternate conformations of the chromophore are colored yellow and green. See text for details.

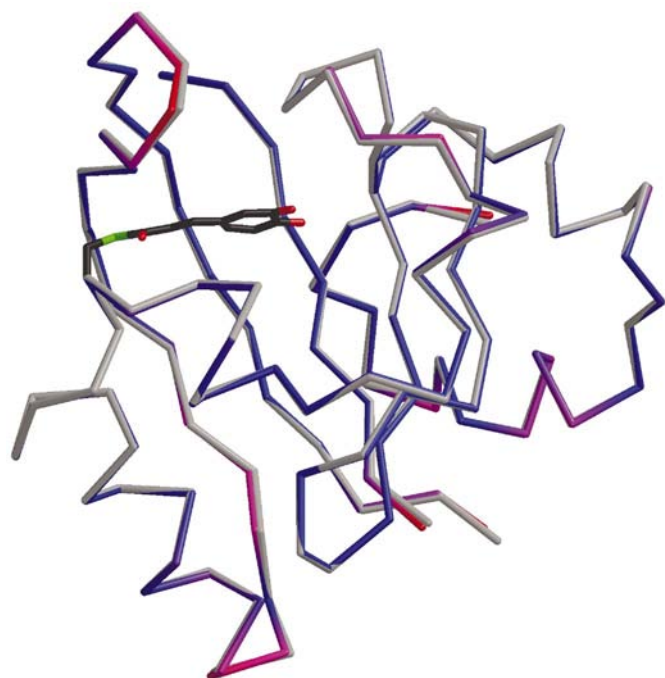


Figure 3
Comparison of C^α traces. WTPYP (gray) is compared with CAFYYP (colored by shift in atomic position). Structures were superimposed using C^α atoms. The atomic shifts are colored from blue (0.0 Å) to red (>0.25 Å). The caffeic acid chromophore is also shown as a stick model with black C atoms.

conformations and water molecules could be modeled. In addition, a strong peak appeared in the CAFYYP $F_o - F_c$ map close to the chromophore, indicating a possible alternate conformation (Fig. 2*b*). This alternate conformation was built into the model and subsequent refinement produced occupancies of 0.67 and 0.33 for the two possible orientations. Inspection of the resulting $2F_o - F_c$ map further confirmed the presence of this alternate conformation (Fig. 2*b*). In the last stages of the refinement, H atoms were added using the riding H atoms model (Sheldrick & Schneider, 1997). From the early

Table 2
Chromophore–protein interactions.

All distances between chromophore ($O3'$ and $O4'$) and protein atoms <3.5 Å are shown, together with the shifts upon chromophore exchange. DISP is the displacement of the atom between the WTPYP and CAFYYP structures. CAFYYP_A and CAFYYP_B represent the most and least occupied alternate positions of the chromophore, respectively.

	WTPYP $O4'$	CAFYYP $O4'$	CAFYYP _A $O3'$	CAFYYP _B $O3'$	DISP
Tyr42 O^η	3.24	2.41	2.81	—	0.10
Glu46 $O^{\epsilon 2}$	2.51	2.63	—	3.01	0.10
Thr50 $C^{\gamma 2}$	3.49	3.38	—	—	0.09
Arg52 $N^{\epsilon 2}$	—	—	3.30	—	0.27
Phe62 C^ζ	—	—	—	3.20	0.07
Val66 $C^{\gamma 1}$	—	—	3.36	—	0.11
Phe96 $C^{\epsilon 2}$	—	—	—	3.03	0.08

stages of the refinement it was apparent that residues 1–3 and residues 114–116 were disordered. During the refinement the maps showed some evidence of several possible conformations for these regions. Attempts were made to build these regions, but in the end we decided we could not determine their conformations with a reasonable degree of confidence and they were left out of the model. Both regions have high B factors in the first PYP structure (Borgstahl *et al.*, 1995). In addition, these regions are underdetermined in the NMR structure of PYP (Düx *et al.*, 1998).

2.3. Figures

Figures were prepared using *MOLSCRIPT* (Kraulis, 1991), *BOBSCRIPT* (Esnouf, 1997), *Raster3D* (Bacon & Anderson, 1988; Merritt & Murphy, 1994) and *GRASP* (Nicholls *et al.*, 1991).

3. Results and discussion

The structure of CAFYYP in space group $P6_5$ was solved by molecular replacement and refined to atomic resolution with *SHELX97* (Sheldrick & Schneider, 1997) (Fig. 2). The

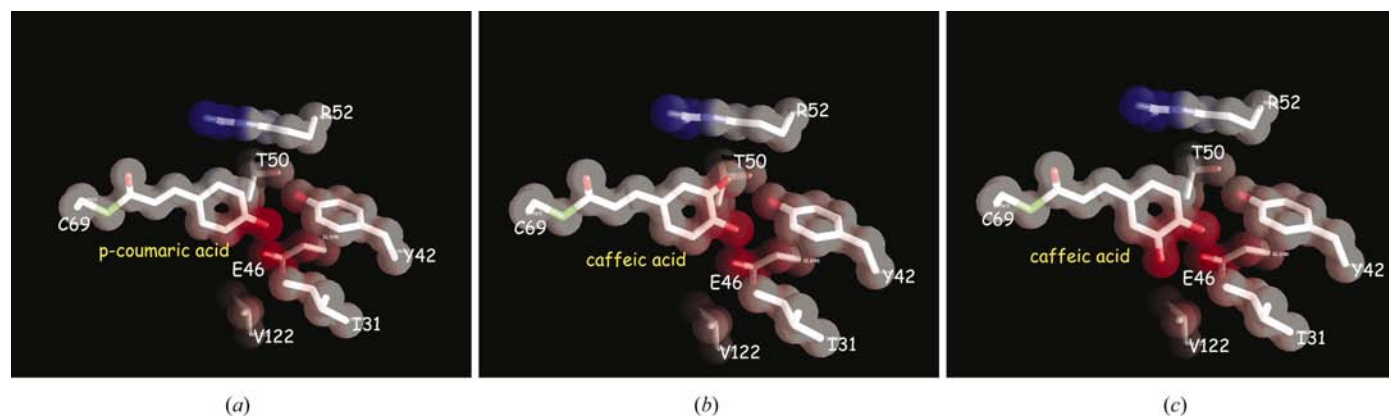


Figure 4
Electrostatic interactions in the chromophore-binding pocket. WTPYP is shown in (a) and the two alternate conformations of the caffeic acid chromophore in CAFYYP are shown in (b) (occupancy 0.67) and (c) (occupancy 0.33). A molecular surface was calculated with *GRASP* (Nicholls *et al.*, 1991) and colored by electrostatic potential. For WTPYP, the phenolic chromophore O atom was assigned a charge of -0.5 and the Glu46 O atoms charges of -0.25 . For CAFYYP, charges were -0.25 for the Glu46 and chromophore ring O atoms.

isomorphous structures of PYP crystallized in space group $P6_5$ (WTPYP; van Aalten *et al.*, 2000) and CAFYP are compared in Fig. 3. Superposition of these structures on their C^α atoms yields an r.m.s.d. of 0.19 Å [estimated mean coordinate error as determined from a Luzzati plot (Luzzati, 1952) is 0.1 Å], indicating that the overall structure of the PYP protein is only slightly perturbed by reconstitution with caffeic acid. Hydrogen-bonding distances and positional shifts between WTPYP and CAFYP are listed in Table 2. The most consistent interpretation of the electron density in the region covering the chromophore in CAFYP assumes that the 3,4-dihydroxycinnamate moiety is present as a mixture of two alternate conformations (see Fig. 2). In both conformations, the phenolic oxygen (O4') makes hydrogen bonds identical to those in WTPYP, namely with Tyr42 and Glu46 (Fig. 4). In the highest occupancy (67%) conformation of the two, the extra (O3') hydroxyl group is pointing outwards, in the direction of the protein surface, and hydrogen bonds to Arg52 N^ε, Thr50 O^{γ1} and Tyr42 O^γ. In the other conformation, which has an occupancy of 33%, the additional hydroxyl group is pointing inwards, to the hydrophobic bottom of the chromophore-binding cavity, and only makes a hydrogen bond to Glu46 O^{ε2} (Fig. 4). The shifts in position, listed in Table 2, show that the side chains interacting with the chromophore barely shift their position upon exchange of the chromophore. Most shifts are smaller than the mean coordinate error. The largest shift is for Arg52 N^{ε2}, the solvent-exposed side chain forming the lid on the chromophore-binding pocket. Thus, it seems that the protein is able to accommodate the non-physiological chromophore 3,4-dihydroxycinnamic acid in the pG state, although a few close contacts do exist with non-hydrogen-bonding atoms (Table 2).

A prerequisite for a detailed understanding of the processes involved in the activation of biological photoreceptors is the availability of high-resolution structural information. For bacteriorhodopsin, the detailed description of its proton-pumping mechanism has been greatly accelerated by the availability of an early low-resolution electron cryomicroscopy structure (Grogorieff *et al.*, 1996; Henderson *et al.*, 1990) and more recent X-ray diffraction structures (Luecke *et al.*, 1999; Pebay-Peyroula *et al.*, 1997). In addition, interactions of the protein with its chromophore, retinal, have been studied by chemical and genetic engineering, which has led to an increasingly detailed description of the structural events in the photocycle.

The photoactive yellow protein has a photocycle similar to that of the bacterial rhodopsins, yet is a water-soluble protein and binds a completely different chromophore. The availability of large quantities of PYP protein and its relatively straightforward crystallization make PYP a suitable system for detailed structural studies of protein–chromophore interactions and the structural transitions that PYP goes through while progressing through its photocycle. Here, we have described the first structure of a photoreceptor reconstituted with a non-physiological chromophore. The derivative used, 3,4-dihydroxycinnamic acid, contains an extra hydroxyl group (O3') in addition to the phenolic hydroxyl group (at the *para*

position of the ring) already present in the native chromophore *p*-coumaric acid. The additional hydroxyl group seems to fit well into the chromophore-binding pocket without significantly perturbing the protein backbone structure. This may be related to the two small 'holes' (or packing defects; see Borgstahl *et al.*, 1995) that are present in the hydrophobic core of PYP close to the chromophore. These holes are each approximately the size of a water molecule, *i.e.* large enough to house a hydroxyl group (Borgstahl *et al.*, 1995) and CAFYP thus makes extra hydrogen bonds with the protein (Table 2) that are not present in the WTPYP structure (van Aalten *et al.*, 2000). However, a few unfavorable close contacts also exist with protein side chains (Table 2), which might cause some strain in the pG state. The caffeic acid chromophore fits into its binding pocket in two alternative conformations. Since the chromophore is well ordered, caffeic acid appears to bind to PYP in two different conformations rather than the existence of a dynamic equilibrium.

In earlier studies (Devanathan *et al.*, 1997; Kroon *et al.*, 1996), the PYP hybrid reconstituted with 3,4-dihydroxycinnamic acid (*i.e.* CAFYP) has been characterized with static and transient UV/Vis absorption spectroscopy. These studies have focused on the ground state of CAFYP (pG) and its long-living blue-shifted intermediate (pB or I2). They showed that compared with native PYP, CAFYP has a significantly faster pR to pB transition, but a much slower return to the ground state (pG) (Devanathan *et al.*, 1997; Kroon *et al.*, 1996). This suggests that although our data show that the overall backbone structure of the pG state is not perturbed, in the pR-like state more steric hindrance may be introduced by the caffeic acid than by the native chromophore, leading to a faster transition to pB. Interestingly, previous spectroscopic characterization of CAFYP by Devanathan *et al.* (1997) revealed that CAFYP is less stable than native PYP against both urea-induced and acid-induced denaturation. This again suggests that although additional hydrogen bonds are present in the CAFYP structure and the extra O4' oxygen appears to fit the structure, the CAFYP pG state may be inherently more strained, perhaps owing to some of the close contacts described above.

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