Electronic Supplementary Material (ESI) for Photochemical & Photobiological Science

Electronic Supplementary Information

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

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Introduction

In this supplementary information is made available derived from additional experiments with the series of 'single-Trp' and 'double-Trp' mutants, and as control the W119F mutant and WT PYP.

Experimental

Sample preparation

Buffers with varying amounts of $(NH_4)_2SO_4$ were prepared by mixing 20 mM Tris buffer pH 8.0 with and without 3.0 M $(NH_4)_2SO_4$. The measurements with $(NH_4)_2SO_4$ were done within one hour, to limit precipitation. For spectra at pH 6.0, 8.0, 9.0 and 10.0 a buffer was used that contained 20 mM Acetic acid, 20 mM Na_2HPO_4, 20 mM Tris, 20 mM Boric acid, and NaCl to set ionic strength at 250 mM, using calculations described elsewhere.[1] The pH was set with 1 M HCl or 1 M NaOH. The pH of the sample was checked in the measurement set-up of the spectrometer. PYP proteins were unfolded in 20 mM Tris buffer with 1 % SDS at pH 8.0. Samples with an OD at 280 nm of 0.26 were incubated for 1 hour at room temperature. As a control for complete unfolding, samples were characterized with UV/Vis spectroscopy. Unfolded PYP absorbs around 346 nm instead of 446 nm. After addition of the detergent the OD at 446 nm was less than 0.0016.

Transient ms/s UV/Vis spectroscopy

UV/Vis absorbance is recorded as described in the main article. PYP samples were prepared as described above, or in 20 mM Tris buffer pH 8.0.

Steady-state fluorescence

Steady-state fluorescence spectra were recorded using an Olis DM45 spectrofluorimeter with a 150 W Xe Arc lamp and an AMINCO Bowman Series 2 Luminescence spectrometer (with a 450 W Xe lamp). Tryptophan fluorescence spectra are recorded as described in the main article. For chromophore fluorescence, the excitation wavelength was set to the absorption maximum of the used protein (see table 2 main article and table S1), and emission was recorded from 450 to 550 nm. The bandpass for the excitation and emission beams was set at 0.50 nm and 2.4 nm, respectively. For mutants F92W, F92W/W119F, F96W, and F96W/W119F the intensity of the excitation light was decreased to minimize bleaching. WT PYP was measured with this low excitation intensity as reference. Excitation spectra of the chromophore were recorded from 250 nm to 485 nm, with the emission set at 495 nm. The same bandpass settings were used as for the chromophore emission spectra. Tyrosine residues were excited at 280 nm (with a 2.4 nm bandpass), and emission was recorded from 290 to 400 nm (with 2.4 nm bandpass). Excitation spectra of tryptophan/tyrosine residues were recorded from 250 nm to 310 nm, with the emission set at 328 nm. The same bandpass settings were used as for the tryptophan fluorescence spectra, described in main article. Buffer for all fluorescence measurements was the same as for tryptophan fluorescence (see main article), except where otherwise indicated in sample preparation (see above). The recorded spectra were corrected for emission of the buffer. The emission spectra of the buffer consist mainly of a raman peak from water and scattering from the excitation light and LED light The emission spectra were normalized via the absorption at the excitation wavelength. The chromophore excitation spectra were corrected for protein concentration at the absorption maximum. The tryptophan excitation spectra were corrected for absorption at 295 nm. The spectra of tryptophan fluorescence (emission and excitation spectra), tyrosine fluorescence, and chromophore fluorescence (emission and excitation spectra) were normalized at the emission maximum of WT PYP, in the emission spectra of tryptophan fluorescence, tyrosine fluorescence, and chromophore fluorescence, respectively.

Results

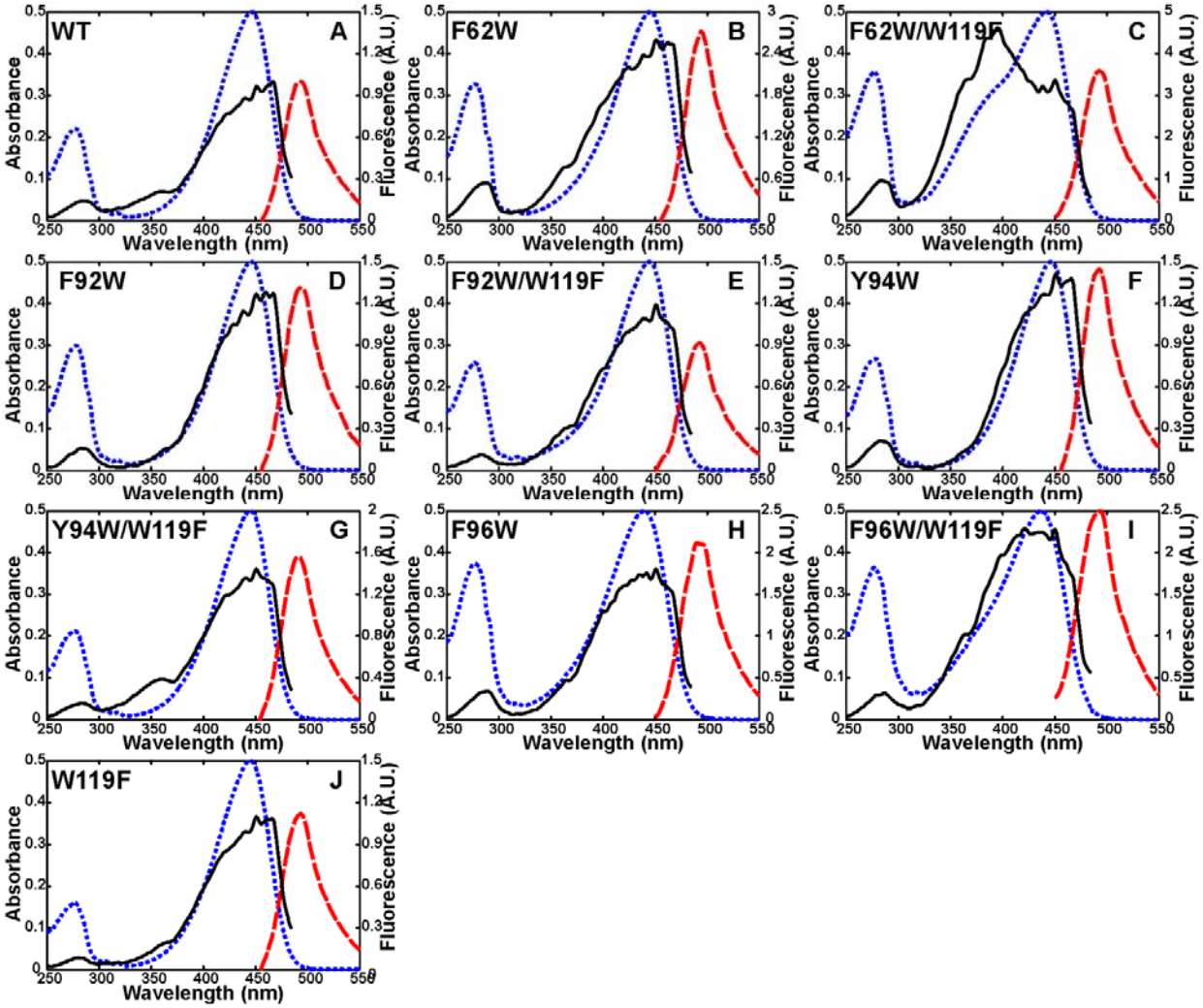

Fig. S1 Absorption spectra (dotted blue), excitation spectra of chromophore fluorescence measured at 495 nm (solid black), and emission spectra with excitation at absorption maximum (dashed red) of WT PYP (A), F62W (B), F62W/W119F (C), F92W (D), F92W/W119F (E), Y94W (F), Y94W/W119F (G), F96W (H), F96W/W119F (I), and W119F (J). Absorption spectra are normalized with their maximum at 0.5. Excitation and emission spectra are normalized at the maximum of WT PYP. All samples have pH 8.0.

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. For each 'single tryptophan' mutant also the corresponding 'double tryptophan' mutant was made, to investigate whether or not each single mutation did significantly alter the PYP photocycle characteristics. The spectral properties of the chromophore in mutants of PYP are a good indicator for structural perturbations in the protein. PYP has a low chromophore emission,[2] indicating that the PYP binding pocket is adjusted to promote photochemistry over fluorescence. Changes in the PYP binding pocket cause higher fluorescence. However, it is also possible that only a small sub-fraction of the PYP proteins in the sample causes this high fluorescence. To determine if this is the case, excitation spectra were measured. If the excitation spectrum does not follow the absorption spectrum, this reveals the presence of different conformations in the protein population. In figure S1 we show the absorption and emission and excitation spectra of WT PYP and of several mutants thereof. Excitation at the absorption maximum gives emission peaking around 493 nm for WT PYP. The chromophore emission spectra of the PYP mutants have the same shape as the spectrum of WT PYP and their maxima are between 491 and 495 nm, but differences are apparent in the quantum yield. For mutants F62W, F62W/W119F, F96W, and F96W/W119F the quantum yield is much higher compared to WT PYP. Excitation spectra of the chromophore fluorescence are measured with the emission wavelength set at 495 nm. In these excitation spectra, the excitation peak overlapped with the main absorption peak. A small additional excitation peak is present at 286 nm. This peak originates from excitation via aromatic residues, of which the fluorescence is transferred to the chromophore via FRET.

However, it cannot be excluded that also direct excitation by UV light of the chromophore occurs.
The excitation spectrum of F62W/W119F has high fluorescence at the same wavelength of the shoulder in its absorbance spectra. This is in agreement with the assumption that a fraction of the protein is in a different conformation. The high chromophore fluorescence of this different conformation suggests that the photocycle yield of this sub-fraction is low or completely absent. F62W has high chromophore fluorescence, which is relatively high upon excitation around 390 nm. This shows that a small part of this mutant protein is also in this different conformation. The main absorption peak of F96W and F96W/W119F is broadened at the high-energy side of the main absorption band and the maximum has a lower absorption which is evident from their UV/visible absorption ratio. These differences are larger in F96W/W119F than in F96W. This suggests that the former is a less stable protein, which is in agreement with its relatively high chromophore fluorescence and the broadened excitation peak.

Table S1 Recovery kinetics

PYP mutant	Absorption maximum	Recovery rate		
		k_1 (s^{-1})	k_2 (s^{-1})	fraction k_1
F6W	444 nm	*2.15*	*0.33*	0.95
F28W	446 nm	*0.37*	*0.092*	0.89
F62W	446 nm	*0.87*	*0.15*	0.96
F92W	446 nm	*0.017*	*0.0077*	0.88
Y94W	446 nm	*1.50*	*0.78*	0.81
F96W	440 nm	*0.0053*	*0.011*	0.79
Y98W	446 nm	*1.70*	*0.16*	0.88
H108W	447 nm	*1.51*	*0.31*	0.96

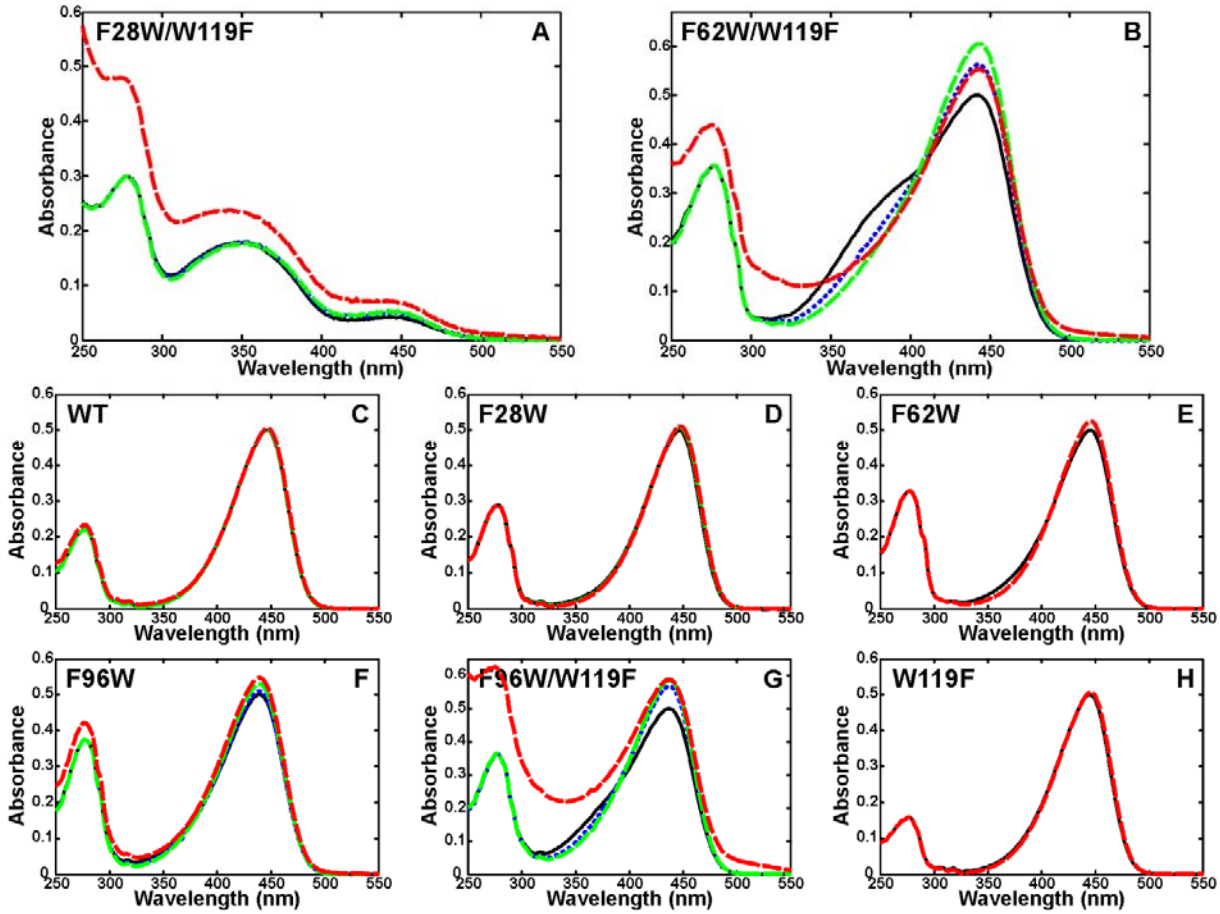

Fig. S2 Absorption spectra at different concentrations $(NH_4)_2SO_4$: 0 M $(NH_4)_2SO_4$ (solid black), 1.0 M $(NH_4)_2SO_4$ (dotted blue), 2.0 M $(NH_4)_2SO_4$ (dashed green), and 3.0 M $(NH_4)_2SO_4$ (dashed red) of F28W/W119F (A), F62W/W119F (B), WT (C), F28W (D), F62W (E), F96W (F), F96W/W119F (G), and W119F (H) in 20 mM Tris pH 8.0. In the graphs is not shown for every mutant the spectra at all $(NH_4)_2SO_4$ concentrations.

All 'single tryptophan' and 'double tryptophan' 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 mutants is decreased, as can be seen in table 2 in the main article and in table S1 for the 'double tryptophan' mutants.
Kosmotropic salts stabilize intermolecular interactions in proteins by salting out hydrophobic groups and so stabilizing proteins.[3] In PYP kosmotrope salts are able to stabilize the main absorption peak.[4, 5] Kosmotrope salt ammonium sulphate stabilizes the main absorption peak of F62W/W119F by reducing the 390 nm shoulder (Fig. S2). The corresponding single mutant, F62W, is also stabilized by addition of ammonium sulphate. The absorption at 446 nm is increased and the absorption between 340 and 400 nm reduced by this addition. This corresponds to a decreasing fraction of the protein with a different conformation from the majority of the protein. Also, ammonium sulphate stabilizes F96W and to a greater extent F96W/W119F, such that the high UV/visible absorption ratio is decreased. These experiments are complicated by the fact that several proteins precipitate in 3.0 M $(NH_4)_2SO_4$.

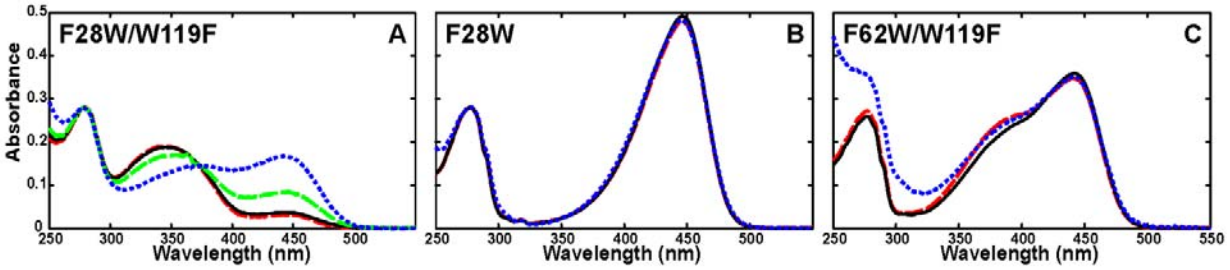

Fig. S3 Absorption spectra at different pH values: pH 6.0 (dashed red), pH 8.0 (solid black), pH 9.0 (dashed green), and pH 10.0 (dotted blue) of F28W/W119F (A), F28W (B), and F62W/W119F (C). The spectrum at pH 9.0 is only shown for F28W/W119F.

Ammonium sulphate does not stabilize the 445 nm peak of F28W/W119F. The 445 nm peak of F28W/W119F can be increased by increasing the pH (Fig. S3A). At pH 10.0 the absorption at 445 nm is higher than the absorption at 355 nm, but the spectra still show a second peak, which represents PYP with a protonated chromophore. At pH >10 the link between C69 and the chromophore spontaneously hydrolyses. Determination of a precise pK for the chromophore in the W28F/W119F mutant is therefore difficult. From the spectra in figure S3A, we estimate a pK of around 10. However, the possible presence of multiple intermediates, such as in G29A,[6] makes this estimation difficult. A pK near the pK of *p*-coumaric acid in denatured PYP of 8.8[7] is not excluded for F28W/W119F. A chromophore pK around 8.8 for F28W/W119F suggests the chromophore is fully exposed. Because of the high pK, it is not possible to convert the chromophore of all F28W/W119F proteins into the deprotonated form. Therefore tryptophan fluorescence measurements on F28W/W119F will not represent the WT environment of W28. The pK of the single mutant F28W is much lower, *i.e.*, more like the pK in WT PYP, because even at pH 6.0 the chromophore is still deprotonated (Fig. S3B). In figure S3C one sees the pH dependence of the absorption of F62W/W119F. The ratio shoulder/main peak has a small pH dependence and is optimal at pH 8.0.

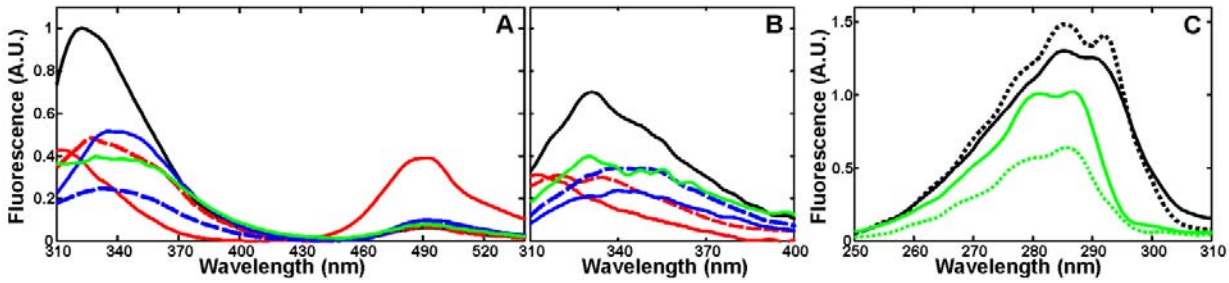

Fig. S4 Tryptophan emission spectra with excitation at 295 nm of WT PYP (black solid), F62W/W119F (red solid), F92W/W119F (red dashed), Y94W/W119F (blue solid), Y98W/W119F (blue dashed), and W119F (green solid). At pH 8.0 in pG state (A) and pB state (B). The spectra are normalized at emission maximum of WT in pG. C) Tryptophan excitation spectra of WT (black) and W119F (green) in pG at pH 8.0 (solid) and pH 6.0 (dotted). Emission is measured at 328 nm.

In table 3 from the main article is listed the fluorescence quantum yield and emission maxima of the 'single tryptophan' mutants, WT and W119F. The emission spectra for the mutants F6W/W119F, F96W/W119F, and H108W/W119F are shown in figure 3 in the main article. Figure S4 shows emission spectra, obtained after excitation at 295 nm for WT PYP and the mutants F62W/W119F, F92W/W119F, Y94W/W119F, Y98W/W119F, and W119F in the pG and in the pB state at pH 8.0. The emission between 310 and 350 nm of W119F, which lacks tryptophan residues, is lower than 40 % of the emission of WT PYP. To see what causes this remaining emission, we measured excitation spectra of W119F and WT at pH 8.0 and 6.0. These excitation spectra are shown in figure S4C. The excitation spectra of WT are in agreement with excitation of tyrosine and tryptophan residues, which has absorption maxima around 274

and 280 nm, respectively.[8] Compare to WT, W119F has a lower emission after excitation above 290 nm. This is in line with the absence of tryptophan residues in W119F. The excitation of the five tyrosine residues in W119F gives a lower emission at 228 nm than for WT. This is caused by tyrosine fluorescence being transferred via FRET to W119 in WT and not in W119F. In sum the excitation spectra of WT is in agreement with excitation of tyrosine and tryptophan residues and the spectra of W119F with only excitation of tyrosine residues. However, the chromophore absorbs also in the UV and it cannot be excluded that part of the emission at 328 nm is from the chromophore. The fluorescence quantum yield of W119F may be higher than expected for a protein without tryptophan residues. It has to be mentioned that the fluorescence is corrected for the absorption at 295 nm, which in W119F is a combination of the absorption of amino acids without tryptophan and the chromophore. For WT PYP the absorption at 295 nm is for a large part by the tryptophan residues. The difference in excitation spectra between pH 8 and 6 for WT and W119F is in the fluorescence quantum yield. The shape of the excitation spectra shows only minor differences between these pH values. This is in line with the pK of the five tyrosine residues in PYP are all above 10.[9]

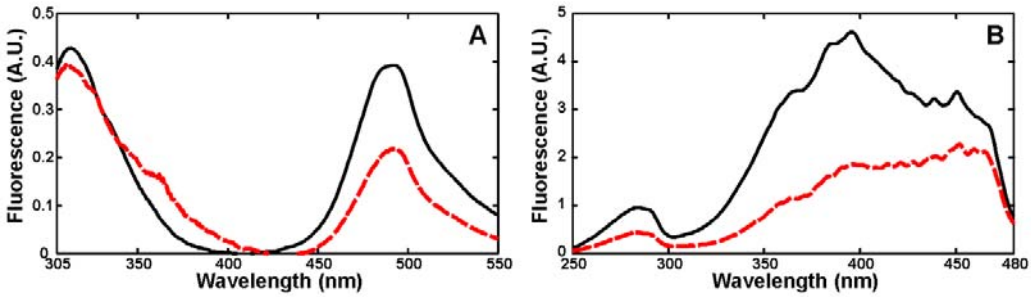

Fig. S5 A) Tryptophan emission spectra with excitation at 295 nm of F62W/W119F PYP at pH 8.0 (solid black) and in 20 mM Tris buffer pH 8.0 containing 2 M $(NH_4)_2SO_4$ (dashed red). B) Excitation spectra of chromophore fluorescence. Emission is measured at 495 nm. Line colors are the same as in A.

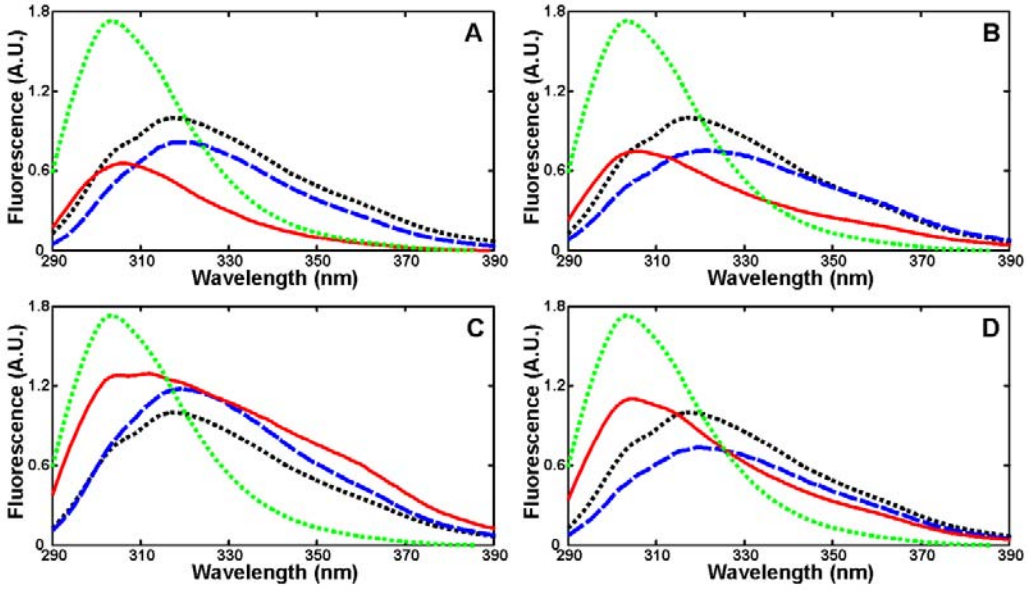

Fig. S6 Emission spectra from tyrosine excitation (280 nm) of WT PYP (A-D, dotted black), W119F (A-D, dotted green), F62W (A, dashed blue), F62W/W119F (A, solid red) , F92W (B, dashed blue), F92W/W119F (B, solid red) , Y94W (C, dashed blue), Y94W/W119F (C, solid red) , F96W (D, dashed blue), and F96W/W119F (D, solid red) at pH 8.0 in pG state. The spectra are normalized at the emission maximum of WT PYP.

The emission spectrum after excitation at 295 nm shows high chromophore, but low tryptophan emission for F62W/W119F (Fig. S4A). The high chromophore emission is caused by the protein fraction with an altered conformation that is represented by the shoulder in the main absorption band. This protein fraction has a large spectral overlap with the emission of the chromophore. In addition, the quantum yield of the chromophore fluorescence is high for this protein fraction. Addition of 2.0 M $(NH_4)_2SO_4$ reduced the FRET from tryptophan to the chromophore (Fig. S5A) in F62W/W119F, but the chromophore emission is still high. More importantly, the emission around 310 nm decreased compare to F62W/W119F in buffer without $(NH_4)_2SO_4$. Excitation spectra of the chromophore of these proteins (Fig S5B) is reduced by adding 2.0 M $(NH_4)_2SO_4$. The addition of 2.0 M $(NH_4)_2SO_4$ decreases the fraction of protein in the altered

conformation, as is evident from the absorption spectra. This reduction leads to lower chromophore emission and less FRET from the tryptophan to the chromophore. However, a small fraction of the protein is still in this different conformation and causes considerable FRET. At higher concentrations of $(NH_4)_2SO_4$ the PYP protein starts to precipitate.
To investigate further the reasons behind the low tryptophan fluorescence of several of the mutants that we constructed, we measured the emission after excitation of the tyrosine residues at 280 nm, as shown in figure S6. For the mutant without tryptophan, W119F, tyrosine fluorescence is clearly observed. As expected, for WT PYP, FRET occurs from tyrosine residues to the tryptophan at position 119. In contrast, in F62W/W119F, F92W/W119F, Y94W/W119F, and F96W/W119F both the tyrosine and the tryptophan emit fluorescence. The emission of F62W/W119F has its maximum at 306 nm. This tyrosine-fluorescence-like maximum could be caused by the fact that the emission above 300 nm is transferred efficiently to the chromophore. Nevertheless, at least a part of the emission between 290 and 306 nm is from tyrosine, because the emission in this region is higher for F62W/W119F than for F62W. For F92W the introduction of an extra tryptophan residue at position 92 leads to a lower tryptophan emission upon 280 nm excitation. W92 does not display efficient FRET from its tyrosine residues and the tryptophan emission of W92 is lower than for W119. The emission from Y94W and Y94W/W119F is higher than for W119, despite the fact that these mutants have one tyrosine residue less. In F96W the emission is lower than in WT PYP, in contrast to F96W/W119F that has a relatively high emission.

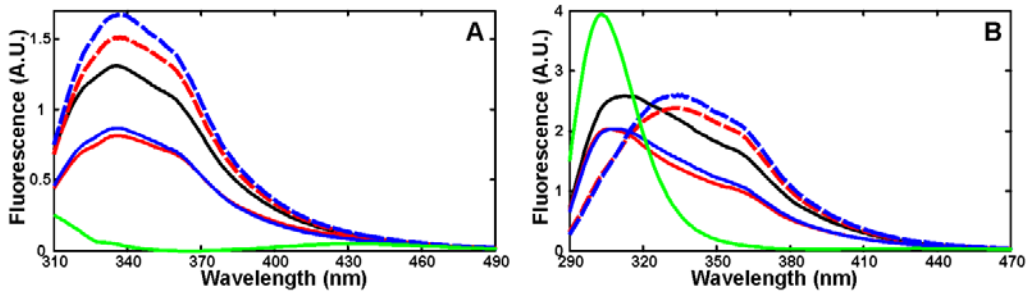

Fig. S7 A) Tryptophan emission spectra with excitation at 295 nm of PYP proteins unfolded with 1 % SDS. Spectra are shown of unfolded WT PYP (solid black), F92W (dashed red), F92W/W119F (solid red), Y94W (dashed blue), Y94W/W119F (solid blue), and W119F (solid green) at pH 8.0. B) Emission spectra from tyrosine excitation (280 nm) of unfolded PYP proteins. Line colours are the same as in A.

The fluorescence of tryptophan displays large differences between WT PYP, F92W/W119F, and Y94W/W119F. Unfolding these PYP proteins leads to almost the same environment of the tryptophan residues at the different locations. We have used 1 % (w/v) SDS in 20 mM Tris pH 8.0 to unfold WT PYP, F92W, F92W/W119F, Y94W, Y94W/W119F, and W119F. From these samples the emission after tryptophan excitation at 295 nm and tyrosine excitation at 280 nm was measured (Fig. S7). The emission of W119F after excitation at 295 nm is low. For intact W119F protein, this emission is 40 % of the emission of WT PYP. The emission from W119F is likely a combination of fluorescence from tyrosine residues and the chromophore, which also absorbs in the UV range. In unfolded W119F protein the emission is separated in fluorescence emitted from the tyrosine residues (at wavelengths shorter than 350 nm) and chromophore fluorescence (between 400 and 500 nm, Fig. S7A). However, it cannot be excluded that the chromophore has also a small contribution in the emission in the UV. FRET to the chromophore and its fluorescence in unfolded PYP is not comparable with intact PYP because the chromophore is protonated in unfolded PYP at neutral pH. The emission spectra from the unfolded proteins WT PYP, F92W/W119F, Y94W, and Y94W/W119F have a similar shape, showing that the environment of these tryptophan residues is similar. However, the quantum yield is lower for W92 and W94, compared to W119. Excitation of the tyrosine residues (Fig. S7B) results in emission spectra of F92W/W119F, Y94W/W119F, and WT PYP that are comparable with respect to shape. The quantum yield in these spectra is lower for F92W and F94W compare to WT. The FRET for all three PYP proteins is incomplete. Introduction of a second tryptophan such as in mutants F92W and Y94W causes an increase of this FRET. All in all the low tryptophan fluorescence is primarily determined by the local environment of W92 and W94. Even in unfolded PYP proteins the fluorescence of W92 and W94 is quenched by nearby residues.

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