Dipolar Relaxation within the Protein Matrix of the Green Fluorescent Protein: A Red Edge Excitation Shift Study

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Received: August 24, 2007; In Final Form: October 15, 2007

The fluorophore in green fluorescent protein (GFP) is localized in a highly constrained environment, protected from the bulk solvent by the barrel-shaped protein matrix. We have used the wavelength-selective fluorescence approach (red edge excitation shift, REES) to monitor solvent (environment) dynamics around the fluorophore in enhanced green fluorescent protein (EGFP) under various conditions. Our results show that EGFP displays REES in buffer and glycerol, i.e., the fluorescence emission maxima exhibit a progressive shift toward the red edge, as the excitation wavelength is shifted toward the red edge of the absorption spectrum. Interestingly, EGFP displays REES when incorporated in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT), independent of the hydration state. We interpret the observed REES to the constrained environment experienced by the EGFP fluorophore in the rigid protein matrix, rather than to the dynamics of the bulk solvent. These results are supported by the temperature dependence of REES and characteristic wavelength-dependent changes in fluorescence anisotropy.

Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (*A. victoria*) and its variants have become popular reporter molecules for monitoring protein expression, localization, and dynamics of membrane and cytoplasmic proteins.[1] Tagging cellular proteins with GFP has allowed direct visualization of signaling and real-time trafficking in living cells.[2,3] GFP possesses characteristics that are highly desirable for use as a reporter molecule.[1] These include its intrinsic, cofactor-independent fluorescence which exhibits remarkable stability in the presence of denaturants and over a wide range of pH. Interestingly, mutants of GFP such as the S65T mutant[4] display enhanced brightness over the wild type GFP.[5]

GFP has a compact barrel-shaped structure made of 11 β strands with an α helix running through the central axis of the cylindrical structure. The fluorophore of GFP responsible for its green fluorescence is localized at the center of the cylindrical structure and is formed spontaneously upon folding of the polypeptide chain by internal cyclization and oxidation of the residues Ser65-Tyr66-Gly67 in the α helix.[6] The fluorophore is in a highly constrained environment, protected from the bulk solvent by the surrounding β strands.[7] This results in a small Stokes' shift and a high fluorescence quantum yield.[8] Although various photophysical aspects of GFP have been extensively studied,[6,9] the phenomenon of solvent dipolar relaxation around the GFP fluorophore is only beginning to be addressed. In this paper, we have used the wavelength-selective fluorescence approach to monitor solvent (environment) dynamics around the enhanced green fluorescent protein (EGFP) under various conditions. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in an organized molecular assembly.[10,11] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed red edge excitation shift (REES).[10−12] This effect is mostly observed with polar fluorophores in motionally restricted environments such as viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises due to slow rates of solvent dipolar relaxation (reorientation) around an excited-state fluorophore, which is dependent on the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. This makes the application of REES in particular, and the wavelength-selective fluorescence approach in general, very useful since hydration plays a crucial modulatory role in the formation and maintenance of organized molecular assemblies such as micelles, membranes, and folded proteins in aqueous solutions.[13] The unique feature about REES is that while all other fluorescence techniques such as fluorescence quenching, resonance energy transfer, and polarization measurements yield information about the fluorophore itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics, which is not possible to obtain by other techniques. Since the dynamics of hydration is directly associated with the functionality of proteins, REES has proved to be a useful tool to explore the organization and dynamics of soluble and membrane proteins under varying degrees of hydration.[14,15] An in-depth discussion of the photophysical framework for REES and the wavelength-selective fluorescence approach is provided in recent reviews.[10,11]

Experimental Section

Materials. Purified EGFP (F64L, S65T) was a generous gift from Prof. G. Krishnamoorthy (Tata Institute of Fundamental

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10.1021/jp076797z CCC: $37.00
Published on Web 12/06/2007

Research, Mumbai, India). AOT (sodium bis(2-ethylhexyl)-sulfosuccinate), ethylenediaminetetraacetic acid (EDTA), and Tris were purchased from Sigma Chemical Co. (St. Louis. MO). Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. The purity of AOT was confirmed by good agreement of its UV absorption spectrum with a previously reported spectrum.[16]

Sample Preparation. The concentration of pure EGFP in 20 mM Tris and 1 mM EDTA, pH 7.5, buffer was estimated using the molar absorption coefficient (ϵ) 53 000 M^{-1} cm^{-1} at 489 nm.[17] Reverse micelles of AOT containing EGFP were prepared by directly incorporating EGFP to AOT in isooctane and appropriate amounts of buffer were added to make reverse micellar dispersions of different [water]/[surfactant] molar ratio (w_o). Samples were stirred for 3 min to get a clear dispersion. The optical density of the samples at the excitation wavelength was low (generally <0.15) in all cases. Background samples were prepared the same way except that EGFP was not added to them. All experiments were done at room temperature ($\sim$23 °C), unless otherwise mentioned.

The molar ratio of fluorophore to surfactant was carefully chosen to give an optimum signal-to-noise ratio with minimal perturbation to the micellar organization and negligible inter-probe interactions. The final EGFP concentration was 500 nM while the concentration of AOT was 100 mM in all cases. This corresponds to a final molar ratio of fluorophore to surfactant of 1:200 000 (mol/mol). At such a low fluorophore to surfactant molar ratio, not more than one EGFP molecule would be present per reverse micelle on an average, which rules out any EGFP aggregation effects, especially keeping in mind the aggregation number of AOT of $\sim$50$-$300 in the range of w_o between 5 and 25.[18]

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 3 nm were used for all measurements. Background intensities of samples in which EGFP was omitted were negligible in most cases and were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases or were within $\pm$1 nm of the ones reported. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation[19]

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples, and average values of anisotropy are shown in Figure 4.

Results and Discussion

Representative fluorescence emission spectra of EGFP in buffer obtained using different excitation wavelengths are shown in Figure 1. The figure shows the dependence of the emission spectrum on the excitation wavelength for EGFP. As shown in the figure, upon excitation at 475 nm, the maximum of fluorescence emission[20] is displayed at 507 nm, in agreement with previous results.[21] Upon changing the excitation wavelength to 495 nm, the wavelength of maximum emission displays a shift from 507 to 509 nm. The comprehensive shift in the maxima of fluorescence emission of EGFP as a function of excitation wavelength is shown in Figure 2. The figure shows that as the excitation wavelength is changed from 475 to 502 nm, the emission maximum exhibits a corresponding shift from 507 to 511 nm, which corresponds to REES of 4 nm. Such dependence of the emission maximum on excitation wavelength is characteristic of REES. This implies that the fluorophore in EGFP is localized in a motionally restricted region in the protein matrix.

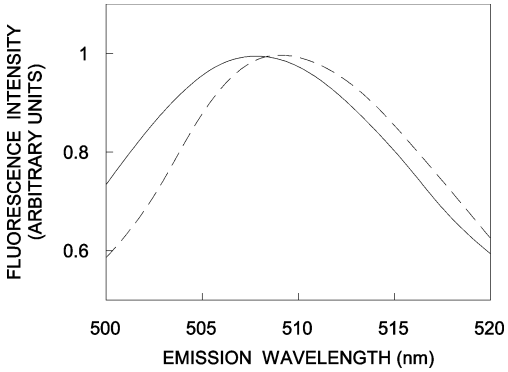

Figure 1. Representative fluorescence emission spectra of EGFP at different excitation wavelengths. The buffer used was 20 mM Tris, 1 mM EDTA, pH 7.5. The excitation wavelengths were 475 (—) and 495 (- - -) nm. All spectra are intensity-normalized at the emission maximum. The concentration of EGFP was 500 nM. See Experimental Section for other details.

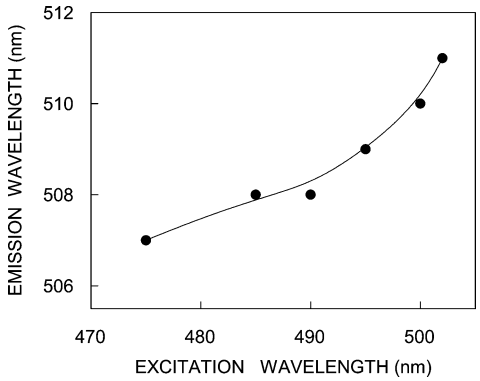

Figure 2. Effect of changing excitation wavelength on the wavelength of maximum emission of EGFP in buffer. All other conditions are as in Figure 1. The line joining the data points is provided merely as a viewing guide. See Experimental Section for other details.

The rate of solvent dipolar reorientation ("solvent" in this case refers to the dipolar protein matrix,[6] see below) will be enhanced at higher temperatures and fluorescence emission under such conditions will be dominated by a solvent-relaxed state.[22] The magnitude of REES therefore is expected to reduce with an increase in temperature.[23] Figure 3 shows the effect of temperature on REES of EGFP in buffer. As shown in the figure, as the excitation wavelength is changed from 475 to 502 nm at a higher temperature (at 62 °C), the emission maximum exhibits a concomitant shift from 510 to 512 nm, corresponding to a reduced REES of 2 nm. This indicates that the rate of solvent dipolar relaxation (reorientation) around the constrained fluorophore in EGFP is enhanced upon increasing temperature.

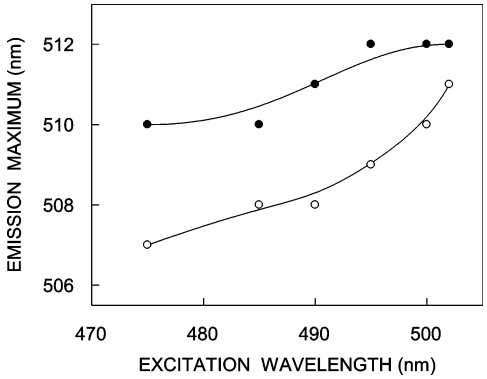

Figure 3. Effect of changing excitation wavelength on the wavelength of maximum emission for EGFP in buffer at 23 (○) and 62 °C (●). All other conditions are as in Figure 1. The lines joining the data points are provided merely as viewing guides. See Experimental Section for other details.

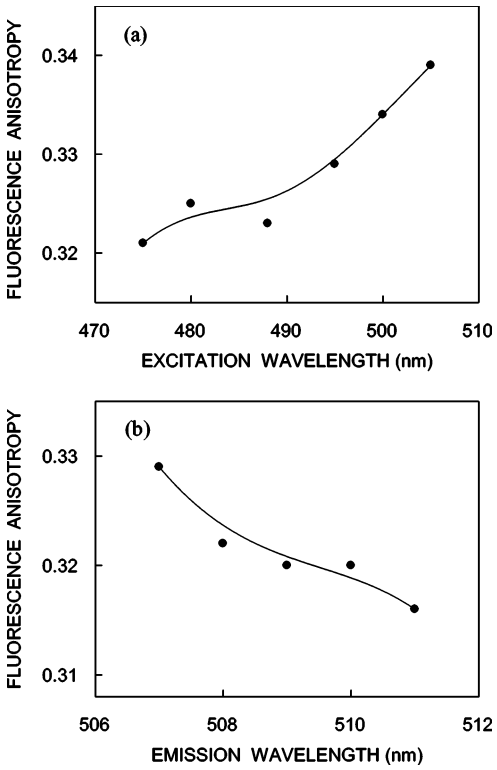

Figure 4. Fluorescence anisotropy of EGFP in buffer as a function of (a) excitation and (b) emission wavelengths. The anisotropy values were recorded at an emission wavelength of 507 nm (in panel a), and the excitation wavelength used was 485 nm (panel b). Data shown are means of three independent measurements. All other conditions are as in Figure 1. The lines joining the data points are provided merely as viewing guides. See Experimental Section for other details.

Alternatively, EGFP may be denatured to some extent at this temperature[24,25] which could also lead to an increase in rate of solvent dipolar relaxation resulting in reduced REES.

In addition to the shift in emission maximum on red edge excitation, fluorescence anisotropy is also known to be dependent on excitation and emission wavelengths in motionally restricted media.[26] The excitation anisotropy spectrum (i.e., a plot of steady-state anisotropy versus excitation wavelength) is shown in Figure 4a. The anisotropy values show a characteristic increase with increasing excitation wavelength. Figure 4b shows the change in fluorescence anisotropy across the emission spectrum. In this case also, the anisotropy exhibits a characteristic decrease with increasing emission wavelength. Taken together, these wavelength-dependent anisotropy changes reinforce the fact that the fluorophore in EGFP experiences constrained dynamics in its immediate environment.

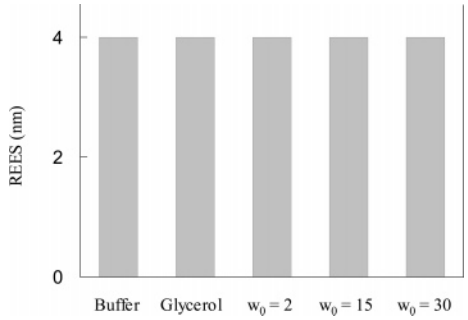

Figure 5. Comparison of the magnitude of REES of EGFP in buffer, 90% glycerol, and AOT reverse micelles. The concentration of EGFP is same (500 nM) in all cases. The last three bars represent REES of EGFP in AOT reverse micelles with varying [water]/[surfactant] molar ratio (w_0). The ratio of EGFP to surfactant was 1:200 000 (mol/mol). See Experimental Section for other details.

As mentioned above, REES is observed predominantly with polar fluorophores in motionally restricted environments such as viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. In this context, our observation of REES of EGFP in buffer, a nonviscous solvent at the temperature of the experiment, may be somewhat surprising. It should be noted here that EGFP is a compact protein, and the fluorophore in EGFP, localized at the center of the barrel-shaped protein, experiences a highly constrained environment.[7] We therefore interpret the observed REES of 4 nm (at 23 °C) to this constrained environment rather than the dynamics of the bulk solvent (buffer) since the EGFP fluorophore remains protected from the bulk solvent by the surrounding β strands. In other words, the contribution of the bulk solvent surrounding the EGFP protein matrix is minimal in the observed REES of EGFP. To test this, we examined REES of EGFP in 90% glycerol, a bulk viscous solvent. Figure 5 shows that the extent of REES of EGFP is the same (4 nm), irrespective of the bulk viscosity of the solvent (i.e., the emission maximum displays a shift from 507 to 511 nm, in response to change in excitation wavelength from 475 to 502 nm).

Reverse micelles have emerged as an appropriate molecular assembly for monitoring constrained dynamics of proteins incorporated in them.[21,27] They offer the advantage of monitoring dynamics of molecules incorporated in them with varying states of hydration.[15] Amphiphilic surfactants, such as AOT, self-assemble to form reverse micelles in nonpolar solvents in which the polar head groups of the surfactant monomers cluster to form a micellar core and are directed toward the center of the assembly, and the hydrophobic tails extend outward into the bulk organic phase.[16,28] Reverse micelles provide an attractive model system for biomembranes since they mimic a number of important and essential features of biological membranes although lacking much of the complexity associated with them. It is known that the dynamics of liquids in confined spaces is different than that of their bulk counterparts,[29] and this constitutes one of the main reasons for the popularity that reverse micelles enjoy as a model system in studies of water dynamics.[30] A unique structural parameter of reverse micelles is w_o, which determines micellar size as well as the unique physicochemical properties of the entrapped water. We monitored REES of EGFP incorporated in reverse micelles of AOT under varying hydration conditions in order to explore whether entrapment in a reverse

micelle affects environmental dynamics around the fluorophore in EGFP. Interestingly, Figure 5 shows that neither entrapment in a reverse micelle nor the hydration state of the reverse micelle influences REES of EGFP. EGFP incorporated in AOT reverse micelles displayed REES of 4 nm (the emission maximum exhibits a shift from 508 to 512 nm in all cases upon changing excitation wavelength from 475 to 502 nm), irrespective of the hydration state ($w_o = 2-30$). This reinforces our earlier conclusion that the extent of REES of EGFP is dependent solely on the dynamics of the core protein matrix and is independent of the viscosity of the surrounding medium. Interestingly, it has been previously shown by measurement of kinetics of proton transfer in EGFP that the dynamics in the interior of the protein is very weakly coupled to viscosity changes of the bulk medium.[31]

GFP exhibits complex fluorescence characteristics and is characterized by multiple absorption bands.[1] Interestingly, EGFP with mutations F64L and S65T displays a single absorption and emission peak.[5] This makes EGFP an ideal choice for REES measurements since ground-state heterogeneity is avoided. An essential criterion for observation of REES is a change in dipole moment of the fluorophore upon excitation.[32] The exact change in dipole moment of EGFP upon excitation is not known. However, the change in dipole moment for the S65T mutant has been estimated to be ~7 D.[33]

In summary, our results show that EGFP displays REES due to slow dipolar relaxation around its fluorophore. The slow dipolar relaxation could be attributed to the rigid protein matrix around the fluorophore. The extent of REES appears to be independent of the viscosity of the medium, implying that the dynamics of the protein matrix, rather than the dynamics of the surrounding medium plays an important role.

Acknowledgment. This work was supported by the Council of Scientific and Industrial Research, Government of India. S.H. thanks the Council of Scientific and Industrial Research for the award of a Junior Research Fellowship. A.C. is an Honorary Professor of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore (India). We sincerely thank Prof. G. Krishnamoorthy (Tata Institute of Fundamental Research, Mumbai, India) for the kind gift of purified EGFP. We gratefully acknowledge Dr. Devaki Kelkar for preliminary experiments and helpful discussion. We thank members of our laboratory for critically reading the manuscript.

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