Journal of Fluorescence, Vol. 12, No. 1, March 2002 (© 2002)

The Influence of Solvent Viscosity on the Fluorescence Decay and Time-Resolved Anisotropy of Green Fluorescent Protein

Klaus Suhling,[1,3] Daniel M. Davis,[1] and David Phillips[2]

Received November 9, 2001; accepted December 23, 2001

This report describes fluorescence decay and time-resolved anisotropy studies of green fluorescent protein (GFP) in various environments. The addition of glucose and fructose, NaCl, or polyethylene glycol changes the viscosity of the medium surrounding the GFP. Both the time-resolved anisotropy and the fluorescence decay of GFP are measured and it is shown that only the time-resolved anisotropy of GFP is affected by the viscosity, but not its fluorescence decay.

KEY WORDS: GFP fluorescence lifetime; anisotropy; TCSPC; viscosity.

INTRODUCTION

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* [1] is extremely popular in the life sciences, principally in imaging applications [2,3]. The fluorescence decay of GFP could be used to report directly on its biophysical environment (e.g., by fluorescence lifetime imaging [FLIM] [4–9]). However, the question of which environmental parameters affect the GFP fluorescence decay has to be addressed.

The viscosity of the medium surrounding a fluorophore can affect the fluorescence lifetime [10] if the molecule can undergo internal twisting. This can provide a non-radiative de-excitation pathway which competes with radiative de-excitation [11].

We have recently presented preliminary data to show that the GFP lifetime scales with the square of the refractive index in mixtures of water and glycerol [12]. However, when adding glycerol to the water, not only the refractive index increases but also the viscosity. Here, we address the influence of viscosity on the fluorescence and anisotropy decay of GFP. We show that the time-resolved anisotropy does indeed depend on the viscosity of the environment, but the GFP fluorescence decay does not.

[1] Department of Biological Sciences, Sir Alexander Fleming Building.
[2] Department of Chemistry, Imperial College of Science, Technology & Medicine, London SW7 2AZ, U.K.
[3] To whom correspondence should be addressed Tel: +44/20/7594 5409; fax: +44/20/7594 5801; e-mail: k.suhling@ic.ac.uk

THEORETICAL BACKGROUND

The Fluorescence Lifetime

The fluorescence lifetime τ_{fl} is the time a molecule remains in its excited state after excitation. It related to the radiative rate constant k_0 and the non-radiative rate constant k_{nr} according to:

$$\frac{1}{\tau_{fl}} = k_0 + k_{nr} \tag{1}$$

Non-radiative de-activation of the excited state shortens the natural radiative lifetime τ_0 to the measured lifetime τ_{fl}. Internal conversion with a rate constant k_{ic} and intersystem crossing with a rate constant k_{isc} contribute to non-radiative de-activation. In addition, internal molecular twisting can provide a non-radiative de-excitation path-

1053-0509/02/0300-0091/0 © 2002 Plenum Publishing Corporation

way with a rate constant k_{visc}, which depends on the viscosity η [11]

$$k_{visc}^{-1} = c\eta^{\alpha} \quad (2)$$

where c and α are constants.

Bimolecular interactions such as diffusion-controlled collisional quenching (e.g., by oxygen) also depend on the viscosity. An increase in viscosity leads to a reduction of collisional encounters between fluorophore and quencher, and thus to a reduction in the non-radiative rate constant [13].

Time-Resolved Fluorescence Anisotropy

The time-resolved fluorescence anisotropy $r(t)$ is defined as

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} \quad (3)$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence decays parallel and perpendicular to the polarization of the excitation, respectively. G is a correction factor that accounts for different transmission efficiencies for the parallel and perpendicular polarization [13,14].

For a spherically symmetrical, unhindered rotor, $r(t)$ decays mono-exponentially and is related to the rotational correlation time τ_{rot}, according to:

$$r(t) = r_0 e^{-\frac{t}{\tau_{rot}}} \quad (4)$$

where r_0 is the initial anisotropy. The rotational correlation time τ_{rot} is directory proportional to the viscosity η and the volume V of the rotating molecule according to a Stokes-Einstein relationship:

$$\tau_{rot} = \frac{\eta V}{kT} \quad (5)$$

where k is the Boltzmann constant and T the absolute temperature [13,14].

MATERIAL AND METHODS

Polyethylene glycol (BDH, average molecular mass 400) and NaCl (BDH) were added to phosphate buffered saline (PBS, pH 7.3, Sigma) in the desired quantities. For the sugar solutions, D(−)-fructose (D-levulose, fruit sugar, Sigma) and D(+)glucose (dextrose, corn sugar, Sigma) were mixed at a ratio at which the optical activity is zero and then diluted with PBS. Recombinant-soluble GFP (enhanced GFP, i.e., F64L, S65T, Clontech) was added to solutions of polyethylene glycol, salt, or sugar to yield concentrations of $5.3 \cdot 10^{-8}$ mol 1^{-1}.

The fluorescence and anisotropy decays were obtained by time-correlated single-photon counting [14,15] using a mode-locked tunable Ti:sapphire laser (Coherent) for excitation. The output was frequency doubled to excite the samples at 470 nm with vertical polarization, and the laser repetition rate of 76 MHz was reduced to 3.8 MHz using a pulse picker (SiO_2 crystal, APE). The fluorescence decays were measured at 510 nm with a 10-nm bandpass using a polarizer oriented at the magic angle 54.7° to eliminate rotational depolarization effects [13,15]. The stop rate was kept below 1% of the start rate to avoid pulse pile-up. The detector was a cooled microchannel plate operated at −3.4 kV (Photek). Instrumental response functions were typically 230 ps full-width half-maximum, and fluorescence decay analysis was performed on reconvolution software from IBH.

The anisotropy decays were calculated according to Eq. (3) by alternately measuring several fluorescence decays at parallel and perpendicular polarization. They were analyzed according to Eqs. (3) and (4) using a G-factor of 1.09, which was determined from measurements at horizontally polarized excitation with the emission polarizer at 0° and 90°.

In addition, a dead-time correction factor was used to account for the fact that at count rates near 1% of the excitation rate (38,000 counts/sec) the dead-time loss for the parallel decay is greater than for the perpendicular decay [16]. This means that, in effect, the parallel decay is acquired for a shorter time. The correction factor is count rate dependent and can be calculated from the stop-to-start ratio and the instrumental dead time (18.1 μs) [16]. It ranges from 1–1.3, and the parallel decay is multiplied by it.

RESULTS

Fluorescence and Anisotropy Decays in Solutions of Different Viscosity

To determine the effect of increasing the viscosity on the GFP fluorescence lifetime, glucose and fructose or NaCl were added to the buffer. Figure 1(a) shows the fluorescence decays of GFP in PBS with 0.33g/ml glucose and 0.24g/ml fructose, and with 0.185g/ml NaCl. They can hardly be distinguished. The bump at 14 ns is due to re-excitation of the fluorophore by a secondary laser pulse. It is fully accounted for by deconvolution with the instrumental response function, which yields average

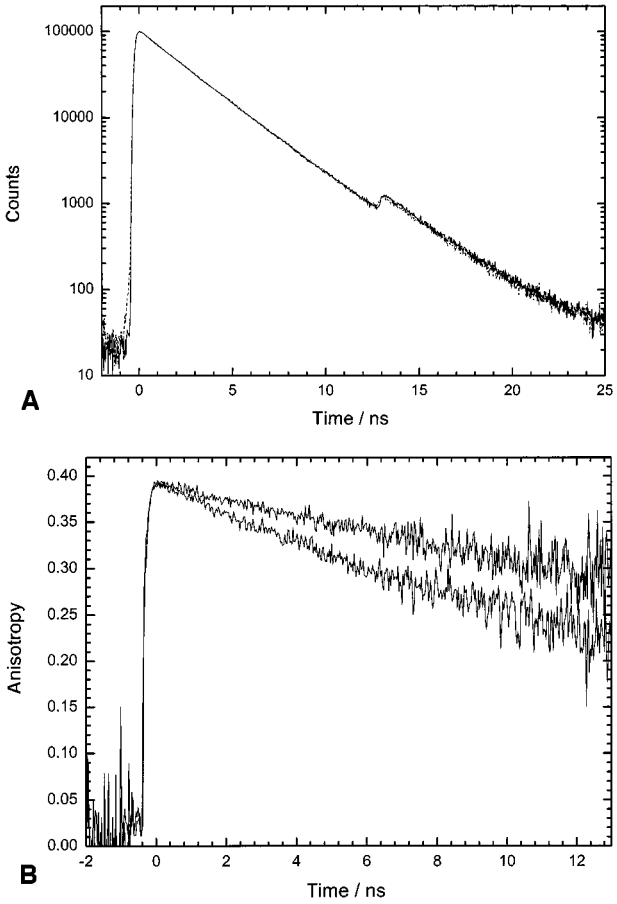

Fig. 1. Fluorescence and anisotropy decays of GFP in solutions of different viscosity. (a) Two fluorescence decays of GFP in buffer, one with 0.185 g/ml added NaCl, and one with 0.33 g/ml glucose and 0.24 g/ml fructose. The fluorescence decays are almost identical, τ_{fl}^{sugars} = 2.64 ns and τ_{fl}^{NaCl} = 2.61 ns (b) The time-resolved anisotropy decays of the same samples. The upper anisotropy decay of GFP is in buffer with 0.33 g/ml glucose and 0.24 g/ml fructose; the lower anisotropy decay is in buffer with 0.185 g/ml added NaCl. τ_{rot}^{sugars} = 43.7 ± 4.9 ns and τ_{rot}^{NaCl} = 23.0 ± 2.3 ns, and the initial anisotropies are r_0 = 0.39.

fluorescence lifetimes of $\tau_{fl}{}^{sugars}$ = 2.64 ns and $\tau_{fl}{}^{NaCl}$ = 2.61 ns, respectively.

The viscosity increase of the GFP environment is, however, reflected in the time-resolved anisotropy decays shown in Fig. 1b. They are visibly different, and a fit to a single rotational correlation time τ_{rot} according to Eq. (4) yields τ_{rot} = 23.0 ± 2.3 ns for the salt solution, and τ_{rot} = 43.7 ± 4.9 ns for the sugar solution. The initial anisotropies are r_0 = 0.39 in both cases.

Fluorescence and Anisotropy Decays in Solutions of Similar Viscosity

The same viscosity was created by different means and the GFP fluorescence and anisotropy decays measured. Figure 2a shows the fluorescence decays of GFP in PBS with 10% polyethylene glycol and with 0.248g/ml NaCl. Deconvolution with the instrumental response function yields an average lifetime τ_{fl} = 2.73 ns in 10% polyethylene glycol, and τ_{fl} = 2.49 ns with 0.248g/ml of added NaCl.

Figure 2b shows the corresponding time-resolved anisotropy decays. They are very similar (τ_{rot}^{PEG} = 26.0 ± 2.3 ns and τ_{rot}^{NaCl} 23.8 ± 3.1 ns), which is due to a similar viscosity of the medium surrounding the GFP fluorophore. The initial anisotropies are r_0^{PEG} = 0.38 and r_0^{NaCl} = 0.40.

To establish whether there is a relationship between the viscosity and the fluorescence lifetime at all, a plot of the fluorescence lifetime τ_{fl} versus the viscosity η for

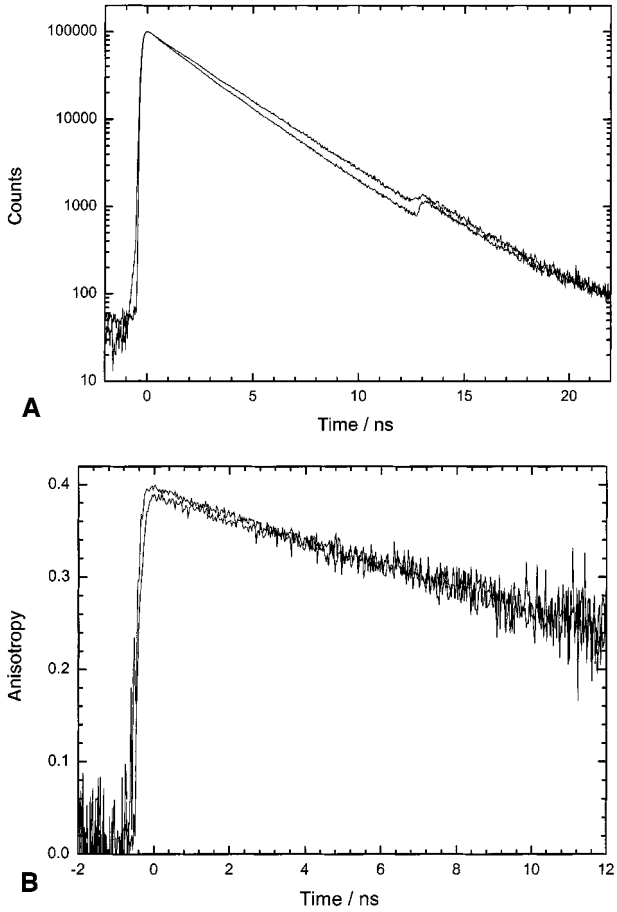

Fig. 2. Fluorescence and anisotropy decays of GFP in solutions of similar viscosity. (a) Two fluorescence decays of GFP in buffer. The upper decay is GFP in buffer with 10% polyethylene glycol, and the lower decay is GFP in buffer with 0.248 g/ml salt. They are clearly different, τ_{fl}^{PEG} = 2.73 ns and τ_{fl}^{NaCl} = 2.49 ns (b) The corresponding time-resolved anisotropy decays. They are very similar (τ_{rot}^{PEG} = 26.0 ± 2.3 ns, r_0^{PEG} = 0.38 and τ_{rot}^{NaCl} = 23.8 ± 3.1 ns, r_0^{NaCl} = 0.40), which is due to a similar viscosity of the medium surrounding the GFP.

a number of salt, sugar, and polyethylene glycol solutions is shown in Fig. 3a. An increase of the fluorescence lifetime with increasing viscosity, as would be expected for internal twisting of the fluorophore [Eq. (2)] [10] or oxygen quenching, cannot be observed. The fluorescence decays of the points marked (1) and (2) are shown in Figs. 1 and 2, respectively.

However, there is a clear relationship between the rotational correlation time τ_{rot} and the viscosity η, as shown in Fig. 3b. The data points lie on a straight line, as predicted by Eq. 5. A straight line fit yields an intercept of zero and a gradient of 14.2 $\pm$ 1.2 ns/cp, from which a GFP radius of 2.40 $\pm$ 0.07 nm is calculated (assuming a spherical GFP).

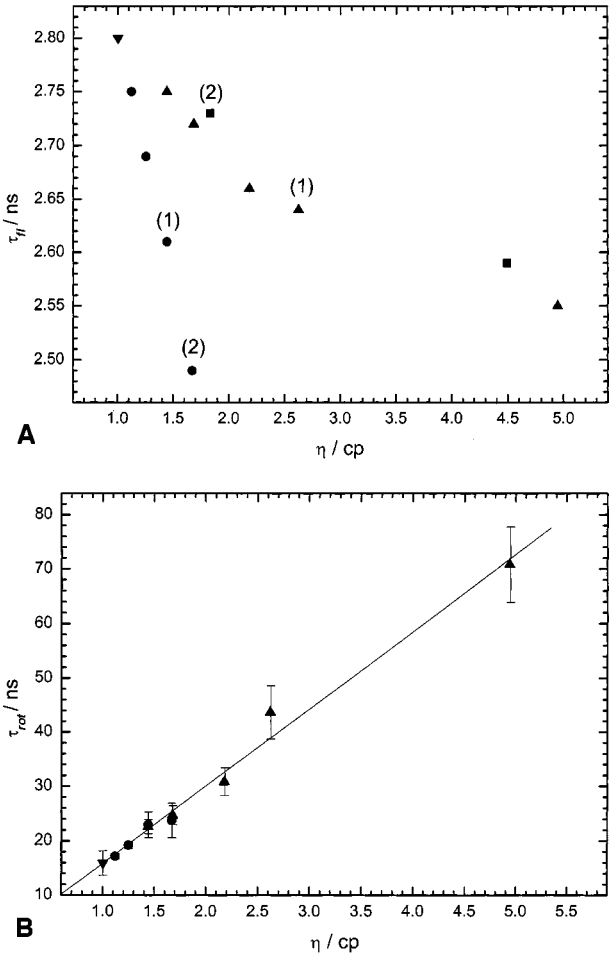

Fig. 3. The GFP fluorescence lifetime and the rotational correlation time plotted against the viscosity. ▼: buffer; ■: polyethylene glycol; ▲: glucose and fructose; ●: NaCl. (a) The fluorescence lifetime τ_{fl} plotted versus the viscosity η shows that the solvent viscosity cannot be predicted from the fluorescence lifetime. (1) and (2) are the fluorescence decays shown in Fig. 1a and 2a. (b) The rotational correlation time τ_{rot} versus the viscosity. The data points lie on a straight line, as predicted by Eq. (5), and the GFP radius calculated from the gradient is 2.40 $\pm$ 0.07 nm.

DISCUSSION

The anisotropy decays of GFP can all be fitted to a single exponential decay law, in agreement with other work [17,18,19,20]. This is consistent with the view that the whole protein undergoes rotational diffusion and that the fluorophore is rigidly fixed inside a barrel-shaped cage, the structure of which is known [21]. It is 4.2 nm long and 2.4 nm in diameter, and the single rotational correlation time measured is a weighted average of three rotational correlation times expected for a rotating cylinder [14]. The initial anisotropies are all close to 0.4, which is expected for absorption and emission dipole moments that are parallel [17,18].

The Stokes-Einstein relationship Eq. (5) holds as the viscosity is increased by adding sugars or salt to the buffer. This slows down the molecular tumbling, which increases the rotational correlation time τ_{rot}. The GFP radius of 2.40 $\pm$ 0.07 nm, determined from a series of measurements in solutions of sugar or salt, is in good agreement with the value of 2.37 nm calculated from a single time-resolved fluorescence anisotropy measurement in water [20].

There is no indication that the GFP fluorescence lifetime depends on the viscosity. If the fluorescence lifetime were shortened by internal molecular twisting according to Eq. (2), an increase in the solvent viscosity should result in a concomitant increase in the fluorescence lifetime. This is not the case. In environments of different viscosities, the fluorescence lifetimes of GFP are similar, as shown in Fig. 1a. Moreover, in solutions of similar viscosities, we obtain different fluorescence lifetimes, as shown in Fig. 2a. A plot of all fluorescence lifetimes τ_{fl} obtained in a series of measurements in solutions of sugar, salt, or polyethylene glycol versus the viscosity η (see Fig. 3a) shows that the solvent viscosity cannot be predicted from the fluorescence lifetime. Fluorescence quenching by oxygen would also be viscosity dependent; an increase of viscosity should increase the lifetime. We find no evidence for such a mechanism either, in agreement with previous work [22]. The GFP's barrel-shaped cage [21] shields the fluorophore and protects it from collisional interactions. Indeed, preliminary data show that the inverse GFP fluorescence lifetime in mixtures of water and glycerol scales not with viscosity but with the square of the refractive index [12]. A more detailed investigation of this effect will be the subject of a future publication.

CONCLUSIONS

The GFP fluorescence decay and time-resolved anisotropy in different solutions of glucose and fructose, salt, or polyethylene glycol were measured. The anisotropy decays could be fitted to a single rotational correlation time. Increasing the viscosity increases the rotational correlation time, but does not affect the fluorescence lifetime. The GFP fluorescence lifetime is independent of the viscosity of the surrounding medium and cannot thus be used to report on the viscosity.

ACKNOWLEDGMENTS

We would like to acknowledge support from the UK's Engineering and Physical Sciences Research Council (EPSRC) and from the Biotechnology and Biological Sciences Research Council (BBSRC).

REFERENCES

1. R. Y. Tsien (1998) *Annu. Rev. Biochem.* **67**, 509–544.
2. M. Chalfie and S. Kain (Eds.) (1998) *Green Fluorescent Protein: Properties, Application and Protocols*, Wiley-Liss, New York, pp. 1–385.
3. K. F. Sullivan and S. A. Kay (Eds.) (1999) *Methods in Cell Biology 58: Green Fluorescent Proteins*, Academic Press, London, pp. 1–450.
4. X. F. Wang, A. Periasamy, B. Herman, and D. M. Coleman (1992) *Crit. Rev. Analytic. Chem.* **23**(5), 369–395.
5. T. W. J. Gadella Jr, T. M. Jovin, and R. M. Clegg (1993) *Biophys. Chem.* **48**, 221–239.
6. J. R. Lakowicz, H. Szmacinski, K. Nowaczyk, K. W. Berndt, and M. Johnson (1992) *Analytic Biochem.* **202**, 316–330.
7. K. Dowling, M. J. Dayel, S. C. W. Hyde, P. M. W. French, M. J. Lever, J. D. Hares, and A. K. L. Dymoke-Bradshaw (1999) *J. Modern Optics* **46**(2), 199–209.
8. A. D. Scully, A. J. MacRobert, S. Botchway, P. O'Neill, R. B. Ostler, and D. Phillips (1997) *Bioimaging* **5**, 9–18.
9. P. I. H. Bastiaens and A. Squire (1999) *Trends Cell Biol.* **9**, 48–52.
10. W. Sibbett and J. R. Taylor (1983) *J. Luminescence* **28**, 367–375.
11. T. Förster and G. Hoffmann (1971) *Zeitschrift für Physikalische Chemie Neue Folge*, **75**, 63–76.
12. K. Suhling, D. M. Davis, Z. Petrášek, J. Siegel, and D. Phillips (2001) *Proc. S. P. I. E.* **4259**, 92–101.
13. J. R. Lakowicz (1999) *Principles of Fluorescence Spectroscopy* 2nd edition, Kluwer Academic/Plenum Publishers, New York, pp. 1–698.
14. D. V. O'Connor and D. Phillips (1984) *Time-Correlated Single Photon Counting*, Academic Press, London, pp. 1–288.
15. D. J. S. Birch and R. E. Imhof (1991) in *Topics in Fluorescence Spectroscopy: Techniques*, Vol 1, Plenum Press, New York, pp. 1–95.
16. K. Suhling, D. McLoskey, and D. J. S. Birch (1996) *Rev. Scientific Instruments* **67**(6), 2238–2246.
17. A. Volkmer, V. Subramaniam, D. J. S. Birch, and T. M. Jovin (2000) *Biophys. J.* **78**, 1589–1598.
18. G. Striker, V. Subramaniam, C. A. M. Seidel, and A. Volkmer (1999) *J. Physical Chem. B* **103**(40), 8612–8617.
19. J. Widengren, Ü. Mets, and R. Rigler (1999) *Chem. Physics* **250**, 171–186.
20. M. A. Uskova, J. Borst, M. A. Hink, A. van Hoek, A. Schots, N. L. Klyachko, and A. J. W. G. Visser (2000) *Biophys. Chem.* **87**, 73–84.
21. M. Ormö, K. Kallio A. B. Cubitt, L. A. Gross, R. Y. Tsien, and S. J. Remington (1996) *Science* **273**, 1392–1395.
22. R. Swaminathan, C. P. Hoang, and A. S. Verkman (1997) *Biophys. J.* **72**, 1900–1907.