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Ultrafast Electronic and Vibrational Dynamics of Stabilized A State Mutants of the Green Fluorescent Protein (GFP): Snipping the Proton Wire

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Abstract

Two blue absorbing and emitting mutants (S65G/T203V/E222Q and S65T at pH 5.5) of the green fluorescent protein (GFP) have been investigated through ultrafast time resolved infra-red (TRIR) and fluorescence spectroscopy. In these mutants, in which the excited state proton transfer reaction observed in wild type GFP has been blocked, the photophysics are dominated by the neutral A state. It was found that the A* excited state lifetime is short, indicating that it is relatively less stabilised in the protein matrix than the anionic form. However, the lifetime of the A* state can be increased through modifications to the protein structure. The TRIR spectra show that a large shifts in protein vibrational modes on excitation of the A* state occurs in both these GFP mutants. This is ascribed to a change in H-bonding interactions between the protein matrix and the excited state.

INTRODUCTION

The green fluorescent protein (GFP) family is widely used in cellular and molecular imaging, owing to the presence of an intrinsically fluorescent chromophore formed autocatalytically from residues S65, Y66 and G67.(1) The cloning and in-vivo expression of GFP has made it one of the most powerful tools in cell biology.(2) In the wild-type GFP (wtGFP) two bands are observed in the electronic absorption spectrum which have been assigned to neutral (A form) and deprotonated (B form) states of the chromophore.(3–5) Excitation into either the dominant A form (λ_{max} 395 nm) or the anionic B form (λ_{max} 475 nm) results in very efficient green (λ_{em} 508 and 504 nm, respectively) fluorescence. The 508 nm emission following excitation of A arises from an anionic species formed in the excited state (I*) by deprotonation of the chromophore and concomitant protonation of the E222 carboxylate group via a proton relay chain (Figure 1), a rare example of excited state proton transfer (ESPT) in living systems. (3,6–8) The directly excited B* and indirectly excited I* states are thought to differ in that the

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former has a chromophore environment optimized for the anionic form while the latter retains the unrelaxed environment of the neutral form.(6)

Numerous mutants of GFP have been produced, many of which are designed to modify the relative population of the A and B forms. For example, the yellow fluorescent proteins (often containing the S65G mutation) yield mutants in which the B form dominates,(9) while the commonly utilised S65T mutation results in fluorescent proteins that are often highly pH sensitive, allowing for either A or B forms to be selected, and conferring on GFP the ability to act as a pH sensor. Remington, Boxer and their co-workers have characterised the structure and photodynamics of a series of such pH sensitive mutants, all containing the S65T mutation. (10–12) They found that at low pH the A* emission was favoured due to a suppression of the proton transfer reaction arising both from disruption of the proton relay network and protonation of the E222 acceptor. The excited state lifetime for A* was generally short, but the A* fluorescence yield increased with decreasing temperature. At higher pH the B state was formed and some A* to I* ESPT was observed.(12) Other mutants also retain the ability of wt GFP to undergo ESPT, but at varying rates (T203V(13)) while yet others undergo ESPT but apparently by a different route to wtGFP (S65T/H148D(14–16)).

In this work we investigate through both time resolved fluorescence (TRF) and time resolved infra-red (TRIR) spectroscopy the photodynamics of S65G/T203V/E222Q GFP (bIGFP), a mutant in which the chromophore is trapped in the neutral, blue emitting, A form. Although not of major interest for imaging applications, due to its blue shifted absorption and relatively weak emission, this mutant affords the opportunity of investigating interactions between the A state and the protein in the absence of proton transfer. These measurements are extended to S65T GFP at low pH, where both A and B forms exist but the ESPT reaction has been blocked, permitting direct excitation and study of the isolated A form. These studies yield a more detailed view on the factors determining the photophysics of GFP, in particular the mechanism which enables the chromophore to emit strongly in the protein even though it is non fluorescent in the denatured form and in aqueous solution.(17) The mechanism of the radiationless decay of the chromophore in solution has been considered in detail elsewhere, both experimentally (18–24) and in theoretical treatments.(25–30) The results described below will provide new data against which to test theoretical calculations of excited state properties in the protein, examples of which are beginning to appear.(25,31)

The remainder of the paper is organised as follows. In the next section mutagenesis and sample preparation will be described, along with the experimental methods used and some additional experimental details. In the third section the TRF and TRIR experimental results for these mutants will be presented and discussed. Conclusions are presented in the final section.

EXPERIMENTAL

Plasmid carrying bIGFP (S65G/T203V/E222Q GFP) was generated through three rounds of mutagenesis (QuikChange mutagenesis kit, Stratagene) using the following primers and the pRSETb plasmid encoding His-tagged wtGFP.

Thr203Val

Forward: 5' CCATTACCTGTCCGTACAATCTGCCCTTTTCG 3'

Reverse: 5' CGAAAGGGCAGATTGTACGGACAGGTAATGG 3'

Glu222Gln

Forward: 5' CCACATGGTCCTTCTTCAGTTTGTAACAGCTGC 3'

Reverse: 5' GCAGCTGTTACAACTGAAGAAGGACCATGTGG 3'

Ser65Gly

Forward: 5' CTTGTCACTACTTTTCGGTTATGGTGTTCATGC 3'

Reverse: 5' GCATTGAACACCATAACCGAAAGTAGTGACAAG 3'

Both the plasmid for wtGFP and for S65T GFP were obtained from Prof. Rebekka Wachter (Arizona State University). Proteins were expressed in BL21-DE3/pLysS cells (Stratagene) and using an overnight, 25°C incubation period after addition of 0.8 mM isopropyl- β -D-thiogalactopyranoside (IPTG, Fisher). Following cell lysis (French press) and centrifugation (45 minutes at 33,000 rpm), the His-tagged protein was purified using Ni-NTA resin (Novagen). Fractions containing pure bGFP were combined and dialyzed against 20 mM potassium phosphate, pH 5.5, 300 mM sodium chloride where both protein stability and a neutral ionization state were optimized. S65T GFP was dialyzed against 30 mM MES pH 5.5, 300 mM NaCl such that the neutral became chromophore dominant. Both were concentrated using Microcon YM10 centrifuge filters (Millipore). An overnight, room temperature chymotrypsin digest (1 mg per 50 mg GFP) cleaved the affinity tag, which was then removed by passing the reaction mixture through fresh Ni-NTA resin. For experiments conducted in D₂O, proteins were exhaustively exchanged with solvent deuterium by repetitive cycles of lyophilization and reconstitution with D₂O.

The extent of chromophore maturation in these proteins was somewhat less than that observed with wtGFP. The neutral chromophore of S65T pH 5.5 absorbed at 392 nm, the anion at 485 nm. The neutral form had weak emission at both 455 and 508 nm, the latter arising from absorbance overlap of the anionic form. Excitation of the anionic form yielded strong emission at 510 nm. bGFP absorbed primarily at 386 nm (neutral) with minimal absorbance at 500 nm (anionic). Excitation yielded emission peaks at 450 and 510 nm respectively, with the latter being exceedingly weak.

The two instruments employed for ultrafast spectroscopy, are based in the Ultrafast Spectroscopy Laboratory of the UK Central Laser Facility, and have been described in detail elsewhere.(32–34) The TRIR spectrometer measures IR difference spectra as a function of time after excitation by sub 200 fs 400 nm ‘pump’ pulses. The pump pulse energy was < 1 μ J weakly focused to a spot size > 200 μ m. The IR probe was a broadband difference frequency signal generated by mixing the signal and idler output from a BBO (β -Barium Borate) based optical parametric amplifier in an AgGaS₂ crystal. The time resolution (cross correlation of visible pump and IR probe) was about 400 fs and the spectral resolution 3 cm⁻¹. Typical sample concentrations were 1 mM and the sample was rastered in the beam to eliminate photodamage. The IR spectrum of water vapor was used to calibrate the TRIR data.

Most measurements were made with the ‘magic angle’ between the polarization direction of the linearly polarized pump and probe beams, to suppress the effects of molecular rotation in the measured dynamics. One set of measurements was also made with pump radiation polarized parallel and perpendicular to the IR polarization. The resultant anisotropy data can be used to determine the angle between electronic and vibrational transition moments, in a manner described elsewhere.(35) This measurement serves as a further means of assigning the observed TRIR transients.

The gated time resolved fluorescence spectra were measured with the ultrafast Kerr gate method,(32,33) which required even weaker excitation pulse intensities than TRIR and sample concentrations of only a few μ M. The spectral resolution was 4 nm and the temporal resolution about 4 ps (determined by the pulse width and the polarisability anisotropy relaxation time of the CS₂ Kerr gate). Thus the Kerr gate method has a lower time resolution than the fluorescence up-conversion method, but yields better spectral resolution.

RESULTS AND DISCUSSION

In Figure 2 absorption, emission and excitation spectra for bGFP and S65T at pH 5.5 are shown. For bGFP the A form dominates both the absorption spectrum and the emission following 385 nm excitation. The Stokes loss (judged from the absorption and emission band maxima) is 65 nm. Excitation at 500 nm results in extremely weak green B* emission. The excitation spectrum suggests that this arises in part from direct excitation of a low population of the ground state B form chromophore. The A form excitation also contributes emission at this wavelength, however this is due to the broad emission profile of A* overlapping the very weak green emission, rather than ESPT. Significantly the Stokes loss for the B form is much smaller than for the A form. From these data it may be concluded that the three mutations have stabilised the chromophore in the neutral form and suppressed ESPT to the extent that it does not compete significantly with the radiative and non-radiative decay of A*. For S65T at low pH the picture is more complicated. Both A and B forms of the chromophore contribute to the absorption and emission spectra. A is dominant in absorption but B* dominates the fluorescence spectrum. However, the excitation spectra recorded in the respective A* and B* emission bands suggest that the enhanced B* component arises from the higher relative fluorescence yield of B*, rather than a fast A* → B* ESPT reaction. Once again the Stokes loss for the A form is larger than for the B form.

This picture is supported by the time resolved fluorescence measurements (Figure 3 and Figure 4). In Figure 3 time gated fluorescence spectra recorded as a function of time after excitation are shown. For bGFP, time gated spectra decrease in intensity with increasing time but the spectral profile remains constant, suggestive of emission from a single excited state, the energy of which is not shifting on the picosecond timescale. The spectral profile is very broad, extending beyond 600 nm (as was also observed in the time gated A* emission from wtGFP (36)). The apparent very weak vibronic structure in the A* emission probably arises from interference effects in the collection optics and is not believed to be a feature of the molecule. In general vibronic structure in the neutral GFP chromophore is only observed at low temperatures.(37,38)

The broad asymmetric spectrum and large Stokes loss all point to a significant difference between absorbing and emitting state geometries for the neutral chromophore in bGFP. However, the spectral profile hardly evolves at all in the 2 – 500 ps time window observed. Thus, if the large Stokes shift reflects a time dependent geometry change in the chromophore (or its environment) following excitation it must occur on an ultrafast, probably sub-picosecond, time scale. We return to this point in the discussion of the TRIR data.

The time gated emission for S65T at low pH reveals two distinct emission bands (Figure 3b). The first is blue shifted with a broad spectrum, very similar to that observed for bGFP, and is thus assigned to the A* state; this emission has a much faster decay time in S65T. The second emission is most clearly resolved at long times and has a maximum at 508 nm, a narrow emission spectrum and a relatively long fluorescence lifetime. These features are characteristic of emission from the B* (anionic) form of the chromophore. This spectrum may have two origins – direct excitation or ESPT from the A* state. Direct excitation certainly plays a significant role as the 508 nm peak can be seen in the earliest time spectra. Indeed, simple subtraction of the gated spectrum of the long lived emission recorded at 400 ps (where the A* emission has decayed away) from all the earlier spectra yields an emission spectrum characteristic of the isolated A* state (inset to Figure 3b). This result suggests that ESPT is not a significant process in S65T at low pH, but rather that B* is directly excited and dominates the emission at later times because of its intrinsically longer lifetime. This conclusion is consistent with the excitation spectra (Figure 2) and results for related mutants studied by Boxer and co-workers.(11,12)

The kinetics of the A* fluorescence are shown in Figure 4. Data were obtained from the time gated spectra integrated between 440 nm and 460 nm, thus avoiding any contribution from the long lived B* emission in S65T GFP. The A* decay of S65T GFP at pH 5.5 is non single exponential but is dominated by a fast (sub 100 ps) component, consistent with the low fluorescence quantum yield of this sample. Similar results have been obtained for other mutants in which the ESPT has been disrupted, including S65T/H148E GFP(39), the deGFP mutants studied by Boxer et al. at low pH(11,12,40) and Y66F GFP(1) which lacks the phenolic hydroxyl group. Thus, in the absence of ESPT the A* state has a sub 100 ps lifetime, which may be compared on the one hand with the sub ps lifetime observed for the model compound (HBDI) in a range of solvents(20) and on the other with the > 1 ns lifetime and high fluorescence yield of the anionic form of the chromophore. These data suggest that while the protein stabilises the A* state compared to HBDI in solution it does not do so to the extent that it has a significant fluorescence quantum yield, but only so that it has a sufficiently long lifetime to allow the ESPT in wtGFP to occur with a high yield. The most significant protein stabilisation of chromophore excited state occurs for the highly fluorescent B* (or I*) excited state, giving it its characteristic nanosecond lifetime and high quantum yield. Evidently the protein matrix is well adapted to suppress radiationless decay in the anionic form of the chromophore.

The minor longer lived component in the A* emission has an identical emission spectrum to the short lived component, and a shorter lifetime than the B* emission. Thus, it is assigned to a subpopulation of directly excited A* states which are stabilised compared to the dominant population. Equivalently, the result suggests a minority protein environment which stabilises A* in S65T GFP to a greater extent than the majority structure. The suggestion that changes in protein structure can stabilise the A* state is supported by the blGFP A* decay, which occurs on a much longer time scale than for S65T (Figure 4). Thus, these data show that radiationless decay of the A* form (which most likely arises from internal conversion following excited state structural reorganisation(41,42)) can be suppressed by mutagenesis. However, the origin of the stabilisation is not definitively determined. Both the T203V and E222Q mutations result in more neutral groups adjacent to the chromophore. T203V is known to favour the stabilisation of the neutral A ground state.(13) These changes in charge state in the chromophore environment may also contribute to stabilisation of the excited A* state in the blGFP mutant.

More mechanistic detail on the processes underlying GFP photodynamics can be obtained from TRIR spectroscopy.(8,35,43) In Figure 5 the TRIR spectra for blGFP, S65T GFP at pH 5.5 and the neutral model chromophore HBDI are presented. TRIR spectra of the model compound and wtGFP have been discussed in great detail elsewhere.(35) The three strong bleaches seen for the model compound can be assigned to vibrational modes of the ground state chromophore mainly comprising (with decreasing wavenumber) the C=O stretch, the C=C stretch and a phenyl ring localised mode (with weaker phenyl and C=N modes at higher frequency). These three ground state modes are all evident in the wtGFP spectrum(35) and in the two mutants presented in Figure 5b and c, although the intensity distribution is significantly modified through interaction with the protein; specifically the relative intensity of the carbonyl and C=C modes to the phenyl mode are increased for the A state in the protein.

The additional complexity in the TRIR spectra on moving from HBDI to wtGFP can largely be explained by the contribution of protein modes (particularly those associated with E222) modified by the proton relay reaction, which leads to a number of new time dependent bands.(8,35,43) No such additional kinetics can occur in blGFP and S65T GFP at pH 5.5 where the proton relay reaction has been blocked, and yet the TRIR of these mutants have a number of features which are absent in neutral HBDI. In particular a broad bleach with a complex profile is observed around 1630 – 1650 cm⁻¹ and a broad transient absorption is observed below 1580 cm⁻¹. By comparison with the spectrum for HBDI much of this bleach and transient absorption should be assigned to protein modes, although chromophore C=C and C=O* both contribute

in this wavenumber range.(39) Evidently excitation to the A* state causes a number of protein modes to shift to lower frequency.

In contrast to wtGFP the time dependence of the TRIR spectra for the two mutants is relatively straightforward with a prompt (pulse limited) simultaneous appearance of both bleach and transient modes of both the chromophore and the protein, followed by recovery of the ground state on the time scale expected from the time resolved fluorescence (Figure 4). This is as expected for mutants in which the ESPT reaction is blocked, so that the excited A* state decays back to the ground state by fluorescence and radiationless internal conversion.

The assignment of the new transients in the bGFP and S65T at pH 5.5 TRIR has been further investigated through a study of the anisotropy of the TRIR spectrum of bGFP. The parallel and perpendicular polarisation resolved data recorded 4 ps after excitation are shown in Figure 5d, along with their ratio. In the absence of molecular reorientation (which can be assumed to be negligible on the 4 ps time scale) the ratio is sensitive to the angle between the electronic transition dipole of the chromophore and the direction of the transition moment of the vibration probed.(44) It can take values between 3 and 0.5 for an angle varying between 0° and 90°. From these data the assignment of the highest wavenumber bleach and the intense polarisation sensitive 1600 cm⁻¹ mode to the chromophore carbonyl and phenyl ring mode respectively can be made unambiguously through a comparison with the data in ref. (35). The band near 1640 cm⁻¹ with an anisotropy reaching 2 probably has a strong component of the C=C stretch. The sharp polarisation sensitive transient absorption on the high frequency side of the broad band around 1560 cm⁻¹ may be associated with the phenyl 1 mode in the excited state (again by comparison with anisotropy data for HBDI and Figure 6 ref.(35)).

The other bleach modes around 1620 to 1660 cm⁻¹ and the majority of the transient absorption near 1560 cm⁻¹ are assigned to overlapping contributions of protein modes perturbed by their interaction with the A* excited state and chromophore localised modes. Both the bands shape and wavenumber dependence of the anisotropy are complex for these bands, suggesting the presence of more than one vibrational mode. These modes are evidently a feature associated particularly with the A* state in mutants where the proton wire has been disrupted (we have made essentially identical observations in S65T/H148E GFP, which also does not support ESPT at low pH(39)). In contrast, the array of shifted modes (i.e. the transients around 1560 cm⁻¹) are not observed in either wtGFP or T203V GFP (both of which do undergo ESPT) even at the earliest times after excitation, prior to significant proton transfer, when the A* state dominates. In these ESPT active mutants the alterations in protein modes not associated with the protonation of E222 are largely restricted to small shifts around 1630 – 1690 cm⁻¹.(8,35, 43) Thus it may be concluded that the large shift in protein modes revealed in Figure 5b and c is associated with a perturbation by the A* state of residues involved in the disruption of the proton wire. These modes fall broadly in the frequency range expected for amide bands. The perturbation is mainly a frequency downshift, which suggests a weakening of the bonds on A* excitation. This may be explained by a change in H-bond interaction strength between the chromophore and its environment immediately following electronic excitation, although in the absence of a crystal structure or isotopic substitution in residues adjacent to the chromophore a more definite assignment is not possible

A significant mutation in disruption of the proton wire is S65T, as was found in the ESPT inactive mutants studied by Remington, Boxer and co-workers.(11,12,40) It does not however necessarily follow that the perturbed modes are directly associated with modes of T65. For example they are also seen in bGFP (which has the S65G mutation) but not in S65T/H148D GFP, in which very rapid proton transfer occurs (probably from the chromophore directly to D148(14)). A more probable assignment of these protein modes is to a group of residues interacting with the chromophore through the extensive H-bonding network in GFP, which has

been perturbed during disruption of the ESPT chain and the subsequent changes in protonation state. A more definitive assignment of these protein modes would require measurements on isotope edited mutants.

CONCLUSIONS

The time resolved fluorescence and transient vibrational spectra of two mutants of GFP which do not support ESPT have been investigated. These mutants show that the A* excited state lifetime is intrinsically quite short in the protein; although significantly stabilised relative to the model chromophore in solution. It was thus concluded that the protein stabilises the A* state only to an extent sufficient for the ESPT reaction to occur with a high yield. The protein matrix in GFP is mainly concerned with promoting the formation of and subsequently suppressing radiationless decay in the I* (or B*) form. However, it was observed that the lifetime in the A* state can be significantly modified by changes in the protein structure near the chromophore, as demonstrated by the slower decay time of the blGFP mutant. This raises the prospect of the creation of blue absorbing GFP mutants with a significant quantum yield, which may have application in dual labelling bioimaging studies and Förster resonance energy transfer measurements. Additionally, a detailed understanding of how electronic excitation can be coupled to specific structural reorganization of the protein matrix will facilitate the design of light activated proteins.

The TRIR spectra of these ESPT inactive mutants were recorded. The spectra were complex showing both the expected chromophore modes and a number of modes assigned to the protein which are not observed in wtGFP or other ESPT active GFP mutants. In particular a significant group of modes are downshifted on a sub picosecond time scale following excitation to A*. These protein modes are quite different to those observed in wtGFP or other ESPT active GFP mutants. It is proposed that this frequency shift arises from a change in H-bond interactions between the excited chromophore and adjacent residues involved in the disruption of the proton relay chain in GFP.

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References

1. Tsien RY. The green fluorescent protein. *Annual Review of Biochemistry* 1998;67:509–544.
2. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside live cells. *Science* 1998;281:269–272. [PubMed: 9657724]
3. Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, Ormo M, Remington SJ. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* 1997;94:2306–2311. [PubMed: 9122190]
4. Bell AF, He X, Wachter RM, Tonge PJ. Structure of Green Fluorescent Protein chromophores probed by Raman spectroscopy. *Abstracts of Papers of the American Chemical Society* 2001;222:U124–U124.
5. Bell AF, He X, Wachter RM, Tonge PJ. Structure of green fluorescent protein chromophores probed by Raman spectroscopy. *Biochemistry* 2001;40:8619–8619.
6. Chatteraj M, King BA, Bublitz GU, Boxer SG. Ultra-fast excited state dynamics in green fluorescent protein: Multiple states and proton transfer. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93:8362–8367. [PubMed: 8710876]

7. Lossau H, Kummer A, Heinecke R, PollingerDammer F, Kompa C, Bieser G, Jonsson T, Silva CM, Yang MM, Youvan DC, MichelBeyerle ME. Time-resolved spectroscopy of wild-type and mutant Green Fluorescent Proteins reveals excited state deprotonation consistent with fluorophore-protein interactions. *Chemical Physics* 1996;213:1–16.
8. Stoner-Ma D, Jaye AA, Matousek P, Towrie M, Meech SR, Tonge PJ. Observation of excited-state proton transfer in green fluorescent protein using ultrafast vibrational spectroscopy. *Journal of the American Chemical Society* 2005;127:2864–2865. [PubMed: 15740117]
9. Wachter RM, Elsliger MA, Kallio K, Hanson GT, Remington SJ. Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. *Structure with Folding & Design* 1998;6:1267–1277. [PubMed: 9782051]
10. Hanson GT, McAnaney TB, Park ES, Rendell MEP, Yarbrough DK, Chu SY, Xi LX, Boxer SG, Montrose MH, Remington SJ. Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry* 2002;41:15477–15488. [PubMed: 12501176]
11. McAnaney TB, Park ES, Hanson GT, Rende MEP, Remington SJ, Boxer SG. Excited state dynamics of a pH-sensitive, dual emission green fluorescent protein variant. *Biophysical Journal* 2002;82:314A–314A.
12. McAnaney TB, Shi XH, Abbyad P, Jung H, Remington SJ, Boxer SG. Green fluorescent protein variants as ratiometric dual emission pH sensors. 3. Temperature dependence of proton transfer. *Biochemistry* 2005;44:8701–8711. [PubMed: 15952777]
13. Kummer AD, Wiehler J, Rehder H, Kompa C, Steipe B, Michel-Beyerle ME. Effects of threonine 203 replacements on excited-state dynamics and fluorescence properties of the green fluorescent protein (GFP). *Journal of Physical Chemistry B* 2000;104:4791–4798.
14. Stoner-Ma D, Jaye AA, Ronayne KL, Nappa J, Meech SR, Tonge PJ. An Alternate Proton Acceptor for Excited State Protein Transfer in Green Fluorescent Protein: Rewiring GFP. *J. Amer. Chem. Soc* 2008;130:1227–1235. [PubMed: 18179211]
15. Shi X, Abbyad P, Shu X, Kallio K, Kanchanawong P, Childs W, Remington SJ, Boxer SG. Ultrafast excited-state dynamics in the green fluorescent protein variant S65T/H148D. 2. Unusual photophysical properties. *Biochemistry* 2007;46:12014–12025. [PubMed: 17918960]
16. Shu X, Kallio K, Shi X, Abbyad P, Kanchanawong P, Childs W, Boxer SG, Remington SJ. Ultrafast excited-state dynamics in the green fluorescent protein variant S65T/H148D. 1. Mutagenesis and structural studies. *Biochemistry* 2007;46:12005–12013. [PubMed: 17918959]
17. Niwa H, Inouye S, Hirano T, Matsuno T, Kojima S, Kubota M, Ohashi M, Tsuji FI. Chemical nature of the light emitter of the *Aequorea* green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93:13617–13622. [PubMed: 8942983]
18. Litvinenko KL, Webber NM, Meech SR. An ultrafast polarisation spectroscopy study of internal conversion and orientational relaxation of the chromophore of the green fluorescent protein. *Chemical Physics Letters* 2001;346:47–53.
19. Litvinenko KL, Webber NM, Meech SR. Internal conversion in the chromophore of the green fluorescent protein: Temperature dependence and isoviscosity analysis. *Journal of Physical Chemistry A* 2003;107:2616–2623.
20. Mandal D, Tahara T, Meech SR. Excited-state dynamics in the green fluorescent protein chromophore. *Journal of Physical Chemistry B* 2004;108:1102–1108.
21. Mandal D, Tahara T, Webber NM, Meech SR. Ultrafast fluorescence of the chromophore of the green fluorescent protein in alcohol solutions. *Chemical Physics Letters* 2002;358:495–501.
22. Webber NM, Litvinenko KL, Meech SR. Radiationless relaxation in a synthetic analogue of the green fluorescent protein chromophore. *Journal of Physical Chemistry B* 2001;105:8036–8039.
23. Kummer AD, Kompa C, Niwa H, Hirano T, Kojima S, Michel-Beyerle ME. Viscosity-dependent fluorescence decay of the GFP chromophore in solution due to fast internal conversion. *Journal of Physical Chemistry B* 2002;106:7554–7559.
24. Gepshtein R, Huppert D, Agmon N. Deactivation mechanism of the green fluorescent chromophore. *Journal of Physical Chemistry B* 2006;110:4434–4442.

25. Sinicropi A, Andruniow T, Ferre N, Basosi R, Olivucci M. Properties of the emitting state of the green fluorescent protein resolved at the CASPT2//CASSCF/CHARMM level. *Journal of the American Chemical Society* 2005;127:11534–11535. [PubMed: 16104693]
26. Martin ME, Negri F, Olivucci M. Origin, nature, and fate of the fluorescent state of the green fluorescent protein chromophore at the CASPT2//CASSCF resolution. *Journal of the American Chemical Society* 2004;126:5452–5464. [PubMed: 15113217]
27. Martinez TJ. Insights for light-driven molecular devices from ab initio multiple spawning excited-state dynamics of organic and biological chromophores. *Accounts of Chemical Research* 2006;39:119–126. [PubMed: 16489731]
28. Toniolo A, Olsen S, Lamothe K, Martinez TJ. Direct photodynamics of green fluorescent protein. *Abstracts of Papers of the American Chemical Society* 2004;228:U209–U209.
29. Toniolo A, Olsen S, Manohar L, Martinez TJ. Conical intersection dynamics in solution: the chromophore of Green Fluorescent Protein. *Faraday Discussions* 2004;127:149–163. [PubMed: 15471344]
30. Toniolo A, Granucci G, Martinez TJ. Conical intersections in solution: A QM/MM study using floating occupation semiempirical configuration interaction wave functions. *Journal of Physical Chemistry A* 2003;107:3822–3830.
31. Frutos LM, Andruniow T, Santoro F, Ferre N, Olivucci M. Tracking the excited-state time evolution of the visual pigment with multiconfigurational quantum chemistry. *Proceedings of the National Academy of Sciences of the United States of America* 2007;104:7764–7769. [PubMed: 17470789]
32. Matousek P, Towrie M, Ma C, Kwok WM, Phillips D, Toner WT, Parker AW. Fluorescence suppression in resonance Raman spectroscopy using a high-performance picosecond Kerr gate. *Journal of Raman Spectroscopy* 2001;32:983–988.
33. Matousek P, Towrie M, Stanley A, Parker AW. Efficient rejection of fluorescence from Raman spectra using picosecond Kerr gating. *Applied Spectroscopy* 1999;53:1485–1489.
34. Towrie M, Grills DC, Dyer J, Weinstein JA, Matousek P, Barton R, Bailey PD, Subramaniam N, Kwok WM, Ma CS, Phillips D, Parker AW, George MW. Development of a broadband picosecond infrared spectrometer and its incorporation into an existing ultrafast time-resolved resonance Raman, UV/visible, and fluorescence spectroscopic apparatus. *Applied Spectroscopy* 2003;57:367–380. [PubMed: 14658632]
35. Stoner-Ma D, Melief EH, Nappa J, Ronayne KL, Tonge PJ, Meech SR. Proton relay reaction in green fluorescent protein (GFP): Polarization-resolved ultrafast vibrational spectroscopy of isotopically edited GFP. *Journal of Physical Chemistry B* 2006;110:22009–22018.
36. Jaye AA, Stoner-Ma D, Matousek P, Towrie M, Tonge PJ, Meech SR. Time-resolved emission spectra of green fluorescent protein. *Photochemistry and Photobiology* 2006;82:373–379.
37. Stavrov SS, Solntsev KM, Tolbert LM, Huppert D. Probing the decay coordinate of the green fluorescent protein: Arrest of cis-trans isomerization by the protein significantly narrows the fluorescence spectra. *Journal of the American Chemical Society* 2006;128:1540–1546. [PubMed: 16448124]
38. Webber NM, Meech SR. Electronic Spectroscopy And Solvatochromism In The Chromophore Of GFP And The Y66F Mutant. *Photochemical & Photobiological Sciences* 2007;9:276–281.
39. Stoner-Ma D, Jaye AA, Ronayne KL, Nappa J, Tonge PJ, Meech SR. In Preparation
40. McAnaney TB, Park ES, Hanson GT, Remington SJ, Boxer SG. Green fluorescent protein variants as ratiometric dual emission pH sensors. 2. Excited-state dynamics. *Biochemistry* 2002;41:15489–15494. [PubMed: 12501177]
41. Kummer AD, Kompa C, Lossau H, Pollinger-Dammer F, Michel-Beyerle ME, Silva CM, Bylina EJ, Coleman WJ, Yang MM, Youvan DC. Dramatic reduction in fluorescence quantum yield in mutants of Green Fluorescent Protein due to fast internal conversion. *Chemical Physics* 1998;237:183–193.
42. Litvinenko KL, Webber NM, Meech SR. Ultrafast excited state relaxation of the chromophore of the green fluorescent protein. *Bulletin of the Chemical Society of Japan* 2002;75:1065–1070.
43. van Thor JJ, Zanetti G, Ronayne KL, Towrie M. Structural events in the photocycle of green fluorescent protein. *Journal of Physical Chemistry B* 2005;109:16099–16108.

44. Rubtsov IV, Wang J, Hochstrasser RM. Vibrational coupling between amide-I and amide-A modes revealed by femtosecond two color infrared spectroscopy. *Journal of Physical Chemistry A* 2003;107:3384–3396.

Figure 1.

Model for the interconversion of the A and B forms of the chromophore via the I form (adapted from Brejc et al.(3)). Structures were based on X-ray data from wt (A) or anionic mutants (B), whereas I is a proposed intermediate.

Figure 2. Absorption (blue), emission (green) and excitation (red) spectra of (a) bEGFP at pH 6.5 and (b) S65T GFP at pH 5.5. Emission and excitation spectra of the neutral form are shown as solid lines, the anionic form as dashed lines. For both mutants, the 508 nm excitation maximum was set to equal the anionic absorption peak.

Figure 3.

Time resolved fluorescence spectra (a) bEGFP 3 ps (red) 10 ps (green) 50 ps (blue) 100 ps (orange) 300 ps (violet) after excitation. (b) S65T at pH 5.5 2 ps (red) 10 ps (green) 20 ps (blue) 50 ps (orange) 150 ps (violet) 400 ps (Indigo) after excitation. Inset, the same spectra as (b) with the long lived component subtracted.

Figure 4. Time dependent fluorescence intensity (440 – 460 nm) for bEGFP (squares) and S65T pH 5.5 (circles). Empty symbols for deuterated solvent, filled for H₂O.

Figure 5.

(a) TRIR Spectra for HBDI in DMSO 2 ps (red) 4 ps (green) 6 ps (blue) 10ps (orange) 30 ps (violet) after excitation (b) S65T at pD 5.5 2 ps (red) 6 ps (green) 10 ps (blue) 30 ps (orange) 100 ps (violet) after excitation (c) bEGFP 2 ps (red) 4 ps (green) 10 ps (blue) 30 ps (orange) 200 ps (violet) after excitation (d) polarisation resolved measurements of bEGFP 4 ps after excitation. Pump and probe parallel (red) and perpendicular (blue) with the ratio (green, dash line) shown on the right hand axis.