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Chapter 4

Modulating the color of a bacterial photosensor: conversion of a yellow into an orange protein

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4.1 Abstract

Photoactive Yellow Protein (PYP) is a bacterial blue-light photoreceptor protein, that owes its color to a p-coumaric acid chromophore, covalently attached to the single cysteine of the protein via a thio-ester linkage. The protein environment shifts the absorption maximum of the free chromophore in solution from 284 nm to 446 nm in the protein (both at pH 8). We analyzed this phenomenon called spectral tuning into greater detail using chromophore analogs (ferulic acid and sinapinic acid) in combination with the E46Q variant of PYP. All three modifications by themselves result in a red-shift of the absorption maximum of PYP. When a protein with the E46Q mutation is reconstituted with the sinapinic acid chromophore, the effects are additive, resulting in a red-shift of 50 nm. This effectively changes the color of the protein from yellow to orange. The same trend was found in fluorescence emission spectra.

We also analyzed photocycle kinetics and pH dependence of the absorption spectra in these hybrids. Surprisingly, regarding the pKₐ of chromophore protonation, it was found that the effect of the chromophore replacement and the mutation are not additive: the pKₐ of protonation of the phenolic oxygen of the sinapinic acid chromophore in the E46Q protein lies in between that of the WT protein with the native chromophore and the WT protein with the sinapinic acid chromophore. Furthermore, the photoactivity in this specific variant, i.e. sinapinic acid in the E46Q protein environment, is impaired: upon excitation, no photocycle initiation is observed. We complement the above findings with quantum mechanical calculations on the charge distribution on the sinapinic acid chromophore. These calculations indeed indicate that there is more, and more evenly distributed, negative charge residing on the phenolic ring, in agreement with a red-shift in the absorption maximum of the protein.

4.2 Introduction

Pigmented proteins that function as receptors 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 protein backbone. GFP is an exception, here the chromophore is formed autocatalytically by a chemical conversion of a tripeptide motif of amino acids. The chromophores absorb UV and/or visible light because of the presence of a conjugated system like an aromatic rings or a polyene chain. The surrounding apo-protein changes the absorption properties of the chromophore through specific interactions, to obtain a holoprotein with the desired absorption...
characteristics. This phenomenon is called spectral tuning. Spectral tuning has been extensively studied in rhodopsins (Ren et al., 2001a; Man et al., 2003) and in Photoactive Yellow Protein (PYP) (Kroon et al., 1996). Here we extend the analyses from the latter reference to study the combination of a pointmutation with chromophore derivatives in PYP. PYP is a small, water-soluble photoreceptor protein first found in Halorhodospira halophila (Meyer, 1985). It absorbs maximally at 446 nm, and after blue-light excitation it enters a photocycle. This cycle consists of several short-lived intermediates, and ultimately the ground state is recovered in ~0.5 seconds (Meyer et al., 1987; Hoff et al., 1994b). As a chromophore, PYP binds a p-coumaric acid molecule, to the single cysteine of the protein through a thioester linkage (Van Beeumen et al., 1993; Baca et al., 1994; Hoff et al., 1994a). In the ground (i.e. dark) state of the protein, the phenolic oxygen of the chromopore is deprotonated, and the vinyl double bond is in its trans configuration (see Figure 1). The negative charge on the oxygen atom is stabilized by the hydrogen-bonding network that consists between Tyr42, Glu46, Thr50 and the chromophore. During the photocycle, the chromophore double bond isomerizes, which is followed by protonation of the chromophore by its hydrogen bonding partner Glu 46 (Kort et al., 1996b; Xie et al., 1996). An E46Q mutant, in which the proton-donor glutamic acid has been replaced by a glutamine residue, has been thoroughly studied (e.g. (Genick et al., 1997b)). This E46Q mutant of PYP is photoactive, but has significantly altered characteristics: its absorption maximum has been red-shifted to 460 nm, and its photocycle is ~3 times faster than in WT PYP.

The heterologous overexpression of PYP in Escherichia coli allows us to attach chromophore derivatives to PYP in vitro, since wild-type E. coli does not attach a chromophore to PYP. The spectral tuning and fluorescence properties of WT PYP, reconstituted with the native chromophore and several chromophore analogs, have been described in (Kroon et al., 1996). Here, we describe spectroscopic characteristics of E46Q PYP, reconstituted with chromophore analogs, and complement these experimental results with quantum mechanical calculations describing the charge distribution on the chromophore in these PYP variants.

4.3 Materials and Methods

Sample preparation
ApoPYP, both WT and E46Q, was produced and isolated as described in (Kort et al., 1996a) as hexa-histidine tagged apo-proteins in Escherichia coli. The apoprotein was reconstituted with the 1,1-carbonyldiimidazole derivative of the respective chromophore (i.e. p-coumaric acid (pCA), 7-hydroxy-coumarin-
3-carboxilic acid (*lock*), 3-methoxy-4-hydroxycinnamic acid (*ferilic acid; fer*) or 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid; *sin*, see also Figure 1) (Hendriks *et al*., 2002). Protein samples were used without removal of their hexahistidine containing N-terminal tag in 10 mM Tris/HCl, pH 8.0.

**Chromophore synthesis**

Chromophore derivatives were synthesized as described in (Kroon *et al*., 1996)

**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.

**Laser-flash photolysis spectroscopy**

To study sub-millisecond events we used an Edinburgh Instruments Ltd. LP900 spectrometer equipped with a photomultiplier, in combination with a Continuum Surelite I-10 YAG-laser and Continuum Surelite OPO (for details, (Hendriks *et al*., 1999b)).

**Steady-state fluorescence spectroscopy**

For steady state fluorescence analyses an Aminco Bowman Series 2 Luminescence Spectrometer was used. The excitation wavelength was set at the excitation maximum of the respective sample. To calculate the fluorescence quantum yield $\Phi_f$, samples were excited at 455 nm, and emission intensity was determined relative to WT PYP.

*Quantum Mechanical calculations to determine the charge distribution on the chromophore (analogs).*

Charge distribution in various chromophore-Glx (*i.e.* Glu or Gln) systems was calculated using the Gaussian98 software package. Together with Glu46, the pCA-chromophore from PDB-entry 2PHY served as a starting point. The orientation of Gln46 originated from a crystal structure of the E46Q mutant resolved by Spencer Anderson (personal communication). The PRODRG server generated coordinates for the sin-chromophore, that was subsequently placed on the original chromophore in the two structures. The systems considered included the thio-ester bond to Cys69 as well. The two amino acids in the system were capped with methyl groups at the $C_\beta$ positions. Hydrogen atoms were added also with the PRODRG server. Four chromophore-Glx systems were generated for the calculation of the charge distribution. The calculations were performed at a Hartree-Fock level of theory, using the 6-31G* basis set to describe the atomic orbitals. Graphics were created using Molscript and Raster3D.
4.4 Results

Spectral tuning
We reconstituted both WT PYP, and the E46Q derivative, with the native PYP chromophore, i.e. \( p \)-coumaric acid, and the three derivatives shown in Figure 1. Both proteins could bind all four chromophores in a 1:1 stoichiometry with apo-PYP, as could be seen by both mass spectrometry and UV/Vis analysis. For WT PYP, these spectra match those in (Kroon et al., 1996). With one methoxy substituent at the meta-position, a red-shift of 15 nm is observed; with two methoxy substituents, an even larger red-shift of 40 nm is obtained.

![Chemical structures of chromophores](image)

**Figure 1:** Chemical structures of chromophores used in this study.
pCA is the native chromophore in *H. halophila* PYP. The *lock* 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.

In WT/sin, at pH 8, a substantial amount of a pB\(_{\text{dark}}\)-like species is seen, with an absorption maximum at 362 nm (Figure 2A, see also below). This pB\(_{\text{dark}}\) state is formed in the dark at low pH, but does resemble the light-induced state pB (Meyer et al., 1987; Craven et al., 2000). E46Q PYP was also reconstituted with all four chromophores, resulting in farther-red absorbing species (see Figure 2B).

![UV/Vis absorption spectra of PYP hybrids](image)

**Figure 2:** UV/Vis absorption spectra of PYP hybrids.
Spectra were taken in the dark, in 50 mM phosphate buffer, pH 7.5. Solid: pCA, dash: lock, dot: fer, dash-dot: sin A: WT PYP backbone, B: E46Q PYP backbone
The same trend can be seen as in WT PYP: locked 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 chromophore results in a wavelength shift of 50 nm. Also note that in this variant, there is less \( pB_{\text{dark}} \) formed at pH 8, as compared to WT/sin (see also below).

**\( pH \) titration**

We measured the pH dependence of spectra of the free sinapinic acid, WT/sin and E46Q/sin (see Figure 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.

**Figure 3: \( pH \) titration of free and protein bound sinapinic acid.**

\( pH \) dependent spectra were measured 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 peak maxima versus pH (open circles), and the fit of the data according to Henderson-Hasselbalch (lines).
by fitting the pH dependent absorption to the Henderson-Hasselbalch equation. The results are shown in the right panels of Figure 3. The pKₐ value for the free acid (pKₐ = 9.6) differs from the pKₐ value of the model thio-ester of sinapinic acid given in (Kroon et al., 1996) (pKₐ = 8.7), indicating that the thioesterification stabilizes the negative charge on the phenolic oxygen atom of the chromophore. For WT/sin we found a pKₐ of 8.7, similar to the pKₐ of 8.7 given in (Kroon et al., 1996), and 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 pKₐ has decreased almost 1 pH unit, to 7.9, *i.e.* it lies in between the pKₐ 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, *i.e.* the degree of cooperativity, of these transitions. 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 disrupted in the two PYP hybrids that contain sinapinic acid as a chromophore.

*Fluorescence*

We measured fluorescence spectra and determined the quantum yield of fluorescence for most of the constructed PYP variants. The emission spectra shown in Figure 4 were obtained after excitation of the samples at their respective excitation maximum. A clear and strict trend can be observed, following the red-shift in the absorption maxima. The quantum yields of fluorescence Φᵦ were determined relative to WT / pCA, with excitation at 455 nm. Results are shown in Table 1. The Φᵦ value was higher for the proteins containing the sin-chromophore than for proteins containing the pCA-chromophore, in agreement with the lower quantum yield for photochemistry, as shown below.

![Figure 4: Room-temperature fluorescence of PYP hybrids.](image)


*Photocycle kinetics*

The kinetics of the recovery of the groundstate upon excitation were determined for all PYP variants. The results are shown in Table 1. As expected, in the variants with the lock-
chromophore, a photocycle was not observed upon blue-light excitation. The variants with the fer-chromophore showed somewhat slower recovery than with the pCA chromophore; the WT/fer variant recovered to the ground state with a rate constant $k = 5.4 \text{ s}^{-1}$, the E46Q/fer variant recovered with a rate constant $k = 7.1 \text{ s}^{-1}$. Surprisingly, in the variants with the sin-chromophore, no significant photocycle activity was observed after excitation.

Calculations of electron distributions
To provide a molecular interpretation for the observed color tuning, we calculated the charge distribution on various chromophore-Glx systems in the ground state. The results are shown in Figure 5. The influence of the methoxy groups added to the pCA chromophore is clearly visible: in these cases, there is more, and more evenly distributed negative charge on the phenolic ring.

![Figure 5: Charge distributions in chromophore-Glx systems.](image)

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 results in more negative charge in the conjugated $\pi$-system of the chromophore. This is confirmed by quantum mechanical calculations on the electron distribution over the chromophore. These show that in the sinapinic acid chromophores, there is more delocalized negative charge on the phenolic ring, in both WT PYP and E46Q PYP.

4.5 Discussion

To test 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-carboxilic acid, ferulic acid and sinapinic acid.
There are two surprising findings regarding this E46Q/sin PYP variant: (i) it has a lower pKₐ for protonation of the phenolic oxygen of the chromophore than the WT/sin protein. This indicates more stabilization of the negative charge on the oxygen in this variant; however, this, presumably subtle, effect could not be confirmed with the quantummechanical calculations. (ii) the proteins containing a sinapinic acid chromophore are not able to undergo a photocycle upon excitation. Possible reasons are either the changed charge distribution and/or steric hindrance because of the methoxy groups on the chromophore. The 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 as cause of the impaired photocycle in this protein.

In conclusion, 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 latter could not (yet) predict the color tuning as a result of the amino acid at the 46 position. Neither could the pKₐ of chromophore protonation be predicted: the E46Q/sin variant shown an unexpected low pKₐ. Note however that this pKₐ can be influenced both by protein stability and charge distribution on the chromophore. There is some controversy on the nature of the pKₐ as being the pKₐ of the chromophore in folded protein or unfolding of the protein resulting in exposure of the chromophore ((Meyer et al., 2003), see also Chapter 7.2.2). However, because of the relative high pKₐ in the variants described here, the observed n-value of 1, and the reported small perturbation in the structure of PYP with a comparable chromophore analog (van Aalten et al., 2002a), it is likely
that these pKₐ values describe the pKₐ of the chromophore in a folded protein.

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