Primary Light-Induced Reaction Steps of Reversibly Photoswitchable Fluorescent Protein Padron0.9 Investigated by Femtosecond Spectroscopy

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SUPPORTING INFORMATION

1. Eight state model of Fig. 1c for pH dependent absorption spectra

The pH dependencies of the *cis*- and *trans*-Padron0.9 absorption spectra (Figs. 1a and b) can be modelled assuming two additional protonating groups in the protein environment, X^H and Y^H, modulating the proton affinity of the chromophore by electrostatic interactions. Such a model involves eight species (see Fig. 1c) coupled by seven equilibria and mass conservation which for the *cis*-isomer are:

$$K_C = \frac{[c^- X^H Y^H]\cdot[H]}{[c^H X^H Y^H]}, \quad K_X = \frac{[c^- X^- Y^H]\cdot[H]}{[c\text{-}X^H Y^H]}, \quad K_Y = \frac{[c^- X^- Y^-]\cdot[H]}{[c^- X^- Y^H]}, \quad K_{CX} = \frac{[c^H X^- Y^H]}{[c^- X^H Y^H]},$$

$$K_{XY} = \frac{[c^H X^H Y^-]}{[c^H X^- Y^H]}, \qquad K_{CY} = \frac{[c^H X^- Y^-]}{[c^- X^- Y^H]}, \qquad K_{XC} = \frac{[c^- X^H Y^-]}{[c^H X^- Y^-]}, \qquad \text{(S1-S7)}$$

$$[C]_{tot} = [C^H X^H Y^H] + [C^- X^H Y^H] + [C^- X^- Y^H] + [C^- X^- Y^-] + [C^H X^- Y^H] + [C^H X^H Y^-] + [C^H X^- Y^-]$$

$$+[C^- X^H Y^-] \tag{S8}$$

where $[C]_{tot}$ is the total *cis*-Padron0.9 concentration. These can be solved, e.g. for the fractional concentration of $[C^H X^H Y^H]$ as a function of pH

$$\frac{[C^H X^H Y^H]}{[C]_{tot}} = \tag{S9}$$

$$10^{-3pH} \Big/ \left\{ 10^{-pK_Y - pK_X - pK_C} + 10^{-pK_C - 2pH} \left(\frac{10^{-pK_{XY}} + 1}{10^{pK_{CX}}} + 1 \right) + 10^{-pK_X - pK_C - pH} \left(\frac{10^{-pK_{XC}} + 1}{10^{pK_{CY}}} + 1 \right) + 10^{-3pH} \right\}$$

From which all the other pH dependent fractional concentration are calculated using Eqs. S1-S7. Spectroscopically only four absorption spectra can be distinguished in Fig. 1a which we attribute to the species $C^H X^H Y^H$, $C^- X^- Y^-$, and the non-distinguishable $C^H X^H Y^-/C^H X^- Y^H/C^H X^- Y^-$ and $C^- X^H Y^-/C^- X^- Y^H/C^- X^H Y^H$, respectively. For the pH dependent absorption spectra of the *trans*-isomer we applied the same model. It turned out that the only protonated *trans*-chromophore species which is populated is $T^H X^H Y^H$ whereas $T^H X^H Y^-$, $T^H X^- Y^H$, and $T^H X^- Y^-$ do not appear. Hence the absorption spectrum of these species could not be determined. All the modeled ground state *cis* and *trans* absorption spectra are shown in Fig. S1a and b, respectively.
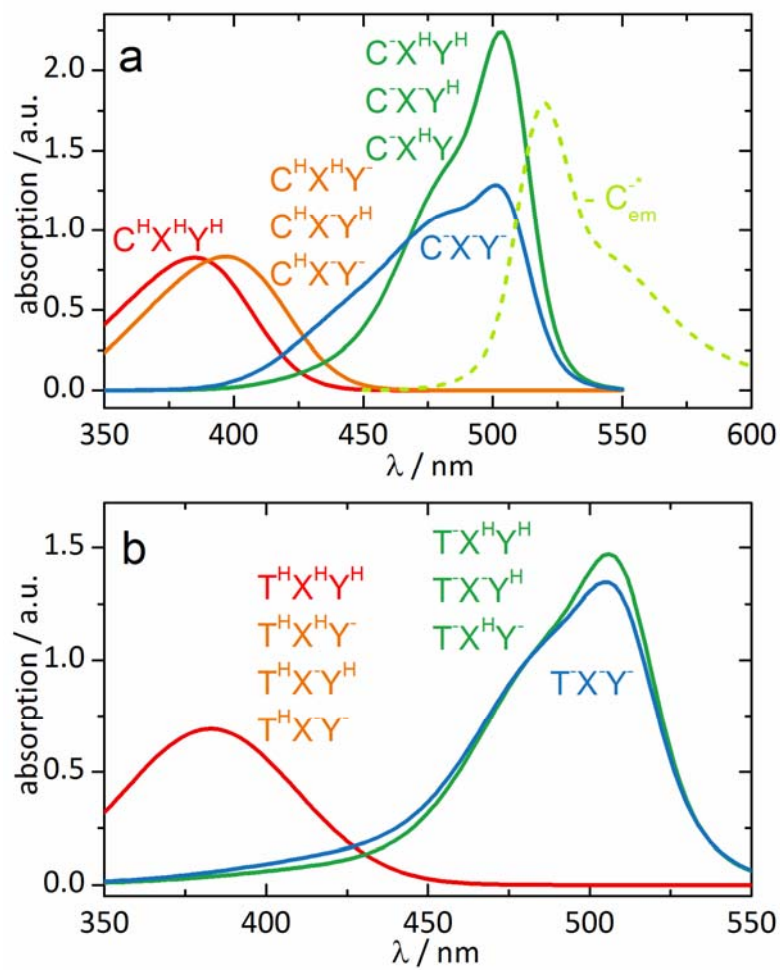
Figure S1. Stationary absorption spectra of various *cis* (a) and *trans* (b) species of Padron0.9 obtained from fitting the pH dependend spectra of Fig. 1 with the eight-state model, (a) also shows the stimulated emission spectrum of C^{-*} calculated from the fluorescence spectrum.

2. Analysis of quasi-stationary population distributions during the fluorescence lifetime of photo-excited cis-Padron

Consider first a kinetic model with only one ground state equilibrium as shown in Fig. S2. We assume the rate constants determining proton transfer in the ground and excited state (k_1, k_{-1}, k_H, and k_r) are much faster than the lifetime of C^{-*} determined by k_f. After the characteristic times for ESPT and ground state proton transfer are over, i.e. at about 100 ps the populations of C^H, C^{-*} and C^- reach a quasi-stationary state in which during the lifetime of C$^-$ their relative concentrations $[C^H]_{qs}$, $[C^{-*}]_{qs}$, and $[C^-]_{qs}$ stay constant. Consider first excitation of C^-. If we neglect the depopulation C^{-*} due to its long lifetime of 1.16 ns and denote y_- as the fraction of C^- initially excited by the pump pulse the quasi-stationary concentrations are.

$$[C^{-*}]_{qs,-} = y_- \tag{S10}$$

$$[C^-]_{qs,-} = -y_- \cdot \frac{K}{1+K} \tag{S11}$$

$$[C^H]_{qs,-} = -y_- \left(1 - \frac{K}{1+K}\right) \tag{S12}$$
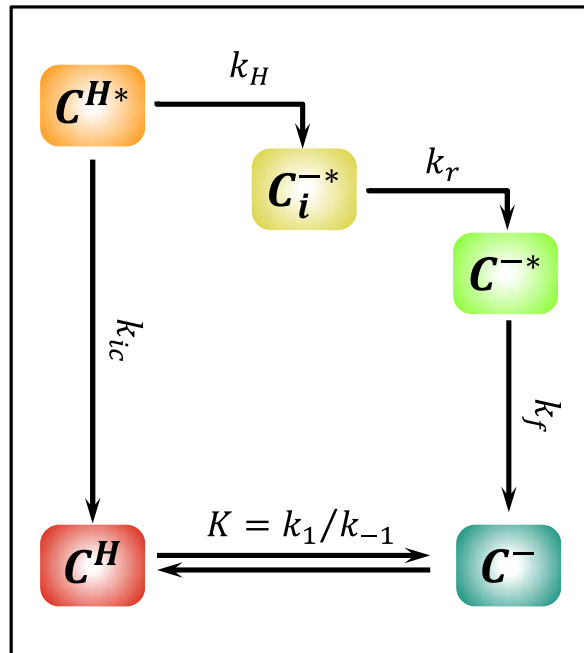

Figure S2: Kinetic model with a single ground state equilibrium.

Note that the concentrations of C^- and C^H appear negative since these populations are bleached by the pump pulse. When $[C^{-*}]$ slowly drops by the reaction $C^{-*} \rightarrow C^-$ the ground state equilibrium will always adapt to the increase of $[C^{-*}]$ such that the concentration ratio between C^H, C^- and C^{-*} expressed by Eqs. (S10)-(S12) remains constant.

This result will be compared in the following with a second experiment where a certain fraction y_H of C^H is excited. In this case the quasi-stationary populations are

$$[C^{-*}]_{qs,H} = y_H \Phi_{ESPT} \qquad (S13)$$

$$[C^-]_{qs,H} = -y_H \Phi_{ESPT} \frac{K}{1+K} \qquad (S14)$$

$$[C^H]_{qs,H} = -y_H \Phi_{ESPT} \left(1 - \frac{K}{1+K}\right) \qquad (S15)$$

where

$$\Phi_{ESPT} = \frac{k_H}{k_H + k_{ic}} \qquad (S16)$$

is the quantum yield for proton transfer from the state C^{H*}.

Eqs. (S10)-(S12) and (S13)-(S15) show that the concentration ratios of the three species in the quasi-stationary phase are independent on whether C^- or C^H is excited, i.e. if the model of Fig. S2 were valid the scaled 126 ps transients of Fig. 5 obtained from C^- or C^H and excitation should be identical.

This clearly is not the case. Instead when normalized at the red edge of the stimulated emission spectrum a difference appears which is just the properly scaled difference between the ground state spectra of C^H and C^- (see Fig. 5). In the following we will show that the model of Fig. 6a is in accord with this result. The ground state distribution in Fig. 6a is characterized by two equilibrium constants

$$K_1 = \frac{[C_1^-]}{[C^H]} \qquad (S17)$$

$$K_2 = \frac{[C_2^-]}{[C^H]} \tag{S18}$$

The equilibration between C_1^- and C^H is assumed to be fast, i.e. settles within 100 ps whereas for C_2^- and C^H it is slow, such that C_2^- stays constant for a long time once the system is disturbed by the pump pulse. The spectra of C_1^- and C_2^- as well as C_1^{-*} and C_2^{-*} cannot be distinguished. The total concentration $[C_{tot}^-] = [C_1^-] + [C_2^-]$ of the deprotonated form then is determined by

$$K_{tot} = K_1 + K_2 = \frac{[C_1^-]+[C_2^-]}{[C^H]} \tag{S19}$$

and the concentration ratio of C_1^- and C_2^- by

$$K_{12} = \frac{[C_2^-]}{[C_1^-]} = \frac{K_2}{K_1} \tag{S20}$$

If the deprotonated cis chromophore is excited C_1^- and C_2^- are bleached according to their abundance dictated by K_{12}. Subsequently, the system relaxes to quasi-stationary concentrations given by

$$[C_{tot}^{-*}]_{qs,-} = [C_1^{-*}]_{qs,-} + [C_2^{-*}]_{qs,-} = y_- \tag{S21}$$

$$[C_1^-]_{qs,-} = -y_- \cdot \frac{K_1}{1+K_1} \cdot \frac{1}{1+K_{12}} \tag{S22}$$

$$[C_2^-]_{qs,-} = -y_- \cdot \left(1 - \frac{1}{1+K_{12}}\right) \tag{S23}$$

$$[C^H]_{qs,-} = -y_- \cdot \frac{1}{1+K_{12}} \left(1 - \frac{K_1}{1+K_1}\right) \tag{S24}$$

If the protonated form C^H is excited the steady state concentrations are

$$[C_{tot}^{-*}]_{qs,H} = y_H \Phi_{ESPT} \tag{S25}$$

$$[C_1^-]_{qs,H} = -y_H \Phi_{ESPT} \frac{K_1}{1+K_1} \tag{S26}$$

$$[C_2^-]_{qs,H} = 0 \tag{S27}$$

$$[C^H]_{qs,H} = -y_H \Phi_{ESPT} \left(1 - \frac{K_1}{1+K_1}\right) \tag{S28}$$

In Fig. 5 the 126 ps transients arising from C^- and C^H excitation were normalized to the stimulated emission intensity (which is proportional to $[C_{tot}^{-*}]_{qs}$) and then the difference (C^H transient minus C^- transient) was calculated. If we do this procedure here with the steady state concentrations we find for the difference in ground state concentrations

$$\Delta[C_{tot}^-]_{norm} = \frac{[C_1^-]_{qs,H}+[C_2^-]_{qs,H}}{[C_{tot}^{-*}]_{qs,H}} - \frac{[C_1^-]_{qs,-}+[C_2^-]_{qs,-}}{[C_{tot}^{-*}]_{qs,-}} = \frac{K_{12}}{1+K_{12}} \cdot \frac{1}{1+K_1} = \frac{K_{12}}{1+K_{12}+K_{tot}} \tag{S29}$$

and

$$\Delta[C^H]_{norm} = \frac{[C^H]_{qs,H}}{[C_{tot}^{-*}]_{qs,H}} - \frac{[C^H]_{qs,-}}{[C_{tot}^{-*}]_{qs,-}} = -\frac{K_{12}}{1+K_{12}+K_{tot}} \tag{S30}$$

i.e. a positive contribution (Eq. (S29)) due to C^- and a negative one of identical absolute value (Eq. (S30)) from C^H. This is exactly observed in Fig. 5. Note that K_{tot} is know from analysis of the pH dependent ground state spectra (Fig. 1) and K_{12} is the only adjustable quantity to define $\Delta[C_{tot}^-]_{norm}$ and $\Delta[C^H]_{norm}$.

3. Fitting parameters for species spectra

Species spectra were fitted by sums of Gaussian functions of the form

$$S(\lambda) = \sum_{i=1}^{k} \frac{A_i}{\sigma_i \sqrt{2\pi}}\, exp\left(-\frac{1}{2}\left(\frac{\lambda-\lambda_{c,i}}{\sigma_i}\right)^2\right) \tag{S31}$$

With amplitude factor A_i, central wavelength $\lambda_{c,i}$ and standard deviation σ_i. The parameters derived from fitting the spectra are summarized in Tables S1-S3.

Table S1. Parameters for describing the species spectra of Fig. S1 by sums of Gaussian functions (Eq. S31).

	λ_c	σ	A
$C^HX^HY^H$	367.5	23.8	29.8
	393.1	17.6	21.8
$C^HX^HY^-$, $C^HX^-Y^H$	382.0	23.8	34.3
	407.6	17.6	19.1
$C^-X^HY^H$, $C^-X^-Y^H$, $C^-X^HY^-$	449.2	23.6	9.43
	487.1	19.8	66.9
	505.8	8.7	28.8
	449.2	23.6	29.3
$C^-X^-Y^-$	487.1	19.8	43.2
	505.8	8.7	12.9
$T^HX^HY^H$	382.8	26.4	45.8
$T^HX^HY^-$, $T^HX^-Y^H$	452.7	46.6	13.3
	491.9	23.5	59.2
	509.7	9.9	15.2
$T^-X^HY^H$, $T^-X^-Y^H$, $T^-X^HY^-$	452.7	46.6	20.3
	491.9	23.5	56.0
	509.7	9.9	12.2
$T^-X^-Y^-$	518.6	9.0	27.6
	538.2	22.8	43.2
	576.8	33.9	14.1
$C_{em}^{-*}{}_{pH10}$	518.6	9.0	27.6
	538.2	22.8	43.2
	576.8	33.9	14.1

Table S2. Parameters for describing the species spectra of Fig. 3b by sums of Gaussian functions (Eq. S31).

	λ_c	σ	A
	452.7	46.6	0.263
T^-	491.9	23.5	0.727
	509.7	9.9	0.158
	405.3	31.3	0.698
T^{-*}	475.2	27.7	0.163
	619.1	58.3	-0.406
	405.3	31.3	0.948
T_r^{-*}	475.2	27.7	0.002
	619.1	58.3	-0.722
T_h^-	492.6	31.0	0.532
	520.1	11.1	0.170

Table S3. Parameters for describing the species spectra of Fig. 6b by sums of Gaussian functions (Eq. S31).

	λ_c	σ	A
C^-	449.2	23.6	0.186
	487.1	19.8	0.854
	505.8	8.7	0.348
C^H	382.0	23.9	0.466
	407.6	17.6	0.261
	441.3	31.9	0.921
	519.8	21.8	1.004
C_i^{-*}	515.7	14.4	-0.552
	532.4	20.2	-0.865
	574.0	33.9	-0.282
	619.4	28.7	0.053
C^{H*}	439.0	22.7	0.468
	564.1	52.1	-0.357
	413.0	41.1	1.010
	477.4	14.4	0.070
C^{-*}	518.5	9.0	-0.242
	538.2	22.8	-0.380
	576.8	33.9	-0.124
	619.4	28.7	0.011