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Binding, tuning and mechanical function of the 4-hydroxy-cinnamic acid chromophore in photoactive yellow protein

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The bacterial photoreceptor protein photoactive yellow protein (PYP) covalently binds the chromophore 4-hydroxy coumaric acid, tuning (spectral) characteristics of this cofactor. Here, we study this binding and tuning using a combination of pointmutations and chromophore analogs. In all photosensor proteins studied to date the covalent linkage of the chromophore to the apoprotein is dispensable for light-induced catalytic activation. We analyzed the functional importance of the covalent linkage using an isosteric chromophore–protein variant in which the cysteine is replaced by a glycine residue and the chromophore by thiomethyl-*p*-coumaric acid (TMpCA). The model compound TMpCA is shown to weakly complex with the C69G protein. This non-covalent binding results in considerable tuning of both the pK_a and the color of the chromophore. The photoactivity of this system, however, was strongly impaired, making PYP the first known photosensor protein in which the covalent linkage of the chromophore is of paramount importance for the functional activity of the protein *in vitro.* We also studied the influence of chromophore analogs on the color and photocycle of PYP, not only in WT, but especially in the E46Q mutant, to test if effects from both chromophore and protein modifications are additive. When the E46Q protein binds the sinapinic acid chromophore, the color of the protein is effectively changed from yellow to orange. The altered charge distribution in this protein also results in a changed pK_a value for chromophore protonation, and a strongly impaired photocycle. Both findings extend our knowledge of the photochemistry of PYP for signal generation.

Introduction

Pigmented proteins that function as a receptor for visible light bind cofactors to acquire absorption in this region of the spectrum. The cofactors, in these cases called chromophores, usually are small organic molecules that bind to the apoprotein. The chromophores absorb UV and/or visible light because of the presence of a conjugated system like an aromatic ring or a polyene chain. The surrounding apoprotein is able to change the absorption properties of the chromophore through specific interactions, in order to obtain a holoprotein with the desired absorption characteristics. This phenomenon, called spectral tuning has been extensively studied in rhodopsins,[1,2] and in photoactive yellow protein (PYP).[3,4] PYP is a small, water-soluble photoreceptor protein first found in *Halorhodospira halophila*.[5] It absorbs maximally at 446 nm, and after blue-light excitation it enters a fully reversible photocycle. During this cycle several short- and long-lived intermediates are formed, and ultimately the ground state is recovered in ~0.5 s.[6,7] As a chromophore, PYP contains 4-hydroxy-cinnamic acid (also known as *p*-coumaric acid; pCA), covalently bound to the single cysteine of the protein through a thio-ester linkage.[8–10] In the ground state of the protein, the phenolic oxygen of the chromophore is deprotonated, and the vinyl double bond is in its *trans* configuration (see Fig. 1). The negative charge on the oxygen atom is stabilized by a hydrogen-bonding network that consists of Tyr42, Glu46, Thr50 and the chromophore.[11] During the photocycle, the chromophore vinyl bond isomerizes, which is followed by protonation of the chromophore by its hydrogen bonding partner Glu46.[12,13] An E46Q mutant, in which this proton-donor glutamic acid has been replaced by a glutamine residue, has been thoroughly studied.[14–18] This E46Q mutant of PYP has retained its photoactivity, but has significantly altered characteristics: Its absorption maximum has been red-shifted to 460 nm, and ground state recovery is ~ 30 times faster (at pH 8) than that in WT PYP.

For all currently known photosensor protein families, apart from the *p*-coumaric acid-containing xanthopsin family,[19] it has been shown that a covalent linkage of the chromophore to the apoprotein in the receptor state is not an essential prerequisite for catalytic activation. These families include the rhodopsins, phytochromes and flavin-containing photoreceptor proteins.[20] In the flavin-containing photoreceptor proteins (cryptochromes, phototropins (and other-domain containing proteins) and BLUF proteins) FAD or FMN, is bound non-covalently to the apoprotein in the ground state. Note, however, that for the phototropin family, it has been shown that there is a transient covalent linkage from the FMN chromophore to a conserved cysteine residue in the signaling state.[21,22] The rhodopsins comprise the protein family with members containing a seven-transmembrane helix-containing apoprotein. A retinal is bound as the chromophore to a lysine residue in this apoprotein through a Schiff-base linkage. For both bacteriorhodopsin and visual rhodopsin it has been established, using site-directed mutagenesis, that functional

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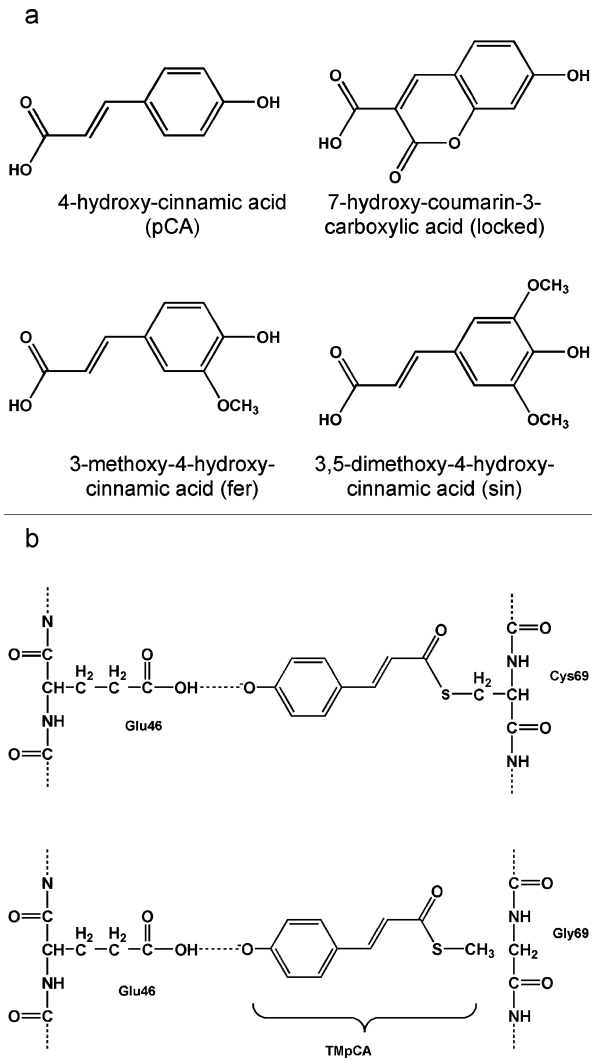

Fig. 1 Chemical structures of the chromophores used in this study. (a) pCA is the native chromophore in *H. halophila* PYP. The locked chromophore cannot undergo *trans–cis* isomerization. The fer chromophore has one methoxy-substitution on the *meta*-position of the aromatic ring, the sin chromophore has two. (b) Schematic representation of the chromophore-binding pocket in PYP and the model compound used in this study for isosteric replacement; upper part: wild type chromophore binding pocket, with the 4-hydroxy-cinnamic acid chromophore covalently bound to Cys69; lower part: the TMpCA model compound in the binding pocket of C69G PYP, thereby providing a situation of isosteric replacement compared to wild type protein.

holoprotein is obtained even in the absence of a covalent linkage: The covalent linkage between retinal and the backbone of the apoprotein is not required for the function of bacteriorhodopsin as a proton pump if proper isosteric replacement is provided, as shown in bothref. 23 and.ref. 24. In similar experiments Zhukovsky *et al.* showed that a covalent bond to the retinaldehyde in mammalian rhodopsin is not essential for binding of the chromophore nor for catalytic activation of transducin.[25] In the phytochromes, a linear tetrapyrole, usually covalently bound to a cysteine *via* a thioether linkage, functions as the chromophore (for a review, see ref. 26). Recently, a subclass of bacteriophytochromes has been discovered, in which the chromophore is bound non-covalently (CphB from *Calothrix* sp. PCC7601[27]). Both the latter, and oat phytochrome reconstituted with a chromophore analogue not able to bind covalently, were shown to be fully functional *in vitro*.[28]

Heterologous overexpression of apo-PYP in *Escherichia coli* allows us to attach chromophore derivatives to PYP *in vitro*.[19] The spectral tuning and fluorescence properties of WT PYP, reconstituted with the native chromophore and several chromophore analogs, and in various PYP mutants, have been described.[3,29–33] In these studies, contributions to the color tuning of either amino acid residues in the chromophore-binding pocket, or of substituents on the aromatic ring of the chromophore, are described. The remarkable shift in absorption maximum—from 284 nm for free pCA in aqueous solvent to 446 nm in the chromophore-binding pocket in the protein—is caused by the joint contribution of: (i) formation of the thiol ester bond between pCA and Cys69, (ii) deprotonation of the chromophore, and (iii) specific protein–chromophore interactions. Still, the precise molecular origin of this shift remains a topic of interest. Here, we extend these analyses and study the combination of a point mutation and chromophore derivatives on the tuning and photocycle characteristics in PYP, to analyze the (combined) effects of changed electron density on the chromophore, and changed hydrogen-bonding between the chromophore and Glu46.

Furthermore, the functional importance of the covalent linkage between chromophore and apoprotein in PYP is studied by changing cysteine 69 to a glycine or an alanine residue. The addition of the methyl thio-ester of the pCA chromophore (methylmercaptyl-4-hydroxycinnamate, also known as thiomethyl-*p*-coumaric acid, TMpCA) to C69G PYP results in a structure that is isosteric with the wild type holo protein. The only differences are the absence of the covalent linkage (see Fig. 1) and possibly changes in the flexibility of the protein backbone at that position. We describe the binding of this chromophore model compound to the apoprotein derivatives, as detected *via* the spectral tuning of the chromophore, and the resulting changes of spectroscopic properties of this system.

Experimental

Sample preparation

Apo-PYP, both of WT, E46Q-, C69A- and C69G-protein, was produced and isolated as described in ref. 19 as hexahistidine tagged apoprotein in *Escherichia coli*. Mutagenesis was performed using the QuickChange kit (Stratagene) and with pHISP as a template.[19] The sequences of the mutagenic primers for C69G were: 5′ GGCAAGAACTTCTTCAA GGACGTGGCCCCGGGCACTGACAGCC 3′ and 5′ GGCTGTCAGTGCCCGGGGCC AGCTCCTTGAAGAAGTTCTTGCC 3′ and for C69A: 5′ CTTCAAGGACGTCGCCC CG GCCACTGACAGCCCGG 3′ and 5′ CCGGGCTGTCAGTGGCCGGGGCGACGTCCTTGAAG 3′. The mutations were confirmed by nucleotide sequence analysis. Except for the C69 variants, the apoprotein was reconstituted with the 1,1-carbonyldiimidazole derivative of the respective chromophore (*i.e.* 4-hydroxy-cinnamic acid (pCA), 7-hydroxy-coumarin-3-carboxylic acid (locked), 3-methoxy-4-hydroxycinnamic acid (ferulic acid; fer) or 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid; sin), see also Fig. 1) as described in ref. 34. Protein samples were used without removal of

their hexahistidine containing *N*-terminal tag in 10 mM Tris–HCl, pH 8.0.

Chromophore synthesis

4-Hydroxy-cinnamic acid derivatives with modifications on the phenolic part of the chromophore were synthesized as described in ref. 3. Thiomethylesters were synthesized from both *p*-coumaric acid (Sigma) and 7-hydroxy-coumarin-3-carboxylic acid (Molecular Probes), resulting in TMpCA and TM7HC, respectively. They were synthesized using the 1,1-carbonyldiimidazole (Aldrich) derivative of the chromophores (prepared as described in ref. 34) and sodiumthiomethoxide (Acros Organics). An equimolar amount of sodiumthiomethoxide (dissolved in water) was added to the activated ester of the chromophore and the mixture was allowed to react overnight at room temperature. The compound was then purified using a silicagel column. The column was washed with 2 column volumes petroleum ether, after which the thio-methyl ester was eluted using a 1 : 1 ethylacetate–petroleum ether mixture. Concentrated TMpCA samples were stored in dry *N*,*N*-dimethylformamide (DMF). The identity and purity of the compounds was confirmed using NMR and mass spectrometry. The extinction coefficient of TMpCA was determined using high-pH hydrolysis of the thioester; the compound was dissolved in 10 mM phosphate, pH 13 and stirred for 30 min, after which no further spectral changes were observed. After hydrolysis the pH was lowered to 5 and the spectrum was compared to the spectrum of *p*-coumaric acid at pH 5, which has a molar extinction coefficient (ε) of 20 mM^{-1} cm^{-1} in its protonated form.[35]

Steady-state and transient (millisecond–second) UV/Vis measurements

Steady-state protein spectra and photocycle kinetics on a millisecond to second time-scale were measured with an HP 8453 UV/Vis diode array spectrophotometer with a time resolution of 100 ms. Samples were excited using a white-light photoflash (Morris AC Slave II, 21 W s^{-1}). Binding of the model compounds was followed spectroscopically using the HP 8543 spectrophotometer and a "Kraayenhoff vessel" enabling simultaneous monitoring and adjustment of pH and temperature. Initial binding experiments were carried out in 50 mM phosphate buffer pH 7, and in 50 mM phosphate plus 50 mM boric acid, pH 9, at temperatures between 25 °C and 5 °C. Experiments to determine the binding affinity for TMpCA and to study the photoactivity of the non-covalent adduct were carried out at −20 °C in 50 mM phosphate buffer, 20% glycerol, pH 7, at −10 °C and −15 °C.

Experiments at 77 K were performed in 10 mM phosphate buffer, 67% glycerol, pH 7 in a Perkin Elmer Lambda UV/Vis spectrophotometer, equipped with a liquid nitrogen cryostat. To investigate photoactivation, the protein sample was transiently illuminated with a blue LED (470 nm).

Results

Spectral tuning of covalently attached chromophores

We reconstituted both WT PYP, and the E46Q derivative, with the native PYP chromophore, *i.e.* 4-hydroxy-cinnamic acid, and the three derivatives shown in Fig. 1(a), resulting in the proteins WT–pCA, WT–locked, WT–fer, WT–sin, E46Q–pCA, E46Q–locked, E46Q–fer and E46Q–sin. Both proteins could bind all four chromophores in a 1 : 1 stoichiometry with apo-PYP, as was evident from both mass spectrometry and UV/Vis analysis (data not shown). For WT PYP, these spectra match those in ref. 3. With one methoxy substituent at the *meta*-position, a red-shift of 15 nm is observed, and with two methoxy substituents, an even larger red-shift of 40 nm. In WT–sin at pH 8, a substantial amount of a pB_{dark}-like species with a protonated chromophore is seen, with an absorption maximum at 362 nm (Fig. 2(a), see also below and Table 1). The pB_{dark} state is formed in the dark at low pH, but does resemble the light-induced state pB.[5,7,36,37] E46Q PYP was also reconstituted with all four chromophores, resulting in farther-red absorbing species (see Fig. 2(b)). The same trend can be seen as in WT PYP: locked chromophore results in a narrower absorption peak, with only a small wavelength shift (to 453 nm, *i.e.* a small blue-shift as compared to *p*-coumaric acid). Ferulic acid results in a red-shift of 20 nm. Sinapinic acid shows the largest red-shift: the combination E46Q–sin has an absorption maximum at 495 nm, resulting in an orange protein. Here, the change of one residue in the protein plus the addition of two methoxy groups to the

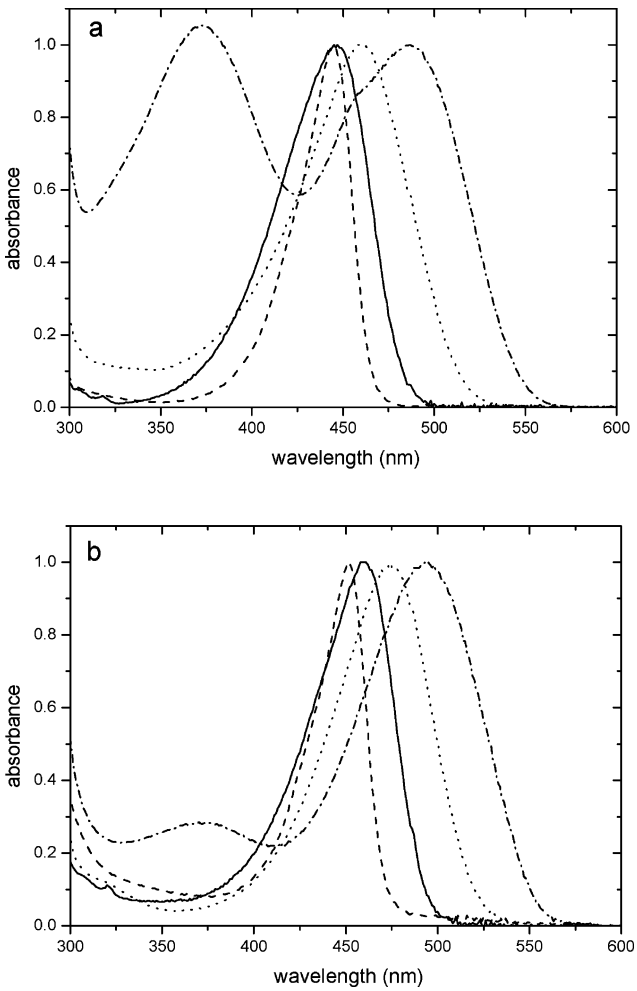

Fig. 2 Normalized UV/Vis absorption spectra of PYP hybrids: (a) WT PYP apoprotein, (b) E46Q PYP apoprotein; pCA (——), locked (---), fer (· · ·), sin (-·-). Spectra of protein reconstituted with different chromophores were taken in the dark, in 50 mM phosphate buffer, pH 7.5.

Table 1 Spectral characteristics of systems used in this study[a]

System	Absorption maximum (protonated/deprotonated)/nm	pK_a (n-value)[b]	Fluorescence emission maximum/nm
WT–pCA	355/446	2.7 (1.9)	492
WT–locked	370/443	ND	464
WT–fer	417/461	3.5	526
WT–sin	380/486	8.7 (0.82)	547
E46Q–pCA	360/460	5.3	499
E46Q–locked	ND/453	ND	486
E46Q–fer	ND/473	ND	529
E46Q–sin	375/495	7.9 (0.79)	550
Sinapinic acid	306/355	9.6 (0.86)	ND
TMpCA	330/385	8.5	ND
TM7HC	362/421	ND	ND
C69G–TMpCA	—/435	6	ND

[a] ND: not determined. [b] n-value: measure for the steepness of the pH-transition.

chromophore results in a wavelength shift of 50 nm. Although the effects of mutation and chromophore derivative are additive, the size of the shifts resulting from the different chromophores do differ between the wild type and the E46Q protein: the E46Q mutation by itself results in a shift of 682 cm^{-1}, in WT, the sinapinic acid chromophore results in a shift of 1845 cm^{-1}, whereas in E46Q, the sinapinic acid chromophore results in a shift of 1537 cm^{-1}. The total shift from WT–pCA to E46Q–sin is 2220 cm^{-1}. Also note that in E46Q–sin, there is less pB_{dark} formed at pH 8, as compared to WT–sin (see also below).

pH titration of covalently attached chromophores

To study the formation of pB_{dark}, as described above, we measured the pH dependence of ground state spectra of the free sinapinic acid, WT–sin and E46Q–sin (see Fig. 3). In all three cases, the clear isosbestic points obtained indicate the involvement of a two-state transition. We determined the pK_a values for protonation of the phenolic oxygen in these compounds by fitting the pH dependent absorption to the Henderson–Hasselbalch equation. The results are shown in the right panels of Fig. 3. The pK_a value for the free sinapinic acid (pK_a = 9.6) differs from the pK_a value of the model thio-ester of sinapinic acid given in ref. 3 (pK_a = 8.7), indicating that the thioesterification stabilizes the negative charge on the phenolic oxygen atom of the chromophore. For WT–sin we found a pK_a of 8.7, indicating that covalent binding of the chromophore to the protein does not provide extra stabilization of the negative charge. Surprisingly, in the combination E46Q–sin, the pK_a has decreased almost 1 pH unit, to 7.9, *i.e.* it lies in between the pK_a of E46Q–pCA and WT–sin. Apparently, the E46Q mutation stabilizes the (extra) negative charge on the phenolic oxygen. Another surprising finding is the low n-value in these titrations, as indicated by its steepness. In all three cases, the n-value was 0.8–0.9, whereas in WT–pCA, an n-value of 1.9 is found. The lower n-value indicates that the hydrogen-bonding network, of which the phenolic oxygen is part, has been (partly) disrupted in the two PYP hybrids that contain sinapinic acid as a chromophore. Surprisingly, in the variants with the sin-chromophore, no significant photocycle activity was observed upon excitation. Possibly, the two methoxy groups "anchor" the chromophore in the protein, in such a way that the photocycle can not be initiated. However, the different charge distribution, as suggested by the altered pK values, may also play an important role, since charge movement upon electronic excitation was shown to play an important role in PYP activation.[38,39]

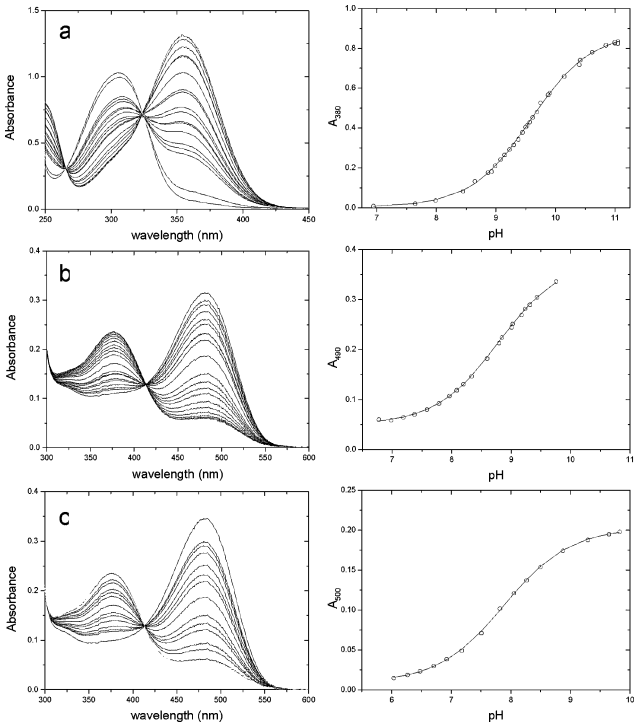

Fig. 3 pH titration of free and protein-bound sinapinic acid. Spectra were measured as a function of pH in the dark after adding small aliquots of concentrated HCl or NaOH. (a) Free acid in solution, (b) WT PYP–sin, (c) E46Q PYP–sin. Left-hand panels: absorption spectra; right-hand panels: absorbance at long-wavelength peak *versus* pH (○), and the fit of the data according to Henderson–Hasselbalch (——). From this, pK_a values of 9.6 (0.86), 8.7 (0.82) and 7.9 (0.79) were calculated for (a), (b) and (c) respectively (values between parentheses are the n-values).

Model compound synthesis for removal of the PYP–chromophore covalent bond

The thiomethyl model compounds TMpCA and TM7HC were successfully synthesized and purified, as judged from NMR and

mass spectrometry analysis (not shown). The absorption spectra of both compounds are shown in Fig. 4. TMpCA shows an absorption maximum at 330 nm at low pH, where the phenolic oxygen is protonated. Its deprotonated form absorbs at 385 nm. This shows that the ester linkage does indeed red-shift the absorption maximum when compared to the free acid.[3] The pK_a of the phenolic oxygen was determined to be 8.5, very similar to the pK_a found in free 4-hydroxy-cinnamic acid;[3] apparently, the thioesterification does not influence the pK_a of this chromophore, in contrast to sinapinic acid, as described above. TM7HC has absorption maxima at 362 nm and 421 nm, for the protonated and deprotonated form, respectively.

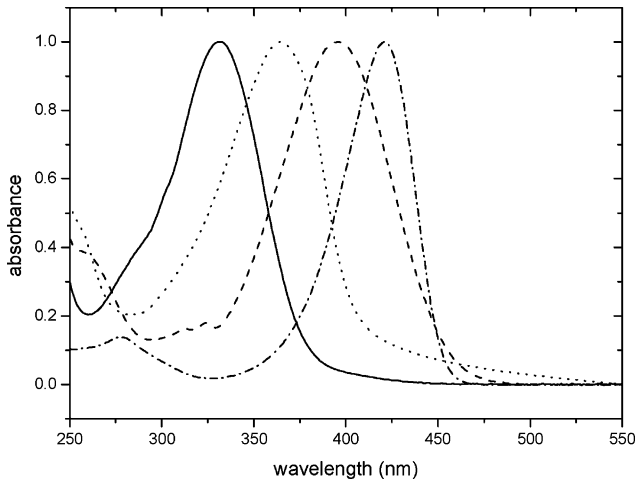

Fig. 4 Absorption spectra of chromophore model compounds, taken in the dark at room temperature. TMpCA, pH 5 (——), TMpCA, pH 10 (---), TM7HC, pH 5 (· · ·), TM7HC, pH 10 (-·-).

The extinction coefficient of TmpCA was determined using high-pH hydrolysis of the thioester. After hydrolysis its absorbance was compared to the absorbance of free 4-hydroxy-cinnamic acid. Accordingly, the extinction coefficient was determined for its protonated form ($\varepsilon_{330} = 22$ mM^{-1} cm^{-1}) and for its deprotonated form ($\varepsilon_{385} = 34$ mM^{-1} cm^{-1}).

Non-covalent binding of chromophore model compounds to C69G PYP

To study the importance of the covalent linkage of the chromophore to apo-PYP, we constructed PYP mutants that lack the single cysteine, *i.e.* the C69A and C69G mutant proteins, and tested complexation of the model compounds and the free acids with these proteins. The C69A protein does not bind TMpCA, nor any of the other available chromophores, at any of the conditions tested (see also below). The model compound TMpCA is shown to complex with the C69G protein, but only at low temperatures. As seen in Fig. 5, complexation results in tuning of the absorbance maximum, resulting in a maximum at 435 nm, a wavelength maximum close to WT PYP. At room temperature, no absorption at 435 nm is seen, but upon lowering the temperature to −20 °C, the absorption at 435 nm significantly increases, indicating temperature-dependent complexation of TMpCA with C69G protein. The absorption maximum of the chromophore–protein complex was 435 nm, regardless if the experiment was performed at pH 7 or pH 9, suggesting that only the deprotonated chromophore can complex with the protein (see also below). The extinction coefficient of the bound chromophore was determined by comparing the absorbance of same amounts of chromophore in buffer and in the presence of C69G PYP. By comparing the difference in absorbance at 330 nm and the absorbance at 435 nm, $\varepsilon_{435(\text{bound})}$ was determined to be 1.75 times $\varepsilon_{330(\text{free})}$ (±0.03, averaged over 3 measurements), resulting in an extinction coefficient for the non-covalently bound chromophore $\varepsilon_{435(\text{bound})} = 40$ mM^{-1} cm^{-1}. This value is only slightly smaller than the extinction coefficient of WT PYP, which equals 45.5 mM^{-1}cm^{-1}.[40] The affinity of TMpCA for C69G PYP was determined by titration of a known amount of protein with small aliquots of the chromophore. Results are shown in Fig. 6; by fitting the titration curve, affinity constants of 90 μM and 75 μM were found at −5 °C and −10 °C, respectively. A pH titration experiment shows that the protein surroundings stabilize the deprotonated form of the bound chromophore; in solution, the pK_a is 8.5, whereas in the protein it is 6 (not shown). This value is intermediate between the values for the chromophore in solution and WT PYP (pK_a = 2.7), indicating that the stabilization by the chromophore surroundings is present, but much weaker than in the WT protein. Upon lowering of pH, absorption at 435 nm decreases, while absorption at 330 nm increases, suggesting that only the deprotonated chromophore can complex with the C69G protein pocket. Control experiments to test the specificity of the chromophore–protein interaction were performed using BSA, WT holo-PYP and WT apo-PYP. In none of these cases (aspecific) complexation of the chromophore with the protein, as deduced from absorption changes, could be observed.

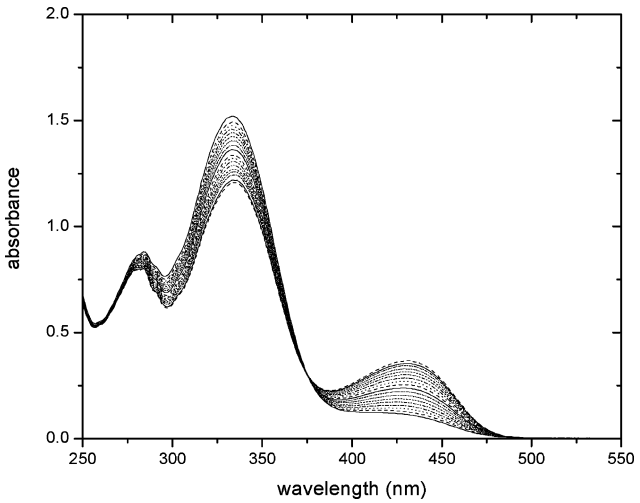

Fig. 5 Temperature dependence of TMpCA-binding to apo-PYP. Spectra were taken in the dark, at temperatures between 25 °C and −20 °C. At decreasing temperature, absorption at 330 nm decreased, whereas absorption at 435 nm increased.

Photoactivity in the C69G–TMpCA protein–chromophore complex

At the conditions where complexation of the model compound with C69G PYP was observed, the possibility of this protein–chromophore complex to undergo a photocycle was tested after excitation with either a short pulse of white light or with blue light from a LED (470 nm), and after prolonged illumination with

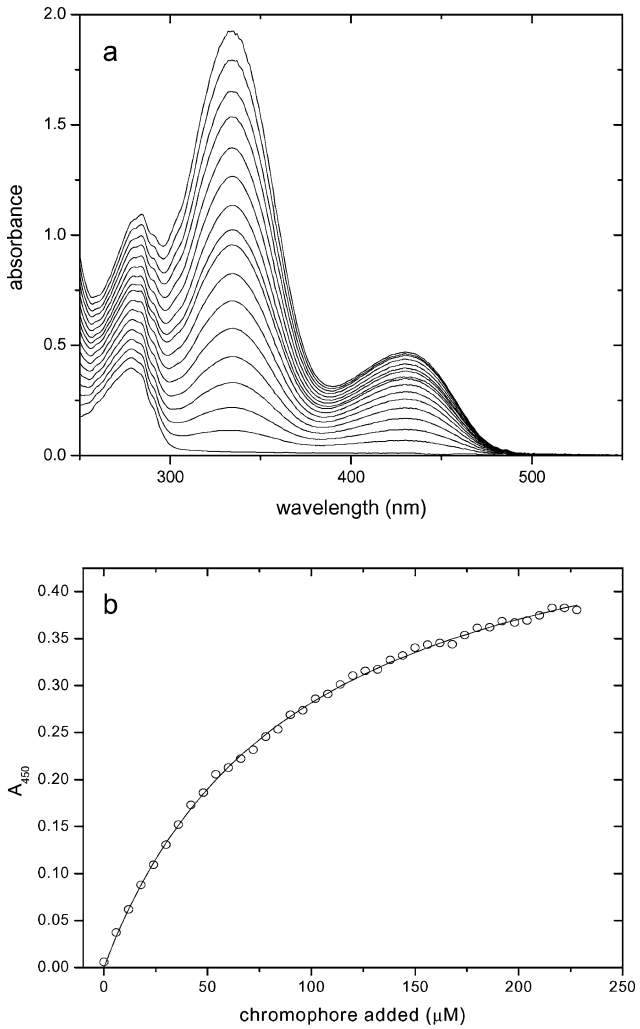

Fig. 6 Titration of C69G PYP with TMpCA. Small aliquots of *trans*-TMpCA in DMF were added to the C69G protein in 50 mM phosphate buffer, 20% glycerol, pH 7. The titration was carried out in the dark, at −5 °C. Open circles represent the measured absorption, the solid line represents the fit.

the blue LED. After short excitation, or illumination for several minutes, no changes were seen in the spectrum of the pigment. Only after illumination for at least 10 min, a small irreversible decrease was observed in the absorbance of the main absorption peak at 435 nm. Simultaneous formation of a photoproduct, however, was not observed, nor was there recovery of the bleached peak. The photochemistry in this pigment was further studied at low, *i.e.* liquid nitrogen, temperatures (77–80 K). Spectra of a C69G PYP–TMpCA–glycerol glass were taken both in the dark and after a 3 minute illumination. The result is shown in Fig. 7: the dark spectrum of the pigment is not affected by the low temperature. A light-induced bleach of the ground state, albeit relatively small, is observed. The narrowness of the bleached band (19 nm FWHM) and the structure at the edges possibly indicate the formation of (a) species with a maximum close to that of the ground state, but with a smaller extinction coefficient. Furthermore, changes in the UV region around 350 nm are visible. The red-shifted product PYP_B[41] is not readily observed.

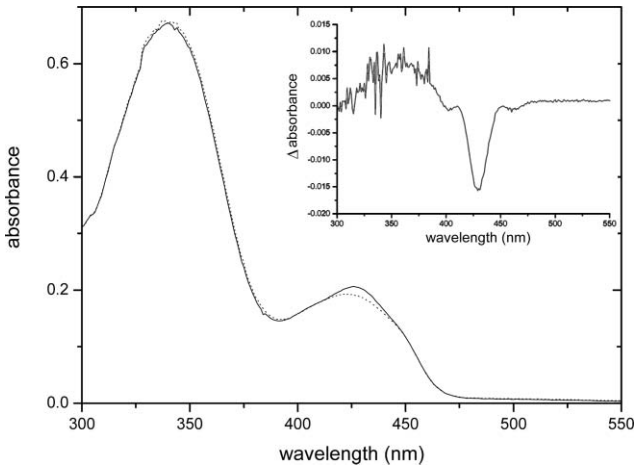

Fig. 7 Photoactivity in the C69G–TMpCA pigment at 77 K. UV/Vis spectra were measured before (——) and after (---) 3 minute illumination with blue light. The inset shows the light − dark difference spectrum.

Discussion

Spectral tuning and pK_a value of covalently attached chromophores

To study the influence of both chromophore and protein modification on the color tuning of PYP, we constructed PYP variants in which the E46Q protein was reconstituted with either the native pCA chromophore, or the analogs 7-hydroxy-coumarin-3-carboxylic acid, ferulic acid and sinapinic acid. Both the mutation and the inclusion of methoxy groups on the chromophore, result in a red-shift in the absorption spectrum of the respective proteins. In the combination E46Q–sin, this shift is so large that it results in a protein that has an orange color instead of yellow. This can be explained by the fact that the electron-donating methoxy groups result in more negative charge in the conjugated π-system of the chromophore. There are two surprising findings regarding this E46Q–sin PYP variant: (i) it has a lower pK_a for protonation of the phenolic oxygen of the chromophore than the WT–sin protein, indicating more stabilization of the negative charge on the oxygen in this variant. (ii) The proteins containing a sinapinic acid chromophore are not able to undergo a photocycle upon excitation. The increased (approximately 10-fold compared to pCA in E46Q PYP) quantum yield for fluorescence of this derivative supports this observation. Possible reasons are either the changed charge distribution and/or steric hindrance because of the methoxy groups on the chromophore. Quantum mechanical calculations do not show great variations in the charge distribution close to the C=C double bond of the chromophore, favoring the explanation of steric hindrance (*e.g.* between one of the methoxy groups and Tyr-42, see also ref. 42) as cause of the impaired photoisomerization in this protein (results not shown). Summarizing, we have shown that the absorption spectrum of PYP can be tuned to the red using electron-donating substituents (methoxy groups) on the aromatic ring of the chromophore, as expected, and as could be shown in quantum mechanical calculations. The E46Q mutation itself also results in a red-shift of the absorption spectra. When these two (mutation and chromophore modification) were combined, it was shown that the red-shifts are additive, resulting in a total shift of 50 nm. The E46Q–sin variant shows an unexpectedly lowered pK_a. Note,

however, that this pK_a can be influenced both by protein stability and charge distribution on the chromophore.[43] However, because of the relative high pK_a in the variants described here and the reported small perturbation in the structure of PYP with a comparable chromophore analog,[42] it is likely that these pK_a values describe the pK_a of the chromophore in a folded protein.

Non-covalent binding of chromophore model compounds to C69G PYP

We synthesized chromophore model compounds that are thiomethylesters of the respective free acids, *i.e.* 4-hydroxy-cinnamic acid and 7-hydroxy-coumarin-3-carboxylic acid. In the latter, the vinyl bond that isomerizes in 4-hydroxy-cinnamic acid is effectively locked in its *trans* configuration. The spectra of these compounds, both in neutral and in deprotonated form, are red-shifted with respect to the spectra of the free acids, showing the importance of the ester linkage in the tuning of these chromophores. When compared to the absorption maximum of denaturated PYP, the absorption maxima of both neutral and deprotonated TMpCA are blue-shifted by $\sim$ 20 nm, showing that in the denaturated state, there are still protein–chromophore interactions that red-shift the absorption maxima. Furthermore, the spectrum is also red-shifted when compared to the methylester of pCA, that has an oxygen atom instead of a sulfur atom (absorption maxima at 310 nm and 360 nm for the protonated and deprotonated form, respectively; result not shown). This shows that the conjugated light-absorbing system includes the sulfur atom at this position, which is consistent with quantum mechanical calculations described in ref. 4. The pK_a of protonation of the phenolic oxygen of the chromophore is very close to the one in the denatured protein or in the free acid, *i.e.* 8.5. We used these model compounds to study the importance of non-covalent binding on photoactivation in PYP variants in which the chromophore-binding cysteine has been replaced by either an alanine or a glycine residue. In the C69A protein, no binding of any of the model compounds was observed, presumably because of steric hindrance between the alanine side-chain and the methyl group of the chromophores (see Fig. 1). Non-covalent binding, followed spectrally, was only observed when the chromophore model compound TMpCA was used in combination with C69G PYP, *i.e.* upon isosteric replacement. Complexation resulted in a large red-shift of the absorption maximum to 435 nm, remarkably close to the absorption maximum at 446 nm in WT PYP. As opposed to the non-covalent binding of chromophores in other photoreceptor proteins, however, the complexation in this case was very weak, with a K_d in the 10–100 μM range. The temperature-dependence of the complexation, *i.e.* more complexation at low temperatures, indicates that this process is enthalpy-driven. The complexation shows that the hydrogen bonds from Glu46 to the chromophore hydroxyl group and from the chromophore carbonyl to the cysteine backbone nitrogen are sufficient to keep the chromophore in the binding pocket, at least in the ground state of the protein. However, although binding can take place in the absence of a covalent linkage between the chromophore and the protein backbone, the photoactivity of the resulting pigment is strongly impaired. At temperatures from 0° C to −20 °C, short blue-light excitation does not result in observable spectral changes, implying a very low—if any—quantum yield for photochemistry. Note that the TmpCA chromophore free in aqueous solution has been shown before not to isomerize (efficiently) upon illumination.[44] Apparently, in the WT protein, the protein surroundings make efficient isomerization possible. Only after prolonged illumination, a small decrease in absorbance of the main absorption peak is observed. However, the absorbance does not recover after incubation in the dark, not even if the temperature is raised to 20° C and then subsequently is decreased again. Because the experiment has to be performed with an excess amount of TMpCA (to saturate the binding site; see above), this implies that the chromophore does remain in the active site after excitation, but cannot return to its ground state. The chromophore may have undergone *trans–cis* isomerization, but the absence of the covalent linkage prevents the following steps in the photocycle. In wild type PYP, at temperatures below 93 K, the photocycle is blocked[45] after formation of the red-shifted intermediate I_0 or, in a branched pathway, the slightly blue-shifted intermediate PYP_H and the red-shifted intermediate PYP_B.[41] Low-temperature photocycle intermediates have been studied using FTIR, where PYP_B and PYP_L were shown to be formed in parallel pathways, and to differ in structure.[46] Both intermediates were also analyzed in (low temperature) crystallographic studies.[47,48] These studies showed that PYP_B and PYP_H have a chromophore in the *cis* configuration. The exact difference between the two intermediates could not be shown, but the studies indicated that all changes are restricted to the region around the C2=C3 bond. We studied if an intermediate similar to one of these can be formed in the C69G PYP–TMpCA pigment after excitation at 77 K. Indeed a slightly blue-shifted intermediate, possibly PYP_H, can be observed. None of the absorbance changes indicate formation of the red-shifted intermediate PYP_B, absorbing at 489 nm in WT PYP. Apparently, the lack of a covalent linkage from the chromophore to the protein backbone prevents formation of this specific intermediate (and the subsequent branched intermediate PYP_{BL}). The small changes at the red edge of the bleach, *i.e.* around 460 nm, may indicate the formation of a PYP_L-like species.[41] Furthermore, increased absorption in the UV region is visible, with a maximum at 355 nm, suggesting formation of a pB-like (protonated) species. Apparently, in this complex, transitions are possible at temperatures where in WT PYP these are blocked, presumably because the chromophore has more freedom to move. However, only a very small fraction of molecules is bleached, even after prolonged illumination.

Conclusions

During the PYP photocycle, the hydrogen bonds between the chromophore and the protein backbone, *i.e.* from Tyr42 and Glu46 to the hydroxyl group of the chromophore and from the chromophore carbonyl group to the nitrogen atom of Cys69, are of paramount importance. Recently, through ultrafast studies on PYP, the importance of these hydrogen bonds has been stressed again. It was shown that already in the very early stages of the photocycle, Glu46 starts "pulling" on the chromophore: The hydrogen bond first weakens in the excited state, then gets stronger during I_0 and pR formation,[49,50] but finally breaks upon formation of pB. The carbonyl group that in the ground state hydrogen bonds to cysteine, rotates upon photo-activation, thereby transiently breaking the hydrogen bond. Also from an

optoacoustic study,[51] chromophore 'contraction' directly resulting from photoisomerization, was observed upon pR formation.

We conclude that the covalent linkage between the chromophore and the protein backbone is of high importance for the functional activity of the protein *in vitro*. This in contrast to other photosensor proteins with an isomerizable chromophore, that can function without a non-covalently bound chromophore. Presumably, the delicate interplay between photoisomerization and changes in the hydrogen bonding network needed in the PYP photocycle requires a covalently linked chromophore.

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