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Protein fluorescence decay: A gamma function description of thermally induced interconversion of amino acid rotamers

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We present a description of fluorescence decay kinetics in complex environments based on gamma functions rather than the conventional approach using exponentials. The gamma function description is tested in measurements on the temperature dependence of the protein human serum albumin (HSA), N-acetyl tryptophanamide (NATA), and 2, 5-dipenyl oxazole (PPO). The monitoring of macromolecular structure and dynamics is demonstrated by means of distinct tryptophan (Trp) rotamer populations and their interconversion in HSA.

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It could be argued that the fluorescence of the amino acid tryptophan in the study of protein structure and dynamics is a story of promise unfulfilled. Since the pioneering work of DeLauder and Wahl [1], Conti and Förster [2], and Grinvald and Steinberg [3] Trp fluorescence has attracted special attention. The fluorescence decay of Trp was known at the outset [4] to be complex. This led to much debate as to the origin of this complex decay with explanations proposed in terms of two solvent-induced noninterconvertible states $^1L_a^e$ and $^1L_b^e$ [4], rotamers [5,6], and electron transfer [7]. A specific ambiguity was identified by the work of Ware [8] who brought to light the fact that a distribution of exponentials could in certain cases describe fluorescence decay just as well as discrete exponential components. This led to much debate [9,10] concerning diphenyl-propane with the distribution treatment also being applied to proteins and Trp. The use of distributions culminated in the widespread acceptance of the applicability of the maximum entropy method to describe fluorescence decay [11]. Presently the existence of three exponentials attributed to rotamers of the side-chain in Trp seems to hold sway, albeit when modified by solvent interactions, excited state charge transfer, resonance energy transfer, etc. [12]. However, in all these approaches the assumption of an underlying exponential character to the fluorescence decay of Trp has gone unquestioned. In this Rapid Communication we challenge these four decades of accepted dogma and propose an alternative description based on gamma functions as well as presenting opportunities for gaining structural insight to protein structure and dynamics. Such work is timely as, in addition to interest in the fundamental properties of Trp, the fluorescence of proteins has gained further importance in recent years with the realization of, and need for, noninvasive detection of molecular processes in living organisms [13].

The fluorescent amino acids, Trp, tyrosine (Tyr), and phenylalanine (Phe), occur in most proteins. Their fluorescence responses, i.e., emission spectra, quantum yields and fluorescence decays, are usually altered by the activity proteins are involved in, thus providing valuable information on events occurring on the nanometer scale. However, fluorescence decay measured in biological systems may differ substantially from the simple decay usually observed in bulk solutions. The thermal motions of fluorescent amino acids, and associated with them fluctuations in energy of interactions with their close surroundings in biological macromolecules, contribute to the complex excited-state kinetics. Consequently, the resulting fluorescence decay is not necessarily a simple exponential and usually requires fitting to multiexponential functions to parametrize the decay. The very nature of this process leaves "the door open" to the adopted decay function not reflecting the actual kinetics and the underlying photophysics can be difficult to resolve as they spread over numerous parameters of often poorly specified physical meaning.

Among fluorescent amino acids, Trp undoubtedly still hides a rich seam of information as an intrinsic probe. Not only is it ubiquitous in proteins and has a dual role as a participant and a potential probe in numerous bioactivities, but also because it can be optically excited and its decay kinetics monitored, without interfering with Tyr or Phe, as they absorb photons of higher energy.

Extensively studied Trp-containing systems include free Trp, its derivatives (e.g., NATA), and numerous proteins [14–18]. Single-Trp proteins (e.g., HSA) are especially important as they allow investigation of Trp fluorescence responses in a relatively well defined environment, but only Trp in azurin [14] demonstrates a single-exponential decay, while in other proteins a good fit can require at least a two-exponential description.

In this Rapid Communication, we consider generic reasons of nonexponential behavior and develop a more general model of the kinetics. We present a Langevin equation-based modeling of kinetics which leads to a gamma distribution of lifetimes and, consequently, a powerlike decay of fluorescence. The conditions leading to this type of kinetics and properties of the recovered lifetime distributions are discussed.

NATA and HSA were chosen as the representatives of a simple (NATA) and a complex (HSA) structure containing effectively a single Trp. We also used a solution of PPO in cyclohexane as a reference fluorophore, since it is commonly accepted to be a lifetime standard [19] as it exhibits a one-exponential decay. A common feature of these three fluorophores is their ability to be excited in lifetime experiments at the wavelength of 295 nm and fluorescence detected at 340 nm. Therefore, all experimental settings and data analysis procedures remain the same. Consequently, any differences observed in decay functions can be attributed only to different kinetics of each fluorophore, not to the different experimental conditions.

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Fluorescence decays obtained for all three species were first analyzed using traditional one-, two-, and three-exponential decay models and then by means of lifetime distribution analysis based on the maximum entropy method (MEM) [20]. In the MEM no model of kinetics is assumed and the recovered data are only a mathematical representation of the decay function.

The simple kinetics of the dye, resulting in a single-exponential fluorescence decay, can be disturbed if its interactions with the environment change due to, e.g., increased thermal motion or as a result of binding of the molecule to other structures such as protein. In both cases the fluorescence decay is likely to lose its exponential character.

Here we introduce a generalization of the simple excited-state kinetics model, which includes thermally induced fluctuations of the ground and excited state levels and the orientational relaxation of the dye molecule with respect to the surrounding solvent molecules and/or a macromolecule it is attached to. Including these factors results in the fluorescence lifetime τ being a time-dependent function, with a behavior which can be expressed by the Langevin equation [21,22],

$$\frac{d\tau}{dt} = -\left[\frac{1}{\tau_R} + \xi(t)\right]\tau + \frac{\tau_F}{\tau_R}. \quad (1)$$

Here τ_R is a relaxation time—a time constant characterizing the rate of relaxation of the Trp with respect to the solvent or a macromolecule ligand. τ_F is the lifetime of the dye when it is fully relaxed with its environment. $\xi(t)$ indicates the white Gaussian noise with an ensemble mean $\langle\xi(t)\rangle=0$ and the correlation function $\langle\xi(t)\xi(t+\Delta t)\rangle=2\sigma\delta(\Delta t)$, which is proportional to the δ function [22], with the constant σ defining the variance of τ_R. The Langevin equation (1) can be converted into the relevant Fokker-Planck equation [22] and its stationary solution ($\tau_R \ll \tau_F$) found as a lifetime distribution function

$$g(\tau;\alpha,\tau_F) = \frac{(\alpha\tau_F)^\alpha}{\Gamma(\alpha)}\left(\frac{1}{\tau}\right)^{\alpha-1}\exp\left[-\alpha\frac{\tau_F}{\tau}\right], \quad (2)$$

where $\alpha=(\tau_R\sigma)^{-1}=(q-1)^{-1}$. Equation (2) is the gamma distribution function. Its two parameters, namely, factor α (or q) and the lifetime τ_F, characterize the observed fluorescence kinetics. The wide distribution of lifetimes for $q>1$ [21] becomes narrower for decreasing q and finally becomes a δ function (implying a monoexponential decay) for $q\to 1$. Also, the decrease in τ_F results in a peak position of the profile being shifted toward shorter lifetimes and the half width of the distribution becoming smaller.

NATA, HSA, PPO, and cyclohexane were purchased from Sigma-Aldrich (Poole, U.K.) and used without further purification. The solutions of 10^{-6} M PPO in cyclohexane and 30×10^{-6} M NATA and HSA in 0.01 M phosphate buffer, pH 7.4, were prepared on the day of measurements. The measurements were performed using a temperature-controlled sample holder connected to a Neslab RTE-11 thermostat (Thermo Scientific U.K.). The Perkin-Elmer Lambda 2 UV/Vis and Perkin-Elmer LS-50 B luminescence spectrometers were used for absorption and fluorescence spectra measurements, respectively.

Fluorescence decays were recorded using the time-correlated single-photon counting (TCSPC) technique on an IBH Fluorocube fluorescence lifetime system (Horiba Jobin Yvon IBH Ltd., Glasgow, U.K.) equipped with both excitation and emission monochromators. An AlGaN version of a pulsed light emitting diode, which we originally developed for use in the visible [23] and also at 280 and 265 nm to excite tyrosine [24] and phenylalanine [25], was used in this work at 295 nm in order to excite tryptophan directly [26]. The pulse duration was ~600 ps (full width at half maximum) pulses and repetition rate 1 MHz. The DAS6 (IBH) data analysis package was used for one- to three-exponential reconvolution analysis. Fluorescence lifetime distribution functions were recovered from the decay data using a Pulse 5 numerical procedure (Maximum Entropy Data Consultants Ltd). The procedure used a set of 250 lifetimes.

The lifetime distribution function $g(\tau)$ was obtained from the MEM approach and compared with Eq. (2). We decided to verify the kinetics models for our Trp data by comparing lifetime distributions, rather than fluorescence decays, for two main reasons: (i) the small changes in fluorescence kinetics are much more visible in $g(\tau)$ function than in $I(t)$, and (ii) the MEM approach reveals $g(\tau)$ without the necessity of making any assumption of the decay model.

Steady-state data show similarities in the absorption and fluorescence spectra between NATA and HSA. PPO absorption and fluorescence bands overlap well with those of Trp and this helps make PPO a useful reference.

PPO in cyclohexane shows a minimal decrease in lifetime, with temperature increasing from 7 to 40 °C. The slight temperature effect is a result of very weak solute-solvent interactions in this nonpolar solvent and perhaps a small contribution of oxygen quenching. Fitting to monoexponential model gave the lifetime of 1.30 ns for low temperatures and about 1.26 ns for temperatures approaching 40 °C. The lifetime distributions obtained from MEM show sharp peaks at lifetimes which agree with the lifetimes revealed in a one-exponential analysis. This result confirmed a single-exponential character of PPO decay for the range of temperatures between 7 and 40 °C, and, more importantly, demonstrated the capacity of our experimental procedure, consisting of lifetime measurement and MEM-based data analysis, to distinguish between pure exponential decays and decays of more complex nature.

In NATA, an increase in temperature not only reduces the mean lifetime but also modifies the nature of the decay. Fitting the decays to one- and two-exponential functions shows decreasing lifetimes, but the χ^2 values are unacceptably high, $\chi^2>1.4$. The results obtained from fitting the decays to a three-exponential function are acceptable by means of statistical fitting criteria, but they neither support two-exponential kinetics (e.g., by obtaining vanishing third component) nor demonstrate a clear trend in the parameters.

It seems that this confusing result can be explained by revealing the lifetime distributions using MEM approach [Fig. 1(a)]. Indeed, the NATA decay cannot be expressed by two or three exponentials because they are characterized by broad lifetime distributions. We observe a dominating peak with the maximum starting at about 4.5 ns for 1.5 °C, which shifts toward shorter lifetimes for higher temperatures. This

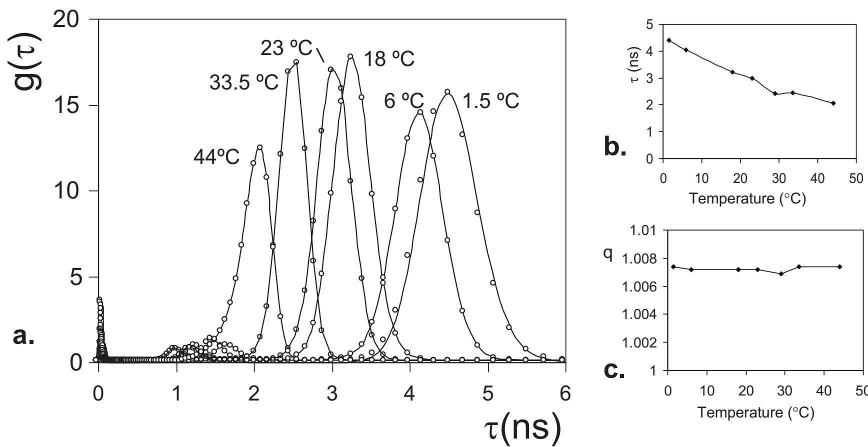

FIG. 1. NATA in a phosphate buffer for the temperatures increasing from 1.5 °C to 44 °C. Lifetime distributions fitted to the gamma function (a) and the evolution of the recovered parameters τ_F (b) and q (c).

observation is consistent with the previous studies on the temperature and detection wavelength effects on NATA decays [27]. Each distribution we observed also shows a much smaller peak which shifts with temperature at about 1.5–1.0 ns, perhaps indicating a much less populated NATA rotamer involved in an electron transfer from an indole ring to carbonyl group. A third small peak observed for very short lifetimes does not shift with the temperature. Therefore, we believe the latter is not fluorescence but the slight effect of scattered excitation light detected along with the fluorescence. Figure 1(a) presents another unusual feature of NATA lifetime distributions. The half width of the main distribution profile decreases with the increase in temperature. This is counterintuitive as we might expect from Maxwell-Boltzmann statistics the increase in temperature to make a lifetime distribution function wider. However, the same effect is observed in the gamma distribution if the parameter τ_F gets shorter and q remains unchanged [21]. Indeed, the parameters of gamma functions fitted to NATA distributions are presented in Figs. 1(b) and 1(c) and confirm our suggestion. τ_F values decrease gradually from 4.5 ns for 1.5 °C to 2 ns for 44 °C, while parameter q remains constant at the level of ~1.0075. A constant and higher than 1 value of q seems to be an important feature of NATA decay.

The decays of Trp in HSA are different from those in NATA due to a substantial change in the Trp surroundings. The dominating factor driving the decay kinetics in this case is the influence of the protein backbone and amino acids located near Trp in the HSA structure. The traditional least-squares one-, two-, and three-exponential analysis confirms that the decay of Trp in HSA needs to be described by at least a three-exponential function in order to satisfy statistical criteria of good fit. This has been thought to be consistent with a rotamer model [15], namely, Trp existing in proteins in three different rotational conformations, each characterized by its own lifetime according to the C_α-C_β bond orientation. However, as our previous HSA studies [17] demonstrated, MEM analysis reveals a broad lifetime distribution of Trp. In this research we have found that lifetime distributions of free HSA molecules were markedly modified by changes in temperature. For the lower temperatures, up to ~20 °C, the experimental distributions show two broad maxima [Fig. 2(a)], while for the temperatures above 20 °C, there are three maxima.

If we assume that each rotamer is not represented by one lifetime but by a gamma distribution given by Eq. (2) with its characteristic time constant τ_{Fi} and parameter q_i some interesting features emerge. We have found that each distribution in Fig. 2(a) (circles) can be well approximated with a sum of three gamma functions (solid lines),

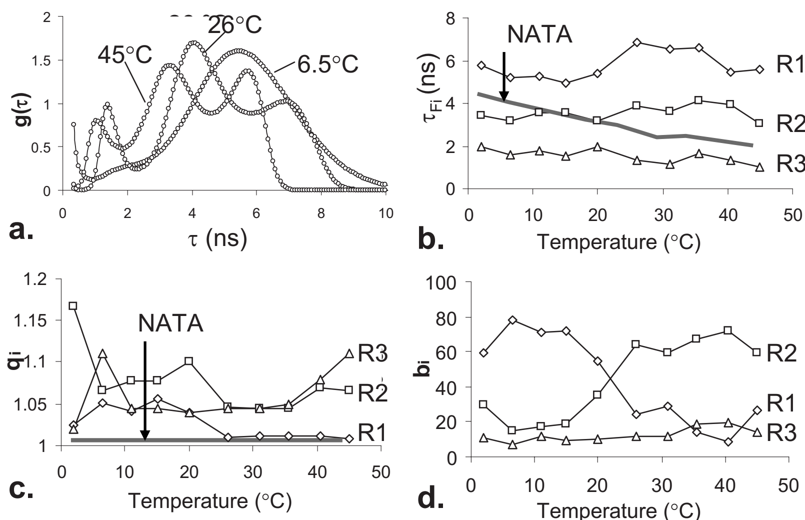

FIG. 2. HSA in a phosphate buffer for the temperatures increasing from 2 °C to 45 °C. (a) Lifetime distributions fitted to the sum of three gamma functions (only three results shown for simplicity) and the evolution of τ_{Fi} (b), q_i (c), and percentage contributions b_i (d) of three rotamers.

$$g(\tau) = \sum_{i=1}^{3} \beta_i g_i(\tau; q_i, \tau_{Fi}), \quad (3)$$

consistent with three rotamers R_1 (long lifetime), R_2 (medium lifetime), and R_3 (short lifetime). The percentage contribution of each rotamer b_i is defined as

$$b_i = \beta_i \int_0^\infty g_i(\tau; q_i, \tau_{Fi}) d\tau / \sum_{j=1}^{3} \beta_j \int_0^\infty g_j(\tau; q_j, \tau_{Fj}) d\tau. \quad (4)$$

All three rotamers are present at each temperature and the apparent additional peak at higher temperatures is a result of increasing contribution of the rotamer R_2 and decreasing contribution of the rotamer R_3 to the total fluorescence.

Changes in the τ_{Fi}, q_i, and b_i parameters with temperature are shown in Figs. 2(b)–2(d). Contrary to the NATA results, it seems that raising the temperature does not cause changes in rotamer lifetimes, which is consistent with Trp being protected by the rigid HSA structure from collisional quenching effects. Simultaneously, the q_i parameters are much larger than those found for NATA, and the parameters q_1 (rotamer R_1), and q_2 (rotamer R_2), seem to be decreasing when temperature increases. It seems likely that the drop in q with temperature is the result of a decreasing relaxation time $\tau_R = (q-1)/\sigma$. If this explanation is correct, the low and constant value of parameter q obtained for NATA is a consequence of a short relaxation time, as expected for a small molecule rotating free in an aqueous environment. In case of HSA rotamers, however, we have larger values of q, different for each rotamer, indicating different flexibilities of the rotamer-backbone binding.

The most pronounced effect of a temperature increase on Trp fluorescence can be seen from Fig. 2(d), which shows changes in the percentage contributions of three rotamers to the total fluorescence. At low temperature ($\sim 0\ ^\circ$C) the relative contributions of rotamers R_1, R_2, and R_3 are, respectively, 7:2:1. At higher temperatures the contribution of R_3 remains on the level of 10%, while the contributions of R_1 and R_2 gradually exchange their values. This result indicates temperature-induced change in the preferred orientation of Trp in HSA with the "transition temperature" at about 22 °C. Fluctuations in the parameters τ_{Fi}, q_i, and b_i, as seen in Fig. 2, result from the high sensitivity of the MEM approach to the slightest systematic errors in the raw data and in this regard we naturally go to some lengths to optimize measurement procedures to minimize them.

We have demonstrated that the use of gamma functions as model fluorescence lifetime distributions of Trp rotamers in protein gives better defined characteristics of Trp behavior in terms of conformational interconversion than fluorescence lifetimes recovered from exponential decays and reveal more nanometer-level structural information on the molecules involved. Indeed, our results show that two parameters characterizing each rotamer, q_i and τ_{Fi}, can reflect the rotamer's flexibility (orientational freedom) and quenching (access of analytes), respectively. The research presented raises many questions regarding the lifetime characteristics of fluorescent probes which are known to show other than a monoexponential character, and the relation between the relaxation time τ_R and the parameter q with respect to the local structural features of bio- and nanomolecular systems.

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