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**Multiscale Transient Absorption Study of the Fluorescent Protein Dreiklang and Two
Point Variants Provides Insight into Photoswitching and Non-productive Reaction
Pathways**

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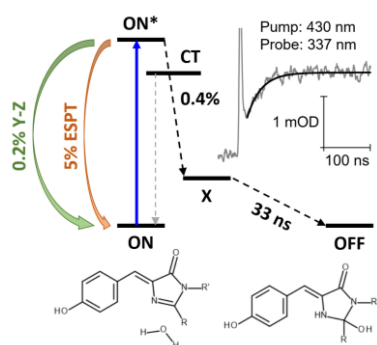
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Abstract:

Dreiklang is a reversibly photoswitchable fluorescent protein used as a probe in advanced fluorescence imaging. It undergoes a unique and still poorly understood photoswitching mechanism based on the reversible addition of a water molecule to the chromophore. We report the first comprehensive study of the dynamics of this reaction by transient absorption spectroscopy from 100 fs to seconds, in the original Dreiklang protein and two point variants. The picture that emerges from our work is that of a competition between photoswitching and non-productive reaction pathways. We found that photoswitching has a low quantum yield of 0.4%. It involves an electron transfer from a tyrosine residue (Tyr203) to the chromophore and is completed in 33 ns. Non-productive deactivation pathways comprise recombination of a charge transfer intermediate, excited-state proton transfer from the chromophore to a histidine residue (His145), and decay to the ground state via micro/millisecond lived intermediates.

TOC Graphic:



Reversibly switchable fluorescent proteins (RSFPs) are homologs of the green fluorescent protein (GFP) capable of repeatedly switch between a fluorescent ON state and a non-fluorescent OFF state under alternating illumination at two appropriate wavelengths. This property makes them highly attractive probes for super-resolution microscopy and other advanced fluorescence imaging techniques.¹ In most cases, fluorescence emission from the RSFP is obtained by excitation at one of the two photoswitching wavelengths. Such spectral coupling, although experimentally simpler, is unfavorable for controlling the protein's ON or OFF state during imaging experiments. Together with its SPOON variant, Dreiklang is currently the only RSFP that does not suffer from this drawback.^{2, 3} In Dreiklang, fluorescence, ON→OFF and OFF→ON photoswitching are elicited by three distinct wavelengths. This is due to a photoswitching mechanism which is entirely different from that of other RSFPs.

X-ray crystallographic studies of several "coupled" RSFPs have shown that the molecular basis of their photoswitching is a *cis-trans* isomerization of the chromophore, coupled with proton transfer.⁴⁻⁶ Subsequently, these RSFPs were studied in detail by time-resolved spectroscopy with visible and infrared probes, as well as by time-resolved crystallography, with particular focus on Dronpa⁷⁻¹⁰ and rsEGFP2.¹¹⁻¹³ Although some questions remain unanswered, it is now well established that *cis-trans* isomerization occurs first on the ps^{9, 11, 13} or ns¹⁰ scale and is followed by ground-state proton transfer on the μs-ms scale.^{9, 10, 12, 13} The photoswitching mechanism of Dreiklang is much less understood. X-ray crystallography combined with mass spectrometry showed that it relies on the reversible addition of a water molecule to the imidazolinone ring of the chromophore,² as illustrated in Scheme 1a. This reaction reduces electron delocalization within the chromophore, causing a strong blue-shift of the absorption of the OFF state which is key for disentangling fluorescence excitation from photoswitching.² In the only time-resolved spectroscopic study of Dreiklang available to

date,¹⁴ some of us reported that, upon excitation of the ON state, excited-state deprotonation of the chromophore's phenol group takes place with a time constant of about 100 fs, followed by excited-state decay on the ps-ns scale and formation of a ground-state photoproduct absorbing at ~450 nm, which we named X. Based on the structure of the active site of ON-state Dreiklang shown in Scheme 1b, we proposed that the proton is transferred to His145 and that this event initiates photoswitching.¹⁴ We further suggested a multi-step mechanism leading to water addition to the chromophore and involving X as an intermediate.¹⁴ The 10-ns time window of this first experimental work however did not allow us to resolve the formation of the final OFF state. On the other hand, Dreiklang has recently been the subject of theoretical studies by the Krylov and Nemukhin teams.¹⁵⁻¹⁷ They identified a low-lying excited state with charge transfer (CT) character, produced by electron transfer (ET) from Tyr203 to the chromophore in the ON state, and proposed that population of this state, hence excited-state ET, is the trigger for water addition.^{16, 17} They further suggested that X is a complex of the cationic chromophore (with protonated imidazolinone ring) with anionic Tyr203 and the water molecule.¹⁷ Excited-state proton transfer (ESPT) was not observed in the simulations,¹⁶ in contrast with the experiments.¹⁴

We report here a new experimental study of Dreiklang aiming at elucidating the interplay between proton and electron transfer and their role in the ON→OFF photoconversion. We prepared for this purpose two point variants of Dreiklang respectively designed to block the aforementioned proton or electron transfers and compared them with the original Dreiklang protein. The study was conducted by transient absorption (TA) spectroscopy in the UV-visible domain with an extended time window from ~100 fs up to ~1 s in order to cover all time scales of Dreiklang's photoinduced dynamics and resolve the formation of the OFF state.

We targeted proton transfer by replacing His145, the putative proton acceptor, with a non-protonatable tyrosine (given its pK_a of ~10,¹⁸ the -OH group of tyrosine is already protonated

at our experimental pH), and electron transfer by replacing Tyr203, the putative electron donor, with a less reducing phenylalanine ($E^\circ(\text{F}^{*+}/\text{F}) \approx 2 \text{ V (vs NHE)} > E^\circ(\text{Y}^{*+}/\text{Y}) \approx 1 \text{ V}^{19}$). Like the original Dreiklang,^{2, 14} its H145Y and Y203F variants exhibit two absorption bands at ~410 nm and ~505 nm in their ON state, corresponding to the neutral (A) and anionic (B) forms of the chromophore, respectively (Figure 1a,b, black lines and Figure S1). Upon irradiation in the A band, H145Y switches to its hydrated OFF state, which is characterized by a blue-shifted absorption band peaking at 341 nm (C; Figure 1a, red line). The ON state then recovers spontaneously in the dark with a characteristic time of ~6 min (Figure S2). This behavior is similar to that previously reported for Dreiklang^{2, 14} and shows that His145 is not essential for photoswitching. In contrast, no significant spectral change is observed for the Y203F variant even after prolonged 410-nm irradiation (Figure 1b, red line), indicating the loss of photoswitching ability. Excitation of H145Y and Y203F in their B band (of the ON state) leads to a strong fluorescence emission at ~520 nm, similar to that of Dreiklang^{2, 14} (Figure S3). However, the two variants exhibit distinct shapes of their fluorescence excitation spectra (Figure 1c). That of Y203F (black line) consists of the same two bands as the absorption spectrum (Figure 1b), albeit with different relative amplitudes. In contrast, the fluorescence excitation spectrum of H145Y (Figure 1c, red line) and that of Dreiklang (blue line) consist essentially of a single anion-like band.

To characterize the excited-state processes in the two variants, we performed broadband fs TA experiments by exciting their respective A forms (of the ON state) at 405 nm (Figures 2 and 3). The data were globally fitted with sums of exponentials whose lifetimes are given in Table 1. The corresponding amplitude spectra (decay-associated difference spectra (DADS)) are shown in Figures S6 and S7 together with kinetic traces at a few representative wavelengths. Like for Dreiklang,¹⁴ we assign the five bands of the initial TA spectrum of Y203F (200-fs black spectrum in Figure 2a) to regions dominated by ground-state bleaching

(GSB) at 365-420 nm, stimulated emission (SE) at 460-620 nm and excited-state absorption (ESA) at < 365 nm, 420-460 nm and > 620 nm. The SE band exhibits a maximum at 515 nm flanked by two shoulders at ~480 nm and ~550 nm which suggest a composite nature: the peak and the red shoulder indicate the presence of a population of excited chromophore that is already deprotonated at 200 fs (compare with the emission spectrum of form B, shown as a gray dash-dotted line), while the blue shoulder is due to the neutral excited chromophore (compare with the emission spectrum of the A* state of GFP,²⁰ shown as a gray dashed line). The first picoseconds of the dynamics (Figure 2a) are mainly marked by a decay in neutral SE (460-500 nm) and a rise in anionic SE (500-620 nm), with a temporary isosbestic point at 500 nm. The apparent rise of the central ESA band (415-485 nm) at this time scale most likely originates from its overlapping with the decaying neutral SE. This signature, which was previously observed in GFP²¹ and Dreiklang,¹⁴ indicates the deprotonation of the chromophore phenol group in the excited state, leading to an anionic excited species I*. The reaction was found to be multiexponential (Table 1). Although we do not have any structural information for Y203F, such multiexponential kinetics in proteins are often assigned to structural heterogeneity.^{9, 14, 22-25} The next step of the dynamics (Figure 2b) consists of a biexponential decay of all bands (Table 1), indicating return from I* to the initial ground state (ON state, form A). Due to the much longer average lifetime of I* ($\langle\tau_{I^*}\rangle = 970$ ps) compared to A* ($\langle\tau_{A^*}\rangle = 1.3$ ps), we assign the A band in the fluorescence excitation spectrum of Y203F (Figure 1c, black line) to the emission of I* formed by ESPT. The quantum yield of ESPT can be estimated by comparing the relative amplitudes of the A and B bands in the absorption and excitation spectra. Taking into account that the lifetime of the I* state of Y203F is shorter than that of the B* state of Dreiklang (excited anion obtained by direct excitation of form B; $\langle\tau_{B^*}\rangle = 2.3$ ns according to ref ¹⁴), we find an ESPT yield of ~40% in Y203F (Figure S8). The same reasoning leads to a much lower ESPT yield of 5% in

Dreiklang, due to the very low amplitude of the corresponding A band (Figure 1c, blue line and Figure S9). Finally, Y203F still exhibits a small TA signal at 20 ns (inset of Figure 2b), after complete excited-state decay attested by the disappearance of SE. It features a central GSB band (390-460 nm) flanked by two TA bands (peaks at 367 and 487 nm) and indicates the formation of a ground-state photoproduct. This species is expected to return to the initial ON state on a longer time scale, since the Y203F variant does not photoswitch (see Figure 1b).

The fs TA spectra of H145Y, displayed in Figure 3, highlight a different excited-state behavior. The initial SE band (black 100-fs spectrum in Figure 3a, 470-620 nm range) is less structured than that of Y203F. It is blue-shifted by 10 nm with respect to the fluorescence of form B (gray dash-dotted spectrum) and extends below 490 nm where only form A contributes. This indicates that, unlike Y203F, the excited neutral chromophore does not undergo ultrafast deprotonation in H145Y. We assign the red shift of the negative SE band relative to the fluorescence spectrum of form A (gray dashed line) to a compensation by the positive ESA contributions around 450 nm. The TA dynamics include three main steps. A slight reshaping of the spectrum (small rise of the 450-nm ESA band and small decay of all other bands, see Figure 3a) is first observed in 145 fs (Table 1). The next changes (920-fs component in Table 1) are mainly concentrated in the region below 485 nm with strong decays of the ESA bands at 330 and 450 nm and of GSB (Figure 3b). The decay of GSB leads to a positive net signal at ~405 nm from 3 ps, due to ESA becoming dominant. SE shows a comparatively much smaller decay on this time scale. We note that a similar asynchronous evolution of the ESA/GSB and SE bands has been observed previously for Dreiklang on the same time scale¹⁴ (Figure S4b). Finally, the third step (Figure 3c) is dominated by the multiexponential decay of the excited state (average lifetime of 1.1 ns; Table 1). Like for Dreiklang¹⁴ and Y203F, a small signal corresponding to a ground-state photoproduct remains

at 20 ns (inset of Figure 3c). It consists of a GSB band at ~410 nm and a TA band at ~500 nm. The 500-nm band is expected to disappear on a longer time scale because it does not correspond to the OFF state which is the only stable photoproduct in the H145Y variant (see Figure 1a).

An important finding at this stage is that the SE band of H145Y only decays throughout the dynamics. This indicates the absence of ESPT in this variant, in contrast with Dreiklang¹⁴ and Y203F, which is in line with the hypothesis that the ultimate proton acceptor in the ESPT in Dreiklang is His145. Moreover, the fact that Y203F exhibits ESPT and no photoswitching while H145Y does not exhibit ESPT but photoswitches shows that ESPT is not involved in the photoswitching mechanism but rather represents a competing reaction pathway.

However, even in the absence of ESPT, one would expect the fluorescence excitation spectrum of H145Y (Figure 1c, red line) to exhibit a prominent A band. Indeed, A* emits at 560 nm, the detection wavelength of our excitation spectrum (see the gray dashed spectrum in Figure 3a), with an oscillator strength that is very close to that of the I* state of Y203F (Figure S10), and it has a similar lifetime of ~1 ns (Table 1). The very weak A band in the fluorescence excitation spectrum of H145Y therefore indicates that only a small fraction of the population excited in the Franck-Condon state (which we will refer to as A_{FC}*) evolves to the fluorescent A* state. Based on the amplitude of the A band and the lifetime of A*, we estimate the quantum yield of formation of A* to be ~5% (Figure S11). Apart from the nature of the fluorescent state (A* instead of I*), this behavior resembles very much that of Dreiklang. Y203F is in fact the only protein in which the population of the fluorescent state (I* in this case) is large enough to lead to a prominent A band in the fluorescence excitation spectrum (Figure 1c). Since the GSB bands of H145Y (Figure 3a) and Dreiklang¹⁴ (Figure S4a) are essentially stable on the time scale of formation of the fluorescent state, we conclude that there must be an ultrafast branching in the initial A_{FC}* state of Dreiklang and H145Y,

leading to the fluorescent state (A^* for H145Y, I^* for Dreiklang) and to a non-fluorescent state. Such a branching would be absent in Y203F.

Searching for the signature of the dark state in our TA data, we noticed that the DADS associated with the 145-fs (DADS1) and 919-fs (DADS2) components of H145Y (Figure S7a) are both very similar to those of the 100-fs and 750-fs components of Dreiklang¹⁴ (Figure S5a) and completely different from the DADS of Y203F (Figure S6a). DADS1 features a large negative band at ~ 470 nm which could reflect the ultrafast rise of the absorption of a dark state. The end of this rise is visible between 440 and 500 nm in the early TA spectra of H145Y (Figure 3a). The next kinetic step is reflected by DADS2 which shows a positive band at 460 nm, a negative band at 400 nm and weaker contributions in the SE region. This corresponds in the raw spectra to an asynchronous evolution of ESA at ~ 450 nm and GSB at ~ 400 nm, which decay significantly, and of SE at ~ 520 nm, which remains essentially stable (Figure 3b and Figure S4b). In Dreiklang, we formerly assigned these features to vibrational cooling of the ESA of I^* following ultrafast ESPT, and to spectral overlap of the ESA and GSB bands.¹⁴ The present study favors a different explanation, namely the ultrafast formation (~ 100 fs) and decay (~ 1 ps) of a dark state, absorbing at ~ 450 nm and resulting from the initial branching mentioned above. The fluorescent state (I^* in Dreiklang or A^* in H145Y) resulting from the same branching then decays in the 100-ps to 1-ns time scale (Figure 3c and Figure S4c).

Furthermore, at the earliest time delays in Y203F (Figure 2a), the apparent rise in the ESA at ~ 450 nm, that we assign to the decay in the overlapping SE of A^* , is accompanied by a significant rise in the SE of I^* at ~ 520 nm, both corresponding to the signature of ESPT. In Dreiklang¹⁴ (Figure S4a), a similar rise in the same ESA is accompanied by a much smaller rise in the SE of I^* . We therefore propose that the ESA rise at ~ 450 nm in Dreiklang is not

solely due to ESPT as in Y203F, but also to the formation of a dark state absorbing at 475 nm, as suggested by DADS1 (Figure S5a).

The suppression of the dark state in Y203F suggests that its formation requires the presence of Tyr203, which is also needed to obtain the OFF state (Figure 1b). On this basis, we propose to assign the experimental dark state to the CT state predicted by theoreticians to be the first precursor of the OFF state.^{16, 17} Its decay in ~1 ps could then correspond to rapid charge recombination between Tyr203 and the chromophore. It may tentatively be hypothesized that the ESA feature at ~450 nm characterizing the CT state would be the signature of a protonated tyrosyl radical resulting from the transfer of an electron from Tyr203 to the excited chromophore. Absorption spectra of this species have indeed recently been obtained in several flavoproteins and show a broad band peaking between 450 and 500 nm.^{26, 27}

The presence of photoproducts at 20 ns, which are different from the expected final states both for Dreiklang (the X species in ref ¹⁴) and its variants, points to the existence of yet unidentified long-lived components in their dynamics. To characterize these slow dynamics, we switched to “classical” TA spectroscopy setups enabling us to probe time scales ranging from nanoseconds to seconds. The “slow” TA data recorded for Dreiklang are shown in Figure 4 and those of H145Y and Y203F in Figures S12 and S13. Like for the fs-ps scales, the signals were fitted with sums of exponentials; the lifetimes are given in Table 1.

For Dreiklang, the kinetics at 337 nm (i.e. at the maximum of the absorption band of the OFF state; blue trace in Figure 4a) shows that the OFF state is formed with a time constant of 33 ns (Table 1). Apart from a slight decay that we assign to back-switching to the ON state by the actinic effect of the probe light, no further evolution is observed at this wavelength (Figure S14), indicating that photoswitching is complete in ~100 ns. The amplitude of the 337-nm plateau enabled us to estimate Dreiklang’s photoswitching quantum yield at 0.4%,

using ruthenium tris-bipyridine as actinometer²⁸ (see Methods in SI for more details). The decay of species X at 457 nm (Figure 4a, green trace) is concomitant with the formation of the OFF state, while GSB is constant on this time scale (gray trace). We deduce that the OFF state is formed directly from X, without any spectroscopically detectable intermediate and with 100% yield. In addition, this step exhibits a strong kinetic isotope effect of 3.7 upon H/D exchange (123 ns in D₂O, see Figure S15), suggesting an involvement of proton transfer. Figure 4b shows the evolution of the TA spectra of Dreiklang between 10 ns (broadband spectrum from ref¹⁴) and 10 μ s. Unexpectedly, the TA band between 430 and 520 nm (10-ns gray spectrum) does not fully decay on the 30-ns scale. The 300-ns spectrum (in green) indeed includes a residual TA at \sim 490 nm, in addition to OFF-state absorption at \sim 337 nm and GSB at \sim 410 nm. This indicates that the TA band at 10 ns actually takes contributions from two species: the 450-nm absorbing X species, precursor of the OFF state, and a longer-lived species absorbing at 490 nm, which we will name Y. Considering the 0.4% photoswitching efficiency, the relative amplitude of GSB versus OFF-state absorption at 300 ns, compared to that in the OFF minus ON difference spectrum, makes it possible to estimate the quantum yield of formation of Y at 0.2% (Figure S17). The subsequent dynamics at the μ s scale show that Y evolves in \sim 7 μ s (Table 1) and with 100% yield towards a new species Z whose absorption remains centered at 490 nm but is broader and more intense (10- μ s blue spectrum in Figure 4b). Finally, the ms-scale dynamics comprise two phases (Figure 4c,d and Table 1). We first observe the decay of the absorption of Z at 515 nm, accompanied by a partial decay of GSB at 400 nm, in \sim 2 ms (Figure 4c). This phase corresponds to the return of Z to the ON state. During the second phase (\sim 500-ms component; Figure 4d), neutral GSB at 400 nm, due to the initial excitation and depopulation of form A (of the ON state), decays partially while anionic GSB appears at 515 nm. We assign this phase to the restoration of acid-base balance

between the A and B forms of the ON state following the partial photoconversion of form A to the OFF state.

The photoswitchable H145Y variant exhibits a time evolution of its TA signals at ns and longer scales qualitatively similar to Dreiklang, but with faster kinetics (Table 1 and Figure S12). In particular, OFF-state absorption is already at its maximum at 30 ns even in deuterated buffer (Figure S16), suggesting that photoswitching is faster than 10 ns. We conclude that the OFF state must already be present in the terminal 20-ns spectrum measured with the fs setup (inset of Figure 3c), although unresolved due to limited signal-to-noise ratio. The quantum yields of photoswitching and Y formation, respectively estimated at 0.6% and 0.4% (Figure S18), are similar to those of Dreiklang. Importantly, the involvement of species Y and Z in the dynamics of H145Y, which does not exhibit ESPT, indicates that they belong to a different reaction pathway. This excludes their assignment to ground-state anionic forms resulting from chromophore deprotonation in the excited state.

In the case of the non-photoswitchable Y203F variant, the 20-ns spectrum has the peculiarity of presenting two positive bands (with peaks at 367 and 487 nm; inset of Figure 2b). The 487-nm band gains intensity and broadens at the μ s scale (average lifetime: 3.4 μ s; Figure S13b and Table 1), a spectral evolution similar to that observed for Dreiklang and H145Y and that we assign to the Y \rightarrow Z transition. A full decay of the 367-nm band is observed on the same time scale (Figure S13b). The simultaneity of the two processes suggests that the 367-nm and 487-nm bands both belong to species Y, which therefore has a different spectrum in Y203F. Further ms-scale dynamics show the decay of Z towards the ON state (average lifetime: 220 ms; Figure S13c,d and Table 1). OFF-state formation and acid-base equilibration in the ON state are not observed in Y203F, in agreement with its non-photoswitchable character. The fact that the Y \rightarrow Z sequence exists in both photoswitchable

and non-photoswitchable proteins and that it occurs in the former at a time scale longer than photoswitching clearly demonstrates that it corresponds to an independent reaction pathway.

The present study highlights the complex photoinduced dynamics of ON-state Dreiklang, which involves four parallel reaction pathways starting from the neutral Franck-Condon excited state A_{FC}^* (Scheme 2). The first two pathways (in black and gray) start with the formation of the CT state in 100 fs, with a quantum yield of ~95%. The black pathway then leads to the OFF state via X with a quantum yield of 0.4%, while the gray one restores the ON state. The third pathway (in green) involves the formation of Y with a quantum yield of 0.2%, followed by its return to the ON state via Z. Finally, the fourth pathway (in brown) consists in the formation of a relaxed A^* state that is quenched by ESPT in Dreiklang, resulting in I^* , but fluoresces in H145Y. Both A^* and I^* are formed with a quantum yield of 5% and then return to the ON-A neutral ground state. We note that three of the four reaction pathways are non-productive, leading to recovery of the initial ON-A ground state.

We would now like to briefly discuss the mechanisms of the different reaction pathways shown in Scheme 2. The suppression of both the CT state and the ON→OFF photoswitching in the Y203F variant supports the proposal of the Krylov and Nemukhin groups that photoswitching is initiated by electron transfer from Tyr203 to the chromophore.^{16, 17} In line with the theoreticians, we also assume that X is likely a complex of the cationic chromophore (with protonated imidazolinone ring) with anionic Tyr203 and the water molecule involved in chromophore hydration.¹⁷ This species would be formed after the primary ET by PT from Tyr203 to the chromophore, followed by back-ET.¹⁷ If this holds true, OFF-state formation from X would then require both addition of the water molecule's hydroxyl ion onto the chromophore's imidazolinone ring and capture of the proton by Tyr203.¹⁷ Such a scenario could explain the strong kinetic isotope effect observed experimentally during this step, if proton capture is kinetically limiting. The main disagreement between experiment and theory

concerns the height of the barrier. We found a characteristic time of 33 ns for OFF-state formation from X. Using the Eyring-Polanyi equation of transition state theory and assuming a transmission frequency of 1, we estimate that the Gibbs free energy barrier to water addition is ~7-kcal/mol, which is significantly higher than the 1 kcal/mol barrier predicted by the calculations.¹⁷

The nature of the green reaction pathway remains unclear. Species Y and Z are characterized by low quantum yields, long lifetimes in the μ s-ms range and red-shifted spectra relative to the initial ON-A state. We tentatively propose that they might correspond to the triplet excited state of the neutral chromophore and one of its products.²⁹ Finally, besides CT state formation and recombination, ESPT is the second most important reaction pathway, with a quantum yield of ~5%. Its suppression in the H145Y variant is consistent with our previous proposal that His145 acts as the proton acceptor.¹⁴ ESPT results in the formation of species I*, assigned to an excited anionic chromophore surrounded by an unrelaxed protein pocket and which then decays to the neutral ground state ON-A on the ps-ns scale.¹⁴ The fact that no deprotonated ground-state intermediate is observed suggests that chromophore re-protonation in the ground state is faster than the decay of I* by fluorescence and internal conversion, as it is also the case in GFP.²¹

In conclusion, the combination of ultrafast (fs-ps) and conventional (ns-s) TA spectroscopy enabled us to cover all time scales of the photoinduced dynamics of Dreiklang in its ON-A state. We were able to establish the characteristic ON→OFF photoswitching time (33 ns) and its quantum yield (0.4%). The study of the H145Y and Y203F variants provides additional new insight into the role of electron and proton transfer processes in Dreiklang's excited-state dynamics. The emerging overall picture is that of a competition between photoswitching and three non-productive reaction pathways. Our results support the involvement of an electron transfer from Tyr203 to the chromophore in photoswitching, as suggested by the Krylov and

Nemukhin groups.^{16, 17} Over 90% of the excited population is lost through rapid charge recombination of the resulting CT state. The excited chromophore can alternatively be deactivated by ESPT involving His145 as the proton acceptor (with 5% yield), followed by a back-PT upon decay to the initial ground state. Finally, a minor non-productive reaction pathway (with 0.2% yield) is responsible for kinetics on the μs -ms scale.

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Supporting Information Available:

Experimental methods. Steady-state absorption and fluorescence emission spectra. Thermal recovery kinetics. Femto-picosecond TA data of Dreiklang. Femto-picosecond DADS and kinetic traces. Graphical estimation of the quantum yields of ESPT and A* and Y formation. Nano-millisecond TA data of H145Y and Y203F. Kinetic isotope effects on OFF-state formation. Residuals of all fits.

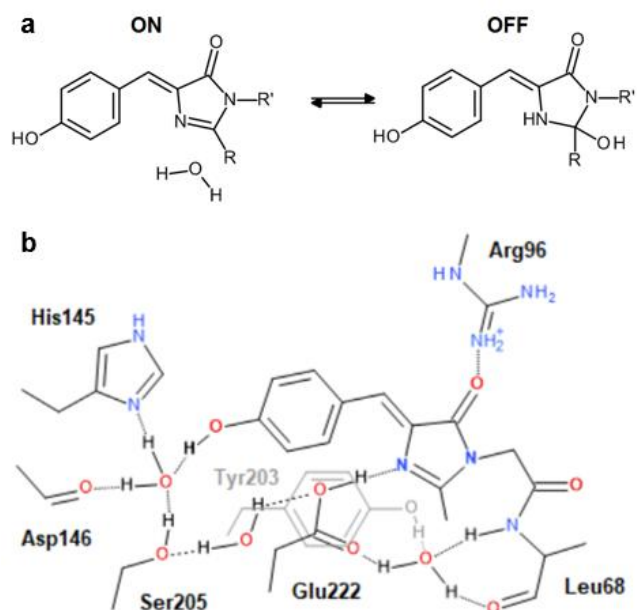
References:

- (1) Jensen, N. A.; Jansen, I.; Kamper, M.; Jakobs, S. Reversibly switchable fluorescent proteins for RESOLFT nanoscopy. *Top. Appl. Phys.* **2020**, *134*, 241-261.
- (2) Brakemann, T.; Stiel, A. C.; Weber, G.; Andresen, M.; Testa, I.; Grotjohann, T.; Leutenegger, M.; Plessmann, U.; Urlaub, H.; Eggeling, C.; Wahl, M. C.; Hell, S. W.; Jakobs, S. A reversibly photoswitchable GFP-like protein with fluorescence excitation decoupled from switching. *Nat. Biotechnol.* **2011**, *29*, 942-947.
- (3) Arai, Y.; Takauchi, H.; Ogami, Y.; Fujiwara, S.; Nakano, M.; Matsuda, T.; Nagai, T. Spontaneously blinking fluorescent protein for simple single laser super-resolution live cell imaging. *ACS Chem. Biol.* **2018**, *13*, 1938-1943.
- (4) Andresen, M.; Stiel, A. C.; Trowitzsch, S.; Weber, G.; Eggeling, C.; Wahl, M. C.; Hell, S. W.; Jakobs, S. Structural basis for reversible photoswitching in Dronpa. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 13005-13009.
- (5) Brakemann, T.; Weber, G.; Andresen, M.; Groenhof, G.; Stiel, A. C.; Trowitzsch, S.; Eggeling, C.; Grubmüller, H.; Hell, S. W.; Wahl, M. C.; Jakobs, S. Molecular basis of the light-driven switching of the photochromic fluorescent protein Padron. *J. Biol. Chem.* **2010**, *285*, 14603-14609.
- (6) Adam, V.; Lelimosin, M.; Boehme, S.; Desfonds, G.; Nienhaus, K.; Field, M. J.; Wiedenmann, J.; McSweeney, S.; Nienhaus, G. U.; Bourgeois, D. Structural characterization of IrisFP, an optical

- highlighter undergoing multiple photo-induced transformations. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 18343-18348.
- (7) Fron, E.; Flors, C.; Schweitzer, G.; Habuchi, S.; Mizuno, H.; Ando, R.; Schryver, F. C.; Miyawaki, A.; Hofkens, J. Ultrafast excited-state dynamics of the photoswitchable protein Dronpa. *J. Am. Chem. Soc.* **2007**, *129*, 4870-4871.
- (8) Warren, M. M.; Kaucikas, M.; Fitzpatrick, A.; Champion, P.; Sage, J. T.; van Thor, J. J. Ground-state proton transfer in the photoswitching reactions of the fluorescent protein Dronpa. *Nat. Commun.* **2013**, *4*, 1461.
- (9) Yadav, D.; Lacombat, F.; Dozova, N.; Rappaport, F.; Plaza, P.; Espagne, A. Real-time monitoring of chromophore isomerization and deprotonation during the photoactivation of the fluorescent protein Dronpa. *J. Phys. Chem. B* **2015**, *119*, 2404-2414.
- (10) Laptенок, S. P.; Gil, A. A.; Hall, C. R.; Lukacs, A.; Iuliano, J. N.; Jones, G. A.; Greetham, G. M.; Donaldson, P.; Miyawaki, A.; Tonge, P. J.; Meech, S. R. Infrared spectroscopy reveals multi-step multi-timescale photoactivation in the photoconvertible protein archetype dronpa. *Nat. Chem.* **2018**, *10*, 845-852.
- (11) Coquelle, N.; Sliwa, M.; Woodhouse, J.; Schiro, G.; Adam, V.; Aquila, A.; Barends, T. R. M.; Boutet, S.; Byrdin, M.; Carbajo, S.; De la Mora, E.; Doak, R. B.; Feliks, M.; Fieschi, F.; Foucar, L.; Guillon, V.; Hilpert, M.; Hunter, M. S.; Jakobs, S.; Koglin, J. E.; Kovacsova, G.; Lane, T. J.; Levy, B.; Liang, M.; Nass, K.; Ridard, J.; Robinson, J. S.; Roome, C. M.; Ruckebusch, C.; Seaberg, M.; Thepaut, M.; Cammarata, M.; Demachy, I.; Field, M.; Shoeman, R. L.; Bourgeois, D.; Colletier, J. P.; Schlichting, I.; Weik, M. Chromophore twisting in the excited state of a photoswitchable fluorescent protein captured by time-resolved serial femtosecond crystallography. *Nat. Chem.* **2018**, *10*, 31-37.
- (12) Woodhouse, J.; Nass Kovacs, G.; Coquelle, N.; Uriarte, L. M.; Adam, V.; Barends, T. R. M.; Byrdin, M.; de la Mora, E.; Bruce Doak, R.; Feliks, M.; Field, M.; Fieschi, F.; Guillon, V.; Jakobs, S.; Joti, Y.; Macheboeuf, P.; Motomura, K.; Nass, K.; Owada, S.; Roome, C. M.; Ruckebusch, C.; Schiro, G.; Shoeman, R. L.; Thepaut, M.; Togashi, T.; Tono, K.; Yabashi, M.; Cammarata, M.; Foucar, L.; Bourgeois, D.; Sliwa, M.; Colletier, J. P.; Schlichting, I.; Weik, M. Photoswitching mechanism of a fluorescent protein revealed by time-resolved crystallography and transient absorption spectroscopy. *Nat. Commun.* **2020**, *11*, 741.
- (13) Uriarte, L. M.; Vitale, R.; Nizinski, S.; Hadjidemetriou, K.; Zala, N.; Lukacs, A.; Greetham, G. M.; Sazanovich, I. V.; Weik, M.; Ruckebusch, C.; Meech, S. R.; Sliwa, M. Structural information about the trans-to-cis isomerization mechanism of the photoswitchable fluorescent protein rsEGFP2 revealed by multiscale infrared transient absorption. *J. Phys. Chem. Lett.* **2022**, *13*, 1194-1202.
- (14) Lacombat, F.; Plaza, P.; Plamont, M. A.; Espagne, A. Photoinduced chromophore hydration in the fluorescent protein Dreiklang is triggered by ultrafast excited-state proton transfer coupled to a low-frequency vibration. *J. Phys. Chem. Lett.* **2017**, *8*, 1489-1495.
- (15) Grigorenko, B. L.; Polyakov, I. V.; Krylov, A. I.; Nemukhin, A. V. Computational modeling reveals the mechanism of fluorescent state recovery in the reversibly photoswitchable protein Dreiklang. *J. Phys. Chem. B* **2019**, *123*, 8901-8909.
- (16) Sen, T.; Ma, Y.; Polyakov, I. V.; Grigorenko, B. L.; Nemukhin, A. V.; Krylov, A. I. Interplay between locally excited and charge transfer states governs the photoswitching mechanism in the fluorescent protein Dreiklang. *J. Phys. Chem. B* **2021**, *125*, 757-770.
- (17) Grigorenko, B. L.; Polyakov, I. V.; Nemukhin, A. V. Modeling light-induced chromophore hydration in the reversibly photoswitchable fluorescent protein Dreiklang. *Molecules* **2023**, *28*.
- (18) Grimsley, G. R.; Scholtz, J. M.; Pace, C. N. A summary of the measured pK values of the ionizable groups in folded proteins. *Protein Sci.* **2009**, *18*, 247-251.
- (19) Nathanael, J. G.; Gamon, L. F.; Cordes, M.; Rablen, P. R.; Bally, T.; Fromm, K. M.; Giese, B.; Wille, U. Amide neighbouring-group effects in peptides: phenylalanine as relay amino acid in long-distance electron transfer. *ChemBioChem* **2018**, *19*, 922-926.
- (20) Jaye, A. A.; Stoner-Ma, D.; Matousek, P.; Towrie, M.; Tonge, P. J.; Meech, S. R. Time-resolved emission spectra of Green Fluorescent Protein. *Photochem. Photobiol.* **2006**, *82*, 373-379.

- (21) Kennis, J. T.; Larsen, D. S.; van Stokkum, I. H.; Vengris, M.; van Thor, J. J.; van Grondelle, R. Uncovering the hidden ground state of green fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 17988-17993.
- (22) Walter, A.; Andresen, M.; Jakobs, S.; Schroeder, J.; Schwarzer, D. Primary light-induced reaction steps of reversibly photoswitchable fluorescent protein Padron0.9 investigated by femtosecond spectroscopy. *J. Phys. Chem. B* **2015**, *119*, 5136-5144.
- (23) Lukacs, A.; Haigney, A.; Brust, R.; Addison, K.; Towrie, M.; Greetham, G. M.; Jones, G. A.; Miyawaki, A.; Tonge, P. J.; Meech, S. R. Protein photochromism observed by ultrafast vibrational spectroscopy. *J. Phys. Chem. B* **2013**, *117*, 11954-11959.
- (24) Larsen, D. S.; van Stokkum, I. H.; Vengris, M.; van Der Horst, M. A.; de Weerd, F. L.; Hellingwerf, K. J.; van Grondelle, R. Incoherent manipulation of the photoactive yellow protein photocycle with dispersed pump-dump-probe spectroscopy. *Biophys. J.* **2004**, *87*, 1858-1872.
- (25) Seifert, M. H.; Georgescu, J.; Ksiazek, D.; Smialowski, P.; Rehm, T.; Steipe, B.; Holak, T. A. Backbone dynamics of Green Fluorescent Protein and the effect of histidine 148 substitution. *Biochemistry* **2003**, *42*, 2500-2512.
- (26) Dozova, N.; Lacombat, F.; Bou-Nader, C.; Hamdane, D.; Plaza, P. Ultrafast photoinduced flavin dynamics in the unusual active site of the tRNA methyltransferase TrmFO. *Phys. Chem. Chem. Phys.* **2019**, *21*, 8743-8756.
- (27) Zhuang, B.; Seo, D.; Aleksandrov, A.; Vos, M. H. Characterization of light-induced, short-lived interacting radicals in the active site of flavoprotein Ferredoxin-NADP⁺ Oxidoreductase. *J. Am. Chem. Soc.* **2021**, *143*, 2757-2768.
- (28) Müller, P.; Brettel, K. [Ru(bpy)₃]²⁺ as a reference in transient absorption spectroscopy: differential absorption coefficients for formation of the long-lived ³MLCT excited state. *Photochem. Photobiol. Sci.* **2012**, *11*, 632-636.
- (29) Byrdin, M.; Duan, C.; Bourgeois, D.; Brettel, K. A long-lived triplet state is the entrance gateway to oxidative photochemistry in green fluorescent proteins. *J. Am. Chem. Soc.* **2018**, *140*, 2897-2905.

Scheme 1. (a) Chromophore structures in the ON and OFF states of Dreiklang and (b) active site of Dreiklang in its ON state^a



^aAccording to the crystallographic structure of ref 2 (PDB ID: 3ST4). We added the water molecule observed between residues Ser205 and Glu222 in the simulations of ref 15. The chromophore is shown in bold and the hydrogen bonds in dotted lines. Tyr203, which is located below the chromophore, is shown in gray.

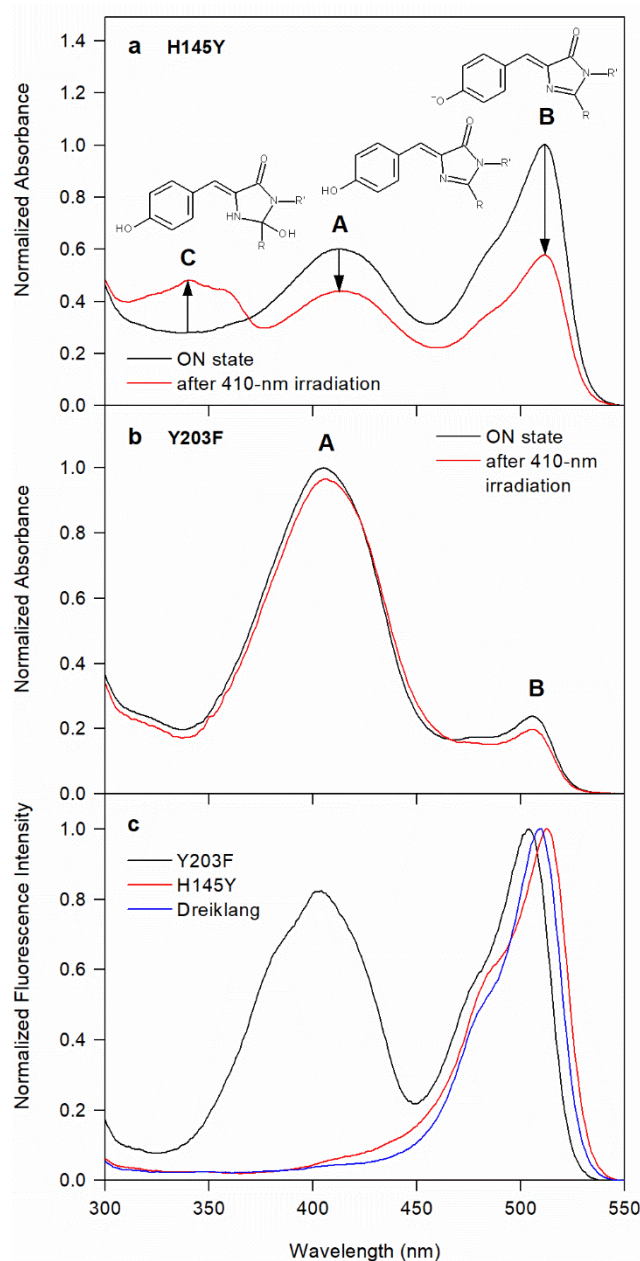


Figure 1. Steady-state spectroscopy of Dreiklang and its H145Y and Y203F variants at pH 7.0. (a, b) Absorption spectra of H145Y (a) and Y203F (b) in the ON state (black) and after irradiation at 410 nm (red). The spectra were normalized with respect to the maximum absorption of each protein in the ON state. We show in (a) the chromophore structures associated with absorption bands A, B and C. (c) Normalized fluorescence excitation spectra of Y203F (black), H145Y (red) and Dreiklang (blue). The fluorescence was detected at 560 nm.

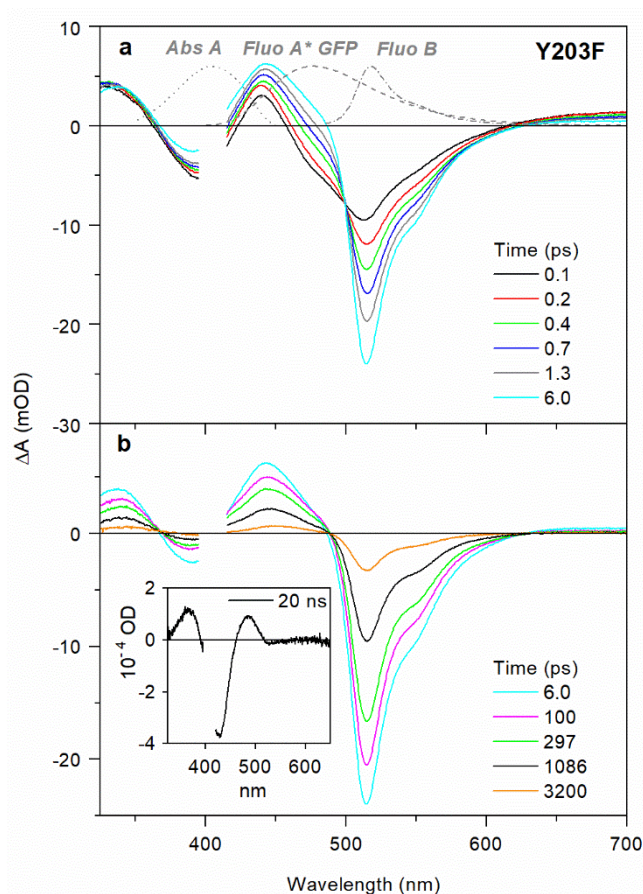


Figure 2. Femtosecond transient absorption spectra of the neutral ON state (form A) of Y203F measured at pH 7.0, after 405 nm excitation. The data were corrected for the chirp of the probe. The region near 405 nm, obscured by pump scattering, was masked. (a) 0.1 to 6.0 ps. The gray dotted, dashed, and dash-dotted lines are the steady-state absorption spectrum of the sample, the fluorescence spectrum of the A* state of GFP (emission of the neutral chromophore measured before excited-state deprotonation, by time-resolved fluorescence spectroscopy; taken from ref 20), and the steady-state fluorescence spectrum of form B of Y203F, respectively. (b) 6.0 to 3200 ps. The inset displays an additional spectrum measured at 20 ns, after complete excited-state decay.

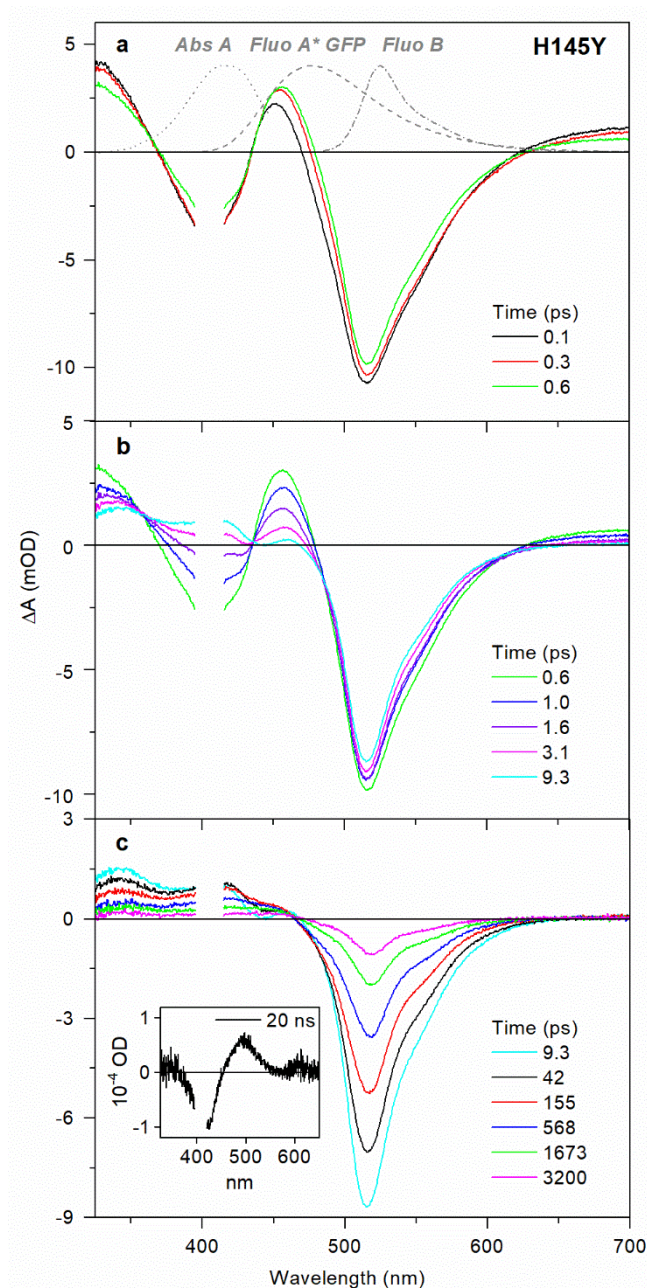


Figure 3. Femtosecond transient absorption spectra of the neutral ON state (form A) of H145Y measured at pH 7.0, after 405 nm excitation. The data were corrected for the chirp of the probe. The region near 405 nm, obscured by pump scattering, was masked. (a) 0.1 to 0.6 ps. The gray dotted, dashed, and dash-dotted lines are the steady-state absorption spectrum of the sample, the fluorescence spectrum of the A* state of GFP (emission of the neutral chromophore measured before excited-state deprotonation, by time-resolved fluorescence spectroscopy; taken from ref 20), and the steady-state fluorescence spectrum of form B of H145Y, respectively. (b) 0.6 to 9.3 ps. (c) 9.3 to 3200 ps. The inset displays an additional spectrum measured at 20 ns, after complete excited-state decay.

Table 1. Kinetic Analysis of the Multiscale Transient Absorption Data of Dreiklang and its H145Y and Y203F Variants^b

Variant	Branching/ESPT ^c	CT decay	Decay of I*/A* ^d	X → OFF	Y → Z	Decay of Z	A/B balance
Dreiklang	100 ± 10 fs	750 ± 30 fs	19 ± 1 ps (51 %) 108 ± 8 ps (39 %) 1.8 ± 0.2 ns (10 %) <τ> = 230 ps	33 ± 1 ns	6.6 ± 0.1 μs	2.29 ± 0.03 ms	476 ± 2 ms
H145Y	145 ± 4 fs	920 ± 30 fs	32 ± 4 ps (26 %) 230 ± 40 ps (29 %) 2.2 ± 0.2 ns (45 %) <τ> = 1.1 ns	< 10 ns	217 ± 3 ns (64 %) 2.9 ± 0.1 μs (36 %) <τ> = 1.2 μs	8.1 ± 0.1 μs	156 ± 4 μs
Y203F	130 ± 10 fs (25 %) 1.0 ± 0.1 ps (65 %) 5.7 ± 0.5 ps (10 %) <τ> = 1.3 ps	-	107 ± 5 ps (21 %) 1.20 ± 0.02 ns (79 %) <τ> = 970 ps	-	115 ± 1 ns (34 %) 1.39 ± 0.01 μs (33 %) 8.7 ± 0.1 μs (33 %) <τ> = 3.4 μs	572 ± 5 μs (52 %) 84.8 ± 0.5 ms (14 %) 608 ± 2 ms (34 %) <τ> = 220 ms	-

^bThe characteristic times of branching, CT decay and decay of I* in Dreiklang are taken from ref 14. All other characteristic times were determined in the present study. For multiexponential processes we give the individual components with their weights in parentheses and the average characteristic time <τ>.

^cBranching between CT and A* for Dreiklang and H145Y. ESPT for Y203F.

^dDecay of I* for Dreiklang and Y203F. Decay of A* for H145Y.

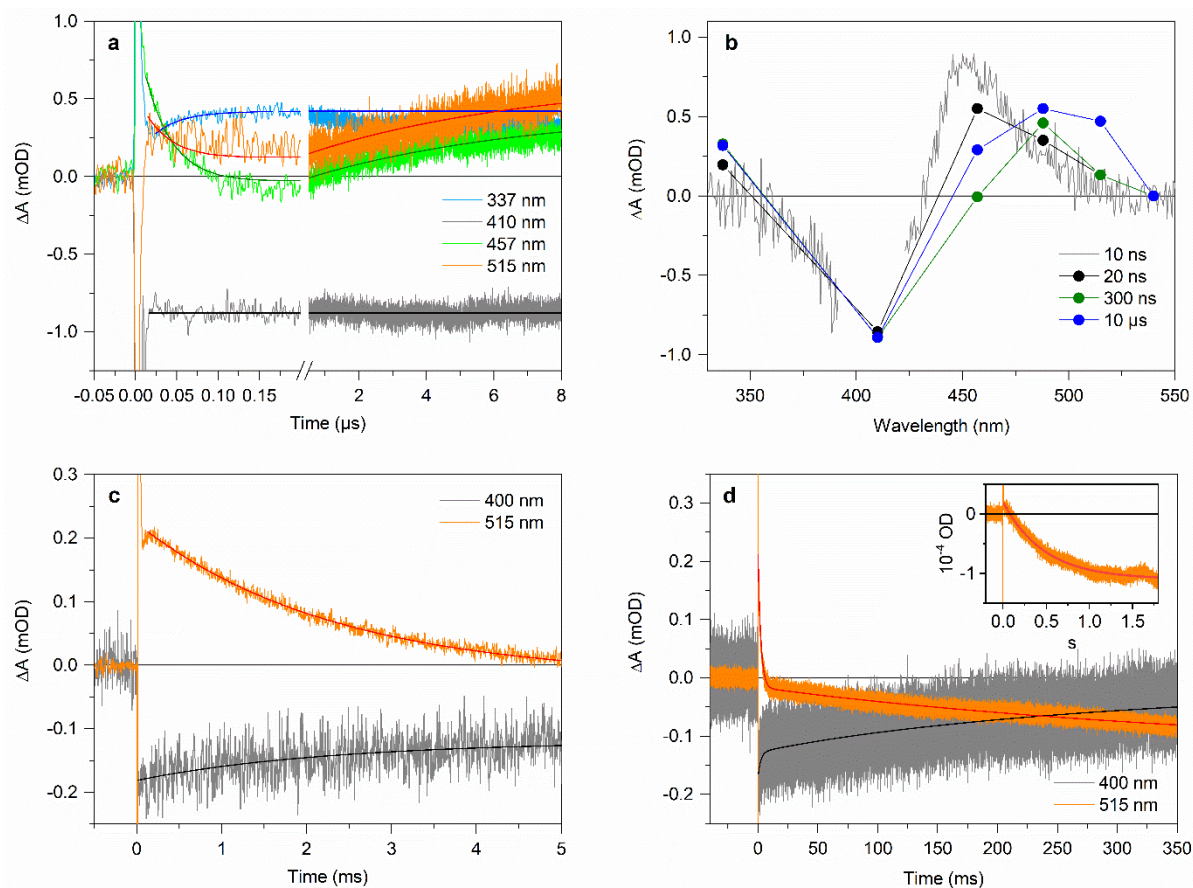
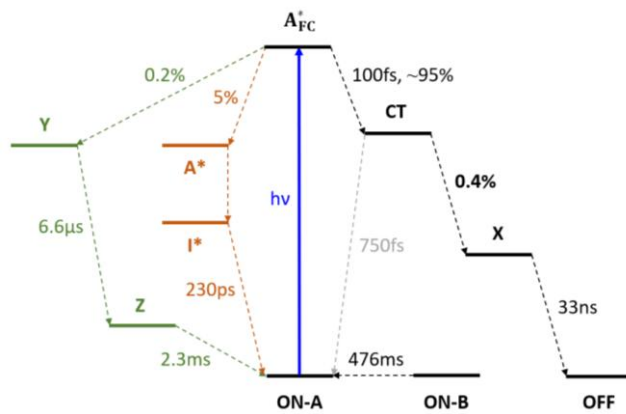


Figure 4. Nano- to millisecond transient absorption data of the neutral ON state (form A) of Dreiklang measured at pH 7.0, after excitation at 430 nm. (a) Kinetics at a few representative wavelengths in the nano-microsecond time range. The initial peaks correspond to unresolved ultrafast signals and the smooth lines to a global biexponential fit with time constants of 33 ± 1 ns and 6.6 ± 0.1 μ s. (b) Spectra at a few representative delays in the same time range, constructed from the individual kinetics. The data were scaled to the 10-ns spectrum from ref 14, shown here in gray. (c, d) Kinetics at 400 nm and 515 nm in the millisecond time range. The initial peaks correspond to unresolved fast signals and the black and red lines to a simultaneous biexponential fit with time constants of 2.29 ± 0.03 ms and 476 ± 2 ms. The inset in d shows the end of the long component at 515 nm, in the second time range.

Scheme 2. Proposed model for the light-induced dynamics of the ON-A state of Dreiklang^c



^cThe quantum yield and characteristic time values are those of the original Dreiklang protein.