

Red-Edge Excitation Study of Nonexponential Fluorescence Decay of Indole in Solution and in a Protein

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Another area in which the interpretation of the data of fluorescence in terms of molecular properties is lacking is that of the red-edge effects Investigation of this spectral region is often important in biological samples because it offers the best possibilities of detecting compositional heterogeneities.

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Proteins are known to be heterogeneous systems with a hierarchy of internal motions. However, those properties are often ignored when the complex fluorescence decay of tryptophan residues is compared to model studies with indole derivatives in solution. Here two simple models are presented, which illustrate different aspects of protein organization: (1) Trp zwitterion in buffer exemplifies ground-state heterogeneity and (2) indole in water/glycerol mixture exemplifies excited-state reconfiguration of solvate. Both systems are known to produce nonexponential fluorescence decay, attributed to the existence of multiple species (rotamers) or to the effects of slow dipolar relaxation, for (1) and (2), respectively. In the latter case a substantial dependence of decay on the excitation wavelength is expected. Indeed such dependence is observed for indole in water/glycerol mixture but not for Trp zwitterion in buffer. Therefore, excitational dependence can be used as a criterion to distinguish effects of multiple conformations in the ground state from effects of excited state reactions on tryptophan decays in proteins. The example of the bee venom peptide melittin indicates that both phenomena are important for interpretation of heterogeneity of decay, and therefore, caution should be exercised when assigning individual decay components to conformational subspecies in proteins.

KEY WORDS: Red-edge excitation; indole fluorescence; protein dynamics; inhomogeneous broadening; decay-associated spectra; melittin.

INTRODUCTION

Intrinsic protein fluorescence provides a powerful tool for a variety of biophysical and biochemical studies. However, despite its great success, there seems to be no clear answer for the basic question: what determines the heterogeneity of decay in proteins? An ever-growing liter-

ature provides a number of explanations involving broadly defined conformational heterogeneity of fluorophore itself and/or existence of multiple internal quenchers [2–6]. Based mainly on model fluorescence and NMR studies done with tryptophan and tyrosine derivatives, several groups have suggested that different rotameric forms are responsible for nonexponential decay [7–10]. In recent years there has been a breakthrough in understanding the mechanisms that govern the decay of rotamers of restricted indole derivatives [11,12]. However, those model studies were carried out in isotropic fast relaxing media. Proteins, in contrast, are heterogeneous

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systems with a hierarchy of internal motions that cover a wide range of correlation times, including the nanosecond time window of fluorescence. This paper describes an attempt to analyze spectroscopic features of fluorescence of the tryptophan residue attributed to intraconformational heterogeneity and restricted flexibility of the protein matrix.

In our analysis, we take advantage of considerations developed to explain inhomogeneous broadening of electronic spectra of dye molecules caused by the distribution in configurational energy in solvate [13]. Such systems are characterized by the following features: (1) decay is nonexponential, (2) decay depends on the excitation wavelength, λ_{exc} , and becomes faster at longer λ_{exc} (red-edge effect), and (3) in extreme cases a rising component in intensity is observed at the longer emission wavelength. Such behavior have been demonstrated for many organic dyes in slowly relaxing polar media [14–20]. However, the excitation dependence of tryptophan analogs was studied only by steady-state spectroscopy [21]. Here an excitation dependence for fluorescence decay in two model systems, Trp zwitterion in fast-relaxing media and indole in media with slow dipolar relaxation, is presented for the first time. As expected, the fluorescence of indole in water/glycerol mixtures follows all three features of the system governed by inhomogeneous broadening presented above. In contrast, Trp serving as a model for ground-state heterogeneity (rotamers [7]) exhibits decay which is mainly independent of the excitation wavelength. Therefore, it is suggested that excitational dependence can be used as a criterion to distinguish alternative mechanisms causing deviations from exponential decay in proteins.

Here, an excitational dependence of fluorescence decay is reported for a bee venom peptide melittin. Melittin contains a single fluorophore, Trp-19, and its conformational changes are well characterized [22]. At low ionic strength melittin exists as an unordered monomer with a highly exposed tryptophan. Increased ionic strength stabilizes a highly ordered α -helical tetrameric structure consisting of identical monomeric molecules. All tryptophans in the tetramer are in equivalent [23,24] and rigid [25,26] environments. In methanol solution melittin exists as an α -helical monomer, a conformation believed to be close to its membrane-bound form at low concentrations. Different forms of melittin were found to exhibit different wavelength dependencies of decay, indicating that both ground-state heterogeneity and excited-state reactions are impotent for interpretation of tryptophan decays in proteins.

Preliminary account of this work has been presented [27].

For consistency of presentation, henceforth the following definitions will be used in the paper:

- **monoexponential decay**, fluorescence kinetics that follows a single exponential;
- **nonmonoexponential decay**, fluorescence kinetics that deviates from a single exponential (can always be formally presented by a sum of exponential components);
- **multiexponential decay**, a type of nonmonoexponential decay in systems where individual exponential components can be related to molecular subspecies (e.g., rotamers);
- **nonexponential decay**, a type of nonmonoexponential decay in systems where more complex time dependence is related to excited-state reactions (e.g., configurational relaxation).

MATERIALS AND METHODS

Melittin (sequencing grade) was obtained from Sigma (St. Louis, MO); L-tryptophan and indole from Aldrich Chemical Co. (Milwaukee, WI); and methanol and glycerol, glass distilled, from EM Science (Gibbstown, NJ). Tryptophan and indole were dissolved in 10 mM sodium phosphate buffer, pH 6.5. Moderate heating was used to help dissolve indole in the stock solution, the appropriate amount of which was added to glycerol to achieve a ratio, by weight, of 9:1 glycerol:water. All melittin samples had a concentration of 16 μM . To obtain a monomeric form, melittin was dissolved in 20 mM Hepes, pH 7.4, while to induce a tetrameric form, concentrated KCl in the same buffer was added to a final concentration of 2 M.

To avoid inner filter effects, samples with an optical density less than 0.1 were used for fluorescence studies. Steady-state measurements were carried out on the SLM-48000 spectrofluorometer with the Xe arc lamp as the excitation source (SLM Instruments Inc., Urbana, IL).

Time-resolved fluorescence decays were collected with a time-correlated single-photon counting apparatus as described earlier [28]. The cavity-dumped output of a synchronously pumped tunable rhodamine-6G dye laser was used to generate a laser pulse, which was then frequency-doubled to a desired wavelength in the range of 280–300 nm. For all types of fluorescence measurements a magic angle configuration of polarizers was used. All measurements were carried out at 20°C unless otherwise indicated. Data were normally collected to a constant peak count of at least 10 K. For clarity of comparison the data in Figs. 1 and 2 were slightly adjusted by multiplying by a normalization factor not to exceed 1.02. No

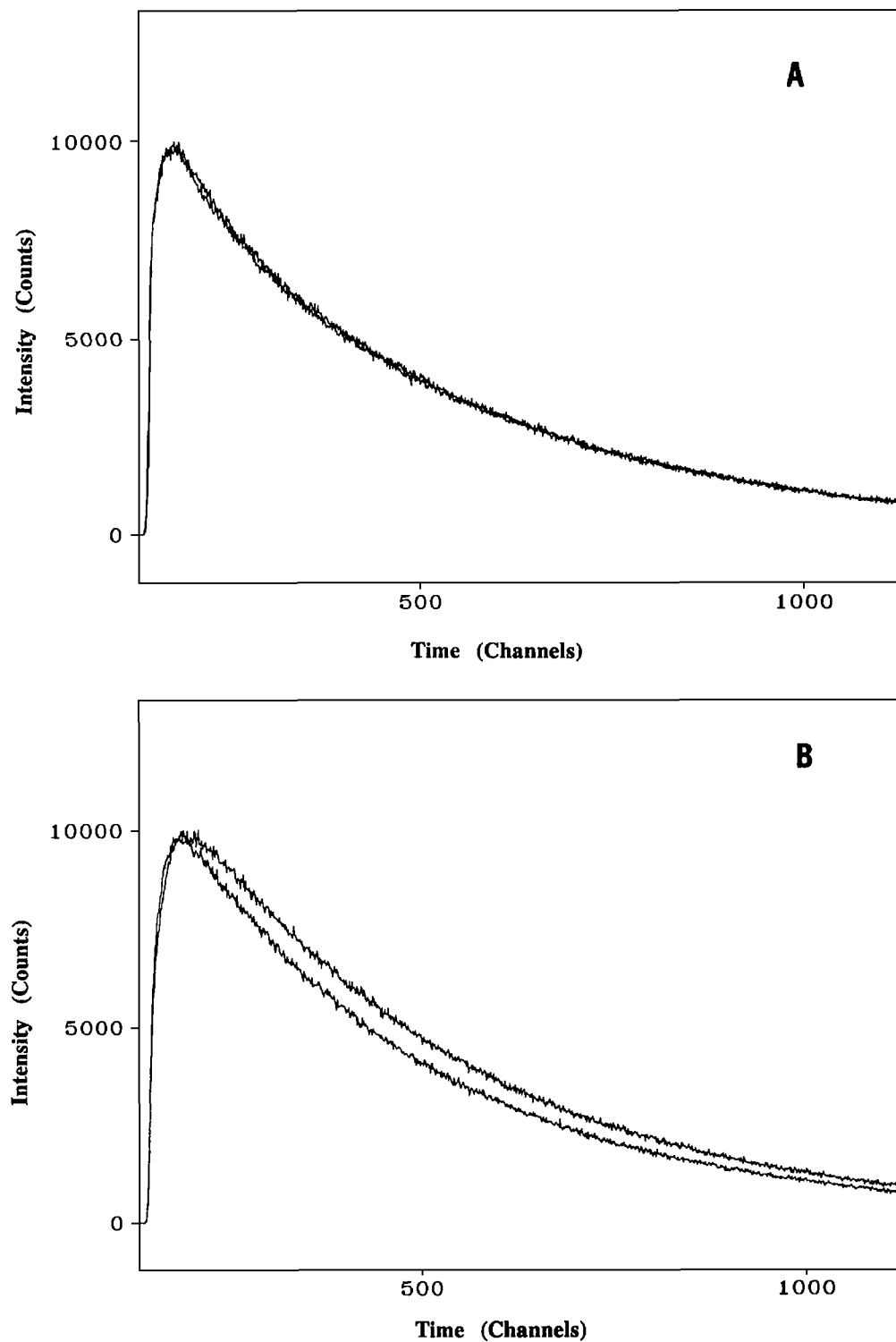


Fig. 1. Fluorescence decay kinetics of two model systems: tryptophan zwitterion in aqueous buffer (A) and indole in a water/glycerol mixture (B) measured at +10°C with 285-nm (upper curve) and 295-nm (lower curve) excitation. The timing calibration was 11 ps/channel. Regardless of the mathematical analysis, raw data clearly indicate that the fluorescence decay for system emulating excited-state reconfiguration of solvate (B) depends on the excitation, while the fluorescence decay for system emulating ground-state heterogeneity only (A) does not.

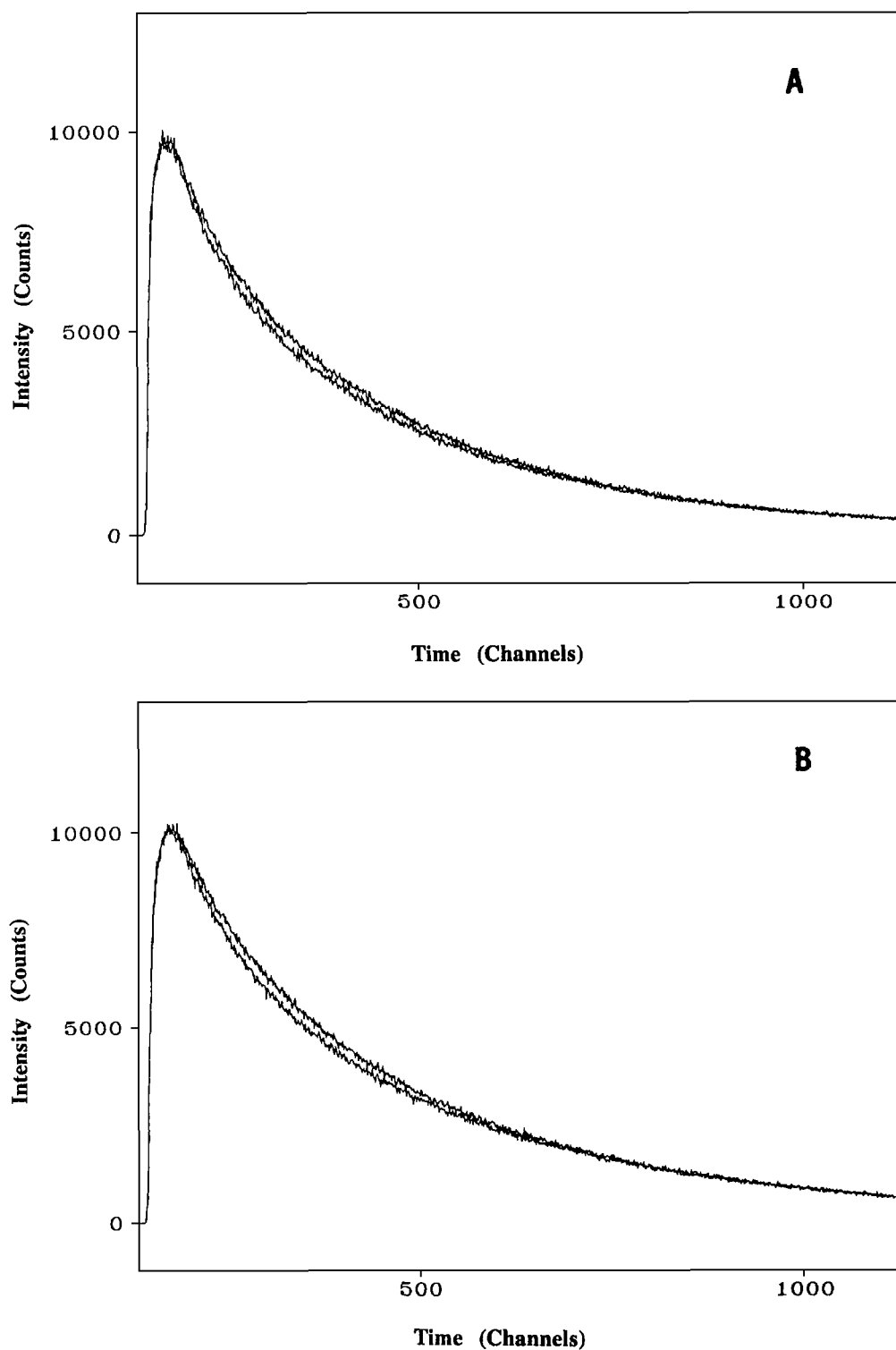


Fig. 2. Fluorescence decay kinetics of monomeric melittin in aqueous buffer (A) and in methanol (B) measured at +20°C with 280 nm (upper curves) and 300 nm (lower-curve) excitation. The timing calibration was 11 ps/channel. Raw data clearly indicate that tryptophan decay in proteins depends on the excitation wavelength.

significant background signal originating from glycerol or buffer was observed, except for an extremely short-lived component representing scattered light.

Experimental data were analyzed by the nonlinear least-squares method assuming that fluorescence decay can be represented as a sum of exponential components: $I(t) = \sum \alpha_i \exp(-t/\tau_i)$. The goodness of fit was assessed by the χ^2 criterion, the randomness of residuals, and their autocorrelation. The nominal errors were estimated assuming normal distribution and did not exceed 0.03 for amplitudes and 5% for the lifetimes. The estimate of the errors of average lifetimes is complicated by the known fact that individual amplitudes and lifetimes are highly correlated. In order to find out how this correlation affects the trends in average lifetime the following procedure was utilized: one of the parameters (usually one of the lifetimes) was fixed at a series of values around the most probable value recovered by unrestricted fit; the rest of the parameters were minimized and the fits and the average lifetimes were compared. In many cases the quality of the fit obtained by such analysis was very close to that of the unrestricted fit. This degeneracy of the fit could not be resolved unequivocally, even in those cases when data are collected to a peak value of 40 K counts. However, this deviation in fit parameters was found not to affect noticeably the value of the average lifetime. More than that, the same trends reported here were still observed even when one of the parameters was fixed at a significantly deviant value and the quality of fit was clearly worsened.

To recover emission or excitation decay associated spectra (EMDAS or EXDAS), a series of kinetics measured at different wavelengths, λ_{em} , and with different excitations, λ_{exc} , was analyzed globally by linking lifetimes and allowing α 's to float independently. The preexponential factors were normalized to yield intensity, calculated as $\sum \alpha_i \tau_i$, to be proportional to the steady-state intensity measured independently. For the presentation in the table the preexponential terms were normalized to a sum of 100%.

RESULTS

The fluorescence decays following excitation pulses of various λ_{exc} but of the same width, approximately 60 ps, are presented in Figs. 1 and 2 (excitation pulses are not shown). Figure 1 contains model data from tryptophan zwitterion in aqueous solution and indole in water/glycerol mixture. Curves collected for the Trp sample show little difference with the change of λ_{exc} . On the other hand, decay of indole in viscous media exhibits a strong

excitation dependence. Similar dependence was observed for tryptophan itself in viscous media (data not shown). The decays of melittin's only fluorophore, Trp-19, show a notable but less pronounced dependence on λ_{exc} as well (Fig. 2). Note that in all cases the lower curve (and thus a faster decay) corresponds to excitation with the longer wavelength.

In order to analyze the decay curves for melittin in different conformations, data were subjected to a nonlinear least-squares deconvolution routine (Table I). In all cases a triple-exponential fit was required to describe the fluorescence decay of proteins. With the change of λ_{exc} to 300 nm, the amplitude average lifetime ($\tau_\alpha = \sum \alpha_i \tau_i / \sum \alpha_i$) for monomeric melittin in both methanol and aqueous solution decreased by 10% of its value measured at 280 nm. The average lifetime of tetrameric melittin appears to be independent of λ_{exc} . There is no obvious correlation in the change of different parameters, and both α 's and τ 's vary with the change of excitation.

In contrast, for the tryptophan zwitterion, the 3% drop in τ_α is clearly associated with a higher contribution of a short component at a longer wavelength, while individual lifetimes are not altered. This observation gives a rationale for combining in one global search data collected with different λ_{exc} with data collected at different λ_{em} . Recovered by such a procedure, EMDAS for tryptophan zwitterion (not shown), correspond well to those reported by Szabo and Rayner [7]: the EMDAS for the short (0.45-ns) component is blue-shifted compared to the EMDAS for the long one (3.2 ns). The opposite is observed with EXDASs, as the relative contribution of the short component continuously increases, from 17% at 280 nm to 23% at 300 nm.

It should be stated that the correlation between different parameters presented in Table I, while influencing the precision of recovery of individual parameters, does not affect the overall trends in the average lifetime (see Materials and Methods for details). Despite certain degeneracy of the fit the dependence of the decay on excitation wavelength, is preserved. This is not surprising, since this dependence is apparent in the raw data before they were subjected to any mathematical analysis (Figs. 1 and 2).

Complex and heterogeneous decay exhibited by all three melittin forms can also be presented, at least formally, by EMDAS. In all cases three spectral components can be recovered with lifetimes close to those recovered by individual analysis (Table I). The case of melittin in methanol is of particular interest (Fig. 3A). The spectra of the two longest components are shifted for 5 nm with respect to each other but remain positive at all wavelength. The shortest component, however, undergoes a

Table I. Best-Fit Parameters of Fluorescence Decay of Trp-19 in Melittin and Trp Zwitterion; Emission Was Measured at 360 nm

Sample	α_1 τ_1 (ns)	α_2 τ_2 (ns)	α_3 τ_3 (ns)	$\lambda_{\text{excitation}}$ (nm)	χ^2	τ_{α} (ns)
Melittin monomer	24%, 0.44	36%, 2.1	40%, 4.3	280	1.09	2.57
	27%, 0.25	30%, 1.4	43%, 4.1	300	1.11	2.26
Melittin tetramer	17%, 0.54	56%, 1.8	27%, 4.1	280	1.04	2.19
	21%, 0.55	57%, 1.9	22%, 4.5	300	1.07	2.19
Melittin in methanol	12%, 0.55	36%, 1.9	52%, 4.7	280	0.98	3.20
	20%, 0.36	33%, 1.6	47%, 4.8	300	1.01	2.88
Tryptophan	16%, 0.44	84%, 3.2		280	1.02	2.74
	21%, 0.49	79%, 3.2		295	1.15	2.65

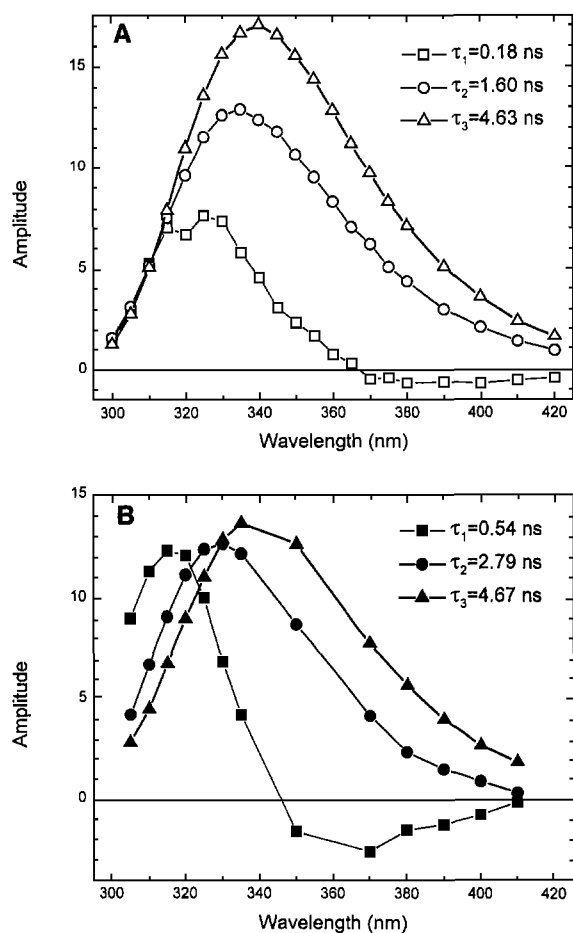


Fig. 3. Emission decay-associated spectra of melittin in methanol (A) and indole in a water/glycerol mixture (B) measured at 285-nm excitation. Global reduced $\chi^2 = 1.07$ (A) and 1.09 (B). In both cases the spectrum of the shortest component changes sign at longer wavelengths. This is indicative of the excited state reaction.

sign change, becoming negative at $\lambda_{\text{em}} > 365$ nm. Such inversion of sign is indicative of an excited-state reaction and was not observed for any components of Trp zwitterion until at least 420 nm (not shown). An EMDAS similar to that of melittin in methanol, but with a more pronounced negative component, is observed for indole in glycerol (Figure 3B).

DISCUSSION

Explanation of the deviations of fluorescence decay from exponential for single tryptophan-containing proteins is important for understanding protein structure and dynamics. Because such complex decays can always be presented as a sum of two or three exponential components, there has been a tendency to interpret the decay as a truly multiexponential one (see Introduction), i.e., to attribute individual components to some conformational sub-species. On the other hand, it is well established that existence of the conformational ensembles in native protein structure and the complex dynamics of the protein itself can lead to nonexponential kinetics. For example, such kinetics were observed in a completely different class of experiments, involving rebinding of substrates to myoglobin [29]. The same considerations of protein dynamics could be invoked to interpret fluorescence decay in proteins as a truly nonexponential process (see Introduction). Thus, multiexponential decay and nonexponential decay represent different views on the nature of the dynamic organization of protein structure. Unfortunately, there is no established mathematical procedure to distinguish between these two cases, and other possible grounds should be explored to arrive at an answer.

In this study two simple models are utilized, for which alternative mechanisms are expected to contribute to the deviation of decay from monoexponential behavior:

- a multiexponential decay for Trp zwitterion in fast-relaxing water environment results from the ground-state heterogeneity (existence of multiple rotamers);
- a nonexponential decay for indole in water/glycerol mixture results from solvent relaxation, demonstrated for other organic dyes [14–16], including *N*-acetyl-Trp-amide [17–20] in viscous media. In this study, the use of indole was important because it obviously has no rotameric forms.

Fluorescence decay curves for these two models were collected under conditions of different excitation (Fig. 1). Even without any mathematical analysis it is clear that while tryptophan exhibits no dependence on excitation, such dependence is important for indole in glycerol. Thus, the dependence of decay on excitation wavelength could be used as a criterion to distinguish between alternative mechanisms causing deviations from exponential decay in proteins. Indeed such dependencies are reported here for monomeric forms of peptide melittin (Fig. 2, Table I).

What is the nature of the excitation dependence of fluorescence? Almost three decades ago, it was shown that, apart from molecular vibrations, there is another cause of the substantial broadening of electronic spectra of dyes in solution, namely, the fluctuations of the structure of the solvation shell surrounding the molecule [30]. The variation of the local electric field caused by the fluctuation of the shell structure leads to a statistical distribution of the frequencies of the electronic transitions of the fluorophores, causing the inhomogeneous broadening of the electronic spectrum (IBES). This effect was experimentally demonstrated from the dependence of the fluorescence spectrum on the excitation wavelength in frozen solutions of several organic dyes, including indole [21,30–32]. Subsequently, it was shown that IBES of complex molecules occurs not only in solid but in liquid solutions as well [33,34]. In the latter case, it can be detected only by time-resolved spectroscopic techniques.

To illustrate the origins of the dependence of fluorescence decay on λ_{exc} for systems with IBES, consider a field diagram of the electronic-configuration states of a solvate in a polar solution (see Fig. 4, adapted from [13]). The free energy of the system (U), consisting of the contributions of energies of orientational interactions of molecules in the first coordination sphere, is a parabolic function of the electric field intensity inside the solvate

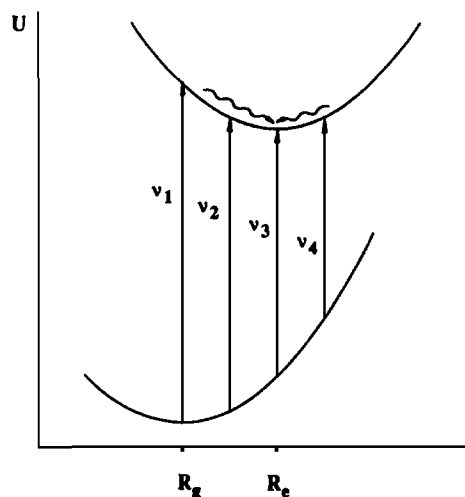


Fig. 4. A field diagram of the electronic-configuration states of a solvate in a polar solution (adapted from [13]) illustrating the origins of the dependence of fluorescence decay on the excitation wavelength. See text for details.

(R) for both the ground and the excited state. For simplicity, the quasi-continuum of vibrational states is neglected. Note that $U(R)$ reaches its minima at different points for the ground (R_g) and excited (R_e) states. If the dipole moment of the fluorophore increases with excitation, R_e will be larger than R_g , because a larger reactive field is needed to compensate for the larger dipole. Since the system obeys the Franck–Condon principle, one can achieve a photoselection of the states with different proximity to the equilibrium in the excited state by radiating the system with light of different frequencies ($\nu_1, \nu_2, \nu_3, \nu_4$). Subsequently, it will take longer for the system to reach an equilibrium (or any other definite state close to equilibrium) after it has been excited with ν_1 compared to ν_2 . If one continues to shift the excitation to the red, one can directly excite solvates already in the equilibrium in the excited state (ν_3), and eventually observe the “up-relaxation” [33,34], exciting at ν_4 . The latter effect is due to the gain in energy of the fluorophore during the configurational relaxation and could be observed in tryptophan in glycerol only at $\lambda_{\text{exc}} > 315$ nm [21].

In accordance with the considerations presented above, we found that the fluorescence decay of indole in a water/glycerol mixture is much faster when $\lambda_{\text{exc}} = 295$ nm than 285 nm (Fig. 1B). In both cases the decay was heterogeneous, while in fast-relaxing solvents (water, methanol) the fluorescence decay is not only monoexponential, but also independent of λ_{exc} or λ_{em} (unpublished data). This heterogeneity of decay by itself is not unexpected, as many organic dyes exhibit similar behavior [14–20]. Interestingly enough, the deviation from the

monoexponentiality decreases with an increase in λ_{exc} . For example, a single-exponential analysis of the data from Fig. 1 shows the reduction of χ^2 from 4.1 for $\lambda_{\text{exc}} = 285$ nm to 2.0 for $\lambda_{\text{exc}} = 295$ nm. Ideally the decay will become monoexponential again if the excitation of ν_3 is reached (Fig. 4), which was estimated to correspond to $\lambda_{\text{exc}} = 305$ –310 nm for indole [21].

Not surprisingly, the decay of fluorescence of Trp-19 in melittin is heterogeneous (Table I). In addition, the decay of both monomeric forms was found to be dependent on λ_{exc} , while the tetrameric form apparently exhibits no such dependence. The plausible explanation for the latter might be that the tetrameric form is too rigid to allow relaxation to influence the decay. Evidence for such rigidity comes from analysis of steady-state red-edge excitation [25,26]. Thus, the overall data on different forms of melittin suggest that both ground-state heterogeneity and excited-state reactions influence tryptophan fluorescence. Because of the extreme complexity of the fluorescence decay (three apparent lifetimes) and correlation between decay parameters leading to degeneracy of the fit, it is not clear at this point whether incorporation of all data collected at all emission and excitation wavelengths will be beneficial for discrimination among different origins of deviation from a monoexponential decay in proteins. This issue will be addressed in future studies with the help of more complex and more realistic model systems than those presented here.

Nevertheless, it is clear that the failure to recognize the possible nonexponential nature of fluorescence decay in proteins could lead to potentially misleading conclusions when results of standard analysis using sum of exponentials are interpreted in terms of ground state heterogeneity only. A common way of presenting fluorescence decay is by creating decay-associated spectra (as described under Materials and Methods). This procedure can formally be applied to systems with nonexponential (rather than multiexponential) decay, such as indole in glycerol/water mixtures (Fig. 3B). Two features of recovered EMDAS appear to be important. First, the shortest component changes sign at longer wavelengths, which is indicative of the excited-state reaction [35]. Second, three components are required to describe the decay. This means that when the resolution is not sufficient to see the shortest component, the decay will still deviate from exponential, but none of the components will have a negative amplitude. Therefore, existence of negative amplitude in EMDAS of protein (e.g., melittin in methanol; Fig. 3A) is sufficient but not necessary to indicate excited state reactions.

In conclusion, it should be emphasized that while excited-state reactions alone will not explain all devia-

tions from exponential fluorescence decay in proteins, ignoring them can lead to misinterpretation of data. Two model systems described here represent the simplest cases of ground state heterogeneity and excited state reaction. Further research should be directed at deepening our understanding of the relative interplay of these phenomena.

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