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

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Supporting Information

ABSTRACT: The reversible photoswitching of the photochromic fluorescent protein Padron0.9 involves a cis–trans isomerization of the chromophore. Both isomers are subjected to a protonation equilibrium between a neutral and a deprotonated form. The observed pH dependent absorption spectra require at least two protonating groups in the chromophore environment modulating its proton affinity. Using femtosecond transient absorption spectroscopy, we elucidate the primary reaction steps of selectively excited chromophore species. Employing kinetic and spectral modeling of the time dependent transients, we identify intermediate states and their spectra. Excitation of the deprotonated trans species is followed by excited state relaxation and internal conversion to a hot ground state on a time scale of 1.1–6.5 ps. As the switching yield is very low (Φcis→trans = 0.0003 ± 0.0001), direct formation of the cis isomer in the time-resolved experiment is not observed. The reverse switching route involves excitation of the neutral cis chromophore. A strong H/D isotope effect reveals the initial reaction step to be an excited state proton transfer with a rate constant of kH = (1.7 ps)−1 (kD = (8.6 ps)−1) competing with internal conversion (kI = (4.5 ps)−1). The deprotonated excited cis intermediate relaxes to the well-known long-lived fluorescent species (k = (24 ps)−1). The switching quantum yield is determined to be low as well, Φcis-→trans = 0.02 ± 0.01. Excitation of both the neutral and deprotonated cis chromophores is followed by a ground state proton transfer reaction partially re-establishing the disturbed ground state equilibrium within 1.6 ps (deuterated species: 5.6 ps). The incomplete equilibration reveals an inhomogeneous population of deprotonated cis species which equilibrate on different time scales.

1. INTRODUCTION

Reversibly photoswitchable fluorescent proteins (RSFPs) are structurally similar to the green fluorescent protein (GFP) but may be reversibly photoswitched between metastable fluorescent “bright” and nonfluorescent “dark” states.1 They exhibit a GFP-like fold, namely, a beta-barrel enclosing an α helix containing the autocatalytically formed chromophore. RSFPs with “positive” switching characteristics are switched to a fluorescent “bright” state when irradiated with fluorescence-inducing light, while “negative” switching RSFPs are switched to a nonfluorescent “dark” state. RSFPs have been used for a number of applications, including protein tracking,3 data storage,4,5 and, most prominently, super-resolution microscopy.5,7

Several studies utilizing X-ray crystallography revealed a light-driven cis–trans isomerization of the chromophore as the molecular key event in the switching process.1,8,9 In all reported RSFPs so far, the bright state adopts a cis configuration, but fluorescent proteins with a trans configuration have been described.10 Hence, a specific chromophore configuration is not sufficient to explain the occurrence of fluorescence. Rather, chromophore torsion, its stabilization within the protein matrix, and protein flexibility in general have been discussed to codetermine the fluorescence yield.11–13

In addition, the protonation state of the chromophore is of key importance. For some RSFPs, cis–trans isomerization seems to be coupled with proton transfer; indeed, also pH-induced isomerization has been shown.14 For most GFP-like proteins, only the deprotonated chromophore fluoresces with high yield, while, for the neutral (protonated) species, fluorescence is weak. The weak fluorescence of the neutral chromophore in many cases is caused by an excited state proton transfer (ESPT) and subsequent emission by the deprotonated form.15,16 Still, many of the mechanistic details of reversible photoswitching are discussed controversially including the role of the protonation state of the amino acid residues in the vicinity of the chromophore.1

The well-investigated green fluorescent RSFP Dronpa exhibits negative switching characteristics.16 The bright state was identified to be the deprotonated cis isomer.5 As an initial step for switching from the dark to the bright state, ESPT has

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been discussed for years\textsuperscript{17} but was recently excluded by Warren et al.\textsuperscript{18} in favor of direct trans to cis isomerization followed by a ground state proton transfer.

Padron is a derivative of Dronpa differing in only a few amino acids (T59M, V60A, N94I, P141L, G155S, V157G, M159Y, F190S), but it shows opposite (i.e., positive) switching characteristics for which only V157G and M159Y are essential.\textsuperscript{2} At thermal equilibrium, Padron adopts the dark state. X-ray studies on the variant Padron0.9 identified the bright state to have a cis configuration and the dark state to have a trans configuration.\textsuperscript{12} Similar to Dronpa, the absorption spectra of both isomers exhibit pH dependencies with clearly distinguishable absorption bands at 400 and 500 nm which are assigned to the protonated and deprotonated chromophores, respectively.\textsuperscript{12} Faro et al.\textsuperscript{19} succeeded in cryo-trapping two different fluorescent intermediates along the on-switching pathway of Padron, showing first the isomerization from trans to cis configuration at 100 K and a subsequent relaxation at 120 K, both in the deprotonated state. An increase in temperature to 180 K eventually led to a protonation equilibrium.

Recently, the short-time dynamics of Padron was investigated by Fron et al.\textsuperscript{20} who found that the excited cis neutral form decays with a time constant of 1 ps presumably associated with ESPT, leading to an unstable intermediate, tentatively assigned to the excited deprotonated cis state which isomerizes with a time constant of 14.5 ps to the trans state. This model does not account for the intense fluorescence observed after excitation of the neutral cis form as shown in the fluorescence excitation spectrum of ref 12. Excitation of the deprotonated trans form was proposed to be followed by a quantitative isomerization to an unrelaxed cis ground state with a time constant of 5.2 ps and subsequent stabilization to the relaxed cis isomer within 0.7 ps. However, this quantitative isomerization from the trans to the cis form is inconsistent with the switching cycles presented by Brakemann et al.\textsuperscript{12} which suggest a significantly lower trans to cis quantum yield.

In this paper, using femtosecond pump–probe spectroscopy, we complement these former measurements and present a comprehensive study of the short-time dynamics of Padron0.9’s ground and exited states. To this end, all possible configurations of cis/trans and protonated/deprotonated chromophores were investigated by shifting the photostationary equilibrium to the desired isomer, choosing a suitable pH, and exciting selectively the desired chromophore choosing the proper wavelength. By using both spectral and kinetic modeling, we propose an as simple as possible model for the complex dynamics and involved states taking into account all available data. From this analysis, we derive species spectra and the dominant relaxation pathways. As a major result, we prove ESPT to be the initial reaction step after excitation of the neutral cis chromophore. The isomerization of cis and trans Padron0.9 cannot be observed directly by our time-resolved method because both switching quantum yields are too low.

2. EXPERIMENTAL SECTION

2.1. Sample Preparation. Samples were prepared from concentrated 5 mM solutions of Padron0.9 in PBS buffer. For experiments at pH 4 and pH 10, an aliquot of 50 μL was diluted in 6 mL of 5 mM aqueous citrate buffer and phosphate buffer, respectively. Deuterated samples at pH 10 were prepared by dissolving disodium hydrogen phosphate in D\textsubscript{2}O. An aliquot of 50 μL of concentrated Padron0.9 solution was diluted in 6 mL of this 5 mM buffer solution, leading to a deuteration grade of >98%. The samples were stored overnight before usage.

The solution was pumped in a closed loop through a flow cell with a cross section of 2 mm x 2 mm equipped with two CaF\textsubscript{2} windows. To ensure a fresh sample in the 150 μm focus of each pump laser pulse, we adjusted the flow rate to 1 mL/s. Part of the loop was an illumination chamber where the solution was irradiated by a collimated high power 1 W LED at 400 or 495 nm to shift the photostationary equilibrium to either the trans or the cis form. LED irradiation started half an hour before the actual measurement and remained on during the whole experiment. Under our experimental conditions, the optical density at an excitation wavelength of 387 nm for the cis (trans) solution was 0.17 (0.12) at pH 4 and 0.1 at pH 10. Excitation at 505 nm was applied for solutions at pH 10 where the optical density was 0.18 (0.26).

2.2. Laser System. The laser system is based on a 1 kHz Ti:sapphire oscillator/regenerative amplifier producing 150 fs pulses at 773 nm with pulse energies of 1 mJ. This output beam was split to generate pump and probe pulses. Pump pulses to photoexcite Padron0.9 at 387 and 505 nm, respectively, were generated either by second-harmonic generation (SHG) or by noncollinear optical parametric amplification (NOPA). These pulses were focused to a diameter of 150 μm to excite the Padron0.9 solution with pulse energies of <200 nJ. This intensity was sufficiently low to prevent multiphoton processes, as was proven in separate experiments with 2 times higher pump intensities which showed no change in the kinetics. The probe pulse was a white-light-continuum generated by focusing a small portion of the 773 nm pulse into a CaF\textsubscript{2} crystal of 4 mm length. This continuum was then split into a reference and a probe beam. The latter was superimposed with the pump beam at the sample. The relative plane of polarization was set to 54.7° to eliminate rotational effects. The reference and probe continua were each detected with a 256-element linear diode array attached to a spectograph. The measured transient spectra were afterward corrected with respect to the shift of temporal overlap of pump and probe pulses due to the group delay dispersion within the white-light-probe continuum. Also, spontaneous emission and stray light signal caught by the array were subtracted.

2.3. Kinetic and Spectral Modeling. Our approach was to use an as simple as possible kinetic model to describe the time evolution of our spectra. Since the contribution of one species to a spectrum at a given wavelength is the product of concentration and extinction coefficient, such a fitting procedure can easily be arbitrary. To avoid that, it is necessary to reduce the number of degrees of freedom. This was done in several steps in which species spectra were determined and represented by sums of Gaussian functions. Once determined, the spectrum of a species was left unchanged when proceeding to the next step of fitting. The analysis started by modeling the ground state absorption spectra of the protonated and deprotonated cis and trans configurations as well as the stimulated emission spectrum. Using these spectra, we modeled the time dependent spectral evolution, which we analyzed in the order of increasing complexity.

To denote the chromophore states, we will use C for the cis isomer and T for the trans isomer. The superscripts minus and H indicate the respective protonation state, and an asterisk is used for excited states.
3. RESULTS AND DISCUSSION

3.1. Steady State Characterization. Ground State Absorption Spectra. Parts a and b of Figure 1 show the pH dependent absorption spectra of cis- and trans-Padron0.9. All spectra are normalized to the protein absorption at 280 nm (not shown). Each series of spectra is dominated by two absorption bands associated with the neutral (CH at 397 nm and T^1 at 384 nm) and the deprotonated (C^- at 503 nm and T^- at 506 nm) chromophores, respectively. The pH dependence of the cis chromophore (Figure 1a) clearly indicates a protonation equilibrium centered at around pH 6. Interestingly, at higher pH, the deprotonation of the cis isomer remains incomplete. This behavior is in contrast to most other related RSFPs, where absorption at 400 nm completely vanishes at high pH, and cannot be explained by a single protonation equilibrium. Instead, one has to assume another protonating group in the vicinity of the chromophore which changes its protonation equilibrium. 

We attribute these to the species CHXHYH, C^-HYH, C^-HY, and C^-X, form a pH dependent equilibrium. However, closer inspection of Figure 1a reveals two other subtleties: a broadening of the C^- spectrum at pH 10 and a blue shift of the protonated species at pH < 5 that for pH 4 peaks at 385 nm. The former effect can be attributed qualitatively to a difference in the absorption spectra of C^-XH and C^-X^- within the four-species model. However, this can be ruled out, as there are quantitative differences: (1) The absorption coefficients differ significantly (the cis band is 20% stronger than the trans band). (2) As shown in Figure 1b, there is residual absorption of T^- at 506 nm, whereas in Figure 1a there is no indication of a deprotonated species at pH 4. Furthermore, protein solutions buffered at pH 4 and switched to the cis (trans) form by LED irradiation at 500 nm (400 nm) showed after neutralization the respective distinctive cis (trans) spectrum. Therefore, we attribute the pH 4 and pH 6 spectra of Figure 1a to protonated cis species which are subjected to a varying electrostatic interaction with yet another protonating group Y of the protein environment, thus forming the reaction scheme presented in Figure 1c. The equations describing the fractional populations of all eight species are presented in the Supporting Information. In a global analysis, we modeled the pH dependent absorption spectra of the cis species using the scheme of Figure 1c. The previous discussion showed that at least four spectra are needed to account for spectral features of Figure 1a. We attribute these to the species C^-IxYH, C^-X^-Y, and the nondistinguishable C^-IX^HYH/C^-IX^HY/C^-IX^-Y^- and C^-X^-Y^-/C^-X^-Y/-C^-X^-Y/-, respectively. Each species spectrum was fitted by a sum of Gaussian functions, two for the C^-I and three for the C^- bands. The final outcome of the modeling is in satisfactory agreement with experiment, as shown in Figure 1a by full lines. Species spectra (Figure S1a, Supporting Information) and corresponding fitting parameters (Table S1, Supporting Information) are presented in the Supporting Information. In Figure 1d, the pH dependencies of the species fractional populations are plotted. One can see that for pH between 4 and 10 there exists a distribution of CH and C^-.
species with different protein environment. In our time-resolved experiments, CH and C− can be distinguished by choosing the proper excitation wavelength. Here Figure 1d shows that the pure protonated species C\H^+Y− can be investigated at pH > 7.5 and the pure deprotonated species C−X^+Y− between pH 7 and 9. In the kinetic analysis of our time-resolved spectra, we assume no additional inhomogeneity. This is also supported by the fluorescence excitation spectrum of cis-Padron0.9 shown by Brakemann et al.12 which for both protonation states shows the same shape as the corresponding absorption spectrum.

In the trans isomer pH dependent spectra (Figure 1b), the presence of protonating groups of the protein environment manifests itself in a more subtle way. The spectra show a protonation equilibrium at pH 4–5. However, the transition appears much sharper than for the cis isomer and cannot be described by a single equilibrium of the form T^H \rightleftharpoons T^− + H^+. Furthermore, at pH > 8, a slight change of the T− band shape is observed. To model both effects, again, two protonating groups X and Y of the protein environment have to be introduced, resulting in a scheme similar to Figure 1c, where C has to be replaced by T. Note that the X and Y groups are not necessarily the same for cis and trans (and if so they can still differ in orientation to the chromophore and in their acidity). For the global analysis of the trans isomer spectra, only three species spectra are needed which we attribute to T−X−Y− and the nondistinguishable T^\H+X^+Y^− / T^\H+X^−Y^+ / T^\H+X^−Y^+ / T^\H+X^−Y^+ and T^\H+X^−Y^− / T^\H+X^−Y^+ / T^\H+X^−Y^+, respectively. The agreement between the measured absorption spectra of Figure 1b and the model fit (full lines) is excellent. The underlying pH dependent fractional concentrations are plotted in Figure 1e, whereas spectra and corresponding fitting parameters (Table S1, Supporting Information) are presented in the Supporting Information. Figure 1e shows that T^\H+X^−Y^+ at low pH and T−X−Y− at high pH can be investigated as pure species. In between the T− species appears as a mixture consisting, however, mainly of T^\H+X^−Y^−. On the basis of Figure 1d and e, we have chosen to perform time-resolved experiments for cis- and trans-Padron0.9 at pH values of 4, 7, and 10.

3.2. Excited States Characterization. Deprotonated States C− and T−. Excited state dynamics of C− and T− was investigated with pump–probe spectroscopy at pH 10 and 7 using 505 nm excitation. Parts d and e of Figure 1 show that at these pH values different deprotonated species exist. The analysis of the pH dependent absorption spectra indicates spectral differences between C−X−Y− and C−X^+Y^−/C−X^+Y^+ as well as T−X−Y− and T−X^+Y^−/T−X^+Y^+. However, a comparison of our time-resolved signals at pH 7 and 10 for both cis and trans shows no significant differences. For the cis isomer, this result is not too surprising, as the fractional concentration of C−X−Y− even at pH 10 is low. In the case of trans-Padron0.9, however, the pH change from 7 to 10 involves a complete transition from mainly T−X^+Y^+ to pure T−X−Y−. As we find no difference in the transient signals for these samples, we conclude that in kinetic terms these species behave identically.

In the following, we present experimental results obtained at pH 10. The measurements were performed for one and the same solution by switching first to the cis (Figure 2a) and after that to the trans isomer (Figure 2b) using LED illumination...
first at 495 nm and subsequently at 400 nm. The transient spectra of both isomers show regions which are dominated by excited state absorption (350–450 nm), bleaching of the ground state (450–520 nm), and stimulated emission (>520 nm). As expected for the bright state, excitation of C− leads to a long-lived (>1 ns) excited state species C−∗, whereas for the dark state the corresponding T−∗ decay is much faster. However, Figure 2b also shows a long-lived transient with identical spectral features as the cis transient of Figure 2a, which is illustrated by superimposing the scaled 193 ps spectrum of Figure 2a on Figure 2b (yellow line).

For each wavelength, the time evolution of the transients in Figure 2a can be fitted by a biexponential decay with time constants \( \tau_1 = 5.3 \pm 0.3 \) ps and \( \tau_2 = 1.2 \pm 0.3 \) ns. Fitting the signal of the trans species (Figure 2b) requires three time constants \( \tau_1 = 1.1 \pm 0.2 \) ps, \( \tau_2 = 6.5 \pm 0.7 \) ps, and \( \tau_3 = 1.2 \pm 0.6 \) ns. The wavelength dependent amplitudes are presented in Figure 2c and d. We attribute the dominating decays to the spectral evolution of excited C− and T−, respectively. The spectrum of the 1.2 ns component in Figure 2c appears as a minor contribution in Figure 2d. Hence, we assign it to contaminations of the undesired cis isomer due to incomplete photoswitching by LED irradiation. On the other hand, the 6.5 ps component dominating in Figure 2d appears to be very similar to the 5.3 ps component in Figure 2c. This again can be due to incomplete switching. However, for the cis signal, one also has to take into account the ground state protonation equilibrium C\textsuperscript{14} \rightleftharpoons C−; i.e., once the C− ground state is bleached by the excitation pulse, it is repopulated by the reaction C\textsuperscript{14} \rightarrow C−. This would result in a similar spectral evolution as represented by the 5.3 ps component in Figure 2c. We will show below that both a trans contamination and the ground state equilibration are responsible for the fast spectral evolution in Figure 2a.

The decay of the photoexcited deprotonated cis isomer appears monoexponential with a time constant of \( \tau_{C−∗} = 1.2 \pm 0.3 \) ns. Fron et al.\textsuperscript{20} investigated the fluorescence emission of Padron0.9 with time-correlated single photon counting and found three decay times of 3.6 ns, 500 ps, and 39 ps, where the 3.6 ns component was attributed to the lifetime of the deprotonated cis state C−∗ and the two faster time constants to relaxation processes within the excited state of the chromophore. Our transients do not show a 39 ps component. Since our observation time window is limited to 200 ps, the 1.2 ns component might be composed of the 500 ps and 3.6 ns components found in ref 20.

In contrast to the spectrum of C− in Figure 2a, the T− stimulated emission in Figure 2b is broad and featureless. Furthermore, the stimulated emission can be described almost entirely by the slow 6.5 ps component, whereas for the bleach and excited state region also the fast 1.1 ps component is required. This result is consistent with Fron et al.\textsuperscript{20} who also observed a fast 0.7 ps and a slower 5.2 ps component. Since the 0.7 ps component did not appear in the fluorescence upconversion experiment, they suggested the formation of a hot ground state or another excited state. Note that in Figure 2d the largest amplitude of the 1.1 ps component appears at the red edge of the T− absorption spectrum at 520 nm which would be consistent with fast recovery of the hot ground state T\textsubscript{1b} by internal conversion. As stimulated emission and exited state absorption last considerably longer and the latter decays biexponentially, it is likely that the initially excited trans species T−∗ rapidly relaxes to a state T\textsubscript{r}−∗ from which internal conversion to the hot ground state occurs at a slower rate as compared to T−∗. The reaction scheme summarizing these considerations is presented in Figure 3a. Assuming that in the

![Figure 3](image-url)
Gaussians. The fitted rate constants are included in Figure 3a, and species spectra are presented in Figure 3b. Parameters for modeling these spectra by sums of Gaussian functions are summarized in Table S2 in the Supporting Information.

**Protonated State CH at High pH: Excited and Ground State Proton Transfer.** The excited state dynamics of CH at pH 10 was investigated using an excitation wavelength of 387 nm. Under these conditions, only one species, namely, CH$^+$X$^-$Y$^-$, is excited (see Figure 1a and d). Since at pH 10 the trans product is completely deprotonated, it cannot contribute to a transient signal even if back switching with the 495 nm LED was incomplete.

Figure 4a shows that 0.5 ps after excitation of CH at pH 10 the transient spectrum consists of ground state bleach at 400 nm superimposed by excited state absorption with a maximum at 445 nm. At wavelengths >510 nm, the stimulated emission evolves within picoseconds from a broad, featureless spectrum to a narrow spectrum with one pronounced peak at 515 nm and a shoulder at 550 nm. As shown in Figure 5, the stationary fluorescence spectra of CH$^+$ and C$^-$ at pH 10 following 395 and 503 nm excitation, respectively, are identical with a distinctly lower fluorescence yield for CH$^+$. A comparison of Figures 4a and 2a also indicates striking similarities of the stimulated emission between the transient spectra at pump−probe delays >100 ps which is clarified by presenting 126 ps transients of both data sets in Figure 5 as well. The two spectra are scaled to match the red edge of the stimulated emission spectrum of CH$^+$, which is shown for comparison. These observations reveal that the emitting species in both cases is the same and can safely be assigned to the deprotonated excited state C$^{-}\ast$. Hence, we attribute the overall spectral evolution in Figure 4a to an excited state proton transfer $^{\ast}\text{CH} \rightarrow ^{\ast}\text{C}^{-}\ast$. Fitting of the transients in Figure 4a by triexponential decays reveals three time constants: $\tau_1 = 1.6 \pm 0.3$ ps, $\tau_2 = 26 \pm 5$ ps, and $\tau_3 = 1.2$ ns (the latter was fixed and corresponds to the excited state lifetime of C$^{-}\ast$). The amplitude spectra for these decays are shown in Figure 4b.

$\tau_1$ and $\tau_2$ appear in the spectral evolution of the stimulated emission, suggesting an intermediate state $^{\ast}\text{CH} \rightarrow ^{\ast}\text{C}_{1}^{-}\ast \rightarrow ^{\ast}\text{C}^{-}\ast$. The 1.6 ps component is also prominent in the decay of the ground state bleach. Hence, we attribute the depopulation of the initially excited $^{\ast}\text{CH}$ state to two competing pathways, namely, internal conversion to the ground state and ESPT. A 1 ps excited state proton transfer to an intermediate deprotonated cis state competing with internal conversion was also suggested in ref 20.

Figure 5 shows that the strong negative signal at 500 nm of both 126 ps transients cannot be entirely explained by the stimulated emission of C$^{-}\ast$. When C$^{-}$ is excited, this is easily explained by ground state bleach of the parent species; however, for CH$^+$ excitation, this is unexpected, as the corresponding bleach appears at 397 nm (see Figure 1a). This discrepancy could be explained by a broader emission

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**Figure 4.** (a) Transient absorption spectra of Padron0.9 at pH 10 generated by 387 nm pump pulse excitation of the protonated cis chromophore CH$^+$ with continuous LED illumination at 495 nm. Open circles are measured spectra, and solid lines were generated from the kinetic model and species spectra of Figure 6 (see text). For each wavelength, transients can be fitted by triexponential decays. The corresponding amplitude spectra and time constants are shown in part b. Corresponding results for the deuterated species CD$^+$ are presented in parts c and d.

**Figure 5.** Transient absorption spectra of cis-Padron0.9 measured 126 ps after C$^-$ (green) and CH$^+$ excitation (brown), respectively; both spectra are scaled to the stimulated emission spectrum of C$^{-}\ast$ (orange). Fluorescence spectra generated with 503 nm (dashed line) and 395 nm (dotted line) excitation are shown for comparison. Blue circles represent the difference between the CH$^+$ and C$^-$ transient spectrum. The red curve is the scaled difference of the C$^-$ and CH$^+$ ground state absorption spectra.
spectrum of the ESPT product as compared to the C− state. However, the similarity of the stationary CH and C− fluorescence spectra (see Figure S) precludes this conjecture. Hence, the CH transient must also have a C− bleach component, which we attribute to a depopulation of C− in a ground state proton transfer reaction reestablishing the equilibrium CH ⇌ C− after being disturbed by the pump pulse. The data of Figure 4a suggest that this ground state relaxation kinetics happens on a similar time scale as ESPT. Moreover, it must also be operative when C− is excited; i.e., the 5.3 ps contribution shown in Figure 2c can partially be attributed to the reaction CH → C− reducing the initial bleach of C− at 500 nm as well as CH absorption at 400 nm.

Excitation of CH is known to induce photoswitching from the cis to trans configuration.12 In separate experiments, we determined the quantum yield to be Φcis→trans(400 nm) = 0.02 ± 0.01. Due to this low value, the formation of trans will be hardly detectable in the transients even if this product is formed within the time window of our experiment. Hence, assume for a while that the only photochemical reactions induced by exciting CH and C− are ESPT, internal conversion, and/or ground state equilibration CH ⇌ C−, respectively. Then, after these obviously fast processes are over, i.e., at pump–probe delays >100 ps, the system reaches a quasi-stationary state in which during the lifetime of C− the relative populations of CH, C−, and C−* stay constant (for details, see the Supporting Information). Moreover, in this quasi-stationary phase, it does not matter whether CH or C− is initially excited, the transient signals are identical apart from a scaling factor. As shown in Figure S for the two 126 ps transients, this is actually not the case. Where CH is excited, there is less bleach (negative absorption) at 430–530 nm as compared to C− excitation; at lower wavelength, the reverse is true. Plotting the difference of both transient spectra (i.e., CH− transient minus C− transient) turns out to be very instructive. As shown by the blue points in Figure S, the resulting spectrum almost coincides with the properly scaled difference of the ground state absorption spectra of C− and CH (red line). This is indicative of a more complex ground state protonation equilibrium between CH and C− in which only a subpopulation equilibrates rapidly, whereas another fraction on the time scale of our experiment does not. An inhomogeneous ground state distribution with two varieties of the deprotonated cis species C− and C−* as shown in Figure 6a, can explain the observed behavior (for details, see the Supporting Information) if one equilibration, e.g., between C− and CH, is fast, i.e., settles within 100 ps, whereas for C−* it is slow, such that changes in the concentration of C−* induced by the pump pulse remain unchanged for a long time. The already identified excited states CH* and C−* are included in Figure 6a as well. In accord with the two ground states, we have to assume corresponding excited states C−* and C−*.

To verify the assumption of ESPT, measurements were repeated with deuterated Padron0.9 using buffered D2O solutions. The results presented in Figure 4c show obvious differences to Figure 4a caused by an H/D isotope effect. A closer analysis reveals an almost identical ratio of ground state bleach (400 nm) to exited state absorption (445 nm) shortly after excitation. Fitting the spectral evolution exponentially with τ1 = 1.2 ns fixed to the C−* lifetime yields two additional time constants of τ2 = 3.0 ± 0.5 ps and τ2 = 10 ± 3 ps with amplitudes plotted in Figure 4d. These decay spectra indicate that the buildup of stimulated emission and refill of ground state bleach happen concurrently. However, τ2 is twice as long as that for the undeuterated sample. These observations support our interpretation of two reaction channels, internal conversion and ESPT, depopulating the excited CH* state. Since upon deuteration ESPT is significantly slowed down, internal conversion is favored and the yield of stimulated emission from C−* is reduced.

Using a global analysis, we simultaneously fitted the spectral changes after excitation of C− in Figure 2a and excitation of CH in Figure 4a and c within the kinetic model of Figure 6a. As discussed, we used two deprotonated ground state species C−* and C−* with corresponding excited states which we assumed to be spectrally equivalent, respectively. The already identified spectra of CH and C− (Figure 1a and Figure S1, Supporting Information), the fluorescence lifetime 1/τ0 = 1.2 ns, and the overall protonation equilibrium constant at pH 10, Kp = K1 + K2 = ([C−] + [C−*])/[CH] = 0.7 (Figure 1d), were used as fixed input. The rate constants defining the ground state equilibrium described by K2 were set to zero, whereas K12 = K2/K1 was an adjustable parameter. Also, we assumed that a fraction of the signal in Figure 2a originates from contamination by the trans isomer described by the established model and spectra of Figure 3. The result of this fitting presented in Figures 2a and 4a and c by solid lines is in excellent agreement with the experimental data. Species spectra of CH*, C−*, and C−* obtained from the fitting procedure are shown in Figure 6b. Parameters for modeling these spectra are summarized in Table S3 in the Supporting Information. The deprotonated state C−* shows stimulated emission which is less structured and broader than the emission of C−*, suggesting that the intermediate state after fast ESPT is further stabilized by rearrangements of the protein environment.

The rate constants obtained from the fitting are included in Figure 6a. The ESPT and the ground state equilibration happen
Figure 7. (a) Transient absorption spectra of Padron0.9 at pH 4 generated by 387 nm pump pulse excitation of the protonated cis chromophore $\text{CH}^+$ with continuous LED illumination at 495 nm. For each wavelength, data can be fitted by triexponential decays. The corresponding amplitude spectra and time constants are shown in part b.

on a ps time scale and show strong H/D isotope effects of $k_{3H}/k_{3D} = 5.2$ for ESPT and $k_{4H}/k_{4D} = 3.5$ for the ground state proton transfer. The concentration ratio of $\text{C}_2^+$ to $\text{C}_1^-$ at equilibrium is determined to be $K_{12} = [\text{C}_2^+]/[\text{C}_1^-] = 0.94$. One could argue that the two $\text{C}^+$ species of the model correspond to $\text{C}^\text{XHY}^-$ and $\text{C}^\text{XYY}^-$ of Figure 1. However, the kinetics we measured at pH 7 was similar to pH 10, although the species $\text{C}^\text{XYY}^-$ does not appear at pH < 9. This indicates that the system apart from the complex protonation equilibrium of Figure 1c is characterized by additional inhomogeneity in $\text{C}^-$. Establishing this inhomogeneity for $\text{C}^-$, one could also assume it for $\text{CH}^+$ to explain the ESPT dynamics of Figure 6a by a sum of processes $\text{C}_1^\text{HH} \rightarrow \text{C}_1^\text{HI}$ and $\text{C}_2^\text{HH} \rightarrow \text{C}_2^\text{HI}$ instead of just one cascade $\text{C}_1^\text{HH} \rightarrow \text{C}_1^\text{HI} \rightarrow \text{C}_2^\text{HI}$. Then, the two time constants $\tau_1 = 1.6 \pm 0.3$ ps and $\tau_2 = 26 \pm 5$ ps could be associated with the lifetimes of $\text{C}_1^\text{HI}$ and $\text{C}_2^\text{HI}$, respectively. However, analyzing the amplitude spectra in Figure 4b, the spectra of $\text{C}_1^\text{HI}$ and $\text{C}_2^\text{HI}$ would have to be very different. Accordingly, the ground state spectra of $\text{C}_1^\text{HI}$ and $\text{C}_2^\text{HI}$ can be expected to be different, too. The fluorescence excitation spectrum of cis-Padron0.9 shown by Brakemann et al.\textsuperscript{12} for the protonated state shows the same shape as the corresponding absorption spectrum. If one assumes this band to consist of two distinct absorption spectra, this observation can be only explained if the ESPT quantum yield for both $\text{C}_1^\text{HI}$ and $\text{C}_2^\text{HI}$ are the same. In view of the large difference between $\tau_1$ and $\tau_2$, this is unlikely because ESPT is competing with efficient internal conversion.

Protonated States at pH 4. The excited state dynamics of $\text{CH}^+$ and $\text{TH}^+$ at pH 4 were investigated using an excitation wavelength of 387 nm. Figure 1d and e show that at pH 4 the prevailing species are $\text{C}_1^\text{HH} \rightarrow \text{C}_1^\text{HI}$ and $\text{C}_2^\text{HH} \rightarrow \text{C}_2^\text{HI}$, respectively. Interestingly, the time-resolved signals for both appear very similar. They only differ in amplitude (the signal for the cis chromophore is larger than that for the trans chromophore by a factor of 1.3) but not notably in shape. Note that both experiments were performed directly one after another by switching from 490 to 400 nm LED illumination and without any variation of the pump–probe laser setup. Figure 7 shows the results for the cis chromophore.

At first, the spectrum consists of a ground state bleach at <400 nm superimposed by excited state absorption with a maximum at 440 nm and broad, featureless stimulated emission at wavelengths >480 nm. For each wavelength, the time evolution of the transients can be fitted by a triexponential decay with time constants of $\tau_1 = 0.9 \pm 0.1$ ps, $\tau_2 = 8.7 \pm 1.5$ ps, and $\tau_3 = 240 \pm 50$ ps. Their corresponding amplitudes are shown in Figure 7b. The spectra indicate that all the population relaxes back to the ground state. Also, there is no hint of an excited state proton transfer which would be evidenced by the narrow stimulated emission band of $\text{C}^\text{HI}^+$ at 515 nm like in Figure 4a. Therefore, we attribute the observed dynamics to a cascade of minor structural changes of the chromophore/protein environment which finally ends in the $\text{C}^\text{HI}$ ground state. Alternatively, the multieponential dynamics could be explained by an inhomogeneous ground state distribution which upon excitation leads to a superposition of exited states with different lifetimes.

4. CONCLUSION

In this work, we present extensive measurements on the light-induced primary reaction steps of the chromophore and the surrounding protein of Padron0.9. As verified by a strong H/D isotope effect, excitation of the neutral cis chromophore $\text{CH}^+$ is followed by an ESPT competing with internal conversion to the ground state. The initially populated ESPT state $\text{C}_i^-$ relaxes into the fluorescent state $\text{C}^-\text{X}^+$ which is directly accessible by excitation of $\text{C}^-$. Excitation of $\text{CH}^+$ and $\text{C}^-$ in both cases is followed by a ground state proton transfer reaction partially re-establishing the perturbed ground state equilibrium $\text{C}^\text{HI} \leftrightarrow \text{C}^-$. The level of equilibration depends on whether $\text{C}^\text{HI}$ or $\text{C}^-$ is excited and points to an inhomogeneous distribution of $\text{C}^-$ species which equilibrate on different time scales. The isomerization to the trans form is too inefficient (2%) to be detectable with our method. The reverse trans–cis switching process is even less efficient as excitation of the deprotonated trans species is dominated by internal conversion to a hot ground state $\text{T}_i^0$ on a ps time scale. This competes with excited state relaxation ($\text{T}_i^0 \rightarrow \text{T}_i^\text{HI}$), tentatively assigned to a combination of chromophore motion and reorganization of the local protein environment.
ASSOCIATED CONTENT

Supporting Information
Protonation equilibria, absorption and emission spectra, analysis of quasi-stationary population distributions during the fluorescence lifetime of photooxidized cis-Padron, and fitting parameters for species spectra are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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