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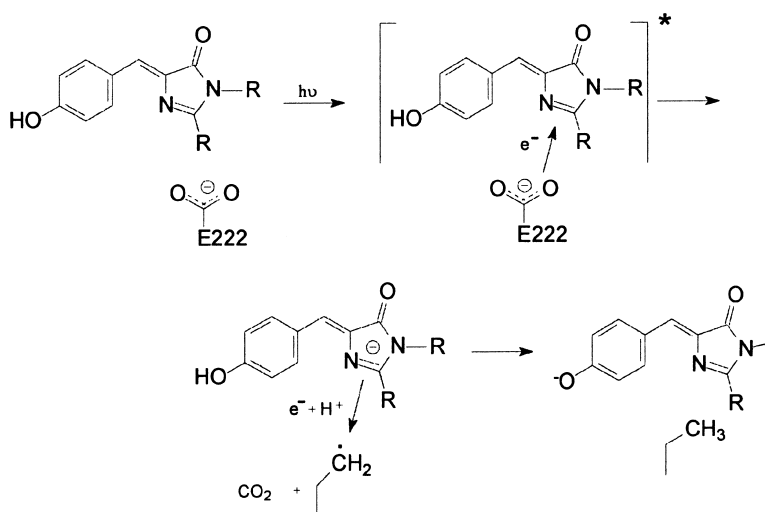# Light-Driven Decarboxylation of Wild-Type Green Fluorescent Protein

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Light-Driven Decarboxylation of Wild-Type Green Fluorescent Protein†

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Abstract: The response of wild-type GFP to UV and visible light was investigated using steady state absorption, fluorescence, and Raman spectroscopies. As reported previously [van Thor, *Nat. Struct. Biol.* **2002**, *9*, 37−41], irradiation of GFP results in decarboxylation of E222. Here it is reported that the rate of the light-driven decarboxylation reaction strongly depends on the excitation wavelength, decreasing in the order 254 nm > 280 nm > 476 nm. The relative efficiencies of decarboxylation are explained in terms of the Kolbe-type mechanism in which the excited state of the chromophore acts as an oxidant by accepting an electron from E222. Specifically, it is proposed that 254 nm excitation populates the S2 (or higher) excited state of the chromophore, whereas 404 and 476 nm excitation populate the S1 excited state of neutral and anionic forms, respectively, and that the relative oxidizing power of the three excited states controls the rate of the decarboxylation reaction. In addition, the role of W57 in the photophysics of GFP has been probed by mutating this residue to phenylalanine. These studies reveal that while W57 does not affect decarboxylation, this residue is involved in resonance energy transfer with the chromophore, thereby partially explaining the green fluorescence observed upon UV irradiation of wild-type GFP. Finally, comparison of Raman spectra obtained from nonilluminated and decarboxylated forms of wild-type GFP has provided further vibrational band assignments for neutral and anionic forms of the chromophore within the protein. In addition, these spectra provide valuable insight into the specific interactions between the protein and the chromophore that control the optical properties of wild-type GFP.

Introduction

The green fluorescent protein (GFP) from *Aequorea victoria* has revolutionized our ability to visualize the key molecular events that occur within living cells.[1−3] This is due to an unusual post-translational modification of GFP that involves the cyclization and dehydrogenation of an internal tripeptide sequence leading to the formation of a fluorescent 4-hydroxybenzylidene-imidazolinone chromophore.[4,5] This chromophore is buried near the center of the folded protein and does not require any cofactors for its formation. By fusing the DNA sequence of GFP to the protein of interest, it is possible to express a target protein inside living cells with a fluorescent protein tag. Using fluorescence microscopy the target protein can then be monitored, generating information on the mobility and localization of the target protein and also on protein−protein interactions by using double-labeling FRET measurements.[1,2] Any experiment of this type requires light absorption by the GFP tag, and

one of our interests is the effect that this has on the structure of the chromophore and the surrounding protein. This information may be valuable in the design of new GFPs with desirable light-dependent properties, as has recently been demonstrated by Patterson et al. for wild-type GFP.[6]

It has been known for some time that the structure of the chromophore within GFP is sensitive to light.[7−10] The wild-type protein is characterized by two absorption bands at 397 nm (A-band) and 475 nm (B-band) that have been ascribed to neutral and anionic forms of the chromophore, respectively. Excitation into either of these two absorption bands, or with UV light, produces the characteristic green fluorescence with an emission maximum at around 508 nm. However, illumination of sufficient intensity and of long enough duration will also result in a photoconversion that is manifested as an increase in the absorption band due to the anionic (B-form) at the expense of the neutral (A-form) absorption band of the chromophore.[8,9] For routine experiments monitoring the location of GFP tags

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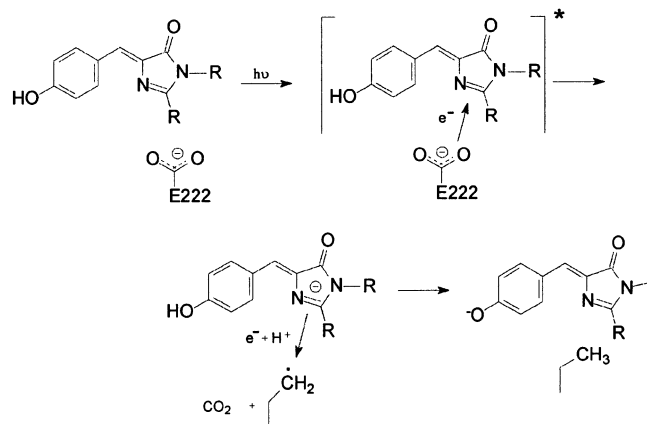
Figure 1. Proposed mechanism for light-induced decarboxylation of wild-type GFP redrawn from ref 17. The two R substituents represent the covalent links to the remainder of the protein.

within cells using fluorescence microscopy, the relatively low power densities and short time periods generally employed may not result in a significant extent of photoconversion. However, for more extreme applications, such as single-molecule experiments[11,12] or fluorescence correlation spectroscopy (FCS),[13,14] the high power densities required can almost certainly be expected to produce light-dependent changes.

Exposure of wild-type GFP to light can trigger both reversible and irreversible changes that are presumably occurring together but are expected to have different kinetics and quantum yields. Boxer and co-workers, using time-resolved fluorescence measurements, demonstrated that the reversible changes correspond to a Förster cycle,[8] leading to the development of a three-state model (A-, B-, and I-forms) for the photoisomerization of wild-type GFP. On the basis of the crystal structures of wild-type GFP and the S65T mutant, the light-induced changes were ascribed to an excited state proton transfer from the phenolic group of the chromophore along a hydrogen-bonding network involving a water molecule, S205, and E222, with the concomitant rearrangement of other neighboring residues.[9,15] Subsequently, Creemers et al. were able to identify the three distinct photoconvertible states of wild-type GFP (A-, B-, and I-forms) with low-temperature hole-burning spectroscopy.[16] More recently, irreversible changes in wild-type GFP have been characterized by a study combining X-ray crystallography and mass spectrometry. Specifically, an irreversible decarboxylation of E222 via a Kolbe-type mechanism was reported with either UV or visible irradiation of GFP, as shown in Figure 1.[17] This study revealed some differences in the ease of photoconversion with 254 and 390 nm light, but these differences were not quantified. It was also proposed that either a higher order transition of the chromophore or the lone tryptophan residue in GFP (W57) may be responsible for the relative ease of photoconversion with 254 nm light.[18]

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In the current study we explore the effect of light of different wavelengths and the role of W57 on the photoinduced decarboxylation of wild-type GFP. The rate of the decarboxylation reaction displays a pronounced dependence on the excitation wavelength (254 nm > 280 nm > 476 nm), and this is explained in terms of the proposed mechanism for this reaction.[17] Specifically, the different oxidizing power of the excited states populated by the distinct wavelengths is proposed to be responsible for the observed wavelength dependence. We also provide evidence that a higher order (S2 or greater) transition of the chromophore itself is responsible for the decarboxylation reaction proceeding with UV light. Furthermore, there is evidence that with visible irradiation the chromophore within GFP acts as a photosensitizer, producing singlet oxygen, which can in turn damage the chromophore. This means that using visible light to produce the photoconverted form of GFP is not only less efficient but also reduces the amount of protein containing intact chromophore. In another set of experiments we have examined the role of the lone tryptophan in GFP (W57) on the photophysics by mutating this residue to phenylalanine. It has been proposed that W57 is involved in resonance energy transfer with the chromophore and is responsible for the chromophore's sensitivity to UV light.[18] Our results indicate that, while this residue is involved in resonance energy transfer, it is not a contributing factor to the decarboxylation reaction. Furthermore, the energy transfer is much more efficient between the W57 and the neutral form of the chromophore, presumably due to the greater degree of spectral overlap. Finally, the ability to generate individual ionization states of the chromophore has provided us with the opportunity to expand upon the Raman band assignments for the neutral and anionic forms of the wild-type protein.

Experimental Procedures

Protein Preparation. Wild-type GFP and W57F were overexpressed in the pRSETb vector (Invitrogen). W57F was generated using the QuikChange site-directed mutagenesis kit (Stratagene). Proteins were induced in BL21 (DE3) pLysS cells with 0.5 mM IPTG and overnight incubation at 25 °C. Following resuspension in 50 mM phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, and 0.1 mM PMSF, cells were sonicated and spun at 5000 rpm for 20 min and the supernatant was loaded onto a Ni-NTA column. After elution with an imidazole gradient, wild-type GFP ran as a single band on a 12% SDS-PAGE gel. However, W57F appeared as a heavy band on SDS-PAGE but with three additional faint contaminating bands. To further purify the W57F mutant, the His-tag was cleaved with chymotrypsin and the protein reapplied to the affinity column and the flowthrough collected. After this additional purification step the mutant protein ran as a single band on SDS-PAGE.

Photoconversion. The photoconversion experiments were performed with either an argon ion laser (476 nm) or a 200 W mercury (Hg) arc lamp (Oriel Instruments) for measurements with 254, 280, and 404 nm light. Typically, the protein was placed in a 500 μL quartz microfluorescence cell with a 1 cm path length for absorption experiments and into a 3 mL quartz microfluorescence cell with 1 cm path length for fluorescence measurements. For 476 nm experiments, the argon ion laser beam was unfocused and had a diameter of approximately 1 cm, leading to a power density of around 400 mW/cm². The Hg lamp produced powers of 6, 8, and 6 mW/cm² at a distance of 10 cm for 254, 280, and 404 nm, respectively. Band-pass filters

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were used at each of these wavelengths to block all other wavelengths. All absorption measurements were performed at 25 °C on a Varian Cary100 UV−vis spectrometer. Fluorescence measurements were performed using a Spex Fluorolog-3 fluorimeter at 25 °C. A concentration of around 10 μM wild-type GFP or W57F was used in all the photoconversion experiments.

Raman Spectroscopy. The Raman spectra were acquired using an instrument optimized for operation in the near-IR that has been described in detail elsewhere.[19] By using 752 nm excitation we remove the problem of sample fluorescence and also the possibility of photoconversion occurring during data acquisition. In earlier studies we have demonstrated that at this excitation wavelength the Raman signals due to the chromophore are strongly enhanced over the background of the protein.[19] The Raman measurements were made by adding approximately 70 μL of protein to a 2 mm by 2 mm quartz cell and collecting the data. After removal of the protein sample, the same volume of buffer was added to the same cell without making any changes and the Raman spectrum measured. Difference Raman spectra were then generated by subtracting the spectrum of the buffer from that of the protein in buffer solution. A typical experiment was completed in 8 min using 700 mW laser power with a protein concentration of around 30 μM. The same concentration of GFP was used for the light and dark measurements to help remove contributions from the protein in the double difference spectrum. The spectra have been baseline corrected to account for small background changes between protein and buffer. The difference spectra were wavenumber calibrated against cyclohexanone and are accurate to ± 2 cm^{-1}. The resolution of the Raman spectrometer is approximately 8 cm^{-1}. All spectral manipulations were carried out using Win-IR software, and data acquisition was performed using WinSpec (Princeton Instruments, Trenton, NJ).

Results

Photoconversion of Wild-Type GFP. We have measured the dependence of the steady state absorption and fluorescence spectra of wild-type GFP as a function of irradiation time with 254, 280, 404, and 476 nm light. The absorption spectra of wild-type GFP as a function of irradiation time with two of these wavelengths (254 and 476 nm) are shown in Figure 2. Prior to illumination the A (protonated) and B (deprotonated) forms of the chromophore, as determined by absorption band intensity at 397 and 475 nm, respectively, are present in a ratio of 3.7:1. Given that the extinction coefficient of the anionic form is approximately double that of the neutral form, we estimate that the population of the anionic form is about 12%. During the course of illumination this ratio shifts in favor of the B-form, the B-form absorption maximum shifts from 475 to 481 nm, and there is a slight increase in absorbance at 278 nm. For both 254 and 476 nm excitation the photoconversion seems to be irreversible, since practically no change in the absorption spectrum of photoconverted GFP was observed even after 30 days in the dark at 4 °C. Similarly, a cycle of denaturation and renaturation of the photoconverted protein resulted in no change in the absorption spectrum (data not shown). Further irradiation of photoconverted wild-type GFP with 476 nm light photobleaches the sample.

The efficiency of photoconversion for wild-type GFP exhibits a strong dependence on the wavelength of light as shown in Table 1. The most efficient conversion is observed with 254 nm irradiation (6 mW/cm^2) with a first-order rate constant of $2.8 \times 10^{-4} \pm 0.8$ s^{-1}, slightly lower than the value of $1.1 \times$

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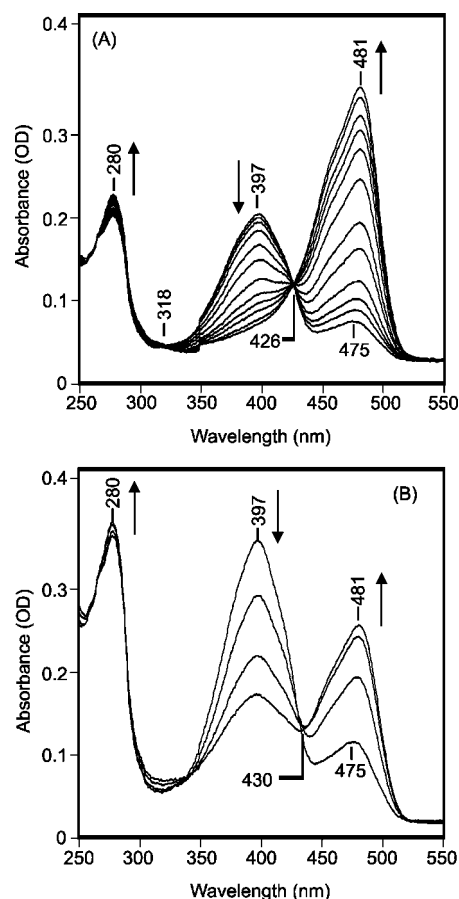
Figure 2. (A) Absorption spectra of wild-type GFP as a function of irradiation time with 254 nm light; (B) absorption spectra of wild-type GFP as a function of time for irradiation with 476 nm light.

Table 1. Wavelength Dependence for the Rate of Photoinduced Decarboxylation in Wild-Type GFP

wavelength of light (nm)	rate of photoconversion (s^{-1})	relative rate[a]
254	2.8×10^{-4}	100
280	5.8×10^{-3}	4.8
404	n.d.[b]	n.d.[b]
476	3.3×10^{-2}	0.85

[a] Corrected for the power density of light source by assuming a linear dependence. [b] No change observed with our light source. Earlier studies used intense laser sources to produce photoconversion data.[6,8]

10^{-3} s^{-1} reported by van Thor et al. with a more intense light source (12.9 mW/cm^2).[18] This rate is reduced 21-fold and 118-fold with 280 and 476 nm excitation, respectively. In contrast to other studies, we do not observe any photoconversion for excitation into the A-band (404 nm, 6 mW/cm^2) even after 6 h of irradiation. In addition, we do not observe any photoconversion with 476 nm if the laser power is reduced to the same value as used for 404 nm measurements. Earlier studies used intense pulsed laser sources to produce photoconversion data for excitation into the A-band.[6,8,17] These observations may mean that there is a wavelength-dependent threshold power density for generating photoconverted GFP and that our 404 nm light source falls below this threshold value. One possible explanation for the difference in efficiency between 254 nm and visible irradiation could be that a two-photon process occurs with visible light whereas a one-photon mechanism dominates for UV light.[20] To test this hypothesis, we measured the rate of photoconversion
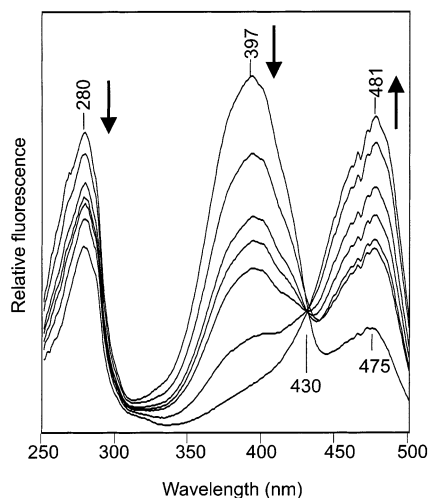
Figure 3. Fluorescence excitation spectra of wild-type GFP measured at 510 nm as a function of irradiation time with 254 nm light.

Figure 4. Comparison of the fluorescence excitation spectra of wild-type GFP (solid line) and W57F (dotted line) measured at 510 nm. The spectra have been normalized with respect to the 397 nm band.

using different power densities of 476 nm light. The linear dependence that we observed (data not shown) indicates that a one-photon process is responsible over the range 50 mW/cm^2 to 1 W/cm^2 at this wavelength, confirming the observations of van Thor et al. with 398 nm pulsed laser excitation.[17]

Another interesting observation is that 254 nm light produces a very clean photoconversion with a well-defined isosbestic point at 426 nm, indicating photoconversion between two forms. In addition, with this wavelength of light the photoconversion can be driven almost to completion (Figure 2A). By comparison, using 476 nm light an isosbestic point at 430 nm is initially observed, but on further illumination the absorbance at 397 nm continues to decrease although no more of the B-form is produced and the isosbestic point is lost (Figure 2B). These observations are consistent with an initial conversion between two forms followed by photobleaching i.e., destruction of the chromophore. One possible mechanism for photobleaching is the production of highly reactive singlet oxygen.[20] We examined this possibility by adding a singlet oxygen quencher, sodium azide (100 mM), to wild-type GFP and repeating the photoconversion experiment with 476 nm light. The addition of quencher produces a considerable reduction in the amount of chromophore damage (data not shown). These observations would be consistent with the GFP chromophore acting as a photosensitizer capable of producing singlet oxygen, which can then react detrimentally with either the chromophore or the surrounding protein.

To relate the effect of illumination directly to the fluorescence properties of wild-type GFP, we have obtained excitation spectra as a function of irradiation time with 254 nm light, as shown in Figure 3. From the excitation spectra we observe that 278, 397, and 475 nm excitation can all produce green fluorescence. For the photoconversion reaction the fluorescence excitation data display the same general trends as the absorption spectra as characterized by a decrease in the A-band (397 nm) with a concomitant increase in the B-band (475 nm). We also note that the ability of UV light to produce green fluorescence is reduced in the photoconverted form, as revealed by the decrease in the 278 nm band in the excitation spectrum. This indicates
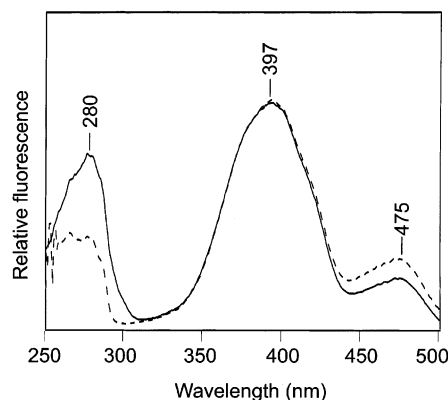
that energy transfer from the 278 nm absorbing species to the chromophore proceeds most efficiently via the neutral form of the chromophore.

Some of the properties of photoconverted wild-type GFP are significantly different from those of nonilluminated GFP. The photoconverted GFP displays a pH dependence (pK_a 5.5), whereas wild-type GFP is insensitive to pH over a wide range from pH 5 to 12.[1] There is also a shift in absorption maximum for the anionic form, from 475 to 481 nm, that presumably reflects some alteration in the environment surrounding the chromophore.

W57F Mutant: Spectral Properties and Photoconversion. There is a single tryptophan residue in wild-type GFP (W57) that lies in a nearly planar orientation about 10−12 Å from the chromophore.[21,22] Since this residue should absorb around 280 nm, it has been hypothesized that it is involved in resonance energy transfer with the chromophore and is thus responsible for the green fluorescence observed when exciting with UV light.[18] To investigate the role of W57 in the photophysics of GFP, we have mutated this residue to phenylalanine (W57F). This particular mutation was chosen because phenylalanine has a significantly lower extinction coefficient and much weaker emission spectrum than tryptophan but is still an aromatic amino acid.

The excitation spectra of wild-type GFP and the W57F mutant are compared in Figure 4. The W57F mutant has an absorption spectrum almost identical to wild-type GFP in terms of the wavelength of the absorption bands. However, there is a major difference in the ratio of the 278 and 397 nm bands. For wild-type GFP this ratio is 1.1:1 (in favor of the 278 nm band), whereas we observe a value of nearly 5:1 for W57F. Given that there is no protein contamination (we estimate >95% purity from SDS-PAGE), and assuming that the extinction coefficients at 280 nm are the same in wild-type and W57F mutant, this means that we have about 20% chromophore formation for W57F. The fluorescence excitation and emission spectra of the wild-type and mutant GFPs are also very similar, but again we observe one key difference. The excitation spectrum monitored at 510 nm exhibits a reduction of about 50% in the 278 nm band relative to wild-type GFP and a new band is revealed at

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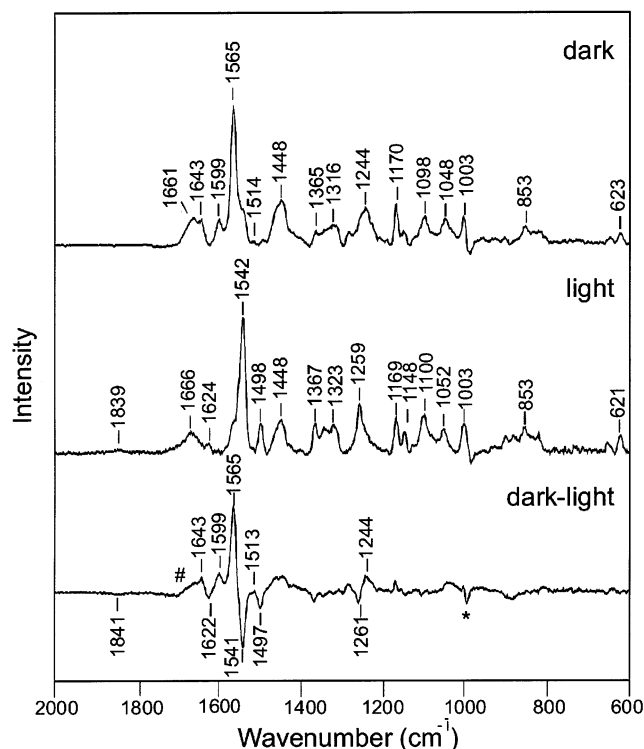
Figure 5. (A) Raman spectrum of wild-type GFP prior to illumination; (B) Raman spectrum of wild-type GFP after 90 min illumination with 254 nm light; (C) difference spectrum generated by subtracting the light spectrum from the dark spectrum. # denotes residual amide I band and * denotes phosphate buffer band.

Table 2. Summary of Raman Data for Wild-Type GFP, the S65T Mutant, and a Model Compound, HBDI, in Aqueous Solution

wtGFP[a]		S65T[b]		model[c]	
neutral	anion	neutral	anion	neutral	anion
1643		1646		1642	
	1624		1618		1631
1599		1599		1603	
					1579(sh)
1565		1560		1567	
	1542		1537		1556
1514				1525	1533(sh)
	1498		1495		1503
1448	1448	1447	1447	1449	1439
1365	1367	1366	1366		1371
1316	1323	1320	1341	1317	1310
	1259		1257		1246
1244		1251		1234	
1170	1169	1170	1170	1178	1171
1150	1148				1144
1098	1100	1082	1082		
1048	1052			1036	1037
1003	1003	1005	1003		

[a] Neutral is dark spectrum, and anion is photoconverted form. [b] Data taken from ref 19. [c] Data taken from ref 23.

properties of the chromophore (Table 2). Since photoconversion leads to formation of the anionic form of the chromophore, derivative shaped features in the difference spectrum are taken to indicate a band that has shifted in wavenumber on ionization. Bands that appear in both the light and dark spectra but cancel in the difference spectrum are completely insensitive to ionization state. Finally, bands that yield positive or negative features in the difference spectrum are assigned to modes that increase or decrease in intensity upon ionization, but are not shifted in frequency.

Raman bands arising explicitly from the neutral form of the chromophore can be identified at 1643, 1599, 1565, 1514, and 1244 cm^{-1}. This list expands the number of neutral form marker bands previously identified,[19] and with the assistance of an earlier isotope labeling study on a model compound,[23] we can now assign these bands in much greater detail. Specifically, the band at 1643 cm^{-1} is assigned to a mode involving the C=C stretch of the exocyclic double bond that links the two rings. The 1599 cm^{-1} band is a phenol mode, and the 1565 cm^{-1} mode is delocalized over the imidazolinone ring with a significant C=N stretching component and perhaps some contribution from the phenol ring. The band appearing at 1514 cm^{-1} is a phenol mode and has weak Raman intensity. This mode has previously been observed only in the FT-IR spectrum of a model compound.[23] The 1244 cm^{-1} band is assigned to the C—O stretch of the phenol ring on the basis of comparison with the Raman spectrum of tyrosine and its sensitivity to protonation. This particular band appears at 1234 cm^{-1} in the neutral form of a model compound and has not previously been assigned in the protein spectrum.[23]

For the photoconverted anionic form of the chromophore, distinctive marker bands appear at 1624, 1542, 1498, 1367, and 1259 cm^{-1}. The band at 1624 cm^{-1} is the counterpart to the 1642 cm^{-1} band of the neutral form of the protein and is assigned to an exocyclic C=C stretching mode. The two bands at 1542 and 1498 cm^{-1} probably correspond to bands at 1556 and 1503 cm^{-1} observed in the anionic form of the model

267 nm. Thus, although W57 is an important contributor in resonance energy transfer with the chromophore, other factors are also involved in determining the response of GFP to UV light.

Finally, we have examined the role of W57 in the photoconversion reaction with UV light. In these experiments we used 254 and 280 nm excitation and compared wild-type and the W57F mutant. In wild-type, 280 nm excitation has a 21-fold lower efficiency than 254 nm light for the photoconversion. Using 254 nm excitation we observe a photoconversion rate of 2.8×10^{-4} ($\pm 0.8 \times 10^{-4}$) s^{-1} for wild-type as compared to 2.0×10^{-4} ($\pm 0.3 \times 10^{-4}$) s^{-1} for W57F. When taken together, these two results indicate that W57 is not involved in the photoconversion reaction. Moreover it indicates that the species responsible for photoconversion absorbs light closer to 254 nm than 280 nm. These factors indicate that a higher order electronic transition of the chromophore itself may be responsible for the photoconversion reaction with UV light.

Raman Spectrum of Photoconverted Wild-Type GFP. The effect of illumination on the Raman spectrum of wild-type GFP has been examined. On the basis of the results discussed above, we employed 254 nm irradiation for the most efficient photoconversion of GFP. The Raman spectra of GFP before irradiation (dark) and after irradiation (light) and a difference spectrum generated by subtracting the light from the dark spectra are shown in Figure 5. Using these spectra we can expand upon the previous vibrational band assignments for neutral and anionic forms of the chromophore. Furthermore, by comparing these spectra with a model compound (HBDI) and with a GFP mutant (S65T) that has a titratable chromophore, we can provide insight into the role of the protein environment in modulating the

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compound and are thus assigned to a mode with some contribution from the $-C=C-C=N-$ portion of the imidazolinone ring and a phenol mode, respectively. These bands are also shifted to lower wavenumber in the protein environment. The 1259 cm^{-1} band is assigned to the C$-$O stretch of the phenol and is found at 1246 cm^{-1} in the anionic form of the model compound.[23]

Discussion

Response of Wild-Type GFP to UV and Visible Light. The potential of photoactivatable GFPs for monitoring the time-dependent movement of labeled proteins has recently been demonstrated by Patterson et al. in experiments tracking interlysosomal membrane exchange using a light sensitive GFP mutant.[6] Similarly, efforts to produce photolabels based on red fluorescent proteins have also been reported.[24,25] A detailed understanding of the photophysics of GFPs will ultimately help to improve upon the design of such photoactivatable GFPs, and with this goal in mind, we have examined the effect of different wavelengths of light on the properties of wild-type GFP. The observed wavelength dependence can be explained in terms of the proposed Kolbe-type mechanism for this decarboxylation reaction.[17] In this mechanism (Figure 1), the excited state of the chromophore acts as an oxidant that will accept an electron from the ionized E222 residue, resulting in the formation of a radical at E222. This radical is highly unstable, and the glutamate will rapidly decarboxylate. This in turn leads to the back transfer of an electron and a proton (or a hydrogen radical) from the chromophore, which becomes deprotonated. The final products of this reaction are the anionic form of the GFP chromophore and the decarboxylated form of E222.

The experimental data on the wavelength dependence of the photoconversion rate are summarized in Table 1. For the proposed Kolbe-type mechanism there is an implicit dependence on the properties of the excited state of the chromophore and its ability to act as an electron acceptor. Using different wavelengths of light will result in different excited states being populated. For 254 nm excitation there is evidence that the S2 (or higher) excited state of the chromophore (in either neutral or anionic form) will be populated. Specifically, our experiments comparing photoconversion rates with 254 and 280 nm demonstrate that the species responsible absorbs closer to 254 nm. Furthermore, fluorescence anisotropy measurements on wild-type GFP revealed a peak at around 250 nm that was ascribed to a higher energy transition of the chromophore.[17] This is consistent with the absorption spectra of a model compound (HBDI) that exhibits peaks at about 240 and 260 nm for the neutral form and at about 250 nm for the anionic form (data not shown). In contrast, with 404 and 476 nm irradiation, the S1 states of the neutral and anionic forms of the chromophore, respectively, will be populated. There is a general agreement from semiempirical and ab initio calculations that the lowest energy transitions (S1) of the chromophore in both neutral and anionic forms can be assigned to a $\pi-\pi^*$ transition.[26−28] Furthermore, these studies have calculated that these transitions involve a significant amount of charge transfer from the phenol ring to the imidazolinone ring.

In general, excited states are expected to be both better electron acceptors and electron donors than their corresponding ground states.[20] This is because in the excited state an electron has been promoted to a higher level, leaving two unpaired electrons, one in the HOMO and one in the LUMO. Since there is an unpaired electron in the HOMO, the electron affinity is increased, making the excited state a better electron acceptor than the ground state. Similarly, because there is an unpaired electron in the LUMO, the ionization potential has been reduced, making the excited state a better electron donor. Importantly, the higher the energy of the electronic transition, the greater the increase in electron affinity (and decrease in ionization potential). The electron affinity of the chromophore is a key determinant of the photoconversion reaction because the light-dependent step is an electron transfer from E222 to the chromophore. On this basis we would predict that the photoconversion rate would follow the order 254 nm > 400 nm > 476 nm. However, the situation is slightly more complicated. Marcus theory predicts that the rate of electron transfer increases proportionally with the driving force of the reaction (free energy change between reactants and products) until a threshold is reached, after which point the rate will start to decrease (inverted region). This means that the important parameter is the difference in free energy between the HOMO of E222 and the half-filled orbital of the chromophore that can accept an electron. Unfortunately, we do not have detailed information on the relative energies of the relevant molecular orbitals. However, if we assume that the inverted region is avoided in this situation, then this would explain the observed wavelength dependence of the photoconversion reaction. Furthermore, we can predict that if the all other factors that may affect the rate of electron transfer, such as distance, orientation, and solvation, remain the same, then by altering the energy of the electronic transitions the rate of electron transfer can be manipulated.

As mentioned above, visible light is much less efficient than UV light at photoconverting wild-type GFP. To make matters worse, there is also evidence that the chromophore acts as a photosensitizer, producing singlet oxygen, which can destroy the chromophore. This is in agreement with an earlier report that used electron spin resonance (ESR) to monitor the production of singlet oxygen from GFP expressing bacteria, which concluded that visible wavelengths were able to produce singlet oxygen.[36] Thus, during experiments using GFP photoactivated with visible light, some of the fluorescent marker will be destroyed during the conversion process. This may affect the interpretation of data obtained by this method. However, it is

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not practical to use UV light for photoactivation, as this will be harmful to the living cells that are being studied.

We have also examined the role of W57 in the response to light of wild-type GFP by studying the properties of a W57F mutant with respect to the photoinduced decarboxylation reaction and to resonance energy transfer with the chromophore. Our data indicate that W57 is not important for the decarboxylation reaction. This adds support to the proposal that the S2 (or higher) state of the chromophore is responsible for the changes observed with 254 nm light. This mutation is also found to reduce the ability of UV irradiation to produce green fluorescence, i.e., resonance energy transfer with the chromophore. However, W57 is not the only determinant since there are still two weaker bands, at 276 and 267 nm, present in the fluorescence excitation spectrum of W57F (Figure 4). These excitation bands may be due either to the chromophore itself or to some of the 14 phenylalanine and 11 tyrosine residues present in W57F.

Raman Spectroscopy. Vibrational spectroscopy is a powerful method for providing detailed information on the structure of GFP chromophores. The first report by Hellingwerf and co-workers made use of the photoconversion of wild-type GFP with UV or visible light to generate difference FT-IR spectra.[18] This study concluded that irradiation did not cause the chromophore to isomerize and that E222 was not protonated during the photoconversion process. Several Raman studies have since appeared on GFPs and model chromophores.[19,23,29−32] These experimental studies have been amply complemented by ab initio calculations of the isolated chromophore.[32−34] However, because wild-type GFP is insensitive to pH, these earlier Raman studies were limited to using a GFP mutant (S65T) with a titratable chromophore to provide assignments for neutral and anionic forms of the chromophore. In the current study the photoconversion process allows the opportunity to study directly the neutral and anionic forms of wild-type GFP.

There are significant differences in the Raman spectra of neutral and photoconverted anionic forms of GFP reflecting changes in the ground state structure of the chromophore and its interactions with the surrounding protein. Inspection of Figure 5 and Table 2 reveals that the band positions of many of the normal modes are altered by deprotonation of the phenolic hydroxyl group within the protein. These shifts in band position provide information on the changes in the ground state structure induced by ionization. The current study helps to clarify some of the previous Raman band assignments,[19] as well as producing some new assignments, for the chromophore within protein.

The changes induced in the chromophore structure as a result of its multiple specific interactions with the surrounding protein can be evaluated by comparing the Raman spectrum of a model compound (HBDI) obtained in aqueous solution[19,23] with the protein spectra presented here (Figure 5). For the neutral form of the chromophore there is a striking similarity between the model compound and the protein, whereas significant changes are evident between the spectra of the anionic form in solution and in the protein. Specifically, for the anion the protein environment causes a decrease in the bond orders of the bridging C=C bond and the imidazolinone C=N stretch, whereas the phenolic C−O bond order increases. In addition, there is a pronounced shift in the phenol ring toward a quinonoid-like structure. For the neutral form, only very weak changes are

observed in the ground state except for an increase in bond order for the phenolic C−O stretching mode. This particular band shifts from 1234 to 1245 cm^{-1} in HBDI upon deprotonation and from 1244 to 1259 cm^{-1} in the protein upon deprotonation. This indicates that there are specific protein interactions with this particular bond in both the neutral and anionic forms. This is perhaps not surprising given the key role of this substituent in the excited state proton transfer known to occur in wild-type GFP.[8]

There are some small reproducible differences between the spectra of the photogenerated anionic form of wild-type GFP and the anionic form of the S65T mutant generated at high pH (Table 2).[19] The absorption maxima for the anionic forms of photogenerated wild-type GFP and S65T are 481 and 489 nm, respectively. This shift presumably reflects some subtle difference in environment around the chromophore caused by the decarboxylation of E222.[17] There are also changes in the Raman spectrum. The band at 1565 cm^{-1} in photoconverted wild-type GFP, assigned to the imidazolinone ring, is shifted to 1560 cm^{-1} in the anionic form of the S65T mutant, and the exocyclic C=C stretching mode shifts from 1624 to 1618 cm^{-1}. Since the crystal structures of S65T and photoconverted GFP are very similar, except for the missing CO$_2$ group in the photoconverted form, these changes in Raman band positions either must reflect subtle global differences in the environment of the chromophore or may indicate some direct interaction with the chromophore and the side chain of E222. The shift in the exocyclic C=C stretching mode fits well to the linear correlation between the position of this band and the absorption maxima we have previously reported.[19] Furthermore, we note that the E222 residue that is decarboxylated is positioned close to the C=N bond of the imidazolinone ring and the shift in band position of this mode on decarboxylation may reflect some interaction between the two.

Concluding Remarks

In the current study we have investigated the photophysical properties of wild-type GFP and a W57F mutant. The key findings are that the photoinduced decarboxylation of wild-type GFP displays a distinct wavelength dependence and that W57 is not involved in this process. These observations are explained in terms of the proposed Kolbe-type mechanism for this reaction and have important implications for the development of photoactivatable GFPs that can be used to study dynamics within living cells. Specifically, visible light is significantly less efficient at generating the photoconverted form of wild-type GFP than UV light and can also lead to photobleaching of the chromophore. However, although much more efficient, the use of UV light is not a realistic option since this will be harmful to the living cells that are being studied. Thus, the solution would seem to be altering the oxidizing powers of the excited state of the chromophore populated upon visible irradiation. This could most readily be achieved through site-directed mutagenesis of residues located close to the chromophore. Patterson et al. used a T203H mutant which required an intense laser source to rapidly produce the desired photoconversion.[6] By designing new mutants aimed at increasing the oxidizing power of the S1 excited state of the neutral form, it may be possible to increase the efficiency of this reaction, making it easier to use photo-activatable GFPs without specialized laser equipment. It is already known that wild-type GFP will tolerate mutations close

to the chromophore, as Patterson et al. were able to replace T203 with many different amino acids and still observe photoconversion.[6] Alternatively, it may be possible to take advantage of the ease of photoconversion with UV light by employing two- or three-photon excitation, as has recently been demonstrated for DsRed.[35]

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