Electronic Structure of the Chromophore in Green Fluorescent Protein (GFP)

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The chromophore responsible for the fluorescence from Green Fluorescent Protein (GFP) of the jelly fish *Aequorea Victoria*[1,2] is formed in an autocatalytic, posttranslational cyclization and oxidation of the tripeptide unit at residues 65−67 (Figure 1).[3] The intense fluorescence requires further interactions between this chromophore and a surrounding protein matrix whose three-dimensional structure has recently been solved.[4,5] Because no exogenous fluorescent dyes are needed, the DNA coding for GFP can be fused with that of any protein whose expression and transport can then be monitored by sensitive fluorescence methods.

The room-temperature absorption spectrum of *Aequorea* GFP exhibits two distinct bands located at 396 and 476 nm. These bands are thought to arise from the protonated (State A) and deprotonated (State B) forms of the chromophore, respectively.[3,6] Chattoraj and co-workers demonstrated that State A can be converted to State B by excited-state proton transfer followed by further protein and/or solvent relaxation.[6] The correlation of these absorption features with specific protonation states is supported by changes in the absorption and fluorescence that accompany single and multiple amino acid changes, and this is further supported by changes in the X-ray structure.[7,8] In particular, the S65T mutant and the Blue (BFP, Y66H/Y145F) Fluorescent Protein (see Figure 1) exhibit absorption spectra that appear to be associated with the lower and higher energy absorption bands, respectively, of GFP (Figure 2, top row of spectra). In this communication, we characterize the electronic structure of these species by Stark effect spectroscopy[9,10] and use this to identify the protonation state and resonance structures of the chromphore.

The absorption and Stark spectra at 77 K for wild-type GFP, BFP, and the S65T mutant are compared in Figure 2.[11] The Stark spectrum (ΔA) line shape of GFP closely matches the second derivative of the absorption spectrum (bottom panel), demonstrating that a change in dipole moment, $\Delta\mu$, dominates the Stark effect.[9,10] Quantitative analysis yields $|\Delta\mu| = 6.8 \pm 0.3$ D for the feature peaked at 21 300 cm^{-1} (470 nm). Analysis of Stark spectra taken with polarized probe light at different experimental angles, χ, between the direction of polarization and the applied electric field gives the angle ζ_A between $\Delta\vec{\mu}$ and the transition moment $\vec{m}$ of $\zeta_A = 21 \pm 7°$. As a first approximation, we can model the absorption as two bands consistent with the Stark data and shown with the dotted lines on the absorption spectrum of GFP in Figure 2. From this deconvolution, $|\Delta\mu|$ for the broad higher energy band must be smaller than ~20 D, since otherwise a larger Stark signal would have been detected in this region of the spectrum; however, it is not possible to say more.

The results for the S65T mutant and BFP are shown in Figure 2 and suggest that they are good models for the lower and higher energy features of GFP, respectively. Neither chromophore is chemically identical with GFP, so the details of the absorption line shape and Stark spectra are not expected to be identical. The low-temperature absorption spectrum of the S65T mutant is structured and narrower than that of BFP and has a similar shape to that of the model for the lower energy band of GFP (see Figure 2). The Stark spectrum (ΔA) line shape of the S65T mutant closely resembles the second derivative of the absorption spectrum; quantitative analysis yields $|\Delta\mu| = 7 \pm 0.5$ D and $\zeta_A = 20 \pm 7°$, both very similar to the lower energy band of GFP. The low-temperature absorption spectrum of BFP is quite broad but exhibits some structure. The Stark effect for BFP is much weaker than that for the S65T mutant. Its line shape closely matches the second derivative of the absorption spectrum; quantitative analysis yields $|\Delta\mu| = 2.5 \pm 0.5$ D and an unusually large value of $\zeta_A = 75 \pm 15°$, i.e., $\vec{m}$ and $\Delta\vec{\mu}$ are nearly perpendicular.[12]

These results provide insight into the electronic structures of the two forms of the wild-type GFP chromophore (Figure 1). The transition moment, $\vec{m}$, likely is along the direction of the bridge connecting the phenol and imidazolinone rings (see top structure Figure 1), as the double bond in the bridge both links the π-systems on the two rings to form the larger conjugated system and is essential to the chromophore structure.[3,13] Both the line shape of the absorption and the line shape and magnitude of the Stark effect spectra for the S65T mutant are very similar to those for GFP, lending support to the assignment of the GFP Stark effect to the lower energy band alone. In contrast to BFP, for the S65T mutant as well as for the deprotonated form of the GFP chromophore, a resonance form of the chromophore exists, whereby the negative charge is associated with the phenol oxygen in one limit and the imidazolinone oxygen in the other limit. A transfer of charge density between the oxygens on the imidazolinone and phenol rings (about 10 Å) would lead to a significant difference dipole moment. In this case, the angle ζ_A should be relatively small ($\Delta\vec{\mu}$ nearly parallel to $\vec{m}$) while $|\Delta\mu|$ should be large, which is what we observe. Similar resonance structures are not favored for the protonated form. For BFP as well as for the protonated form of the GFP chromophore, the main changes in charge density upon excitation occur on the heteroatoms of the imidazolinone ring.[14] Since these atoms are close in proximity, $|\Delta\mu|$ is expected to be small, and $\Delta\vec{\mu}$ should be nearly

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(11) Wild-type GFP was grown, extracted, and purified by Dr. M. Chattoraj as described. (Chattoraj et al. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8362−8367). BFP (Y66H/Y145F) and the S65T mutant were kindly provided by Dr. S. J. Remington (University of Oregon). Stark spectra were taken at 77 K as described in refs 9 and 10. The samples were in a 50:50 (v:v) glycerol: buffer (50 mM Hepes, 0.3 mM NaCl) solution at pH 6.5 (GFP), pH 8.0 (BFP) and pH 7.5 (S65T), and had an optical density of about 0.15 for a 25 mm path length.

(12) When ζ_A is larger than the magic angle, the variation in ΔA with χ is relatively small, making it difficult to obtain precise values for ζ_A (Lockhart, D. J.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 107). In the present case the situation is aggravated by the weakness of the Stark effect.
(13) We are not aware of polarized single-crystal spectroscopy on GFP to determine the transition moment direction relative to the molecular axes; this would be a very useful experiment.
(14) While this manuscript was being reviewed, the results of a quantum chemical calculation for wild-type GFP were published that are consistent with this intuitive picture (Voityuk et al. *Chem. Phys.* **1998**, *231*, 13−25). The authors did not consider the Stark effect, however, making a comparison between calculation and experiment difficult.

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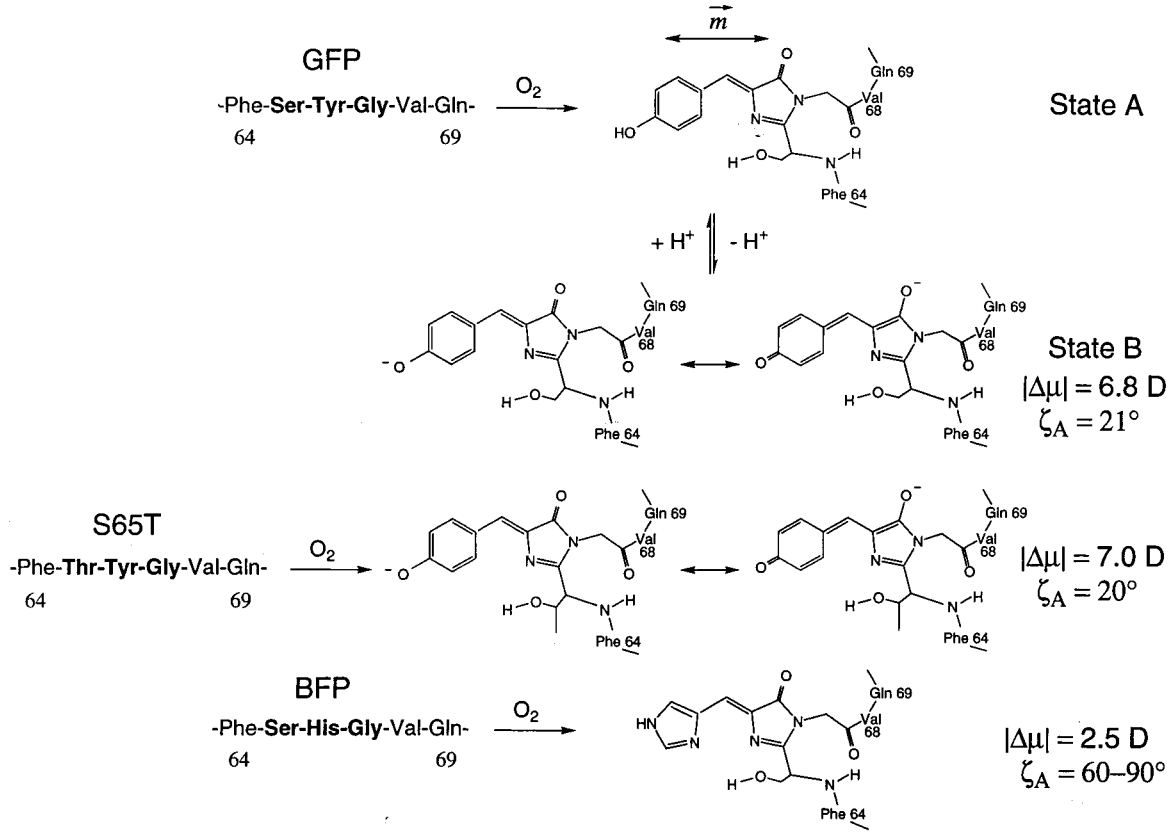

Figure 1. Primary sequences of the chromophore and nearby residues as well as the chromophore structures in GFP, the S65T mutant, and BFP (Y66H/Y145F). The arrow above the GFP structure is only an approximation to the transition moment direction (*vida infra*).[13]

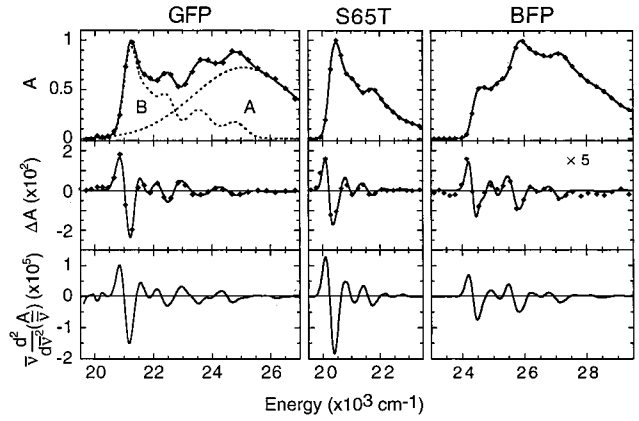

Figure 2. Absorption (top panels, ◆), Stark (middle panels, ◆), and second derivative of absorption (lower panels) of GFP (left), the S65T mutant (center), and BFP (right). The simultaneous best fits of the absorption spectrum and Stark spectrum with the second derivative are shown (top and middle panels, solid lines). Simultaneous fitting of the absorption and Stark spectra yields the deconvolution of the GFP absorption into a structured band at lower energy and a broad band at higher energy, shown as dotted lines. The spectra were scaled to a peak absorption of unity and an applied field strength of 1 MV·cm^{-1} to facilitate comparisons.

perpendicular to the direction of the transition moment, consistent with the Stark data.

These results also provide a basis for understanding the excited-state dynamics of GFP. Chattoraj et al.[6] observed that excitation of State A leads very rapidly (within a few picoseconds at room temperature) via excited state proton transfer to a state that is electronically like B. However, the quantum efficiency of conversion of A* to B, a photoconversion process that imposes limits on the utility of wild-type GFP, is much less than would be expected given the extremely rapid rate of this process. This suggests that some further process must occur before the intermediate is stabilized to form State B, something akin to solvation. The results presented here show that excitation of State A involves a rather small charge displacement, whereas the excited state dipole moment of B is much different from its ground state, and the direction of the dipole in A* is roughly perpendicular to that in B*. Because the electronic properties of A* and B* are very different, the protein environments that stabilize either state are different. Because the initially formed intermediate is environmentally like A/A* and electronically like B/B*, the protein must reorganize around the intermediate, a process that likely involves the motion of multiple residues, for example, as suggested by Brecj et al.[7] Such processes in proteins are likely to involve substantial barriers and occur on multiple time scales.[15] Thus, the Stark data not only provide insight into the electronic structures and protonation state of the forms of the chromophore in GFP, but also provide an explanation for the efficiency of the photoconversion process.

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