Ultrafast dynamics of green fluorescent protein: evidence for excited state proton transfer and a rigid local chromophore environment

S R Meech

School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, Norfolk, NR4 7TJ, UK

D Stoner-Ma, A A Jaye, P J Tonge

Department of Chemistry, Stony Brook University, Stony Brook, New York, 11794-3400, USA

P Matousek, M Towrie

Central Laser Facility, CCLRC Rutherford Appleton Laboratory, Chilton, Didcot, Oxon., OX11 0QX, UK

Main contact email address: *s.meech@uea.ac.uk*

Introduction

Understanding the photochemistry of green fluorescent protein (GFP) remains an important goal across many disciplines. This arises due to the wide ranging applications for which GFP is suitable, such as fluorescence imaging in vivo. In particular, there has been much interest in elucidating the structural changes which occur in the chromophore and the surrounding protein residues in the excited state. This is with a view to the production of specific protein mutants, in which the excited state chemistry can be controlled. It has been hypothesized from fluorescence and crystallography studies that photoexcitation with 400 nm light results in an excited state proton transfer (ESPT) from the chromophore phenol hydroxyl group, through a hydrogen-bonding network, to a buried protein residue, Glu222. In this report, we describe the use of picosecond time-resolved IR spectroscopy to provide the first direct evidence that Glu222 is the terminal proton acceptor in the ESPT reaction[1]. In addition, we show by time-resolved fluorescence of GFP, that the protein residues surrounding the chromophore are orientationally restricted during the fluorescence photocycle[2]. Both of these findings have an important impact on the current knowledge of GFP photochemistry.

The absorption spectrum of wild-type GFP (wtGFP) reveals two characteristic bands at 395 and 475 nm arising from two separate ground state structures of the chromophore; protonated (neutral) and deprotonated (anionic). The relative intensities of these bands represent the populations of the A and B structures of the protein respectively (Figure 1). Excitation into either band results in strong green fluorescence at 508 nm (Φ = 0.8).

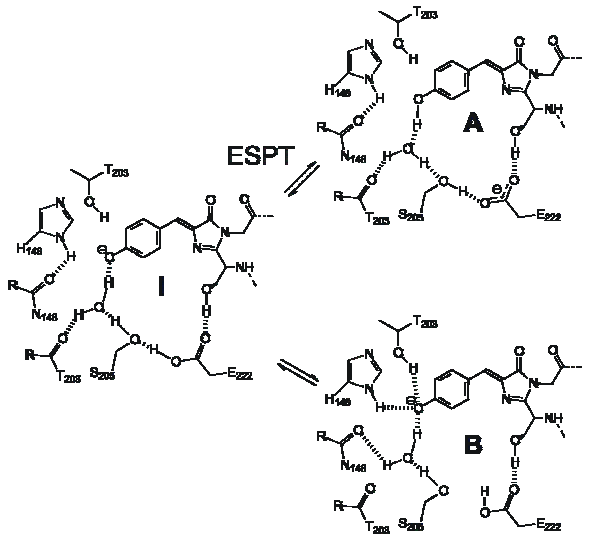

Figure 1. A schematic showing light-driven conversion from the neutral (A form) and the anionic (B form) chromophore via an intermediate state, I. Adapted from Brejc *et al.*[3].

Using fluorescence upconversion techniques, Boxer and co-workers[4] were able to show that photoexcitation at 400 nm resulted in conversion from the A-form of the protein to the I- form (Figure 1). The I-form may then convert to the B-form, but this is an infrequent process, even in the excited state. The photophysics of this process are represented by the cycle in Figure 2.

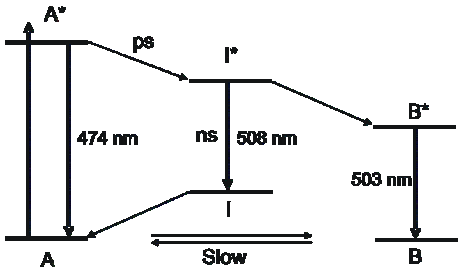

Figure 2. Photocycle of GFP following excitation at 400 nm.

Also illustrated in Figure 1 is the hydrogen-bonding network that links the phenol hydroxyl group and Glu222. This network was first proposed by Remington[3] on the basis of crystal structures. Based on these studies, we have the picture that when GFP is excited with 400 nm light to the A* state, the system can undergo non-radiative decay, fluoresce back to A or transfer a proton through the hydrogen-bonding network to form I*. Time resolved fluorescence dynamics have shown that decay from A* is a multi-exponential process and occurs on the picosecond time scale[5]. Although the fluorescence kinetics of GFP are well characterized, the spectral evolution, which may contain information on local structure changes during the ESPT reaction, has not received much detailed attention. Using a Kerr-gated fluorescence spectrometer, described elsewhere, we were able to record the entire fluorescence spectrum of GFP and a mutant Thr203Val on the picosecond time scale[2].

Although time-resolved fluorescence studies are expected to be a sensitive probe of chromophore-protein interactions, they are not able to probe specific structural changes following photoexcitation. For this reason, we have used time-resolved IR as a means to monitor vibrational modes of the chromophore and protein during the ESPT reaction. Steady state IR has been used previously to monitor these changes[6], but failed to see evidence of proton acceptance by Glu222. It was later discovered that the reason for this was due to decarboxylation of Glu222, occurring at high power densities of the pump laser and preventing the observation of a protonated Glu222 band[6]. The time-resolved measurements below are performed at power densities which minimize the effects of this phototransformation. The PIRATE technique and the TRIR instrument are described elsewhere[1].

Results and Discussion

Ultrafast Vibrational Spectroscopy

Time-resolved IR spectra of wtGFP are shown in Figure 3. Immediately apparent from these data is the spectral complexity, arising from overlap of instantaneous ground state bleaches and transient absorption. Fortunately, the modes of the chromophore structure are well understood and facilitate analysis of these TIR spectra. In particular, we can assign modes at 1596, 1637 and 1680 cm^{-1} to the phenol ring mode, exocyclic C=C and C=O respectively, from the basis of the TIR model chromophore spectra (see [1]). Two long-lived modes that are not present in the chromophore are those at 1565 and 1706 cm^{-1}. These can thus be assigned to modes resulting from the protein. Significantly, these two bands appear with a fall or rise time (respectively) of ~50 ps and persist for over 500 ps, however, the original ground state is recovered in < 1 ms. The appearance of these modes on this time scale could be assigned to the ESPT reaction but in principle, they may arise from another competing process, or from the formation of I*. To rule out these possibilities, we measured the TIR spectra of Ser65Thr which does not undergo ESPT and Thr203Val/Glu222Gln, which exists solely in the I form ground state. Neither of these mutants showed any long lived features at 1565 or 1706 cm^{-1} (see [1]). The positions of these modes are consistent with titration of carboxylate to carboxylic acid in the protein. It has been demonstrated, by difference FTIR experiments, that irreversible photoconversion of wtGFP results in the loss of a band at 1565 cm^{-1}, assigned to the antisymmetric stretch of the Glu222 carboxylate (unpublished results). From these TIR studies, we can additionally assign the 1706 cm^{-1} band to the protonated Glu222 group, which grows at the expense of the Glu222 carboxylate band.

Further information on the mechanism of ESPT can be obtained from comparing the dynamics of the 1565/1706 cm^{-1} modes with the excited state fluorescence dynamics of A* reported previously[4]. We find that the exponential decay and rise for the 1565 and 1706 cm^{-1} bands respectively, are in good agreement with the fluorescence decay (see [1]). This is indicative of the rate determining step arising from initial deprotonation at the chromophore hydroxyl.

Thus we have shown evidence for the first time that Glu222 is the terminal proton acceptor in the ESPT reaction. We have also confirmed that the dynamics of bands associated with Glu222 are consistent with the fluorescence decay of A*.

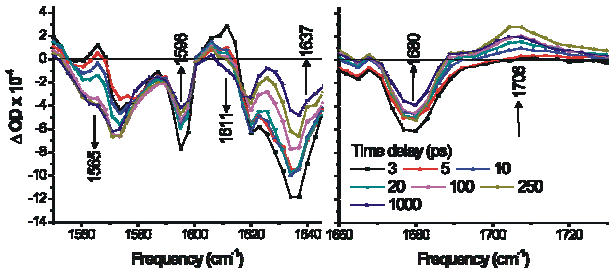

Figure 3. TIR spectra of wtGFP taken at the time delays indicated using the PIRATE system.

Time-resolved Fluorescence Spectroscopy

One of the questions surrounding the excited-state chemistry of GFP is how does the protein scaffold enhance the fluorescence of the internal chromophore? This question arises from the observation that denatured GFP and the model chromophore free in solution are essentially non-fluorescent. One possible mechanism by which GFP may enhance chromophore fluorescence is by suppression of internal conversion through a torsional coordinate. The energetic barrier to this decay mechanism in the model chromophore was proposed to be volume conserving and near zero at 295 K.

The steady state emission spectrum of wtGFP exhibits a strong peak at 508 nm from I* emission and a very weak shoulder on this at 460 nm from A*. Site-specific mutations in and around the chromophore produce a variety of emission profiles over nearly the entire visible spectrum. Therefore one may expect that any subtle reorientations of polar or polarisable residues surrounding the chromophore during ESPT will modify the emission spectrum of the chromophore on the picosecond timescale. Furthermore, Stark shift experiments show a large dipole moment change between A and I* states. Reorientation of polar residues is expected to stabilize this dipole, again leading to a time resolved spectral shift. To investigate this hypothesis, we recorded time resolved fluorescence spectra in the CLF facility. The results of these experiments for wtGFP and a mutant that exists solely in the A ground state (Thr203Val) are shown in Figure 4. The I* emission increases after excitation at the expense of A*. The isoemissive point at 488 nm is evidence for only two fluorescent states of the chromophore.

The main focus in these experiments is the time dependence of the spectra, however, we can also assess the decay kinetics from the same data set. We compare the A* kinetics recorded here with those measured previously[5] by convoluting the fluorescence decay from up-conversion measurements with the instrument response function (fwhm = 4 ps). As can be seen by the fit of our data using this method (Figure 4), we are in excellent agreement.

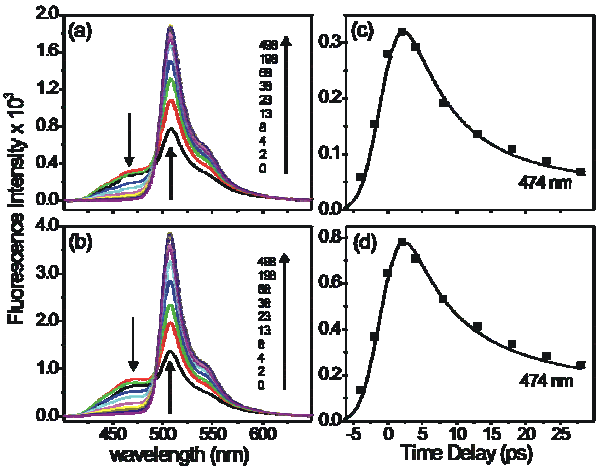

Figure 4. (a) and (b) are time-resolved fluorescence spectra of wtGFP and Thr203Val respectively as a function of pump-gating pulse time delays (indicated by the key). (c) and (d) kinetic plots of the A* emission at 474 nm for wtGFP and Thr203Val respectively (data points). The solid black line is a convolution of the instrument response function with fluorescence decay from another source[5].

To further analyze the data, the spectra are separated into A* and I* components. The description of this procedure and a detailed description of results can be found elsewhere[2]. The first conclusion from these analyses is that neither the I* nor the A* emission spectra show any time dependence for either protein mutant. This is an interesting and surprising result in the light of Stark spectroscopy which shows that the observed change on excitation of the A→A* transition is 2.5 D, and larger for A→I*. In solution it is expected that the medium would relax in response to the changed electrostatic interaction leading to a shift in the emission spectrum to lower energy. The rate of this shift depends on the dynamics of the surrounding medium whereas the magnitude of the shift depends on the magnitude of the dipole moment change and the dielectric

properties of the surrounding medium. The absence of such a shift over the picosecond time scale for wtGFP and Thr203Val in either of the A* or I* states could be described with two explanations. Firstly, the protein environment is unperturbed by the change in chromophore dipole moment. However, this is inadequate given that the protein matrix clearly enhances fluorescence of the chromophore and that mutant proteins can have drastically altered emission profiles. The alternative explanation is that the protein environment must be suitably rigid so that no relaxation occurs on the time scale of the excited state life time which continues for nanoseconds in I*. Thus the lack of spectral shift in response to significant dipole moment change in the excited state is consistent with an environment in which reorientation of polar or polarisable residues surrounding the chromophore are suppressed due to the rigidity of the protein manifold.

Conclusions

We have performed ultrafast vibrational and fluorescence spectroscopy on wtGFP and some of its mutant proteins. From the series of vibrational experiments, we have observed ESPT in wtGFP for the first time and determined Glu222 to be the terminal proton acceptor. In particular, modes at 1565 and 1706 cm^{-1} are consistent with titration of carboxylate to carboxylic acid in the protein on a picosecond time scale which we assign to the buried protein residue Glu222. We are currently expanding our IR studies by incorporating site-specific isotopic labels into GFP and manufacturing mutants with different excited state properties.

In addition to the IR work, we have used time resolved fluorescence spectroscopy as a way of probing the chromophore-protein interaction throughout the fluorescence photocycle. It is observed that neither the I* or A* emission spectral profiles show any time dependence. We suggest that this is evidence for a high rigidity in the protein which prevents reorientation of residues surrounding the chromophore on the time scale of the excitation. The next question that begs asking, and that we hope to answer, is to what degree does the rate of ESPT depend on the rigidity of the local chromophore environment?

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