

Ultrafast Excited State Relaxation of the Chromophore of the Green Fluorescent Protein

Konstantin Leonid Litvinenko, Naomi Marica Webber, and Stephen Roy Meech*

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

(Received October 1, 2001)

Excited state relaxation in a synthetic analogue of the green fluorescent protein chromophore is investigated. Evidence is presented for rapid ground state recovery through internal conversion, with a minor channel populating a long lived bottleneck state. The rate constant for internal conversion is observed to be weakly dependent on medium viscosity over a wide range, but appears to be thermally activated. The rate constant for internal conversion does however depend on the charge of the chromophore. Some speculations on the nature of the protein chromophore interactions which might suppress this radiationless channel are made.

The green fluorescent protein (GFP) is established as one of the most important fluorescence probes in molecular biology and bioimaging.[1,2] The chromophore is formed in a spontaneous cyclization reaction to yield a bioluminescent emitter in the jellyfish *Aequorea victoria*. The isolated GFP exhibits strong, highly stable fluorescence. Crucially using gene fusion techniques the GFP can be used as a specific fluorescence label of living systems. It has found numerous applications in biology and biotechnology.[1–3] The spectrum of GFP can be modified by specific mutations to yield tailored fluorescence properties. This has proved advantageous in matching the absorption characteristics to specific laser lines, and in dual labelling experiments.[4] However, for a proper understanding of the behaviour of GFP mutants it will be necessary to build up a detailed picture of their photophysics. This is the subject of the present paper. Some existing studies of intact GFP suggest that its photophysical behaviour is not straightforward: a complex proton transfer pathway has been characterised by ultrafast spectroscopy;[5,6] the native protein has a fluorescence quantum yield, Φ_f, of 0.8, while the isolated chromophore in solution has $\Phi_f < 10^{-3}$;[7] the protein switches between dark and bright states under illumination;[8] the fluorescence is sensitive to pH.[9–12]

In the following we will be particularly concerned with the observation that the chromophore in free solution has a quantum yield that is at least a thousand times smaller than that observed in the protein.[7] To this end the free chromophore of GFP has been synthesized. Since the original synthesis and spectroscopic characterization of the free chromophore, by Niwa and co-workers,[7] it has been the subject of a number of theoretical and experimental investigations.[13–17] Here the pathway of excited state radiationless decay will be investigated, using both ultrafast polarization spectroscopy and the temperature dependence of the stationary state fluorescence. The temperature dependence and viscosity dependence of the decay rate will be analyzed in an attempt to furnish some mechanistic details.

Experimental

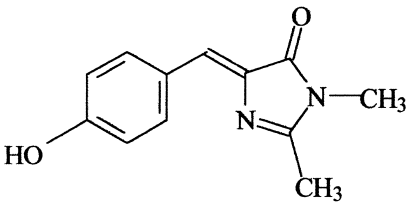
The source of pulses for the ultrafast laser experiments was a regeneratively amplified Kerr lens mode-locked titanium sapphire laser (Clark MXR). Output pulses at a repetition rate of 5 kHz, were of 40 μJ energy and $<$ 100 fs duration. The operating wavelength was 792 nm. The pulses were frequency doubled in a 1 mm thick BBO crystal to yield approximately 10 μJ pulses at 396 nm. The 396 nm pulses were divided at a beam splitter and routed through a conventional pump – probe geometry. Immediately before the sample both beams were passed through polarizers and half waveplates, yielding the necessary polarization control. Dispersion associated with these optics broadened the pulse to $<$ 250 fs at the sample position, as measured by a cross correlation experiment, utilizing the residual fundamental pulses as the second frequency. The 250 fs time resolution is faster than any of the relaxation dynamics reported below.

Polarization spectroscopy measurements were made as follows. The sample was excited by a linearly polarized pump pulse, resonant with the $S_0 \rightarrow S_1$ electronic transition, and probed at the same wavelength with a plane polarized pulse oriented at 45° with respect to the pump polarisation. The probe intensity was observed through a polarizer oriented at −43.5°. The slight (1.5°) misalignment from extinction introduces a heterodyne beam, which increases the signal to noise ratio and linearizes the response.[18,19] The signal transmitted by the second polarizer was detected by an amplified photodiode, the output of which was routed to a lock-in amplifier, referenced to the frequency of a mechanical chopper placed in the pump beam. Since the 5 kHz repetition rate is rather low for a lock-in measurement some experiments were made incorporating a boxcar averager to measure the diode signal, and the averaged boxcar output voltage was routed to the lock-in amplifier. With the stated arrangement of pump - probe polarization, the signal as a function of probe delay, $S(t)$, measures the decay of the pump induced dichroism, and contains contribution to the ground state recovery from both population relaxation, $K(t)$, and orientational relaxation, $r(t)$:[18,19]

$$S(t) \propto K(t)\, r(t). \tag{1}$$

In the case of ultrafast and complete repopulation of the ground state on a time scale much faster than molecular reorientation, $S(t)$ reports the ground state recovery time. Conversely if the excited state decays rapidly into some bottleneck state, then the orientational "hole" left in the ground state population by the polarized excitation source may be "filled" by molecular reorientation, and the reorientation dynamics are recovered. It will be seen that the GFP chromophore yields data somewhere between these two limiting cases.

The samples were solutions of the chromophore, **I**, in a range of alcohols (chart 1). The chromophore was synthesized and purified



I

Chart 1.

following literature methods.[20] Purity was checked by mass spectrometry, chromatography and NMR. The anion form of **I** was generated for most measurements, by addition of 1% 1 M (1 M = 1 mol dm^{-3}) NaOH. The concentration was adjusted to have an OD of < 1 at the excitation wavelength in a 2 mm path length cuvette. Measurements were made with the OD varied between 1.8 and 0.15, and no concentration effect on the dynamics was observed. The maximum of the $S_0 \rightarrow S_1$ transition was around 440 nm in all solvents. Measurements were also made for neutral and cationic forms of **I**. There are significant shifts in the absorption spectrum on protonation and deprotonation. For example the neutral form absorbs around 370 nm. This shift is attributed to deprotonation of the phenolic OH group in basic solution.[7,16] The absorption spectra are shown in Fig. 1, along with the spectrum of a mutant form of the GFP. It is seen that the spectrum of the neutral and anionic forms of **I** correlate with the transitions usually assigned to the neutral (A form) and anionic (B form) states of the

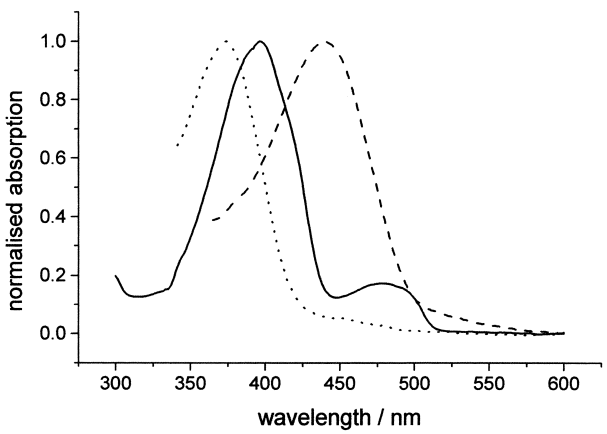


Fig. 1. Electronic absorption spectra of **I** in the neutral (dotted line) and anionic forms (dashed line). The spectra are compared with the GFPuv mutant (solid line). Note that the neutral and anion forms of **I** correlate with their counterparts in the protein, but are shifted to higher energy.

chromophore in the protein environment.[7,21] However, for both transitions the $S_0 \rightarrow S_1$ energy gap is some 1500 cm^{-1} smaller in the protein, suggesting significant protein chromophore interactions.

Fluorescence measurements were made in a conventional photon counting fluorimeter, and the sample was contained in a temperature controlled cryostat.

Results and Discussion

The temperature dependence of the stationary state fluorescence spectra of a solution of the anion of **I** in basic propanol are presented in Fig. 2a. The most obvious and notable fact is the large monotonic increase in fluorescence intensity as the sample is cooled from room temperature into the glassy phase. The large increase in fluorescence intensity, $> 10^3$ times, is consistent with the earlier observations of Niwa and coworkers[7] at room temperature and 77 K. In addition it is noted that the mean frequency of the spectrum and the spectral shape are not a strong function of temperature.

If the assumption is made that the quantum yield is deter-

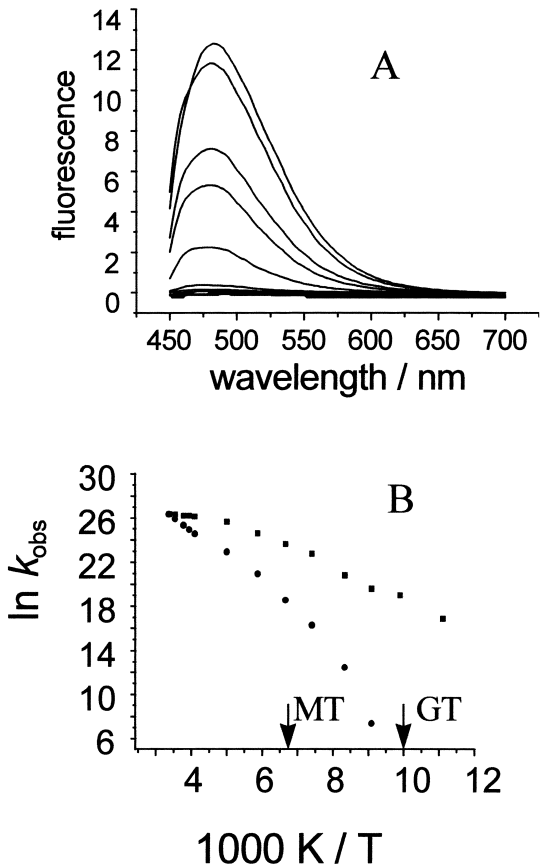


Fig. 2. (A) Fluorescence spectra of **I** in basic propanol solution measured as a function of temperature between 293 K, where the data is barely resolved from the baseline, and 77 K, the most intense spectrum. (B) The calculated radiationless rate constant, k_{obs}, is plotted as a function of reciprocal temperature (■). Also plotted are the data for T/η (●) (see text); the experimetal and solvent viscosity data are normalised at 273 K. On the $1/T$ axis the melting and glass transition temperatures of propanol are marked.

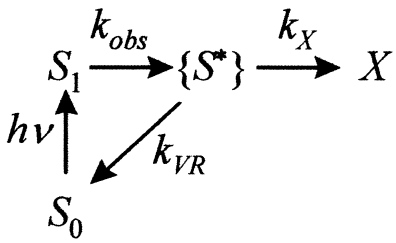
mined by temperature independent radiationless and radiative rate constants, and one temperature (and/or viscosity) dependent radiationless rate constant, k_{obs}, then:

$$k_{obs} = k_f\,[(1-\Phi_f^r)/\Phi_f^r], \quad (2)$$

where k_f is the radiative decay rate (assumed, on the basis of the fluorescence lifetime of GFP,[22] to be equal to 0.25×10^9 s^{-1}), and Φ_f^r is the quantum yield, measured relative to the value at 77 K. The calculated k_{obs} are plotted in Fig. 2b as a function of the reciprocal temperature. For temperatures between 160 K, above the melting temperature, down to 77 K, below the glass transition, k_{obs} is approximately thermally activated, with an activation energy of 12 kJ mol^{-1}. At the higher temperature range, where the precision is lower due to the very low quantum yield, the slope appears to decrease somewhat.

A very natural interpretation for rapid radiationless relaxation in molecules with structures similar to I is excited state isomerization, in which large scale structural changes in the excited state cause close approach of ground and excited states, leading to rapid internal conversion.[23] In the case of I an obvious candidate for such a structural change is rotation about the bridging double bond. When large groups reorient in the excited state it may be expected that the reorganization is opposed by solvent friction. In a hydrodynamic model solvent friction is proportional to viscosity, η, and an inverse relationship between k_{obs} and viscosity is expected. In the simplest case of a diffusive isomerization along a flat co-ordinate a reciprocal dependence is predicted.[24] More complex forms of the excited state potential lead to more complex dependencies. In Fig. 2b the data for T/η, which has been measured over a wide temperature range for propanol,[25] are compared with the data for k_{obs}. It is immediately apparent that the relationship between the two is not a close one. In addition the quantum yield is found to continue to increase, even at temperatures below the glass transition temperature. We will return to these points after discussing the time resolved data.

In Fig. 3 the polarization spectroscopy measurements for the anion of I in (basic) water, ethanol and butanol are presented, on the tens of picoseconds time scale. The measured dynamics reveal two relaxation components. One component is fast, with a mean relaxation time of a few picoseconds. This is too fast for reorientational dynamics in a molecule the size of I, so this fast component is assigned to population relaxation, $K(t)$, specifically internal conversion followed by vibrational relaxation to repopulate the ground state. The second component is evidently much slower, being far from complete on the tens of picoseconds timescale of Fig. 3. It is suggested that this arizes from a minor excited state relaxation channel, leading to population of a long lived bottleneck in the ground state recovery cycle. These data for the anion of I are qualitatively similar to those for the neutral form, except that the ground state recovery is faster in the latter case.[26] Thus the data of Fig. 3 are interpreted in terms of the Scheme 1.



Scheme 1.

in which X is an unidentified bottleneck state and $\{S^*\}$ some intermediate state, populated by internal conversion from S_1, from which rapid vibrational relaxation occurs in competition with a slower conversion to X. It is possible to separate the population and orientational relaxation components of $S(t)$ by making measurements with the angle between pump and probe polarizations set to the "magic angle" of 55°.[18] Such measurements confirm that the state X is indeed long lived, exhibiting negligible relaxation in 300 ps.[26]

The slower of the two relaxation times seen in Fig. 3 is shown in more detail on the longer time scale of Fig. 4. Clearly the slow relaxation time is dependent on the solvent. Since

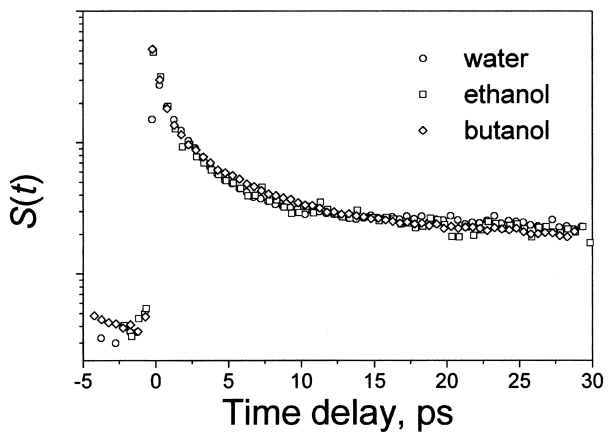


Fig. 3. Ultrafast polarisation spectroscopy measurements of the anion of I in protic solvents of different viscosity. Data are normalized at 1 ps. The fast relaxation time is essentially independent of viscosity. The slow relaxation appears as an offset on this time scale.

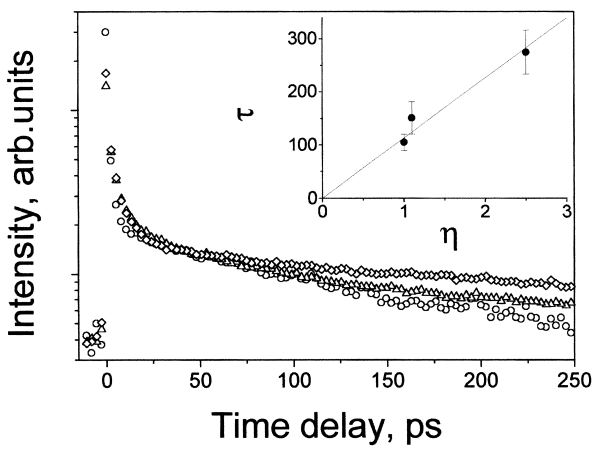


Fig. 4. The slow exponential recovery time observed on the hundreds of picosecond time scale (symbols have the same meaning as in Fig. 3). Inset is a plot of the measured relaxation time as a function of solvent viscosity.

no such relaxation was seen in the "magic angle" experiment[26] it seems reasonable to ascribe the slow relaxation time to molecular reorientation of I. Thus, the orientational hole created in the ground state population by the polarized excitation pulse, and subsequent relaxation of the excited state to the bottleneck state, is filled by orientational motion of the ground state. Assuming a modified Stokes – Einstein – Debye model for the orientational relaxation one expects that the orientational relaxation time will be proportional to viscosity. The data of Fig. 4 yield good agreement with this model (see inset). A more detailed analysis of the orientational relaxation of I is presented elsewhere.[26]

The behavior of the fast and slow relaxation times of I are in sharp contrast. While the long time data are readily fit by a linear viscosity dependence (Fig. 4), the fast relaxation time appears independent of viscosity (Fig. 3). This viscosity independence is not consistent with the rapid internal conversion arizing through an excited state isomerization reaction involving twisting about either of the bridging carbon - carbon bonds. For example the rotation of the imidazole ring would certainly be expected to show some sensitivity to solvent friction, even over the limited viscosity range of the data in Fig. 3. In an effort to extend the viscosity range we investigated the fast relaxation time in sucrose solutions, of concentration 0 – 2 M. This permits variation of the medium viscosity with minimum changes in the solvent – solute interactions. The data are shown in Fig. 5. Once again, even over this very wide viscosity range, the fast relaxation time is essentially independent of viscosity. The increase in relaxation time is at most a factor of 1.2, while the viscosity increase is 20 fold.

These results seem to rule out large scale intramolecular motion as the origin of the radiationless mechanism. This is consistent with the data of Fig. 2b, in which the increase in Φ_f with decreasing temperature did not follow the dramatically increasing solvent viscosity in the supercooled liquid phase. In addition, Φ_f continued to increase even below the glass transition temperature. Taken together, these results point to a thermally activated, rather than viscosity controlled, radiationless relaxation mechanism. Whatever the identity of the coordinate which couples the excited state to the ground state, it can be concluded that it is only weakly coupled to medium viscosity. Thus, although the present data do not identify the coordinate, they do eliminate a number of possibilities.

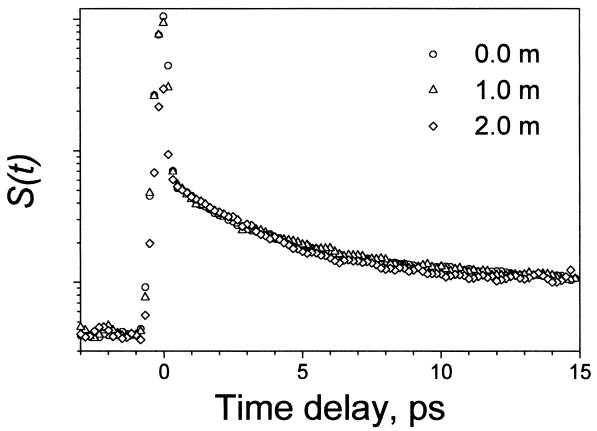


Fig. 5. The fast timescale measurements for the anion of I are presented in sucrose solutions of increasing viscosity (0 M; η = 0.9 cP, 1.0 M; η = 2.8 cP, 2.0 M; η = 21 cP).

Theoretical calculations may yield further information on the mechanism of the radiationless decay. The electronic structure of molecules similar to I (usually replacing the methyl groups with H atoms), in the ground and excited state, have been considered by a number of groups.[14–17,27] Two papers have addressed the effect of conformational change in the excited state, specifically the barrier to rotation about either or both of the two bridging bonds.[14,15] For the anion of I, Voityuk et al. found that the planar form is an energy minimum in the ground state and a maximum in the excited state, whereas the opposite is true for the 90° twisted form.[15] Thus ground and excited states approach during an excited state isomerisation, possibly leading to rapid internal conversion, as observed experimentally (Fig. 3). However, this mechanism is not consistent with the viscosity independence of the relaxation time (Figs. 3 and 5) discussed above. An additional mechanism was considered by Weber et al., a concerted simultaneous ("hula") twist about both bridging bonds.[14] In this case, for both neutral and anionic forms of I, the excited and ground states were calculated to approach at a twist angle of 90°. The attractive feature of this mechanism is that the volume of solvent displaced by the concerted rotation is small, and so might not be expected to be strongly dependent on medium viscosity. In this sense the concerted mechanism is more consistent with the present data. The data of Fig. 2 suggest that the radiationless mechanism is thermally activated, implying the existence of a barrier. Since the calculation of Weber et al. were performed only for twist angles of 0° and 90° it is difficult to comment further on this aspect, although, at least for the anion form, their data do suggest the existence of a barrier to excited state isomerisation. It should however be noted that, while the concerted twist motion appears to be consistent with the present data, it is only one possible coordinate for promotion of the rapid internal conversion. There may well be other excited state coordinates which also lead to facile internal conversion.

In contrast to this remarkable independence of the ground state recovery time of the anion on medium viscosity, it has been found that the ground state recovery time in a single solvent is a strong function of the charge state of the chromophore. In Fig. 6 the ground state recovery for the anion, cation (protonated at the un-methylated N atom by addition of HCl) and neutral forms of the chromophore are shown. It is readily seen that the order of the ground state recovery time is I $< I^+ < I^-$. Interestingly this order correlates inversely with the order of the transition energies of the $S_0 \rightarrow S_1$ transitions (largest for I). The change in the radiationless rate observed may occur either by changes in the height of the barrier to internal conversion, or by changes in the probability of the S_1 to S_0 transition. Measurements as a function of temperature show that the activation energy is not greatly changed between different forms of I. This result in turn suggests that the origin of the changes seen in Fig. 6 is a dependence of the coupling of S_1 and S_0 states on the charge state of the chromophore. It is difficult to comment further on this result. Theoretical calculation suggest that the shape of the ground and excited state potential energy surfaces do indeed depend on the charge,[14] but

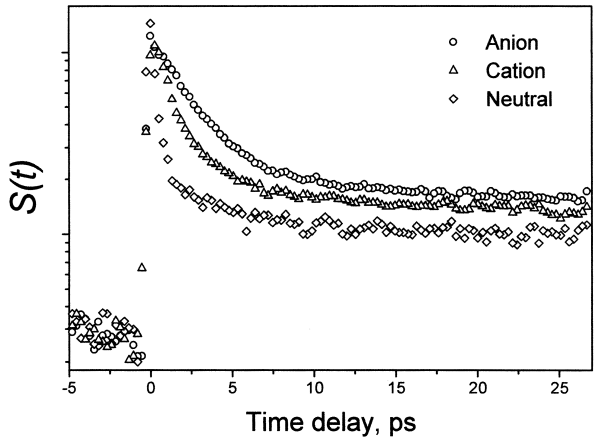


Fig. 6. Fast time scale measurements for the three charge forms of the chromophore, all in aqueous solution.

more detailed calculation are required to estimate the coupling strength.

An important question is how the present results concerning the free chromophore relate to the properties of GFP, and in particular how does I, which is non fluorescent in room temperature solution, become highly fluorescent when incorporated in the protein? From the fact that the denatured GFP is only weakly fluorescent it is known that the local protein structure is important. The result that the ground state recovery time of I is essentially independent of viscosity suggests that there is more to the fluorescence enhancement than simply the provision of a rigid environment by the protein matrix. This conclusion is consistent with the observation of Kummer et al., that some mutants of GFP undergo rapid internal conversion with no obvious change in the local packing around the chromophore.[28] If enhanced friction along the coordinate leading to internal conversion is responsible for rendering GFP fluorescent, it must arise from a highly specific protein structure around the chromophore.

An alternative explanation is that protein-chromophore interactions modify the electronic structure of the chromophore. The fluorescence intensity data of Fig. 2 are consistent with a thermally activated radiationless process in I. From Fig. 1 it is apparent that the electronic transition energy in the protein is considerably smaller (about 1500 cm^{-1}) than in the free chromophore. If this corresponds to a stabilization of the excited state energy, and the barrier in the pathway to internal conversion does not benefit from the same stabilization, then this effective increase in barrier height would reduce the radiationless rate constant, allowing fluorescence to become a competitive channel. To further test this model requires a knowledge of the interaction of the chromophore in its ground and excited states (which have appreciably different electronic structures[15,29]) with the H-bonding and charge environment of the protein. It seems possible to address such questions through quantum chemical calculations for the chromophore in the protein matrix.

Conclusion

The ground state recovery dynamics and temperature dependent fluorescence of a model of the chromophore of GFP have been investigated (preliminary results were presented elsewhere[26,30]). The fluorescence quantum yield increases dramatically with decreasing temperature. The increase continues through the supercooled phase into the glass phase. The temperature dependence is approximately described by an Arrhenius form. The relationship between measured quantum yield and medium viscosity is not a close one. The transient dichroism measurements at room temperature are dominated by an ultrafast component, which is ascribed to rapid ground state recovery, i. e. internal conversion followed by vibrational relaxation. There is an additional minor slow component. The fast component is nearly independent of viscosity, inconsistent with internal conversion arising through large scale intramolecular reorganisation in the excited state. However, the precise magnitude of the radiationless rate constant depends on the charge state of the chromophore. The slow component reveals a linear viscosity dependence, so can be ascribed to reorientational motion of I.[26] This result in turn suggests that a minor excited state relaxation channel in I is population of a long lived bottleneck state.

Collectively these data are consistent with an efficient internal conversion process arising from coupling of excited and ground states via a coordinate which is only weakly coupled to solvent frictional forces, and which exhibits a barrier. The coordinate cannot be identified from the present data, but the concerted rotation discussed by Weber et al. is one plausible candidate.[14] We have speculated that the ability of the protein to render the chromophore highly fluorescent may be related to particular protein structures which suppress intermolecular motion, or to intermolecular interactions which change the energetics of the electronic states. In the latter case it was suggested that the barrier to internal conversion is increased in the protein matrix. It is certainly true that the energy of the electronic transitions of I are significantly perturbed by the protein (Fig. 1). Such speculations may be tested by quantum chemical calculations on I in its protein environment.

We are grateful to Dr Richard Stephenson and his research group for assistance with the synthesis of I. This work was supported by grants from BBSRC and EPSRC. NMW is grateful to EPSRC for a studentship.

References

1 R. Y. Tsien, *Annu. Rev. Biochem.*, **67**, 509 (1998).
2 F. G. Prendegast, *Methods Cell Biol.* **58**, 1 (1999).
3 "Green Fluorescent Protein Properties, Applications and Protocols," ed by M. Chalfie and S. Kain, Wiley (1998).
4 S. R. Kain, M. Adams, A. Kondepudi, T. T. Yang, W. W. Ward, and P. Kitts, *Biotechniques*, **19**, 650 (1995).
5 M. Chattoraj, B. A. King, G. U. Bublitz, and S. G. Boxer, *Proc. Natl. Acad. Sci. U. S. A*, **93**, 8362 (1996).
6 H. Lossau, A. D. Kummer, R. Heinecke, F. Pollinger-Dammer, K. Kompa, G. Bieser, T. Jonson, C. M. Silva, M. M. Yang, D. C. Youvan, and M. E. Michel-Beyerle, *Chem. Phys.*, **213**, 1 (1996).
7 H. Niwa, S. Inouye, T. Horano, T. Matsuno, S. Kojima, M. Kubota, M. Ohashi, and F. I. Tsuji, *Proc. Natl. Acad. Sci., U. S. A.*, **93**, 13617 (1996).
8 P. Schwille, S. Kummer, A. A. Heikal, W. E. Moerner, and

W. W. Webb, *Proc. Natl. Acad. Sci., U. S. A.*, **97**, 151 (2000).
9 S. H. Bokman and W. W. Ward, *Biochem. Biophys. Res. Commun.*, **101**, 1372 (1981).
10 W. W. Ward, C. W. Cody, R. C. Hart, and M. J. Cormier, *Photochem. Photobiol.*, **31**, 611 (1980).
11 W. W. Ward, H. J. Prentice, A. F. Roth, C. W. Cody, and S. C. Reeves, *Photochem. Photobiol.*, **35**, 803 (1982).
12 M. A. Elsiger, R. M. Wachter, G. T. Hanson, K. Kallio, and S. J. Remington, *Biochemistry*, **38**, 5296 (1999).
13 A. F. Bell, X. He, R. M. Wachter, and P. J. Tonge, *Biochemistry*, **39**, 4423 (2000).
14 W. Weber, V. Helms, J. A. McCammon, and P. W. Langhoff, *Proc. Natl. Acad. Sci., U. S. A.*, **96**, 6177 (1999).
15 A. A. Voityuk, M.-E Michel-Beyerle, and N. Rösch, *Chem. Phys. Lett.*, **296**, 269 (1998).
16 A. A. Voityuk, A. D. Kummer, M.-E Michel-Beyerle, and N. Rösch, *Chem. Phys.*, **269**, 83 (2001).
17 A. A. Voityuk, M.-E Michel-Beyerle, and N. Rösch, *Chem. Phys.*, **231**, 13 (1998).
18 D. S. Alavi, R. S. Hartman, and D. H. Waldeck, *J. Chem. Phys.*, **92**, 4055 (1990).
19 A. J. Cross, D. H. Waldeck, and G. R. Fleming, *J. Chem. Phys.*, **78**, 6455 (1983).
20 S. Kojima, H. Ohkawa, T. Hirano, S. Maki, H. Niwa, M. Ohashi, S. Inouye, and F. I. Tsuji, *Tetrahedron Lett.*, **39**, 5239 (1998).
21 G. Bublitz, B. A. King, and S. G. Boxer, *J. Am. Chem. Soc.*, **120**, 9370 (1998).
22 M. A. Perozzo, K. B. Ward, R. B. Thomson, and W. W Ward, *J. Biol. Chem.*, **263**, 7716 (1988).
23 L. Seidner and W. Domcke, *Chem. Phys.*, **186**, 27 (1994).
24 G. Oster and Y. Nishijima, *J. Am. Chem. Soc.*, **78**, 1581 (1956).
25 A. C. Ling and J. E. Willard, *J. Phys. Chem.*, **72**, 1918 (1968).
26 K. L. Litvinenko, N. M. Webber, and S. R. Meech, *Chem. Phys. Lett.*, **346**, 47 (2001).
27 A. A. Voityuk, M.-E. Michel-Beyerle, and N. Rösch, *Chem. Phys.*, **272**, 162 (1997).
28 A. D. Kummer, J. Wiehler, H. Rehaber, K. Kompa, B. Steipe, and M. E. Michel-Beyerle, *J. Phys. Chem. B*, **104**, 4791 (2000).
29 V. Tozzini and R. Nifosi, *J. Phys. Chem. B*, **105**, 5797 (2001).
30 N. M. Webber, K. L. Litvinenko, and S. R. Meech, *J. Phys. Chem. B*, **105**, 8036 (2001).