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Tryptophan fluorescence as a reporter for structural changes in photoactive yellow protein elicited by photo-activation†

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Light-activation of photoactive yellow protein (PYP) is followed by a series of dynamical transitions in the structure of the protein. Tryptophan fluorescence is well-suited as a tool to study selected aspects of these. Using site-directed mutagenesis eight 'single-tryptophan' mutants of PYP were made by replacement of either a tyrosine, phenylalanine or histidine residue by tryptophan, while simultaneously eliminating the endogenous W119. Surprisingly, only three of these eight mutants turn out to emit measurable tryptophan fluorescence: F6W/W119F, F96W/W119F and H108W/W119F. Significantly, all three show altered tryptophan fluorescence upon formation of the pB state. As F96 is located very close to the chromophore, the F96W/W119F mutant protein is particularly suitable for further studies on the dynamical changes of the polarity in the chromophore-binding pocket of PYP. Furthermore, WT PYP can be photo-activated by a UV photon *via* the highly conserved W119 and subsequent Förster resonance energy transfer. Placing a unique tryptophan residue elsewhere in the protein shows that position 119 is favoured for UV-activation of PYP.

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Introduction

Per-Arnt-Sim (PAS) domains are protein domains found throughout all three Kingdoms of life.¹ Most of these PAS domains are involved in signal transduction and function as sensors and/or transducers.^{1,2} This sensor function is often based on an altered ligand or cofactor interaction with the (apo) PAS domain, and often elicits altered protein–protein interaction. It is proposed that in various PAS domains, despite very different ligands, the mechanism of propagating the output signal to the surface of the PAS domain is conserved.^{3–5}

The prototype of the PAS fold is photoactive yellow protein (PYP),² which is a small (125 amino acids in total) water-soluble photo-active protein from the purple sulphur bacterium *Halorhodospira halophila*,⁶ which functions as a photo-receptor for a light-avoidance response in this organism.⁷ PYP displays all the important functional characteristics of PAS domains, such as signal sensing, binding of a cofactor, and signal transduction through protein–protein interaction. In *H. halophila* it interacts with an as yet poorly characterized downstream transducer protein.⁸ Furthermore, it contains a

p-coumaryl chromophore, linked to C69 *via* a thiol–ester bond.^{9–11}

In the ground state (pG) of the protein, this chromophore is in the *trans* configuration and deprotonated, with an absorption maximum at 446 nm.¹² The hydrogen-bonding network around the chromophore stabilizes the negative charge of its phenolate group.^{13,14} Upon absorption of a photon, the chromophore isomerizes across its C(8),C(9) ethylenic bond within a few picoseconds.^{15,16} Also, the hydrogen bond between the carbonyl group of the chromophore and the backbone nitrogen atom of Cys69 is disrupted,^{17,18} followed by rotation of the carbonyl group around the long axis of the chromophore to form the intermediate *I*₀.¹⁹ Through further relaxation of the chromophore, the pR₁ and pR₂ intermediates are then formed.^{20,21} Next, an intra-molecular proton transfer takes place from E46 to the phenolate moiety of the chromophore,²² thereby forming the intermediate pB'.²³ From pB', pB is formed, which is the presumed signalling state of PYP.²⁴ The recovery to the ground state completes the photocycle and includes de-protonation and re-isomerization of the *cis*-chromophore and refolding of the protein.^{25,26} A wide range of techniques have demonstrated that the formation of the signalling state of PYP is accompanied by large structural changes that lead to partial unfolding of the protein.^{20,23,24,27–33} These large structural changes are initiated by intra-molecular proton transfer and presumably assist in altering interactions with a downstream transducer.²³

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The incentive of the hydroxylic proton from E46 to migrate to the phenolate chromophore is amongst the most poorly understood aspects of the photocycle of PYP. Structural changes around the chromophore in the preceding steps of the photocycle may induce this transfer. X-ray crystallographic studies on early photocycle intermediates in PYP reveal only small structural changes, mostly around the chromophore.¹⁹ The pR state is formed as two coexisting sub-species.^{34,35} One with two hydrogen bonds from the chromophore to Y42 and E46, respectively and a second pR sub-state which lacks the hydrogen bond to E46. However, tryptophan fluorescence measurements from the unique tryptophan in PYP only correspond to a pR state with both hydrogen bonds intact.³⁶

Molecular dynamics simulations of the pR state(s) revealed that a water molecule can enter the chromophore-binding pocket *via* E46.³⁷ A subsequent study has shown that protonation of the chromophore occurs over a lower barrier if a water molecule replaces the positively charged R52.³⁸ In WT PYP the chromophore is directly protonated by E46. The water molecule can stabilize the negative charge on E46 even better by changing the position of R52. In agreement with these simulations, experiments show that dehydration of PYP strongly affects the transition from pR to pB'.³⁹ R52 appears to start moving away from the chromophore only a few nanoseconds after photon absorption.¹⁹ In all these simulations R52 has a positive charge which is important for the migration of water molecules into the chromophore-binding pocket and the protonation of the chromophore.^{37,38} This importance of the positive charge on R52, deduced from simulation studies, has not yet experimentally been confirmed. PYP mutants in which R52 is replaced by a non-charged amino acid have unperturbed spectra and photocycle kinetics.^{21,40} Indeed, in neutron crystallography of PYP, R52 is observed to be deprotonated.⁴¹

We are interested in resolving the structural changes preceding intra-molecular proton transfer in the different functional regions of PYP.⁴² Tryptophan fluorescence is particularly suited as a tool to study this for proteins in solution, as both its wavelength of maximal emission and its quantum yield are sensitive indicators of the local structure and polarity in the micro-environment surrounding the side chain of this amino acid.^{43,44} The emission maximum of tryptophan, for instance, shifts to the red when its side chain is exposed to a more hydrophilic environment. WT PYP contains a single tryptophan at position 119. The dynamical changes of its fluorescence emission have already been analysed in detail. This revealed that W119 is more exposed to an aqueous environment in the signalling state.⁴⁵ Nevertheless, W119 is still at quite some distance from the chromophore. To measure structural changes around the chromophore and in other functional regions of PYP, we have generated a series of site-directed PYP mutants in which we have mutated W119 into a phenylalanine and additionally converted a specific phenylalanine, tyrosine or histidine residue into tryptophan (*i.e.*, 'single tryptophan' mutants). In Fig. 1, the structure of PYP is shown with the mutated residues highlighted. Accordingly, we can probe local changes in protein structure specifically at

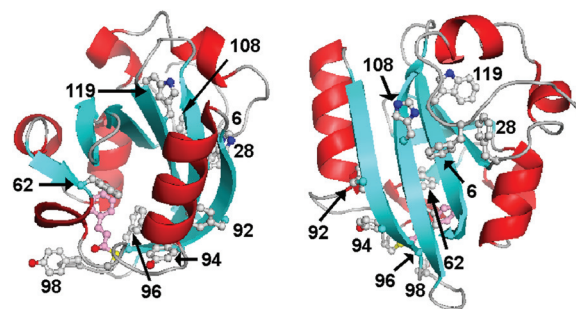


Fig. 1 Structure of PYP (2phy.pdb)¹³ with the locations of the mutated residues.

these mutated sites. Here we present the initial characterization of these mutants to determine their suitability for follow-up studies. Suitable mutations should have a minimal impact on PYP function and the fluorescence characteristics of the introduced tryptophan should change measurably during photocycle transitions. Our initial characterization assayed the photocycle recovery kinetics and the changes in the tryptophan fluorescence characteristics between pG and pB at pH 6 and 8. This revealed several unexpected yet interesting characteristics in some of the mutants.

Experimental

Site-directed mutagenesis

Site-directed mutagenesis was performed with the Quick-Change kit (Stratagene) and using the plasmid pHisp as a template.⁴⁶ The sequences of the mutagenic sense primers are listed in Table 1. The corresponding anti-sense primer is the reverse-complemented of the sense primer. The mutation was confirmed by DNA sequencing.

Purification of photoactive yellow protein

WT PYP and its mutant derivatives were produced and isolated as described previously for WT PYP.⁴⁶ Apo-PYP was reconstituted with the 1,1'-carbonyldiimidazole derivative of *p*-coumaric acid, as described elsewhere.³² The reconstituted holoproteins were purified by using a Pharmacia Äkta FPLC system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in two subsequent steps, with Ni-affinity chromatography and anion exchange chromatography, respectively. For WT PYP, in the Ni-affinity chromatography, 20 mM sodium phosphate/acetate buffer, pH = 7.0, containing 150 mM NaCl, was used as the loading buffer. The proteins were eluted with a pH gradient by using the same buffer with a pH of 4.0 as the elution buffer. Immediately after Ni-affinity chromatography, the proteins were dialyzed against 50 mM Tris buffer, pH 8.0. Anion exchange chromatography was performed with 20 mM Tris, pH 8.0, as the loading buffer and a gradient of 0 to 1 M NaCl. For PYP mutants in the Ni-affinity chromatography 20 mM sodium phosphate buffer, pH = 7.0 was used with an imidazole gradient up to 0.5 M for protein elution. For dialysis and

Table 1 Primer sequences

Mutation	Sense primer
F6W	GACAAAATGGAACACGTCAGCCTGGGGTAGCGAGGACATCG
F28W	CGACGGCCTGGCCTGGGGCGCCATCCAGC
F62W	GGTCATCGGCAAGAAGCTGGTTCAAGGACGTGGCC
F92W	GCAACCTGAACACGATGTGGGAGTACACCTTCGATTACC
Y94W	GCAACCTGAACACGATGTTCGAGTGGACCTTCGATTACCAAATGACGCCAC
F96W	CGATGTTCGAGTACACCTGGGATTACCAAATGACGCC
Y98W	CGATGTTCGAGTACACCTTCGATTGGCAAATGACGCCACGAAGG
H108W	CCCACGAAGGTGAAGGTGTGGATGAAGAAGGCCCTCTCCG
W119F	CCGGCGACAGCTACTTCGTCTTCGTCAAGCGC

anion exchange chromatography the same buffers were used as for WT PYP. The purified holo-proteins were used without removal of the genetically introduced N-terminal hexa-histidine containing tag. The purity index (OD_{280}/OD at maximum) of WT PYP, W119F, and the 'single-Trp' mutants was better than 0.5, except for F28W/W119F, F62W/W119F, F96W/W119F (see the Results section). The 'double-Trp' mutants have purity index better than 0.7.

Transient ms/s UV/Vis spectroscopy

UV/Vis spectra were recorded from 250 to 600 nm, using an HP8453 UV/Vis spectrophotometer. For time-resolved spectra an integration time of 0.1 s was used. Measurements were performed at 25 °C in 20 mM Tris buffer, pH 8.0. PYP samples have an OD = 0.5 at their absorbance maximum. A Schott KL1500 LCD lamp was used to illuminate the sample for 5 s. The ground state recovery rate was determined by fitting a bi-exponential function through the recovery part of the data, using techniques described elsewhere.⁴⁷

Steady-state fluorescence measurements

Steady-state fluorescence spectra were recorded on an Olis DM45 spectrofluorimeter (equipped with a 150 W Xe Arc lamp) and an AMINCO Bowman Series 2 Luminescence spectrometer (with a 450 W Xe lamp). The excitation wavelength was set at 295 nm (2.4 nm bandpass) and emission was recorded from 300 to 580 nm (2.4 nm bandpass), in a buffer containing 20 mM acetic acid, 20 mM Na₂HPO₄, 20 mM Tris, 20 mM boric acid, and NaCl to set the ionic strength at 250 mM. The pH was adjusted to 6.0 with 1 M HCl or to 8.0 with 1 M NaOH. The PYP samples were analysed at room temperature and at $OD_{max} = 0.5$. The samples were illuminated with LED light of 462 nm (FWHM: 22 nm; light intensity in the sample is $\sim 120 \mu\text{Einstein m}^{-2} \text{s}^{-1}$) to produce a pB/pG steady state mixture. The contribution of the pG component in the steady state was determined with UV/Vis measurements. The recorded spectra were corrected for emission of the buffer and absorption at the excitation wavelength. The pG component of the pB/pG steady state spectra is subtracted from the recorded spectra. The fluorescence quantum yield of mutant proteins is determined by comparing their fluorescence between 310 and 400 nm to that of PYP WT in the pG state at pH 8.0, for which it is known that the quantum yield is 0.01 at 295 nm excitation.⁴⁵

Results

One way to follow (the dynamics of) structural changes in a protein is *via* analysis of tryptophan fluorescence. Upon excitation at 295 nm, the emission spectrum in the region from 300 to 400 nm reports about the micro-environment of its tryptophan residue(s).^{43,44} As expected, the fluorescence of PYP mutant W119F, in which the only tryptophan is replaced by a phenylalanine, shows minor emission in this wavelength range (see below). The chromophore of PYP emits fluorescence in a different spectral range, *i.e.*, between 450 and 600 nm.^{48,49} Upon excitation of the aromatic amino acids, absorbed excitation is transferred partially onto the chromophore *via* Förster resonance energy transfer, which then leads to emission by the chromophore above 450 nm.⁵⁰

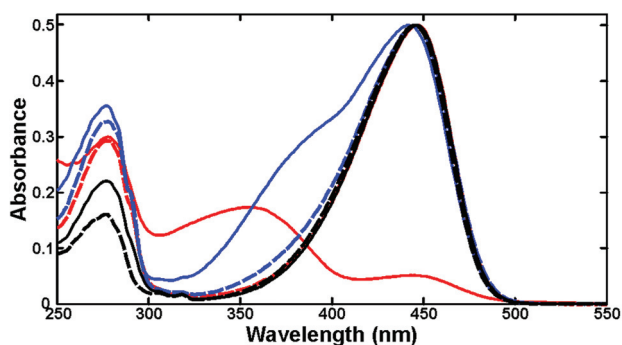
Characterisation of PYP mutants

We created a series of 'single tryptophan' PYP mutants in which we have mutated W119 into a phenylalanine and converted a selected phenylalanine, tyrosine or histidine residue into tryptophan. All the mutant proteins were overproduced in *Escherichia coli*, purified and characterized with respect to their UV/Vis spectra, using well established methods for this protein.^{25,40,46,51,52} PYP mutants F6W/W119F, F92W/W119F, Y94W/W119F, Y98W/W119F, H108W/W119F, and W119F have similar absorption spectra as the wild type protein (Fig. S1, ESI[†]), be it that the absorption maximum of several mutants has shifted a few nm. Their absorption maxima are listed in Table 2. The absorption spectrum of F96W/W119F is slightly different from WT. Its visible absorption peak is broadened at the high-energy side. In addition, its 280/446 nm absorption ratio is relatively high and its absorption maximum is slightly blue-shifted. All in all, this suggests that F96W/W119F is a less stable protein, which is in agreement with the observed stabilisation of its main absorption peak by the kosmotropic salt (NH₄)₂SO₄ (Fig. S2, ESI[†]) and a relatively high chromophore fluorescence quantum yield (Fig. S1, ESI[†]).

The absorption spectra of F28W/W119F and F62W/W119F differ from the spectra of WT as shown in Fig. 2. Surprisingly, the spectra of the single mutations F28W and F62W are unaltered. F62W/W119F has a shoulder at 390 nm in its main absorption band. For Y42 mutants (Y42F, Y42A, and Y42W) this has been observed before.^{53–55} The shoulder in the absorption spectrum of F62W/W119F has several

Table 2 Recovery kinetics

PYP mutant	Absorption maximum	Recovery rate		
		k_1 (s ⁻¹)	k_2 (s ⁻¹)	Fraction k_1
WT	446 nm	2.42	0.67	0.90
F6W/W119F	445 nm	2.11	0.26	0.96
F28W/W119F	355/445 nm	0.12	—	1
F62W/W119F	442 nm	0.21	0.010	0.96
F92W/W119F	444 nm	0.0088	0.0054	0.58
Y94W/W119F	445 nm	0.83	0.58	0.79
F96W/W119F	438 nm	0.0036	0.0098	0.88
Y98W/W119F	445 nm	0.77	0.22	0.90
H108W/W119F	445 nm	2.13	0.33	0.97
W119F	445 nm	1.29	0.69	0.89

**Fig. 2** Normalized absorption spectra of WT PYP (black solid), F28W (red dashed), F28W/W119F (red solid), F62W (blue dashed), F62W/W119F (blue solid), and W119F (black dashed) in 20 mM Tris, pH 8.0.

characteristics in common with the shoulder of the main absorption band of Y42F,⁵⁶ such as only a small pH dependence and the fact that the main peak can be stabilized by (NH₄)₂SO₄ (ESI[†]). On the other hand, the high-energy shoulder of F62W/W119F has high chromophore fluorescence (Fig. S1, ESI[†]). F28W/W119F in contrast has decreased absorption around 446 nm and a peak at 355 nm at neutral pH. The absorption at 355 nm is typical of the protonated chromophore.⁹ Indeed, the chromophore in F28W/W119F has a pK around 10 (ESI[†]), *i.e.*, much higher than WT PYP.⁵⁷ This large pK shift makes W28 unsuitable as a reporter for structural changes. Therefore, we did not use this protein in subsequent fluorescence experiments.

F62W has high chromophore fluorescence (Fig. S1B, ESI[†]). Also, addition of 3.0 M (NH₄)₂SO₄ leads to a small increase in absorption at 446 nm and a reduction in absorption between 340 and 400 nm (Fig. S2, ESI[†]). On the other hand, we do not observe a shoulder in its absorption spectrum (Fig. S1B, ESI[†]). The high fluorescence and stabilisation by (NH₄)₂SO₄ suggests that F62W contains a very small fraction of protein in the conformation that causes the shoulder in the absorption spectrum of F62W/W119F. Also, the single mutants W119F and F28W have unperturbed absorption spectra. It is therefore the combination of the two mutations that gives these large effects on the UV/Vis spectra of these proteins.

All mutants were photoactive, with transient absorption spectra similar to those of WT PYP. Their recovery rate was analysed using a bi-exponential decay function. The rate of the recovery reaction in all ‘single tryptophan’ mutants is decreased, as can be seen in Table 2. A large deceleration of the recovery rate is only observed in F28W/W119F (20 fold), F62W/W119F (12 fold), F92W/W119F (275 fold), and F96W/W119F (680 fold). These large decreases may affect the functional conformational changes in the protein; especially in light of the fact that this series includes all mutants with altered absorption spectra.

Tryptophan fluorescence

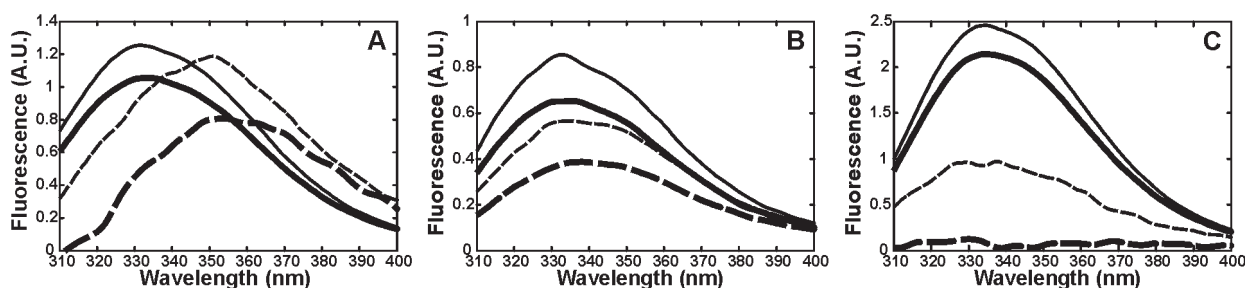
We measured the tryptophan fluorescence from the ‘single tryptophan’ mutants, WT, and mutant W119F in the pG and the pB state, at pH 6.0 and pH 8.0. The fluorescence of the pB state is calculated from the spectrum of a light induced pB/pG steady state mixture *via* correction for the pG contribution. The fluorescence is corrected for small variations in absorption at the excitation wavelength of 295 nm. From the corrected emission in the range from 310 to 400 nm we calculated the quantum yield, using the quantum yield of WT PYP in pG at pH 8.0 (*i.e.*, $\Phi = 0.01$) as the reference.⁴⁵ The quantum yields are listed in Table 3 together with the emission maxima. Mutant W119F, which lacks tryptophan residues, has a quantum yield of 0.005 at pH 8.0. Comparison of excitation spectra of W119F and WT PYP with emission measured at 328 nm (Fig. S4C, ESI[†]) indicates that the remaining emission of W119F is from tyrosine residues.

PYP mutants F6W/W119F, F96W/W119F, and H108W/W119F have high enough quantum yields to use their tryptophan fluorescence as a reporter for structural changes. The tryptophan residue in each of these three mutant proteins senses changes in its environment upon pB formation; however, these changes are of a different nature in the three proteins. The tryptophan emission spectra of these three mutants are shown in Fig. 3. For W6 at pH 8.0 the quantum yield is decreased and the emission maximum is red-shifted in the pB state compared to pG. At pH 6.0 these emission maxima show a small blue shift, as compared to pH 8.0. Upon formation of pB, the quantum yield of W96 fluorescence decreases and its spectrum shifts slightly to the red at pH 8 and 6. For W108 the quantum yield is decreased in the pB state as compared to the pG state at pH 6.0, while at pH 8.0 tryptophan fluorescence disappears in pB. Thus we also observe differences in the fluorescence of W6, W96, and W108 between pH 6.0 and pH 8.0. For all three mutant proteins the fluorescence quantum yield is slightly increased at pH 6.0 compared to pH 8.0 in both pG and pB with a difference in the position of the maxima of at most 3 nm between the two pH values. The fluorescence of W6 and W108 has larger differences in the pB state between pH 6.0 and pH 8.0 than in the pG state. The fluorescence of W96, however, has a similar pH dependence in the pG and pB states.

F62W/W119F, F92W/W119F, F94W/W119F and F98W/W119F have a quantum yield that is lower than, or close to,

Table 3 Fluorescence quantum yield and emission maximum

PYP mutant	pH 8.0				pH 6.0			
	pG		pB		pG		pB	
	Quantum yield	Emission max.	Quantum yield	Emission max.	Quantum yield	Emission max.	Quantum yield	Emission max.
WT	0.010	323	0.007	331	0.015	321	0.015	327
F6W/W119F	0.012	333	0.009	354	0.014	331	0.014	351
F62W/W119F	0.003	313	0.002	312	0.003	313	0.003	314
F92W/W119F	0.006	327	0.003	320	0.007	361	0.006	361
Y94W/W119F	0.006	336	0.003	340	0.007	340	0.005	338
F96W/W119F	0.008	336	0.005	338	0.010	333	0.007	335
Y98W/W119F	0.003	332	0.004	339	0.003	333	0.004	342
H108W/W119F	0.024	335	0.001	—	0.027	334	0.011	338
W119F	0.005	330	0.005	330	0.005	357	0.003	344

**Fig. 3** Tryptophan fluorescence emission spectra of PYP mutants F6W/W119F (A), F96W/W119F (B), and H108W/W119F (C) at pH 6.0 (light) and 8.0 (bold) in pG (solid) and pB state (dashed). Excitation is at 295 nm.

the quantum yield of W119F. These low quantum yields make these mutant proteins less suitable as reporters for structural change. Upon excitation of the aromatic amino acids in PYP, the emission above 450 nm is caused by FRET from tryptophan to the chromophore.⁵⁰ The emission spectrum after excitation at 295 nm of F62W/W119F PYP shows high chromophore-, but negligible tryptophan emission (Fig. S4, ESI†). This high chromophore emission can be caused by a more efficient FRET from tryptophan to the chromophore in combination with a high chromophore quantum yield. Indeed, we measured a high chromophore quantum yield for F62W/W119F, especially upon excitation of the shoulder in the main absorption peak (Fig. S1, ESI†). The low emission between 320 and 380 nm is in line with an efficient FRET from the tryptophan to the chromophore. Such an efficient energy transfer is presumably caused by the shoulder in the absorption spectrum of pG. This shoulder causes large spectral overlap with the tryptophan emission. Decreasing the shoulder by addition of 2 M $(\text{NH}_4)_2\text{SO}_4$ indeed decreases the amount of FRET, resulting in a 44% decrease of the emission at 492 nm (Fig. S5, ESI†). However, the emission between 300 and 400 nm does not increase in parallel.

F92W/W119F, Y94W/W119F and Y98W/W119F have negligible tryptophan fluorescence, but all three mutant proteins show normal chromophore fluorescence. Upon excitation at 280 nm tyrosine residues emit fluorescence at 300 nm. In most proteins the tyrosine fluorescence is transferred *via* FRET to

the tryptophan residues.⁵⁸ In F62W/W119F, F92W/W119F, Y94W/W119F and F96W/W119F this tyrosine-to-tryptophan FRET is not complete (Fig. S6, ESI†). In unfolded PYP, W92 and W94 do have tryptophan fluorescence (Fig. S7, ESI†), showing that the environment of W92 and W94 in native PYP is causing the absence of the fluorescence emission.

Discussion

In order to be able to use the aromatic amino acid tryptophan as a reporter of the local polarity within the PYP protein, a series of eight ‘single tryptophan’ mutants was constructed, while the corresponding eight ‘double tryptophan’ mutants, and a PYP mutant without tryptophan, served as the controls. The tryptophan fluorescence of these ‘single tryptophan’ mutants was analysed in detail.

W119F mutation

W119 is located relatively far from the chromophore. Carroll *et al.* have shown, with laser excitation of W119F PYP, that the photocycle dynamics and chromophore photochemistry are essentially the same for this mutant and for WT PYP.⁵⁹ This is in agreement with our present results. The mutation W119F causes only small changes in visible absorption spectra and in the recovery rate of the protein. In addition, we show in the ESI† that W119F is a stable protein with a slightly higher

chromophore fluorescence compared to WT PYP. Another mutation of W119, W119G, also does not alter the nature of the photocycle dynamics and the chromophore environment of PYP.^{21,60–63} It appears therefore that we can safely use the W119F mutation to create ‘single tryptophan’ mutants. Nevertheless, W119 is a highly conserved residue,⁴² possibly because of its function in UV sensing,⁵⁹ rather than being essential for the chromophore photochemistry and photocycle dynamics (see further below).

Absorption and kinetic characteristics: small changes

The mutations F6W, Y94W, Y98W, and H108W, also in combination with W119F, cause only small changes in visible absorption spectra and in the recovery rate of the protein. Here it is of interest to note the following. The phenyl ring of F6 has cation/ π interaction with K123. Loss of this interaction in mutants F6A and $\Delta 6$ (in which the six N-terminal residues have been deleted) slows down the recovery rate of the protein 50 times.⁶⁴ Other mutations at the same position (*i.e.*, F6D, F6H, F6L, and F6Y) have smaller effects on the recovery rate.⁶⁴ The small inhibition of the recovery rate of mutant F6W suggests that the cation/ π interaction with K123 is intact in this mutant and that substitution of F6 with tryptophan has no large effect either on the photocycle kinetics or on the conformation of PYP.

In H108F PYP, the kinetics of the photocycle are slightly affected: the recovery rate is slightly decreased.⁶⁵ FTIR measurements suggest that this is due to smaller structural changes upon the formation of the pB state.⁶⁶ The H108W mutant has also a slightly lower recovery rate than WT.

Absorption and kinetic characteristics: significant changes

The chromophore in PYP F28W/W119F has a much higher pK than WT PYP. At pH 8, most F28W/W119F protein molecules contain a protonated chromophore. An increased pK of the chromophore is also observed in G29A.⁶⁷ G29 is the first amino acid of the PAS core of PYP (*i.e.*, F28 is the last one of the N-terminal cap).⁴² In G29A, E46 is displaced by steric hindrance caused by the increase in size of A29. Likely, this displacement of E46, which results in changes of its hydrogen bonding, causes the increased pK of its chromophore.⁶⁷ A possible explanation of the high pK in F28W/W119F therefore is that the mutation F28W also gives a small change in the position of W28 and G29. Adding the mutation W119F may result in a larger change in conformation around residue G29, which then may lead to displacement of E46, and results in a less stable protein with a high pK of its chromophore.

PYP F62W/W119F has a shoulder at 390 nm in its main absorption band. Such an intermediate spectral species is also observed in several Y42 mutants,^{53–55} the best studied of which is Y42F. In these mutants the chromophore in the dark (pG) state is in equilibrium between the yellow form and the blue-shifted intermediate spectral form. This equilibrium can be shifted towards the yellow form by addition of kosmotropic salts.⁵⁶ This stabilisation of the yellow form is also seen in F62W/W119F. The equilibrium between yellow and

intermediate spectral pG species in F62W/W119F and in Y42F is relatively insensitive to pH.⁵⁶ The explanation for the high fluorescence quantum yield of the blue-shifted pG form in F62W/W119F is different: whereas the normalized fluorescence excitation and absorption spectrum of Y42F coincide, including the ratio between the main peak and the shoulder,⁵⁶ which is in agreement with the supposed photoactivity of the intermediate species of Y42F,⁶⁸ the high fluorescence of the blue shoulder in F62W/W119F suggests minimal or no photoactivity for this blue-shifted pG species. These differences in chromophore fluorescence between the intermediate spectral species in F62W/W119F and Y42F indicate that they have a different spatial structure. El-Mashtoly *et al.* have reported that in WT PYP in the pG state (in the dark) 5% of the protein carries the chromophore in the intermediate spectral form.⁶⁹ Considering the fact that the structure of the blue shifted pG state of F62W/W119F differs from that of the corresponding intermediate of Y42F, this low-abundance intermediate of WT PYP may also have a low photoactivity.

F62 is part of the chromophore-binding pocket and points towards the phenyl ring of the chromophore at a distance of 3.9 Å.¹³ Presumably, the mutation F62W slightly disturbs the position of the chromophore and/or its hydrogen bonding network, thus resulting in a slightly increased fraction of the blue-shifted pG intermediate. This effect is then enhanced in the F62W/W119F double mutant. The small increase in chromophore fluorescence for W119F PYP suggests that it is slightly less stable than WT PYP (see above). Possibly, the F62W/W119F double mutant is destabilized synergistically. In Y42F, this intermediate most likely originates from the stronger and shorter hydrogen bonds between E46 and the chromophore, resulting in protonation of the chromophore.⁶⁸ The shoulder of F62W/W119F has a similar absorption maximum of the intermediate as in Y42F. This indicates that the chromophore in the intermediate spectral form in F62W/W119F is also protonated, be it that the structure of this intermediate is not identical in the two proteins, as is evident from their fluorescence properties (see above).

The residues F92, Y94 and F96 are located in the $\beta 4$ strand and form an aromatic array which points towards the chromophore. Y98 is also part of this aromatic array, but is located in the adjoining loop and its side chain is exposed to the solvent. Alanine substitution of the aromatic residues in the $\beta 4$ strand results in ~ 100 , ~ 30 , ~ 3000 , and ~ 4 fold lower recovery rate than in WT PYP, for F92A, F94A, F96A, and Y98A, respectively.⁷⁰ Tryptophan substitution of these residues causes a lower decrease in recovery rate, except for F92W. The recovery rates of F92W, Y94W, F96W, and Y98W are 144, 1.6, 457, and 1.4 times lower than WT, respectively. Also the absorption spectra of these mutants show smaller changes compared to those of the corresponding alanine substitution mutants. This re-emphasizes the importance of iso-steric replacements. However, the mutations F92W and F96W do cause a substantial decrease of the recovery rate (Table S1, ESI[†]). F92W and F92W/W119F show normal absorption spectra, in contrast to F96W and F96W/W119F (Fig. S1, ESI[†]). F96W has a 6 nm blue

shifted absorption maximum and its absorption peak is broadened at the high-energy side. F96A also has a blue-shifted and broadened absorption peak⁷⁰ and these changes are larger than for F96W. The broadened absorption peak is most likely caused by the decreased stability of the protein (see the Results section). This shows that F96W and F96W/W119F are more stable than F96A, be it still less stable than WT PYP.

Photocycle-modulated tryptophan fluorescence

Signalling state formation in the PYP mutants F6W/W119F, F96W/W119F, and H108W/W119F leads to changes in tryptophan fluorescence, both with respect to emission maximum and fluorescence quantum yield. The red shift in the emission of W6 and W96 suggests that their side chains are more exposed in the pB state than in the pG state. W6 is located in the N-terminus. In pG, W6 is located close to the central β -sheet.¹³ The N-terminal cap moves away from the β -sheet upon formation of the pB state.³⁰ From a study with fluorescein labelled PYP in the N-terminal cap it is known that the structural changes in the N-terminus start in the regions around residue 5 upon formation of pB' and they are propagated in pB to the rest of the N-terminal cap.⁷¹ In the pB structure determined with DEER spectroscopy, NMR, and TR-SAXS/WAXS, the first 7 N-terminal residues are separated from the rest of the protein and completely exposed to the solvent,⁷² whereas in the ground state residue 6 is close to the β -sheet and the N-terminus is more compact.¹³ This is in line with the large red shift we observe for W6 upon the formation of pB. W96 is located in the chromophore-binding pocket. The red shift in the fluorescence emission of W96 is consistent with the exposure of the chromophore-binding pocket to the solvent.^{23,32}

Upon formation of the pB state, the environment of W108 is changed such that its fluorescence is efficiently quenched. In the pB state, residue 108 is close to N89, K110, and K123.⁷² Asparagine and lysine residues are known as efficient quenchers for tryptophan fluorescence.⁷³ K110 and K123 are at a larger distance from residue 108 in the ground state than in the signalling state. On the other hand, N89 is located closer to residue 108 in pG, compared to the pB state. As to quenching, not only distance is important, but also orientation,⁷⁴ the question of which of these three residues is most important cannot be answered based on the available data. Alternatively, the chromophore could act as a quencher of tryptophan fluorescence. For WT the orientation of the chromophore and W119 is favourable for quenching in pG, but not in pB.⁷⁵ W108 has a different orientation than W119 with respect to the chromophore, and the spectral overlap between W108 and the chromophore will increase upon the formation of the signalling state. An efficient quenching of the W108 fluorescence by the protonated chromophore in pB is one possible explanation for the large decrease in fluorescence.

The changes in the environment of W6 and W108 between the pG and pB states are larger at pH 8 than at pH 6. The signalling state of PYP exists in multiple subspecies of which the degree of population depends on pH.³¹ At pH 8 a 'medium

pH' pB sub-species is dominant, while at pH 6 also a significant amount of the 'low-pH' pB species (pK 5.9) is present.⁵² The 'low pH' pB sub-species presumably is unfolded to a smaller extent than the 'medium pH' pB sub-species.

Quenching of tryptophan fluorescence

Surprisingly, PYP mutants F62W/W119F, F92W/W119F, Y94W/W119F, and Y98W/W119F emit (almost) no tryptophan fluorescence. A fraction of the pG state of F62W/W119F is present as the blue-shifted pG intermediate species. The absorption maximum of this intermediate species (~ 390 nm) causes a large overlap with the emission of tryptophan, which allows efficient FRET between them. The emitted tryptophan fluorescence in this mutant is (almost) completely transferred to the chromophore. An increase in spectral overlap, which thereby leads to efficient FRET, is also observed for the acid-denatured state of PYP, pB_{dark}.⁵⁰ Stabilisation of the main absorption peak of F62W/W119F by 2 M $(\text{NH}_4)_2\text{SO}_4$ leads to lower chromophore fluorescence but the tryptophan fluorescence does not increase in parallel. Either the intermediate species is still too abundant, or the fluorescence of W62 is also significantly quenched in the ground state conformation. We have not measured the fluorescence of W62 at higher concentrations of $(\text{NH}_4)_2\text{SO}_4$, because addition of this salt leads to precipitation of the protein.

For W92, W94, and W98 it is not so clear why there was no tryptophan emission. Therefore, we performed extra measurements, of which the results are shown in the ESI.† The absence of tryptophan fluorescence in F92W/W119F and Y94W/W119F is caused by the location and/or orientation of the tryptophan residues. Unfolding of these proteins by denaturation leads to the emergence of tryptophan fluorescence from W92 and W94. To explain this, knowledge about the exact position of potential quenchers is crucial. The amino acids lysine, tyrosine, glutamine, asparagine, glutamate, aspartate, cysteine, and histidine are all known as efficient quenchers of tryptophan fluorescence.⁷³ A part of the residues that quench the fluorescence of W92 and W94 are located next to these residues, because the quantum yield of W92 and W94 in unfolded protein is lower than the yield of unfolded WT PYP (Fig. S7, ESI†). This indicates that W92 and W94 are subject to more quenching from nearby amino acids than W119. Good candidates for the quenching of the fluorescence from W92 and W94 are E93, Y94 (for W92) and Y98.

Also the chromophore of PYP can be a quencher of the tryptophan fluorescence. Consistent with this, it is observed that all the tryptophan residues that show low fluorescence are located close to the chromophore. In WT PYP, the fluorescence of W119 is partly quenched by the chromophore.⁷⁵ W62, W92, W94, and W98 are located closer to the chromophore than W119. On the other hand, not only distance determines the efficiency of FRET, but also the orientation is important.⁷⁵ Only one tryptophan located near the chromophore (*i.e.*, W96) emits fluorescence, but with a lower quantum yield than W119.

Mutants of interest

The three best-suited single tryptophan mutants for ultrafast studies on intra-molecular proton transfer in PYP are: F6W/W119F, F96W/W119F and H108W/W119F. Of these, W6 and W108 are located at a relatively large distance from the chromophore but W96 is located in the chromophore-binding pocket. Although F96W/W119F has slightly altered absorption characteristics and a decreased recovery rate, it is the best-suited mutant from this series to test – in a follow-up study – the detailed dynamics of the fluorescence of W96 in the ns to ms time domain, *i.e.*, during the lifetime of pR₁, pR₂, pB', and pB. The other single tryptophan mutants with the aromatic amino acid positioned close to the chromophore showed severely quenched tryptophan emission.

Not only the presence of water molecules will influence the tryptophan fluorescence. In WT PYP the fluorescence of W119 is quenched by energy transfer to the chromophore, which depends on the mutual orientation and spectral overlap.⁷⁵ Both, the orientation and spectrum of the chromophore, change during the photocycle. Otto *et al.*⁷⁵ have calculated the spectral overlap and the angular factor from the transition dipole moment orientations for WT PYP in pG and pB' and predicted tryptophan fluorescence lifetimes, which corresponded well with measured values.⁷⁵ The availability of crystal structures of photocycle intermediates and of structures determined with MD and QM/MM simulations provides an opportunity to predict tryptophan fluorescence based on calculations of quenching by the chromophore, other residues, and the presence of water molecules. Accordingly, one may resolve dynamically which intermediate structure emits measured tryptophan fluorescence. Mutants F6W/W119F and H108W/W119F are of interest to test the feasibility of this approach.

Special position of W119

In most proteins that contain tryptophan and tyrosine residues, the tyrosine fluorescence is transferred to the tryptophan residues *via* FRET.⁵⁸ This happens also in WT PYP, but not in F62W/W119F, F92W/W119F, Y94W/W119F and F96W/W119F. In these PYP mutants this tyrosine-to-tryptophan transfer is incomplete. This shows that position 119 for tryptophan is better for FRET from tyrosine residues to the tryptophan, as compared to alternative locations of a tryptophan residue near the chromophore. Furthermore, fluorescence from W119 is less quenched by nearby residues than that from W92 and W94, as is easily demonstrated in the denatured (*i.e.*, unfolded) form of these proteins. The position of tryptophan in WT PYP is therefore optimal for the collection of UV photons. FRET from W119 to the chromophore can then lead to initiation of the photocycle, making PYP also a UV/blue light photoreceptor.⁵⁹ From our results it is clear that the position of the single tryptophan residue is important for its fluorescence properties and that tryptophan at position 119 has an efficient FRET from the tyrosine residues and a relative high emission. That the position of the tryptophan residue is important for PYP function is also suggested by the high

degree of sequence conservation of this residue in PYP proteins.⁴²

Conclusions

We have analysed tryptophan fluorescence of a series of 'single tryptophan' mutants of photoactive yellow protein (plus a series of control proteins) in both the ground state (pG) and the signalling state (pB) of this eubacterial photosensory receptor protein. This analysis has revealed that in particular the mutants F6W/W119F, F96W/W119F, and H108W/W119F are well suited to monitor changes during the photocycle of PYP. Of these F96W/W119F seems to have the most potential in future studies to unravel the temporal dynamics of the change in polarity (or entry of water molecules) in(to) the chromophore-binding pocket when the PYP protein transits into its signalling state. Furthermore, the current study independently confirmed the specific functionality of tryptophan 119 in the absorption of UV light by this photoreceptor protein.

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