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A new approach to interpretation of heterogeneity of fluorescence decay: Effect of induced tautomeric shift and enzyme→ligand fluorescence resonance energy transfer

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Abstract

Fluorescence decays in protein–ligand complexes are described by a new efficient model of continuous distribution of fluorescence lifetimes, and compared with multi-exponential models. Resulted analytical power-like decay function provides good fits to highly complex fluorescence kinetics. Moreover, this is a manifestation of so-called Tsallis q-exponential function, which is suitable for description of the systems with long-range interactions, memory effect, as well as with fluctuations of the characteristic lifetime of fluorescence. The proposed decay function was used to study effect of the interaction of *E. coli* purine nucleoside phosphorylase (PNP-I, the product of the *deoD* gene) with its specific inhibitor, viz. formycin A (FA), on fluorescence decays of ligand and enzyme tyrosine residues, in the presence of orthophosphate (P_i, a natural co-substrate). The power-like function provides new information about enzyme–ligand complex formation based on the excited state mean lifetime, heterogeneity parameter (q) and a number (N) of decay channels obtained from the variance of gamma distribution of fluorescence decay rates. With FA, which exists as a 85:15 mixture of the N(1)–H and N(2)–H tautomeric forms in aqueous solution, fluorescence intensity decay ($\lambda_{exc}/\lambda_{em}$ 270/335 nm) is described by $q\sim 1$ and $N\sim 200$. Consequently power-like decay function converges to the single-exponential form, and lifetime distribution to the Dirac delta function. In contrast, selective excitation of the N(2)–H tautomer at higher wavelength led to a highly heterogenic fluorescence decay characterized by $q>1$ and 10-fold lower number of decay channels. Heterogeneity of fluorescence decays of both PNP-I and FA is enhanced by PNP–FA–P_i complex formation, reflecting a shift of the tautomeric equilibrium of FA in favor of the N(2)–H species, and fluorescence resonance energy transfer (FRET) from protein tyrosine residue (Tyr160) to the bound N(2)–H tautomer. Moreover, proposed model is simple, and objectively describes heterogeneous nature of studied systems.

Keywords: Fluorescence decay; Lifetime distribution; Enzyme–ligand interactions

1. Introduction

There are many natural physical processes which follow a power-like distribution. The power law behavior is commonly observed in a variety of systems ranging from cosmology, fluids, to econophysics and social science, among others [1,2]. In particular, complex systems characterized by long-range correlations, long-range microscopic memories, or fluctuations of some parameters of the system, display non-exponential behavior. They can be described in terms of non-extensive statistics introduced in 1988 by Tsallis [3]. In this approach a power-like formula (one parameter q-exponential function),

$$e_q^t \equiv \left[1-(1-q)\frac{t}{\tau}\right]^{\frac{1}{1-q}} \overset{q\to 1}{\longrightarrow} e_{q=1}^t \equiv \exp\left(-\frac{t}{\tau}\right) \quad (1)$$

is a generalization of the Boltzman–Gibbs function, replaces the usual exponential function, and reproduces it for $q\to 1$.

Abbreviations: FA, Formycin A; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; L-Tyr, L-tyrosine; NATyrA, *N*-acetyl-L-tyrosinamide; N, number of decay channels; P_i, orthophosphate; PNP-I and PNP-II, purine nucleoside phosphorylases from *E. coli* — the products of the *deoD* and *xapA* genes, respectively; q, parameter of heterogeneity; $\langle t\rangle$, mean decay time; τ_0, mean value of lifetime distribution.

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The non-exponential features of luminescence decays were first quantitatively studied by Edmond Becquerel [4], who noticed that some decays are better described by power law (Eq. (2)) then by a sum of two exponential terms,

$$I(t) = (1 + at)^{-p} \quad (2)$$

where values of p are between 1 and 2. The empirical formula proposed by Becquerel (Eq. (2)) can be reproduced from Eq. (1) through the identifications $p \equiv 1/(q-1)$ and $a \equiv (q-1)/\tau$.

A new model of fluorescence decays in proteins and protein–ligand complexes presented here, as well as power-like decay function derived from it, are in some sense a continuation of Bequerel's empirical approach, and a first application of the power-like law of decay to analysis and interpretation of fluorescence decays in proteins and protein–ligand complexes. This may be interesting and efficient alternatives to classical multi-exponential analysis [5,6].

Proteins and their complexes with ligands (substrates, inhibitors) can be treated as good examples of complex systems, which exist in a large variety of energetic states corresponding to different conformations [7]. Therefore proteins are ideal samples to study relaxation in complex systems such as alloys of the disordered energetic states.

Decays of fluorescence intensity of proteins often exhibit a non-exponential behavior, the origin of which is usually interpreted in terms of multiple (ground state) conformations (e.g. equilibrium between rotamers of the aromatic aminoacids) or excited-state processes (e.g. dipolar relaxations) [8]. The rotamer model is based on the interpretation of each discrete exponential component with the aid of a particular protein conformation, including dynamic equilibrium between rotational isomers of tryptophan [9–12] and tyrosine [13,14] residues. However, there are many difficulties with application of this model, because X-ray or NMR data sometimes do not indicate multiple occupancy of the rotameric states [15]. There are many proteins for which it is difficult to build a molecular model with the alternative orientations of fluorophores. Also the case of multitude conformational substates in proteins with possible time-dependent interconversions between them [16], which are not slow in comparison with fluorescence lifetimes, can not be properly described by this model. Even for the indole moiety in solution, it is difficult to find unique conformations corresponding to discrete fluorescence decay components [17,18]. Additionally, the mobile polar molecules in close proximity to the fluorophore reorientationally relax, and their relaxation occurs from many partially relaxed states. The relaxation processes affect the kinetic of the fluorescence decay and lead to a decay in a non-exponential form. All the foregoing suggest that continuous lifetime distributions are more relevant models than a sum of discrete exponential terms. There are many other cases, where distribution of decay times is expected instead of the limited number of discrete components [19], e.g. a fluorophore in a mixture of solvents, a case of co-existence of the range of environments in solution, or in proteins with many fluorophore residues.

As an example of protein we chose bacterial (*E. coli*) purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1), which uses orthophosphate (P_i, a co-substrate) for reversible cleavage of the *N*-glycosidic bond of purine β-(deoxy)ribonucleosides to yield α-(deoxy)ribose-1 phosphate and the free purine base. PNP-I from *E. coli* (the product of the *deoD* gene) contains six active sites per native homohexamer. It has been reported to accept adenosine with comparable efficiency to guanosine and inosine, the usual physiological substrates for trimeric PNPs from mammals and *E. coli* PNP-II (the product of the *xapA* gene). Formycin A (FA), close structural analogue of adenosine (but with a non cleavable C–C glycosidic bond, see Fig. 1) is a good competitive inhibitor of the *E. coli* PNP-I, with K_i=5 μM vs. inosine, and totally inactive vs. the enzymes from calf spleen and human erythrocytes (see Ref. [20], and literature cited therein).

Since each of the subunits of the *E. coli* PNP-I contains six tyrosine residues, and no tryptophan, absorption (λ_{max} 277 nm) and fluorescence emission (λ_{max} 304 nm) spectra of PNP-I are typical for proteins containing tyrosine. On the other hand, it has long been known that FA is fluorescent, and its absorption (λ_{max} 294 nm) and fluorescence emission (λ_{max} 340 nm) spectra are red-shifted relative to the tyrosine spectra of PNP-I [20,21]. This permits selective excitation of ligands in the enzyme–ligand complexes at the wavelengths above 295 nm, where tyrosine residues do not absorb. Selective observation of enzyme fluorescence is possible at 290–310 nm, and fluorescence of ligand at the wavelengths above 360 nm, where tyrosine emission is usually negligible [21].

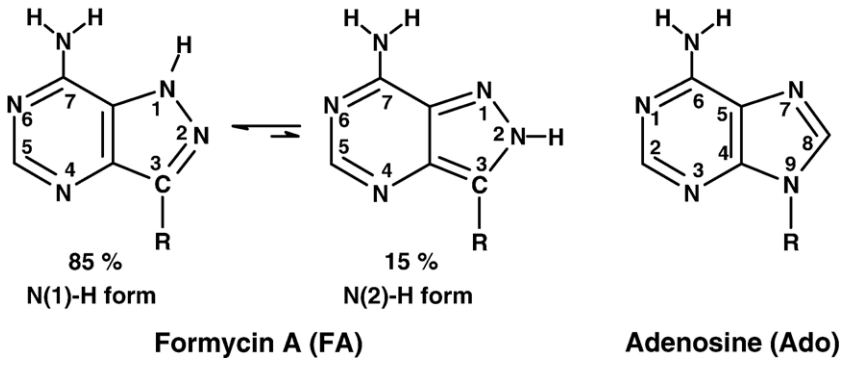

Fig. 1. The structurally similar natural forms of formycin A and adenosine (R = riboside). In aqueous media. FA consists of an equilibrium mixture of the N(1)–H and N(2)–H tautomeric forms, at proportions indicated. Note the different numbering of the two rings according to IUPAC.

It is known from long ago (see literature cited in Ref. [20]) that FA in aqueous medium exists in 85:15 equilibrium mixture of the N(1)–H and N(2)–H prototropic tautomers (Fig. 1) with no effect of excitation on the tautomeric equilibrium. The two tautomeric forms differ in absorption and emission properties, so that both absorption spectra, and fluorescence and phosphorescence emission spectra of the N(2)–H tautomer are red-shifted relative that of the N(1)–H form [20–22]. It was unequivocally shown with the aid of fluorescence [21] and phosphorescence spectroscopy [22] that addition of *E. coli* PNP-I to the aqueous solution of FA lead to selective binding of the N(2)–H tautomeric form, and to a shift of the tautomeric equilibrium in favor of the N(2)–H species. Affinity of the ligand for the enzyme was enhanced in the presence of P_i [21,22]. The maxima of fluorescence excitation and emission spectra of FA, as well as its fluorescence quantum yield, depend on the interaction with *E. coli* PNP-I [20,21]. Now we have extended our study on the time-resolved fluorescence of PNP–FA complexes. We report here a new model of continuous distribution of fluorescence decay rates given by gamma distribution, and results of its application to analysis of the effect of interactions of FA with *E. coli* PNP-I, in the presence of P_i, on the kinetics of fluorescence decay of FA and tyrosine residues of the enzyme.

2. Materials and methods

2.1. Materials

Purine nucleoside phosphorylase (PNP-I) from *E. coli* (the product of the *deoD* gene) was purified to apparent homogeneity (final specific activity 105 U mg^{-1}) as described earlier [23]. Formycin A (FA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), mono- and dibasic sodium phosphates were products of Sigma Chemical Co. (USA). Concentrations of PNP-I and FA were determined spectrophotometrically on a Varian Cary-50 instrument (Australia) at pH 7, using for PNP-I, λ_{max} 277 nm (ε 42.2×10^3 M^{-1} cm^{-1}), and for FA, λ_{max} 294 nm (ε 10.3×10^3 M^{-1} cm^{-1}). Concentrations of the enzyme are expressed in terms of the native hexamer.

2.2. Time-resolved fluorescence measurements

Fluorescence intensity decays of *E. coli* PNP-I (tyrosine protein), FA, and their ternary complex (PNP–FA–P_i) in the presence of 10 mM phosphate (P_i) buffer (pH 7.0) at 25 °C in 50 mM HEPES buffer (pH 7.0) were obtained by time-correlated single photon-counting measurements, performed on an IBH time-resolved spectrofluorimeter (IBH Consultants, Glasgow, UK) equipped with a high frequency flash lamp. Fluorescence excited by the vertical polarized pulse focused in the 5×5 cm cuvette was collected by a suprasil lens, filtered from the contaminating excitation using Corning 290- and a 325-nm long-wavelength-pass filters for excitation at 270 and 315 nm, respectively, and passed through a Glan–Thompson polarizer set at a magic angle (54.7°) and a monochromator operated at 4-nm spectral resolution. The fluorescence signal, free of polarization effects, was given directly by the magic angle signal, after subtraction of background fluorescence (<0.5%). A Ludox solution was used to collect the instrument response function. Measurements were performed in the presence of natural (0.25 mM) concentration of oxygen, which is too low to affect tyrosine fluorescence decay. This was shown previously by measurements of bimolecular oxygen-quenching constants and fluorescence lifetimes for L-tyrosine (L-Tyr), *N*-acetyl-L-tyrosinamide (NATyrA), and tyrosine-containing peptides and proteins [24].

Typical fluorescence intensity decay data were analyzed by the Marquardt–Levenberg method of nonlinear fitting of one-, two-, or three-exponential function (Eq. (3)), and power-like function (Eq. (6)), each convoluted interactively with the instrument response function (IRF) using homemade procedure under Matlab. The quality of fits was evaluated by the reduced chi-square values, and the structure observed in the plots of residuals normalized to error, i.e. residuals $= \frac{I_{exp}-I_{theo}}{\sqrt{I_{exp}+I_{theo}}}$, where I_{exp} is the number of experimental counts, and I_{theo} is the number of counts resulted from convolution of the fitted function with IRF. Thus the I_{theo} is also subjected to experimental error, and, therefore, must be taken into account in normalization of residuals.

2.3. Theoretical model

In the multi-exponential model of fluorescence decay, intensity decays $I(t)$ are fitted using

$$I(t) = \sum_i \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \tag{3}$$

where α_i are the amplitudes associated with the decay time values τ_i. The mean decay time $\langle\tau\rangle$ is given by $\langle\tau\rangle = \sum_j f_j\tau_j$, where fractional intensities f_j are given by $f_j = \alpha_j\tau_j / \sum_i \alpha_i\tau_i$. Multi-exponential model of fluorescence decays is usually applied for a homogenous system of fluorophores or non-interacting fluorophores. However this is not the case of fluorescence decay in proteins and their complexes. In general, fluorescence decay of such complex systems is expected to be performed via large number (N) of decay channels. Each of decay channels associated with partial decay rates (γ_i) represents a combination of all internal states of the system, and it reflects a set of energetically discriminated configurations of the system long before and long after the decay takes place. This in turn, as was shown previously [5,25] might be represented as a distribution of lifetimes. In this case the total decay rate γ is expected to be a sum of a number (N) of partial rates γ_i ($\gamma = \sum_{i=1}^{N} \gamma_i$). The consequence of assuming that the total rate is a composition of many partial rates lead to its distribution in the form of gamma distribution [5],

$$P_N(\gamma)d\gamma = \frac{1}{\Gamma\left(\frac{N}{2}\right)}\left(\frac{N}{2\langle\gamma\rangle}\right)\left(\frac{N\gamma}{2\langle\gamma\rangle}\right)^{\frac{N}{2}-1}\exp\left(-\frac{N\gamma}{2\langle\gamma\rangle}\right) \tag{4}$$

shown by maximum entropy method as the most probable under experimental constraints on the mean value of the decay rate ($\langle\gamma\rangle = \langle 1/\tau\rangle$), normalization ($\int_0^\infty P_N(\gamma)d\gamma = 1$), and the mean

value of the logarithm $\langle \ln(\gamma) \rangle$. The latter express the fact that distribution is determined only for $\gamma > 0$.

Taking into account relaxation from many contributing components, the fluorescence decay $I(t)$ is determined by

$$I(t) = I_0 \int_0^\infty P_N(\tau) \exp\left(-\frac{t}{\tau}\right) d\tau \quad (5)$$

with normalization $I_0 = I(t=0)$. This lead to power-like decay function

$$I(t) = A\left[1-(1-q)\frac{t}{\tau_0}\right]^{\frac{1}{1-q}} \quad (6)$$

where A is the amplitude (for normalized decay data $A = \tau_0/(2-q)$), τ_0 is the mean value of lifetime distribution, and q is the parameter of heterogeneity defined as

$$q = 1 + \frac{2}{N} = 1 + \frac{\langle (\gamma - \langle \gamma \rangle)^2 \rangle}{\langle \gamma \rangle^2} \quad (7)$$

describing the number of decay channels (N) and relative variance of fluctuations of $\gamma = 1/\tau$ around the $1/\tau_0$ value [26]. The mean decay time $\langle t \rangle$ is given by

$$\langle t \rangle = \frac{\tau_0}{3-2q} \quad (8)$$

It is worth noticing that the normalization of power-like decay function leads to constraint on the q values ($q < 2$), which, through Eq. (7), implies constraint on N ($N > 2$). Furthermore, requirement of existence of the mean value of decay time (Eq. (8)) implies that $q < 3/2$, hence $N > 4$. In the limit, when the number of decay channels goes to infinity, i.e. $q \to 1$ (Eq. (7)), the decay function from a power-like form (Eq. (6)) converges to a single-exponential form (Eq. (1)).

3. Results and discussion

3.1. Effect of enzyme–ligand interaction on tyrosine fluorescence decay of the enzyme

E. coli PNP-I and its ternary complex with formycin A (FA) and phosphate (P_i) in aqueous solutions were chosen as good examples of highly complex fluorescence intensity decays resulting from excitation of tyrosine residues in the enzyme, the N(1)–H and N(2)–H tautomeric forms of FA (Fig. 1), free in solution (see Ref. [20], and literature cited therein), and the latter bound selectively in the six active sites of the enzyme [21,22].

Tyrosine fluorescence decay of such a complex system can not be properly described using a single-exponential model [21] due to many interacting fluorophore residues, which prevent consideration of the individual decay times. In addition, the kinetics of fluorescence decay is affected by fluorescence resonance energy transfer (FRET), i.e. the resonance interaction by week (Förster) coupling between transition dipoles of emission and absorption of protein tyrosine residues (Tyr160) and the N(2)–H form of FA bound by the enzyme [22].

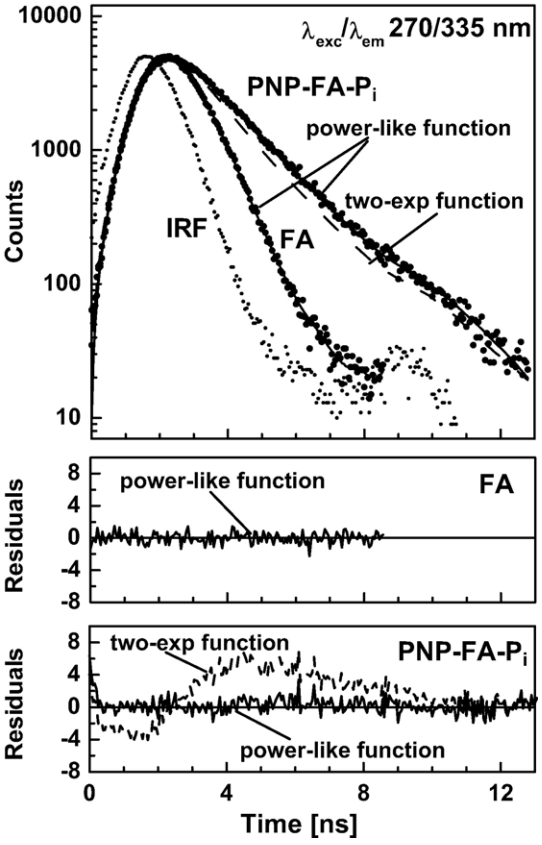

Fig. 2. Fluorescence intensity decays ($\lambda_{exc}/\lambda_{em}$ 270/335 nm) of FA and ternary complex of *E. coli* PNP-I, FA and P_i (PNP–FA–P_i), and instrument response function (IRF) shown by dotted curves. The solid and dashed curves represent the theoretical value of the best fits of power-like function (Eq. (6)) and double-exponential function (Eq. (3)), respectively. The lower panels show normalized residuals (Section 2.2). Note that the best fit of power-like function to fluorescence intensity decay of FA was obtained with heterogeneity parameter $q = 1.01 \pm 0.02$ (Table 1), and therefore experimental data may be approximated by single-exponential function with reduced $\chi^2_R = 0.92$ (see Section 3.2 for further details).

Moreover, crystal data [27] and previous luminescence studies [21,22] indicate that both the base moiety of FA and the phenol ring of Tyr160 are involved in π−π interactions with the aromatic residue of Phe159. The latter is located between FA and Tyr160, almost perpendicularly to the base moiety of FA from one side and to the phenol ring of Tyr160 on the other side. Such π−π stacking implies involvement of higher order multipole couplings, as well as exchange interactions. Therefore, a model involving continuous lifetime distribution seems to be more appropriate than a one considering a sum of the individual decay components. Indeed, we observed that the decay function given by Eq. (6) enables a good fit of enzyme tyrosine fluorescence decay data of both the PNP-I enzyme free in solution and in the ternary PNP–FA–P_i complex (see Fig. 2 as an example) with $\chi^2_R = 1.02$ and 1.1, respectively, and high accuracy of the fitted parameters (Table 1). For both cases poor fits were obtained with single-exponential model ($\chi^2_R = 6.28$ and 2.64, respectively), somewhat improved with the double-exponential model ($\chi^2_R = 1.02$ and 1.32), but the residuals (data not shown) confirmed that power-like decay function (Eq. (6))

Table 1
Results of fittings of power-like decay function (Eq. (6)) to the fluorescence decays of 35 μM *E. coli* purine nucleoside phosphorylase (PNP-I), 35 μM formycin A (FA), and ternary complex of 35 μM *E. coli* PNP, 35 μM FA and 10 mM phosphate (PNP–FA–P_i) at 25 °C in 50 mM Hepes buffer pH 7.0; the excitation and emission wavelengths ($\lambda_{exc}/\lambda_{em}$, nm/nm) were as indicated, and the standard deviations (in brackets) refer to the last digit[a]

Compounds	$\lambda_{exc}/\lambda_{em}$ (nm/nm)	q	τ_0 (ns)	$\langle t\rangle$ (ns)	N
PNP-I	270/310	1.25(2)	1.65(3)	3.13(5)	9
FA	270/335	1.01(2)	0.56(1)	0.57(3)	200
	315/335	1.08(2)	0.66(1)	0.79(3)	25
	315/365	1.13(2)	0.58(1)	0.78(3)	15
PNP–FA–P_i	270/310	1.187(6)	0.87(1)	1.39(3)	11
	270/335	1.105(6)	1.18(3)	1.49(4)	19
	270/365	1.099(6)	1.23(2)	1.53(3)	20
	315/335	1.066(6)	1.29(2)	1.49(3)	30
	315/365	1.057(6)	1.38(2)	1.56(3)	35

[a] Parameter of heterogeneity (q) and the mean value of lifetime distribution (τ_0) were obtained from fittings of power like decay function (Eq. (5)) as described in Section 2.2. The number of decay channels (N) and the mean decay time $\langle t\rangle$ were calculated using Eqs. (6) and (7), respectively.

has fitted experimental data better than double-exponential function [21]. Furthermore, the increasing number of exponential terms in the multi-exponential model (e.g. from two to three) led to an improvement of the goodness of fit, but without physical interpretation of the decay components.

Tyrosine fluorescence decay ($\lambda_{exc}/\lambda_{em}$ 270/310 nm) of the PNP-I enzyme free in aqueous solution is described by a power-like function with the mean decay time $<t>=3.13\pm0.05$ ns, parameter of heterogeneity $q=1.25\pm0.02$, and number of decay paths $N=9$ (Table 1). Relatively higher heterogeneity of fluorescence decay, and lower number of deactivation paths were observed, as compared as with that for L-Tyr ($q=1.009\pm0.009$, $N\sim200$) and NATyrA ($q=1.07\pm0.02$, $N\sim30$) in aqueous solution [5]. These observations suggest that tyrosine residues in the enzyme are subjected to various hindrances, which restrict their movement and interactions, and, thus, slow down relaxation processes. Ternary complex formation strongly affected tyrosine fluorescence decay of the enzyme, selectively observed at 310 nm. Enzyme–ligand interactions led to 2-fold lower value of mean decay time (1.39±0.03 ns), slightly reduced value of $q=1.187\pm0.006$, and consequently higher value of N (Table 1). As expected, resonance energy transfer from tyrosine residues to FA in the enzyme–ligand complex is an additional deactivation path of the excited tyrosine residues. This is the main path responsible for a much faster decay of tyrosine fluorescence, and an increase of the number of relaxation paths of the excited electronic state of tyrosine fluorophores in the enzyme–ligand complex.

The fluorescence intensity decays of ternary complex of *E. coli* PNP-I, FA and P_i (PNP–FA–P_i) were also analyzed using one- or two-exponential functions with pre-exponential $\alpha(\tau)$ factors given by the Gaussian or Lorentzian distribution of lifetime values of components, using a software provided by the Center for Fluorescence Spectroscopy (University of Maryland at Baltimore, USA). As judged from the reduced chi-square values, one-exponential function was not acceptable, while two-exponential model with a sum of two Gaussian or Lorentzian distribution function led to good fits ($\chi^2_R\approx1.1$), similar to those obtained for the power-like model (see above). These results were confirmed by observed residuals (not shown). It is worth to emphasize that the Gaussian or Lorentzian lifetime distributions are used without a theoretical basis for the $\alpha(\tau)$ distribution. Other problems occur if part of the distribution exists below $\tau=0$, and the $\alpha(\tau)$ parameters need additional normalization (to avoid the so called cut off problem). An alternative approach would be to use $\alpha(\tau)$ amplitudes not described by any particular function. This approach may be superior in that it makes no assumption about the shape of the distribution [28]. However, the use of analytical form (e.g. power-like function) minimizes the number of floating parameters in fitting algorithms, and fittings are usually much more efficient.

Summarizing physical features of models proposed here, we would like to accentuate that the power-like model seems to be better physically justified. Power-like decay function is resulted from distribution of decay rates in the form of gamma function. The latter is the most probable (expected) distribution under certain constraints which seems to be a natural consequence of the studied problem. One expects that each decay rate can be realized in a studied system, i.e. the fluorescence decay of ternary complex can be obtained via whichever decay rate. Therefore, we put a constraint of normalization, so that the distribution of decay rates as any other distribution was normalized. Additionally, as in experiments, where we measure only certain average values, we put the constraint of existence of the average value of decay rate ($\langle\gamma\rangle$). Furthermore, to avoid the cut off problem, we expect existence of distribution only for positive decay rate values. The latter led to a constraint, which can be expressed by existence of the average value of logarithm of decay rates ($\langle\ln(\gamma)\rangle$). All of the above bear out physical content of gamma distribution and, consequently, power-like decay function.

3.2. Fluorescence decay of free ligand

It is worth noting that for FA, which in aqueous media exist in the 85:15 equilibrium mixture [21] of the N(1)–H and N(2)–H tautomeric forms (Fig. 1) in the ground and excited electronic states, relative contribution of the emission from both tautomers depends on both the excitation and emission wavelengths. Absorption and emission spectra of the N(2)–H form are red-shifted relative to the spectra of the N(1)–H form. Excitation at wavelengths above 315 nm almost exclusively goes to the N(2)–H form, while selective observation of its fluorescence is possible at wavelengths above 360 nm, where emission from the N(1)–H form is negligible [20].

In the case of FA in aqueous solution, emission ($\lambda_{exc}/\lambda_{em}$ 270/335 nm) is dominated by the major N(1)–H tautomeric form (~85%) of FA [21]. Therefore, one should expect at least double-exponential decays for aqueous solutions of FA. In contrast, depopulation of its excited state is performed via ~200 paths (Table 1) reflecting a dynamical nature of fluorophores and their environment, sufficient to probe a high number of different environments (decay paths). Therefore, fluorescence

intensity decay may be approximated by a single-exponential function [21], and consequently by power-like function with heterogeneity parameter $q \approx 1$ (Fig. 2, top and middle panels; Table 1). In fact, the latter occurs in the case of lifetime distribution of zero width, which converges to Dirac delta function and leads to single-exponential decay. In that case fluorescence lifetime results rather from the statistical average of many fluorescence lifetimes than from a unique value related to specific conformation of the fluorophore. In fact, this reflects its ability to a sufficiently rapid averaging of an environmental effect.

When excitation of FA in aqueous solution has been moved to higher wavelength (λ_{exc} 315 nm), the N(2)–H tautomeric form of FA was exclusively excited at the red-edge of its absorption spectrum [20]. Resulted decays of fluorescence intensities ($\lambda_{exc}/\lambda_{em}$ 315/335 or 315/365 nm), in contrast to that observed at $\lambda_{exc}/\lambda_{em}$ 270/335 nm (Fig. 2, top panel), appeared highly heterogenic (data not shown), hardly described by a sum of two exponential terms ($\chi^2_R = 1.2$). On the other hand, these decays are fitted very well by power-like decay function ($\chi^2_R = 1.04$), and increase of heterogeneity may be judged by higher values of heterogeneity parameter q, and consequently by 10-fold lower number of the decay paths (N) (Table 1).

Several factors, however, may be responsible for increased heterogeneity of fluorescence decay of the N(2)H tautomeric form relative to single-exponential decay of fluorescence resulted from 270-nm excitation of 85:15 mixture of both tautomeric forms of FA in aqueous solutions at pH 7. In the light of current studies on the charge recombination of model donor–acceptor complexes in polar solvents [29], it seems that the most important role may play electron transfer (ET) dynamics, which is consistent with the effect of the excitation wavelength on the decay heterogeneity of FA fluorescence. The latter reflects differences between distribution of the electron density in the tautomeric forms of FA, and, consequently, their ability to interact with electron active molecules, e.g. components of buffering medium, unless they have an energy favoring ET. Another example of a higher heterogeneity is provided by fluorescence intensity decays of the N(2)–H tautomeric form selectively bound in the active site of the enzyme (Section 3.3, below), where local heterogeneity around FA is defined by enzyme–ligand interactions, including both hydrogen bonding and stacking interactions exhibited by solid state structure of the enzyme–ligand complex [27].

It is worth noting that an increase of excitation energy, i.e. decrease of the excitation wavelength leads to a larger number of possible inter- and intra-molecular interactions responsible for deactivation of the excited electronic state. In that case, the number of ET active molecules is relatively large, fluorophores can average environment sufficiently rapidly and mono-exponential fluorescence decay may be expected. On the other hand, at lower energy of excitation smaller number of the ET active states may be involved in deactivation processes, and non-exponential decay is expected. The latter reflects the higher role of the local environment around each fluorophore molecule.

3.3. Effect of enzyme–ligand interaction on fluorescence decay of the ligand

Attention was next directed to the effect of enzyme–ligand complex formation between PNP and the N(2)–H tautomeric form of FA on the decay of FA fluorescence, which at λ_{exc} 270 nm resulted from direct absorption of photons or resonance energy transfer from tyrosine residue (Tyr160) of the enzyme [22]. As expected, fluorescence decays at $\lambda_{exc}/\lambda_{em}$ 270/335 or 270/365 nm are not satisfactorily described by the single-exponential model [21]. Even selective excitation of FA (λ_{exc} 315 nm) in the enzyme–FA complexes also led to non-exponential fluorescence decays (λ_{em} 335 or 365 nm). Double-exponential model, based on the assumption that each exponential term represents fluorescence decay of one tautomeric form, and relative values of fractional intensity factors quantitatively reflect a shift of the tautomeric equilibrium upon binding to the enzyme [21], also exhibited bad fit ($\chi^2_R = 1.32$) of fluorescence decays of the PNP–FA–P_i ternary complex (Fig. 2, top and bottom panels). Further improvement of the goodness of fit was obtained by addition of the third exponential term (data not shown), but without its reliable interpretation.

Analysis was significantly improved by continuous lifetime distribution model (Section 2.3) and power-like decay function (Eq. (6)) derived from it. The best fits of power-like function to fluorescence intensity decay of the enzyme–ligand mixture (see example in Fig. 2, top and bottom panels) showed that ternary complex formation with the N(2)–H tautomeric form of FA led to 2-fold higher value of the mean decay lifetime $<t>$ and significant increase of the heterogeneity parameter ($q = 1.105 \pm 0.006$) of ligand fluorescence decays (λ_{exc} 270/335 nm) relative to single-exponential fluorescence decay of free FA in the same solution ($q \approx 1$) (Section 3.2). Concomitantly, enzyme–ligand interactions led to 10-fold smaller number of the relaxation paths N (Table 1) as compared as with that for free FA.

As expected, effect of ternary complex formation on fluorescence decay of FA is due to significant limitation of its freedom in the enzyme active site, and resonance energy transfer from tyrosine residues of the enzyme. When excitation was shifted to 315 nm, where enzyme do not absorb and absorption is dominated by the N(2)–H tautomeric form of FA bound in the enzyme–ligand complex, number of decay paths associated with specific interactions of the N(2)–H tautomeric form with the enzyme has increased (Table 1). These was further enhanced when observation of emission was moved from 335 to 365 nm (Table 1), where tyrosine fluorescence is negligible low, and emission is dominated by the N(2)–H tautomeric form of FA. This is in line with the model of selective binding of the N(2)–H form in the active site of the enzyme.

4. Concluding remarks

Since power-like function (Eq. (6)) is determined by the mean value of lifetime distribution (τ_0) — characterizing the average rate of the excited-state decay, and heterogeneity

parameter (q) — objectively reflecting physical heterogeneity of the system, it maybe advantageous for the systems where microscopic interpretation of exponential terms in the multi-exponential models is unknown. Such form of the decay function is obtained directly from statistical treatment of the fluorescence decays based on the gamma-distributed fluctuations of the fluorescence decay rates, which has been shown as the most probable. Observations of this work suggest that proposed model could possible be considered in the Fluorescence Lifetime Imaging Microscopy (FLIM) by the use of the images of τ_0 and q of the lifetime distribution, which both may reflect heterogenic nature of fluorescence probes in the cell or tissues.

When heterogeneity parameter $q \rightarrow 1$, i.e. number of decay paths $N \rightarrow \infty$, power-like function leads to a mono-exponential function describing an ideal decay, as it was observed here for FA fluorescence decays at $\lambda_{exc}/\lambda_{em}$ 270/335 nm, where emission from both tautomeric forms was detected. In that case kinetics of fluorescence decay results rather from the statistical average of many decay paths, than from a unique conformation of the fluorophore. The shift of excitation or emission wavelength to the values where the N(2)–H tautomeric form of FA is selectively excited (λ_{exc} 315 nm) or emission is almost exclusively dominated from the N(2)–H form (λ_{em} 365 nm), fluorescence decays are heterogenic with q taking values between 1.13 and 1.08, and consequently with N values between 15 and 25, respectively. The origin of this may be related to the electron transfer phenomena, and should be further investigated in details. The enzyme–FA complex formation led to an increase of the fluorescence decay heterogeneity, similar to that observed for the N(2)–H tautomeric form of free FA. This is in line with the fact that the latter is exclusively bound by the enzyme.

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