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# Ultrafast Excited-State Dynamics of the Photoswitchable Protein Dronpa

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Dronpa is a photoswitchable green fluorescent protein (GFP) evolved from a Pectiniidae coral.[1] Its bright form absorbs at 503 nm and emits at 518 nm. Irradiation of the bright form with intense light leads to its conversion into a nonfluorescent form characterized by an absorption band at 388 nm (Figure 1). The quantum yield for this forward photoswitching ($\Phi_{FSW}$) is $3.2 \times 10^{-4}$. Conversely, irradiation of the dark form with UV light results in efficient back photoswitching ($\Phi_{BSW} = 0.37$) to the bright form. The bright and dark forms correspond to a deprotonated (B, anionic) and protonated ($A_2$, neutral) form of the chromophore, respectively. It has been proposed that the back photoswitching mechanism involves efficient excited-state proton transfer (ESPT) from the excited state of $A_2$ to an intermediate I (and not to B directly) (Figure 1).[1,2] Interestingly, the protonated form (termed $A_1$) existing at pH < p$K_a$ (=5)[1] is different from $A_2$ since it lacks photoconversion properties, although their absorption spectra are nearly identical.[1,2]

In this communication, we report our results on an ultrafast transient absorption spectroscopy investigation of Dronpa, with the particular goal of identifying the rate for the initial step of photoswitching from the neutral dark form $A_2$ to the intermediate I, which eventually evolves to the anionic fluorescent form B. We have also measured protonated Dronpa at low pH ($A_1$) in order to help in the assignment of the kinetic components.

First, deprotonated Dronpa (B) in Tris buffer at pH 8 was irradiated at 495 nm (200 fs, fwhm cross correlation) and probed with a white light continuum. The absorbance changes were detected with a CCD camera (see Supporting Information, SI). The resulting transient spectra were characterized by ground-state bleaching and stimulated emission peaking at 510 nm and a small excited-state absorption band with a maximum at around 420 nm (Figure S1). The kinetics of both bands were fitted by a monoexponential decay of 3.2 ± 0.1 ns, close to the fluorescence lifetime of B measured by single photon timing (3.6 ns).[2]

The next series of experiments was performed on excitation at 395 nm, with the aim of comparing the photoconverted protonated Dronpa at pH 8.0 ($A_2$) with the pH-induced protonated form in 50 mM phosphate/citrate buffer at pH 5.0 ($A_1$). Since our CCD camera is not sensitive enough in the spectral region of interest, we used a setup in which the probe light transmitted through the sample was detected by a PMT and in which the signal was further improved by a heterodyne technique (see SI). This signal acquisition does not directly yield ΔOD and needs a different interpretation. For example, under these conditions, ground-state depletion and subsequent recovery will appear as an instantaneous increase of signal followed by a decay. This is in contrast to the CCD-based measurements, in which ground-state depletion results in negative

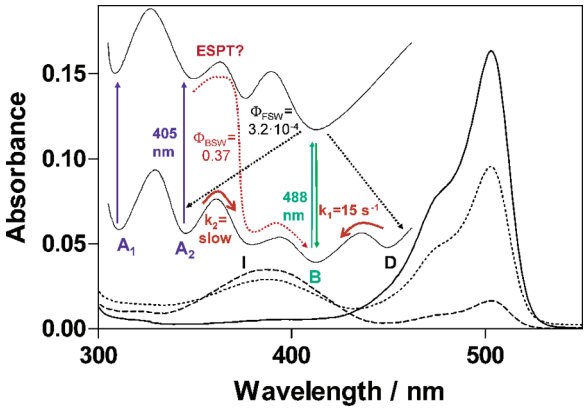


***Figure 1.*** Absorption spectra of Dronpa (2 μM) at pH 8 (—), at pH 5 (· · ·), and photoconverted at pH 8 (- - -). Inset: photoswitching scheme.

ΔOD (see SI). Samples were placed in a flow cell to avoid back photoconversion and/or irreversible photodestruction. For the measurement of the photoconverted form, the sample was irradiated continuously with 527 nm light to preserve it in the $A_2$ form. Global analysis on the data obtained for $A_1$ (Table 1) at pH 5 revealed that three time constants were needed to fit the data satisfactorily in the 50 ps time window, with values of 2.0 ± 0.3, 13 ± 2, and 90 ± 5 ps. The 2 ps component is important at 380 and at 410 nm, where it appears as a decay with 80% amplitude. At these wavelengths, we expect to observe the instantaneous ground-state depletion of $A_1$ and its recovery with a time constant equivalent to the excited-state lifetime of $A_1$. We assign this kinetic component to the internal conversion of $A_1$ ($k_{IC} = 5 \times 10^{11}$ s$^{-1}$). The 2 ps component also shows up as a decay at 450 nm (30% amplitude), which we attribute to stimulated emission from the excited state of $A_1$.[2] In the range of 415−430 nm, the signal also rises in 2 ps, which suggests that excited-state absorption of $A_1$ occurs in that spectral region. As for the 13 and 90 ps components, we tentatively attribute them to changes in the protein environment around the chromophore at different time scales, which might be linked to excited-state relaxation.[3a]

The traces recorded for the photoconverted protonated Dronpa were first analyzed individually by fitting two or three exponential functions, followed by global analysis. Very similar time constants as for the pH 5 protonated form were retrieved, plus an additional component of 4.0 ± 0.5 ps and a longer one of ca. 700 ps (Table 1). The 2 ps component, attributed before to the excited-state lifetime of the protonated form, showed a similar spectral contribution as above, that is, a decay at 370−390 and 450 nm and a rise at 415−430 nm. The 13 and 90 ps components were also present, but with different spectral distribution (vide infra). The 4 ps component showed up as a decay with an amplitude of 30% at 420 and 430 nm (Figure 2, left). Since this component was absent

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***Table 1.*** Amplitude (%) of the Kinetic Components Extracted from Global Analysis of the Femtosecond Transient Absorption Data

| Wavelength | pH5 | | | Photoswitched | | | | |
|---|---|---|---|---|---|---|---|---|
| global fit | 2 ps | 13 ps | 90 ps | 2 ps | 4 ps | 13 ps | 90 ps | long |
| 370 nm | not determined | | | 59 | 0 | 37 | −4 | 0 |
| 380 nm | 80 | 0 | 20 | 58 | 0 | 38 | −4 | 0 |
| 390 nm | not determined | | | 31 | 0 | 18 | −30 | 22 |
| 410 nm | 80 | 8 | 12 | 0 | 0 | −37 | 0 | 63 |
| 415 nm | −57 | 17 | 26 | −36 | 0 | 13 | 0 | 51 |
| 420 nm | −51 | 0 | 49 | −37 | 30 | 0 | 33 | 0 |
| 430 nm | −25 | 22 | 53 | −3 | 30 | 0 | 67 | 0 |
| 450 nm | 27 | 27 | 46 | 32 | 0 | 39 | 29 | 0 |

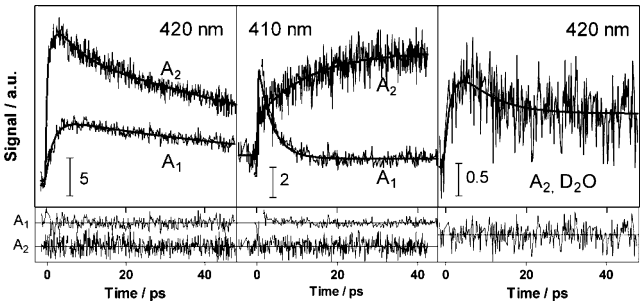


***Figure 2.*** Transient absorption signals, fits, and residuals of the two protonated forms of Dronpa ($A_1$ and $A_2$) with $\lambda_{ex} = 395$ nm in H- and D-containing buffer. Scale bars allow comparison of signal intensity.

in the pH-induced protonated form ($A_1$), which shows no photoswitching to B, we attribute it to the initial photoconversion step from $A_2$ to I. This time constant is consistent with the quantum yield of the photoconversion[1] ($\Phi_{BSW} = 0.37 = k_{BSW}/(k_{BSW} + k_{IC})$). If we assume that $k_{IC}$ is $5 \times 10^{11}$ s$^{-1}$, the value found for the protonated form $A_1$, we can estimate $k_{BSW}$ as $3 \times 10^{11}$ s$^{-1}$, which corresponds to a time constant of 3.4 ps, close to the measured 4 ps.

The 700 ps component, which has a main contribution at 390−415 nm, might be related to relaxation of the protein environment after photoconversion since it was not present at pH 5.

Although some of the time constants of the data set of $A_1$ are very similar to those of $A_2$ (except for the components related to photoconversion), it is interesting to note that their spectral contributions are quite different; that is, the shape of the traces is different at some wavelengths. For example, Figure 2 (center) shows the traces at 410 nm, in which the dissimilarity is evident. From this data, it is difficult to assign the difference to a particular structural feature, but it serves to illustrate that $A_1$ and $A_2$ are distinct species. Owing to their different photoconversion properties, these two protonated forms have been suggested to differ in their protein environment, although their steady-state spectroscopic features are very similar.[2] It appears from our data that the difference is more evident when probing their excited-state properties.

Finally, to clarify if the first step for photoconversion involves ESPT, we performed the measurements at 420 and 430 nm in a photoconverted deuterated sample, where ESPT is expected to slow down. These measurements yielded rather noisy signals with small amplitudes, presumably due to the lower efficiency for photoconversion in the deuterated sample. At 420 nm, we find a trace with a similar shape to that of the non-deuterated sample, that is, a 2 ps rise followed by a decay of $8 \pm 2$ ps and a longer component (>90 ps) (Figure 2, right). The 2 ps component was not slowed down in $D_2O$, consistent with its attribution to internal conversion of the protonated form. At 430 nm, we recorded a signal that could be fitted with a single exponential of $10 \pm 1$ ps (Figure S2). The 2-fold slower rate in the deuterated sample (from 4 to 8−10 ps) confirms that ESPT is involved in the first step for photoconversion. The isotope effect is smaller than for GFP, for which a 3- to 6-fold slower rate was found.[3] A different ESPT pathway that involves a different, less extensive reorganization of the H-bonding network in Dronpa as compared to GFP might be the cause for the smaller isotope effect. Based on the recently solved crystal structure of the deprotonated form of Dronpa, it has indeed been proposed that the proton relay mechanism is different for Dronpa than for GFP.[4] It has also been proposed that, similar as for other GFPs,[5] photoisomerization is involved in the photoswitching of Dronpa due to the similarity of its crystal structure with that of asFP595.[6] However, a recent study shows that photoisomerization cannot exclusively account for the large spectral shift observed upon photoconversion.[7]

In conclusion, we have spectroscopically characterized the excited states of Dronpa and identified the rate for the first step of photoconversion of $A_2$, which involves ESPT due to its sensitivity to H/D exchange. We also demonstrated that the acid-induced protonated form $A_1$ and the photoconverted protonated form $A_2$ are two distinct spectroscopic species. Further work on this topic, in combination with X-ray data of the photoconverted form and time-resolved Raman data, should provide a more detailed view of the nature of the intermediates and of the extent of H-bonding reorganization upon photoconversion.

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**Supporting Information Available:** Materials and Methods; transient absorption spectra and traces at pH 8 ($\lambda_{ex} = 495$ nm); kinetic traces of deuterated Dronpa, $\lambda_{ex} = 395$ nm. This material is available free of charge via the Internet at http://pubs.acs.org.

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