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Ultrafast fluorescence of the chromophore of the green fluorescent protein in alcohol solutions

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Abstract

The ultrafast fluorescence dynamics of solutions of the chromophore responsible for emission from the green fluorescent protein are measured by fluorescence up-conversion. Decays are non-exponential but well fit by a sum of two-exponentials. All decays have a prompt rise time. The two decay times are approximately independent of wavelength, but their weights are wavelength dependent, in a manner consistent with a spectral narrowing with time. The longer decay time has a weak dependence on medium viscosity.

1. Introduction

The green fluorescent protein (GFP) is established as one of the most important fluorescence probes in molecular biology [1,2]. The photophysics of GFP and many of its mutants have been studied intensively by ultrafast spectroscopy, and the fundamental mechanism of fluorescence has been established [3,4]. It has however been noted that some mutants of GFP are only weakly fluorescent, in contrast to the high quantum yield (Φ_f) of 0.8 found for many mutants [5,6]. Even more surprisingly, both the denatured protein and the isolated chromophore in free solution are essentially non-fluorescent ($\Phi_f < 10^{-3}$) [7]. How the protein is able to so efficiently suppress radiationless decay in the chromophore is an important question.

In this Letter we report preliminary observations of the excited state dynamics of the isolated chromophore of GFP, so as to better characterise the mechanism of radiationless decay. These data complement recent studies of the ultrafast ground state recovery (GSR) of the chromophore [8,9]. These showed that the radiationless decay leads to rapid repopulation of the ground state (internal conversion, IC) within a few picoseconds, and was, within the signal to noise, essentially independent of viscosity. The latter point showed that the mechanism for IC does not involve large scale

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intramolecular reorganisation, which would be opposed by solvent friction [8,9]. These GSR measurements set an upper limit for the excited state decay time, but do not yield any information on the dynamics on the upper surface.

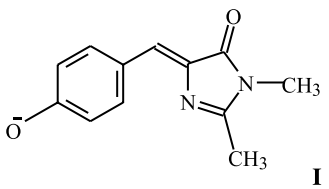
In this Letter excited state dynamics of the chromophore **I** are reported. They have been observed by means of fluorescence up-conversion, as a function of both the emission wavelength and solvent viscosity. The chromophore **I** is identical to the chromophore in GFP, except that in the protein it is attached to the backbone at the positions occupied by the methyl groups. However, the absorption spectra of **I** in solution are shifted compared with the protein, by $\approx 1500\ cm^{-1}$ [7,10]. The anion form of **I** has been studied here, as it is the form ultimately responsible for fluorescence in GFP [3,7]. Studies of the excited state dynamics of the neutral form of **I**, which has an even faster GSR time than the anion [9], are in progress.

The results presented below are of interest not only for the characterisation of the excited state dynamics in the GFP chromophore, but also because they complement a number of recent theoretical investigations of GFP. To date most calculations of the electronic spectra and excited state dynamics of GFP have focussed on the structure **I** [11–16]. The more demanding task of specifically including the protein in the calculation has been addressed less frequently [16,17].

2. Experiment

The apparatus for fluorescence up-conversion has been described in detail elsewhere [18]. For the present measurements the titanium sapphire laser was fitted with long wavelength reflecting mirrors, to generate excitation wavelengths between 420 and 460 nm. Under these conditions the width of the cross correlation of excitation and gate beams at the sample position was 200 fs. The cross correlation was measured by up-conversion of a solvent Raman signal. The 200 fs cross correlation is taken as the effective instrumental time resolution, although faster dynamics can be detected by the deconvolution analysis.

Compound **I** was synthesised following literature methods [19]. Solutions were prepared with an optical density of approximately 1 in a 0.5 mm quartz cell. In all solutions ca. 2% by volume of 1 M base was added, to generate the anion **I**. Due to the scarcity of the compound it was necessary to record the data in a static cell, rather than the flowing cell or free jet more frequently used. As a result the excitation intensity had to be reduced to a level below that at which optical heating could be observed; typical excitation beam energies were < 3 mW (at the laser repetition frequency of 82 MHz). Under these conditions a maximum up-conversion signal of 300 counts per second was found. Data were recorded under 'magic angle' conditions to suppress contributions due to orientational motion. Each data point was an average over 5 s, and points were recorded every 30 fs for the first 3 ps, and less frequently for longer times. All measurements were repeated, and stated errors indicate the spread of these measurements. Data were fit to one and two exponential functions which were convoluted with a Gaussian, which had been extracted from a fit to the cross correlation, measured as described above. In all the cases studied here a single exponential function was inadequate to describe the data.



I

Scheme 1.

3. Results and discussion

The electronic absorption spectra of **I** in basic *n*-propanol solution are shown in Fig. 1; electronic spectra for other solvents have been discussed elsewhere [20]. The absorption is a single band, and is calculated to arise from a single electronic transition, of mainly HOMO to LUMO character [21]. The emission spectrum (which is very weak, and is obtained by subtracting the solvent Raman

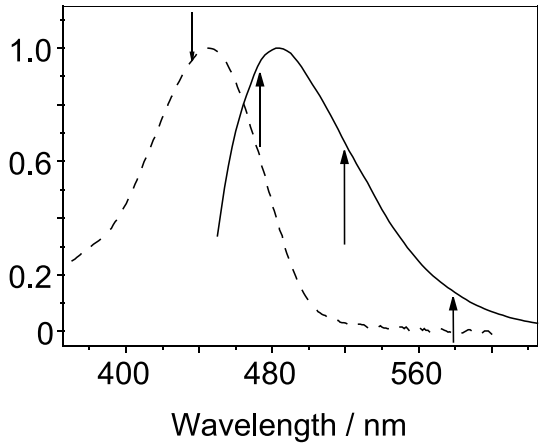


Fig. 1. Absorption (dashed line) and emission spectra (solid line) of the anion of **I** in *n*-propanol (+1% 1 M NaOH). The excitation wavelength and the wavelengths at which up-conversion measurements were made are indicated by arrows.

contribution) is Stokes shifted by $> 2000\ \mathrm{cm}^{-1}$. The Stokes shift is a relatively weak function of solvent [20]. The excitation wavelength and the emission wavelengths at which up-conversion data were recorded are marked. It was established that the spectra did not change during the experiment, indicating the absence of appreciable photochemistry.

The up-conversion data in methanol solution, recorded as a function of emission wavelength, are shown in Fig. 2. Three features are immediately apparent. At all wavelengths the data are not well described by a single exponential. The traces are however well fit by a sum of two exponential terms. At no wavelength is a rise time observed. The fluorescence decays on the blue and red edges are somewhat faster than those observed at the peak of the emission spectrum.

A non-exponential decay could arise from an inhomogeneous distribution in the ground state – for example *cis* and *trans* isomers of **I** may be formed. However, all of the data presently available suggest that **I** exists in a single form. For example measurements as a function of pH reveal a well-defined isosbestic point, consistent with equilibrium between a single ground state in its neutral and ionised forms [20]. In addition preliminary X-ray measurements suggest that the synthesis yields a single isomer, of the structure

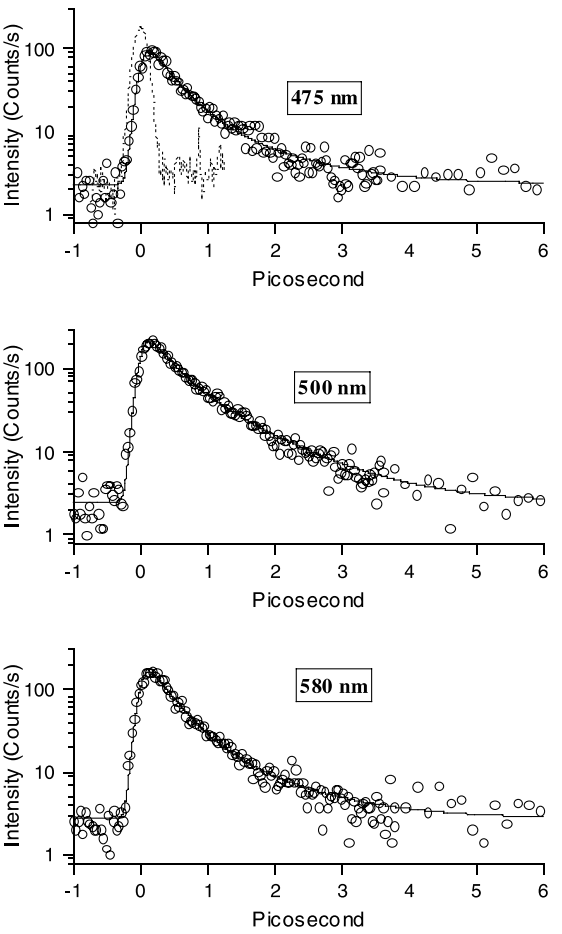


Fig. 2. Fluorescence decay data for the anion of **I** in basic methanol, measured as a function of the emission wavelength. The data are fit to a sum of two exponential terms (solid line). Also shown in the 475 nm data is the instrument response function to illustrate time resolution.

shown in Scheme 1. A wavelength dependent non-exponential decay may also arise from solvent stabilisation of the excited state on the time scale of the decay. However, the expectation in such cases is for a time-dependent shift of the emission spectrum to lower energy, leading to a rise time at the red-edge [22]. This is not observed (Fig. 2); rather, the entire Stokes shift develops on a time scale below the present time resolution. Thus, the most likely origin of a non-exponential decay of the form seen in Fig. 2 is an intrinsically non-exponential character in the mechanism leading

to IC. This possibility will be expanded on further below.

In Fig. 3 the fluorescence decay data recorded at a single wavelength in three solvents of widely different viscosity are presented. There is a small but reproducible increase in the decay time as viscosity increases. However, as can be seen more readily from Table 1, the effect is small. For the major, fast, component the decay time increases by at most 35%, while for the slow component the increase is a factor of 2. This result suggests a weak dependence of the excited state decay on solvent viscosity.

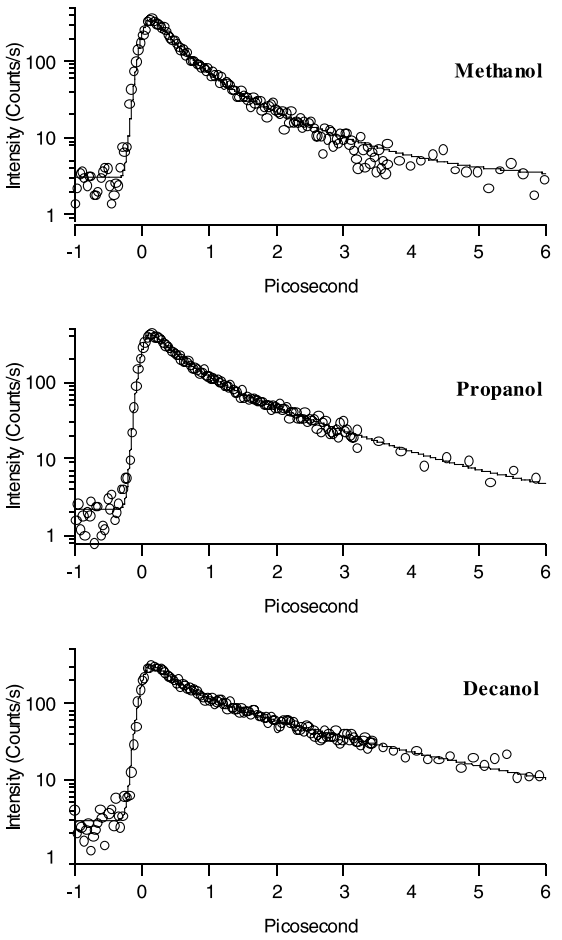


Fig. 3. Fluorescence decay data for the anion of **I** in three basic alcohol solvents. The data were recorded at 520 nm. The data are fit to a sum of two exponential terms (solid line).

In our previous measurements the mean recovery time of the fast component of the non-exponential GSR was reported to be effectively independent of solvent viscosity [8,9]. The difference between these two data sets, GSR times which are essentially independent of viscosity [8,9], while the excited state lifetimes show a weak viscosity dependence (Table 1), may be real, since the GSR measurement also includes relaxation on the ground state surface, which is not observed in fluorescence. However, the difference may also arise from the lower dynamic range of the GSR experiment, which is restricted at early time by ultrafast dynamics not necessarily associated with GSR (the 'coherent artefact') and at long times by orientational relaxation to fill the persistent bleaching of the ground state population [9]. Inspection of the GSR data in [9] for the anion in fact suggests a small increase in recovery time at high viscosity, similar to that seen for the slow component in the fluorescence decay (Table 1), although it is within the error bar of the GSR measurements [9]. Taken together the fluorescence and GSR data are consistent with a weak viscosity dependence of the IC mechanism. This dependence is considerably weaker than the linear viscosity dependence seen in the relaxation of triphenylmethane dyes in alcohols, interpreted as arising from a barrierless reorientation of the phenyl rings [23], or the η^x dependence (where x is 2/3 or 1/2) observed empirically in some excited state isomerisation reactions [24].

Table 1 also reveals additional information on the unusual wavelength dependence of the decay, which appears faster at the blue and red edges, than around the peak of the emission (Fig. 1). The effect is seen to arise mainly from a variation in the relative weights of the two components across the emission spectrum, rather than wavelength dependent decay times; the two decay constants recovered are approximately independent of wavelength. This behaviour would correspond to a continuous narrowing of the spectrum with time, on the time scale of the fastest relaxation. Such behaviour has only rarely been reported. The two examples we have found also concern molecules which exhibit ultrafast IC – bacteriorhodopsin [25] and the photoactive yellow protein [26,27].

Table 1

Solvent	Viscosity (cp)	Wavelength (nm)	τ_1[a] (fs)	τ_2[b] (ps)	Wt[c] τ_1 (%)
Methanol	0.5	475	350	1.3	88
		520	340	1.1	75
		580	360	1.1	81
n-Propanol	2.2	475	360	1.5	77
		520	370	1.4	70
		580	440	1.6	84
n-Decanol	14.3	475	350	2.0	64
		520	390	2.1	64
		580	450	2.0	76

[a] Error estimated from repeated measurements of ±70 fs.
[b] Error from repeated measurements ±0.2 ps.
[c] Weight of short lived component.

The dynamics observed suggest the following mechanism for the excited state relaxation of **I**. The absence of a rise time at any wavelength, or a temporally resolved Stokes shift, indicates that the initial fluorescent state is formed directly from the Franck–Condon (FC) state on a time scale faster than the present time resolution. The resonance Raman spectrum of **I** shows a dominant mode at 1556 cm^{-1} (mainly associated with stretching of C=C and C=N bonds) [28]. It is expected that such modes correspond with vibrational progressions in absorption and emission, and hence that the vibrational eigenstates relating to these high frequency modes are excited. However, the time resolution (or excitation bandwidth) in the present experiments is insufficient to realise impulsive excitation of these high frequency modes, so vibrational coherence, or wave packet dynamics, are not observed. A similarly prompt appearance of fluorescence or stimulated emission has been reported in bacteriorhodopsin, which has an even larger Stokes shift than **I** [26,29].

We associate the subsequent dynamics with ultrafast relaxation on the excited state surface and IC to repopulate the ground state. One interpretation of the biphasic decay observed is that the fast (< 500 fs) process corresponds to relaxation from the initial conformation to a relaxed conformation in the excited state, and the slower (> 1 ps) component corresponds to IC from the relaxed conformation. Analogies with molecules similar to **I**, and some theoretical calculations [11,16] suggest that the co-ordinates relevant to ultrafast relaxation will involve rotation about the bridging double bond, causing the excited and ground states to approach, or cross. The fact that the fluorescence and GSR dynamics are not a strong function of viscosity shows that motion along this co-ordinate is not strongly coupled to solvent friction [9,10]. One candidate, the co-operative rotation of both bridging bonds (or the 'hula twist') was identified in the calculations of Weber et al. [16]. Unlike rotation of one of the aromatic rings, this co-operative motion does not displace a large amount of solvent, so is only weakly coupled to medium friction. Thus, the < 500 fs component may be associated with relaxation from the planar initial state along the co-operative twist co-ordinate. This relaxation also leads to a spectral narrowing (Table 1). This ultrafast relaxation on the excited state potential surface is followed by an $S_1 \rightarrow S_0$ IC, which occurs on a 1–3 ps time scale, and is weakly dependent on solvent friction. The IC is expected to occur most efficiently where the excited and ground state surfaces approach or cross. Earlier studies, at lower temperatures, of stationary state fluorescence pointed to an activated radiationless process with a low activation energy [8]. The fact that ultrafast IC is observed suggests that the activation barrier from the relaxed conformation to the S_0/S_1 intersection (the 'funnel'), is very small.

An alternative interpretation, closely related to the above sequential model, is as follows. Following the initial FC excitation intramolecular vibrational relaxation takes place on the upper surface, leading ultimately to the relaxed state, on a sub-picosecond timescale. If the coupling strength of ground and excited surfaces decreases as the relaxation proceeds, the result will be a non-exponential relaxation at early times, followed by the slow relaxation corresponding to decay of the relaxed state. It is not possible to distinguish these two models from the present data. In principle a distinction is possible from GSR measurements, as the sequential model predicts that the GSR measurements should exhibit a (sub 500 fs) delay time. Unfortunately, the time resolution of our recent GSR data was only 250 fs, and the time zero data are perturbed by a strong, nearly instantaneous, signal not associated with GSR [8,9]. Higher time resolution experiments are planned.

4. Summary

The excited state dynamics of the chromophore responsible for the fluorescence of GFP have been investigated in free solution by the fluorescence up-conversion method. The fluorescence decays are well fit by a sum of two exponential terms. The decay constants are independent of wavelength and at no wavelength is a rise time observed. The wavelength dependence of the decay arises from a wavelength dependent weight of the two components, in a manner consistent with a spectral narrowing with time. Neither decay component is a strong function of solvent viscosity.

These data are interpreted as initial FC excitation of high frequency modes, followed by relaxation on the excited state surface on a sub 500 fs time scale. The radiationless decay proceeds from the relaxed state on a 1–3 ps time scale, possibly over a low barrier. Some speculations on the nature of the structural relaxation in the excited state are made, on the basis of the weak dependence on solvent friction observed, and the theoretical calculations of Weber et al. [16]. The similarity of the data reported here for the GFP chromophore and those found for bacteriorhodopsin [25] and the photoactive yellow protein [26,27], both of which exhibit excited state structural change leading to rapid IC, was noted. More detailed studies of the ultrafast fluorescence of **I**, in all its charge states, are currently in progress.

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