A Double Decarboxylation in Superfolder Green Fluorescent Protein Leads to High Contrast Photoactivation

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Supporting Information

ABSTRACT: A photoactivatable variant of superfolder green fluorescent protein (GFP) was created by replacing the threonine at position 203 with aspartic acid. Photoactivation by exposure of this mutant to UV light resulted in conversion of the fluorophore from the neutral to the negatively charged form, accompanied by a ~95-fold increase in fluorescence under 488 nm excitation. Mass spectrometry before and after exposure to UV light revealed a change in mass of 88 Da, attributed to the double decarboxylation of Glu 222 and Asp 203. Kinetics studies and nonlinear power-dependence of the initial rate of photoconversion indicated that the double decarboxylation occurred via a multiphoton absorption process at 254 nm. In addition to providing a photoactivatable GFP with robust folding properties, a detailed mechanistic understanding of this double decarboxylation in GFP will lead to a better understanding of charge transfer in fluorescent proteins.

The discovery and subsequent manipulation of naturally occurring fluorescent proteins (FPs) has revolutionized the field of biological microscopy by enabling the visualization of biomolecules in vivo. In addition to emission spectra that span much of the visible region of the spectrum, many FPs have photoconversion functionalities that make them useful for tracking biomolecule dynamics in live cells and have been used for superresolution imaging techniques. Perhaps the most well-known FP with photoconversion functionality is photoactivatable green fluorescent protein (PA-GFP), which can be irreversibly photoactivated from a dark to a brightly emissive state with high contrast upon exposure to near-UV light. This photoactivation is thought to be initiated by an excited state electron transfer process, which has been proposed to occur in many FPs, and has been exploited in the development of other photoconvertable FPs including PA-GFP, DsRed, PA-mCherry, and LSS-mOrange. Here, we show that this charge transfer (CT) pathway can be altered by a single amino acid substitution to impart the photoactivatable functionality onto superfolder GFP (sfGFP), which is otherwise not photoactivatable. In addition to providing a basis for the modulation of photoactivation in other FPs, we think that this photoactivatable mutant will provide a convenient model for understanding the mechanisms of CT in FPs.

Photoconversion in FPs was first observed in the wild type form of GFP, whose embedded fluorophore is formed from the residues Ser 65, Tyr 66, and Gly 67 (hereafter SYG). In the ground state, the GFP fluorophore can be either neutral or negatively charged depending on the protonation state of Tyr 66. The neutral form (A state) absorbs maximally at 400 nm and is present in a ~ 6:1 ratio relative to the negative form (B state), which absorbs maximally at 490 nm. Excitation of either of these states leads to green fluorescence with a high quantum yield (~0.6), either directly from the excited B state or from a deprotonated form of the excited A state after excited state proton transfer. Although fluorescence is the dominant decay pathway of these excited states, several infrequent CT pathways exist that lead to interesting fluorophore chemistry, including bleaching, oxidative reddening, and decarboxylation of Glu 222. Upon repeated illumination, these pathways can lead to the accumulation of various photoproducts and has been the subject of a recent review regarding photoinduced chemistry in FPs. For example, the accumulation of decarboxylated Glu 222 in GFP causes an increase in the B state absorption at the expense of the A state. This is ultimately due to the change in fluorophore pH that accompanies the decarboxylation of Glu 222. Optimization of the residues surrounding the fluorophore led to the development of PA-GFP, which exhibits a high fluorescence contrast (under 488 nm excitation) upon accumulation of decarboxylated Glu 222 and the concomitant population shift from neutral to charged fluorophore.

In contrast to the SYG fluorophore, the one formed from Thr 65, Tyr 66, and Gly 67 (Figures 1A and 1B; hereafter TYG) has several interesting properties. First, mutants containing the TYG fluorophore typically have a dominant B state absorption at physiological pH. This largely has to do with the extra methyl group on Thr 65, which forces Glu 222 to donate a hydrogen bond to the –OH side chain of Thr 65, ultimately allowing Glu 222 to be neutral even at high pH. The neutral Glu 222 allows the negatively charged form of the fluorophore to be favored, compared to the mutants with Ser...
where Glu 222 is typically negative and destabilizes the B state. Additionally, the TYG fluorophore matures more quickly, has a higher extinction coefficient, and is more photostable, which has earned mutants containing the TYG fluorophore the title of “enhanced” GFPs due to their improved use as fluorescence tags for microscopy experiments.

While the TYG fluorophore offers spectroscopic benefits, it is inherently incompatible with the high contrast photoactivation that is observed in PA-GFP due to its increased B state absorbance. Additionally, if Glu 222 is indeed neutral in GFP mutants with Thr 65, then decarboxylation of this group would not represent as drastic of an electrostatic change (compared to the decarboxylation of negative Glu 222) and would not be expected to shift the fluorophore equilibrium significantly. Furthermore, the reduction potential of neutral glutamic acid is much lower than that of the negatively charged carboxylate, which means that neutral Glu 222 will be less likely to undergo the initial CT step that is necessary for decarboxylation. To illustrate these points, we attempted to photoactivate superfolder GFP (sfGFP), which has desirable folding properties and contains the TYG fluorophore. Figure 1C shows the absorption spectrum of sfGFP before (black) and after (red) 10 min of exposure to 254 nm light with a power density of \( \sim 7 \) mW/cm\(^2\). As expected, because of the dominant B state absorption before UV exposure, we observed only a minimal increase in the B state absorption (\( \sim 10\% \)) after 10 min of UV exposure.

We then became interested in making mutations that would allow the TYG fluorophore in sfGFP to be photoactivated with a similar contrast to that of PA-GFP, which contains the SYG fluorophore. Because the SYG and TYG fluorophores generally have different hydrogen bonding networks that drastically affect the pK\(_a\) Glu 222,\(^{16,17}\) we reasoned that a negatively charged residue near TYG would be necessary to force the equilibrium toward the A state such that its absorption spectrum resembled that of SYG. To do this, we replaced Thr 203 with Asp 203 because of the proximity of position 203 to Tyr 66 (Figures 1A and 1B), and because of the well-known influence of the position 203 side chain on the fluorophore pK\(_a\).\(^{19,20}\) We found that this T203D (which abbreviates the replacement of Thr at position 203 with Asp) mutation was enough to force the TYG equilibrium entirely to the A state (Figure 1D; black). Titration of the T203D mutant revealed that the B state of the fluorophore was only present above pH 9 (Figure 2, black circles), with the protein unfolding before the end of the titration. This suggests that the TYG fluorophore in the T203D mutant has a pK\(_a\) of at least 10, which is at least 3 pH units higher than the pK\(_a\) of wild-type sfGFP (Figure 2, green circles; pK\(_a\) = 6.7) and almost 2 pH units higher than the highest

![Figure 1](image1.png)

**Figure 1.** (A) Top-down view of the crystal structure of sfGFP (2b3p) showing the embedded TYG fluorophore and residues Glu 222 and Thr 203. (B) A closer view of the fluorophore in sfGFP. Interactions between Thr 203 and the phenolate of Tyr 66 are shown, as well as the interaction between Glu 222 and Thr 65. (C) The absorption spectrum of sfGFP before and after 10 min of exposure to UV light. (D) The absorption spectrum of sfGFP T203D before and after 10 min of exposure to UV light.

![Figure 2](image2.png)

**Figure 2.** pH titrations of the TYG fluorophore. B state absorption relative to total protein concentration plotted against the solution pH. T203D titrates with a pK\(_a\) of at least 10 before UV exposure (black circles), and decreases to 7.2 after UV exposure (red circles). For comparison, sfGFP (green circles) titrates with a pK\(_a\) of 6.7.
Upon the generation of an excited A state (represented by the asterisk), Glu 222 (green) transfers an electron (red) to the fluorophore, which results in the generation of a radical intermediate and the release of CO$_2$ (green). The alkyl radical is quickly quenched, presumably by back transfer of an electron and a proton of the fluorophore, resulting in the stable decarboxylated Glu 222 and a deprotonation of the phenolic oxygen of the fluorophore. Scheme adapted from ref 9.

Bell et al. showed that the efficiency of the CT-initiated decarboxylation outlined in Scheme 1, which they attributed to the population of higher excited states for the protein chain that lacks the N-terminal methionine. The smaller mass corresponds to the same chain minus the N-terminal methionine residue, which is commonly lost during ionization. Grigorenko et al. performed electronic structure calculations which showed that CT states between Glu 222 and the excited fluorophore could feasibly be populated by several pathways. Additionally, the authors showed that one of the key residues in the T203D mutant occurred following a similar process to the CT-initiated decarboxylation outlined in Scheme 1.18 Following the generation of an excited state in GFP, Glu 222 can donate its electron to the fluorophore, which leads to the release of CO$_2$ and the formation of an alkyl radical at position 222. The radical is then quenched by back electron transfer from the fluorophore and extraction of a proton, presumably from a water or nearby titratable residue. This process results in a methylated alanine at position 222 and a change in the electrostatic environment around the fluorophore that now favors the B state. While no intermediate species predicted by this reaction scheme have been directly observed, considerable experimental and theoretical evidence supports the Kolbe-type decarboxylation. van Thor et al. reported crystal structures and experimental and theoretical evidence supports the Kolbe-type decarboxylation as it occurs in fluorescent proteins with the SYG fluorophore. Scheme adapted from ref 9.

The authors showed that both Glu 222 and the newly inserted Asp 203 are both negatively charged. We note that Patterson and Lippincott-Schwartz made the T203D mutant before UV exposure. Interestingly, we observed a very high contrast in the B state absorption, similar to that of PA-GFP. Additionally, after UV exposure the pK$_a$ of the T203D mutant decreased to 7.2 (Figure 2, red circles), which is similar to the wild-type sfGFP pK$_a$. We also looked for differences in infrared absorption in the carbonyl stretching region before and after the UV exposure and observed only a slight bleaching of signal in the 1650 cm$^{-1}$ region (not shown), consistent with the loss of deprotonated carboxylic acid carbonyl stretching. This, coupled with the large increase in the pre-exposure mass from 27800.0 Da to 27887.5 Da, corresponds to the full protein mass of the T203D mutant before UV exposure. The most abundant peak, with a mass of 27887.5 Da, corresponds to the full protein chain including a hexa-histidine affinity tag. The smaller mass corresponds to the same chain minus the N-terminal methionine residue, which is commonly lost during ionization. Figure 3B shows the resulting mass spectrum of the T203D mutant after it was exposed to UV light. The most abundant peak, with a mass of 27887.5 Da, corresponds to the full protein chain including a hexa-histidine affinity tag. The smaller mass corresponds to the same chain minus the N-terminal methionine residue, which is commonly lost during ionization. Figure 3A shows the resulting mass spectrum of the T203D mutant before UV exposure. The most abundant peak, with a mass of 27887.5 Da, corresponds to the full protein chain including a hexa-histidine affinity tag. The smaller mass corresponds to the same chain minus the N-terminal methionine residue, which is commonly lost during ionization. Figure 3B shows the resulting mass spectrum of the T203D mutant after it was exposed to UV light as described above. Interestingly, the most abundant mass in this spectrum (27800.0 Da) corresponds to the pre-exposure mass from Figure 3A minus 87.5 Da. There is also a mass loss of 87.3 Da for the protein chain that lacks the N-terminal methionine. These mass changes are strongly indicative of the loss of two CO$_2$ groups, which can be explained by the decarboxylation of both GFP and the newly inserted Asp 203. Further inspection of Figure 3B shows several other masses that were not present in Figure 3A, which correspond to the singly decarboxylated species and a small amount of protein that did not decarboxylate at all. The observation that both GFP and Asp 203 became decarboxylated is further support that both residues are negatively charged, considering that anionic carboxylate is a better electron donor than neutral carboxylic acid.

This finding suggests that Asp 203 can also donate an electron to the excited fluorophore to initiate a decarboxylation similar to the one outlined in Scheme 1. If Asp 203 can also initiate the CT, then the site of initial CT could be a tunable wavelength in the order of 254 nm >280 nm >476 nm, which they attributed to the population of higher excited states that are stronger oxidizers and thus can accept an electron from GFP 222 more efficiently. In both studies by van Thor et al. and Bell et al., the authors observed that the rate of photoconversion followed first order kinetics, and the independent studies generally agreed upon the observed rate constant.
parameter in the design of fluorescent proteins with increased photoconversion efficiencies. To assess the utility of the Asp 203 mutant as a high contrast fluorescence agent, we recorded the fluorescence spectrum under 488 nm excitation before and after the UV exposure. This is shown in Figure 4A, where we observed a ~95-fold increase in the integrated fluorescence after 10 min of UV exposure, which is similar to the activation efficiency by moving the charge donor site closer to the fluorophore, then it might be expected that the CT processes to the excited state of the fluorophore, and exhibit high contrast photoactivation. In addition to the kinetics of decarboxylation, we were also interested in the power-dependence of the photoconversion rate, because of the information it contains about the reactive excited state (denoted by the asterisk in Figure 1). In Figure 4C, we show the kinetics of the photoconversion at different irradiation powers between 0.6–57.7 mW/cm². In all cases, the growth of the B state absorption as a function of time could not be well-described by a first-order kinetic process. Rather, the photoactivated fluorophore has a pK_a of 7.2 (Figure 2), which means there is still an A state population, even after the double decarboxylation of Glu 222 and Asp 203. Plotting the B state absorption as a function of the irradiation time (Figure 4C, green data points) for this photoconversion yielded a curve that was not well-described by first-order kinetics (see Figures S1 and S2), which is in contrast to the kinetics that have been observed for photoconversion reactions in GFP and PA-GFP. However, as mentioned above, the presence of two CT processes in the same system occurring with different efficiencies, either in parallel or in series, might lead to a complex rate equation for this double decarboxylation. Preliminary attempts to quantify the kinetics suggest that the reaction might obey second-order kinetics. Further experiments are underway to build a fully descriptive kinetic model for this process, which will lead to an understanding of the determinants of CT efficiencies to the GFP fluorophore.

In addition to the kinetics of decarboxylation, we were also interested in the power-dependence of the photoconversion rate, because of the information it contains about the reactive excited state (denoted by the asterisk in Scheme 1). In Figure 4B, we observed a clean isosbestic point at 432 nm, which indicated that the UV exposure induced a photoconversion between only two states. Fifteen minutes of exposure at this irradiation power caused conversion from a fluorophore population that existed almost entirely in the A state to a fluorophore population with a ~2:3 ratio of A to B state. We note that this does not imply an incomplete photoconversion. Rather, the photoactivated fluorophore has a pK_a of 7.2 (Figure 2), which means there is still an A state population, even after the double decarboxylation of Glu 222 and Asp 203. Plotting the B state absorption as a function of the irradiation time (Figure 4C, green data points) for this photoconversion yielded a curve that was not well-described by first-order kinetics (see Figures S1 and S2), which is in contrast to the kinetics that have been observed for photoconversion reactions in GFP and PA-GFP. However, as mentioned above, the presence of two CT processes in the same system occurring with different efficiencies, either in parallel or in series, might lead to a complex rate equation for this double decarboxylation. Preliminary attempts to quantify the kinetics suggest that the reaction might obey second-order kinetics. Further experiments are underway to build a fully descriptive kinetic model for this process, which will lead to an understanding of the determinants of CT efficiencies to the GFP fluorophore.

More relevant factor in determining the efficiency of CT should simply be the distance between the donor and acceptor. Stark spectroscopy has revealed that the imidazolinone ring is electron deficient in the excited A state, which supports its role as the initial electron acceptor as drawn in Scheme 1. If this holds true in the T203D mutant, then it should be the case that CT from Asp 203 is less efficient than CT from Glu 222 due to its increased distance from the imidazolinone ring of the fluorophore (0.8 vs 0.6 nm, estimated from crystal structure 2b3p; Figure 1A). As such, it seems likely that the kinetics of this double decarboxylation process might not obey a first-order rate law if there are indeed two electrons that transfer with different efficiencies.
display linear power-dependence with 254 nm irradiation over a similar range of irradiation power. Rather, the logarithmic power dependence that we observed in Figure 4D suggests that the reactive form of the excited A state is generated by an excited state absorption process. We note that while here we have only analyzed the initial rates, we observed the same logarithmic power dependence when analyzing the rate constants obtained from higher-order exponential fits (data not shown). However, the nature of the reactive excited state is currently unknown, and undoubtedly plays a major role in the CT efficiencies between the fluorophore and Asp 203 and Glu 222 (and thus the observed kinetics). Until we have information from experiments that can probe the dynamics of the excited fluorophore, we can only speculate about the mechanism of this photoconversion and the observed kinetics.

Altogether, these observations on the photoconversion properties of sfGFP mutant T203D lead to several questions regarding the Kolbe-type decarboxylation that has been proposed to occur throughout the FP family. First and foremost, does the decarboxylation of Asp 203 occur via a similar process to the one outlined in Scheme 1? If so, then it might be the case that CT to the excited state of the fluorophore can be tuned to achieve more efficient photoconversion. Because of the generality of this Kolbe-type decarboxylation among the FP family, the tunability of CT distance could be exploited to improve other photoactivatable FPs that function similarly to PA-GFP.

Future studies of the kinetics of this decarboxylation will focus on elucidating the pKₐ of both Glu 222 and Asp 203, because those values ultimately determine the concentrations of negative carboxylates, and thus the reactive species. High resolution crystal structures of the T203D mutant could provide information about the complex hydrogen bonding network surrounding the fluorophore in the ground state. Moreover, the nature of the excited state that acts as the initial electron acceptor could be probed using ultrafast, pulsed excitations and electronic structure calculations. Specific questions of interest are: (1) Is the reactive excited state generated by excited state absorption? (2) What is the nature of the reactive excited state? Is the reactive excited state in this T203D mutant the same as it is in PA-GFP? (3) How do the pKₐ values of Glu 222 and Asp 203 depend on either group being decarboxylated? For instance, would the decarboxylation of Glu 222 change the pKₐ of Asp 203, and thus the concentration of reactive acid? A detailed mechanistic view of the double decarboxylation reported here will allow for a better understanding of CT reactions that occur in a wide range of FPs. Mutations of this nature could provide a tunable parameter in the design of FPs with new properties, which has so far been unexplored.

### EXPERIMENTAL METHODS

The gene for sfGFP was generously provided by Ryan Mehl and was mutated at position 203 using a QuickChange Mutagenesis kit from Stratagene following the manufacturer recommended procedure. The mutated sequences were verified by Sanger sequencing, and all sfGFP genes were expressed and purified as detailed elsewhere. Purified proteins

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**Figure 4.** Spectroscopic and kinetic properties of photoconversion. (A) Fluorescence of the T203D mutant before (black) and after (red) 10 min of UV exposure indicating a ~95-fold increase in the fluorescence under 488 nm excitation due to the photoconversion. (Inset) Enhanced view of the black spectrum that is partially masked by the x-axis. The area under the red spectrum is ~95 times more than that under the black spectrum. (B) Absorption spectra of the TYG fluorophore in the T203D mutant as a function of UV irradiation time, plotted at 1 min intervals for a total of 15 min. The black spectrum was taken before irradiation and the greener spectra correspond to later time points. (C) The B state absorption as a function of time for several different UV irradiation powers. Best-fit lines are drawn over the time course that corresponds to the linear portion of the curve to estimate the initial rates. (D) The initial rates of photoconversion from panel C plotted against irradiation power.
were exposed to UV light (254 nm peak wavelength) from a Spectroline XX-15G lamp at distances of 42.2, 22.1, 12.1, 7.6, and 4.1 cm to achieve power densities of 0.6 mW/cm$^2$, 1.9 mW/cm$^2$, 6.9 mW/cm$^2$, 17.2 mW/cm$^2$, and 57.7 mW/cm$^2$, respectively. Titrations were carried out by buffer exchanging the purified protein into a master buffer of 50 mM phosphate, 50 mM citrate, and 100 mM NaCl at pH 7.5. The protein was then concentrated to ~1 mM and diluted by 150x with the master buffer (adjusted to the desired pH with NaOH or HCl) into a transparent 96-well plate. Visible absorbance was measured on a Biotek Epoch 2 plate reader. Fluorescence spectra were recorded with a Horiba Fluorolog 3 spectrometer.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.7b01101.

Single and double exponential fits to time-dependent spectral changes of sfGFP T203D (PDF)

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


