Energy Migration and Transfer Rates Are Invariant to Modeling the Fluorescence Relaxation by Discrete and Continuous Distributions of Lifetimes

Stanislav Kalinin and Lennart B.-Å. Johansson*

Department of Chemistry, Biophysical Chemistry, Umeå University, S-901 87 Umeå, Sweden

Received: September 24, 2003

Fluorescent groups typically exhibit nonexponential photophysics when incorporated into biomolecular structures, e.g., proteins and lipid membranes. Models assuming discrete and continuous distributions of lifetimes can each accurately describe the observed relaxation. In the analyses of energy transfer and PDDEM (partial donor−donor energy migration) experiments, one frequently needs to model the nonexponential decays of noninteracting donor and acceptor groups. The present paper aims at exploring whether calculated transfer/migration rates depend on modeling the photophysics' decay by discrete or continuous distributions of lifetimes. Discrete or continuous distribution models of the decay, generated synthetically, were analyzed as well as true experimental data. Two proteins were studied. In one of the systems, we examined energy transfer from Trp (donor) to BODIPY (acceptor) in ribosomal protein S6, obtained from *Thermus thermophilus*. In the second system, we examined PDDEM between different BODIPY derivatives that were pairwise specifically incorporated in mutant forms of plasminogen activator inhibitor type 2. Interestingly, the rates of electronic energy migration/transfer and distances determined within pairs of interacting chromophores reveal small or negligible influence on using discrete or continuous distributions of lifetimes.

Introduction

Donor−acceptor energy transfer (DAET) (for a summary, see refs 1 and 2) and donor−donor energy migration (DDEM)[3,4] are used in bio-applications, e.g., in studies of proteins[4−7] and lipid membranes.[8,9] As a rule, rather than an exception, the fluorescence relaxation of donor and acceptor groups in absence of electronic dipole−dipole coupling is not a single-exponential function. For years, the origin of this fact has been and still is subject to discussion. In practice, the fluorescence decay is most often fitted to a sum of exponential functions,[2] while models that assume distributions[10,11] are also used. The former approach would mean that chromophoric groups experience a few distinct physicochemical environments, each representing a unique fluorescent lifetime. This is reasonable for proteins of well-defined structures in which the bound chromophoric group occupies a few preferential orientations in the binding site. However, the explanation seems less reasonable for loosely attached groups that undergo local rotational motions in the binding site or those for which the binding site is flexible. Usually the local rotational correlation times and fluorescent lifetimes are on similar time scales, which makes the assumption of lifetime distributions physically more tractable. Unfortunately the models assuming discrete or continuous distributions of lifetimes can be statistically fitted very well to most realistic experimental data, implying that one cannot definitely distinguish the nature of lifetime distributions. To distinguish between the models when using the time-correlated single-photon-counting (TCSPC) technique, one would need stable experimental conditions to provide extremely good statistics with number of counts in the order of 10^6 or higher.[12]

In energy transfer experiments, the fluorescence relaxation is measured and compared in the presence and absence of chromophoric interaction. Because the coupled system contains distance information, the determination of the transfer rate is of a particular interest. In a special case previously considered (see, e.g., refs 2, 13−16), the lifetime distribution of the coupled system contains information about the distribution of donor−acceptor distances. However, there are instances where the assumption of one distance (R) separating two interacting groups is adequate, e.g., in studies of native protein structures.[5,4] In the following, we have investigated energy transfer/migration for a single distance between interacting groups. For donor and acceptor groups that in absence of coupling exhibit nonexponential photophysics, it is reasonable to ask whether the obtained rates and distances depend on the model describing the fluorescence relaxation. The present paper aims at investigating this question by means of analyzing both synthetic and true experimental data.

By assumption of continuous distributions, we have generated synthetic data of interacting chromophores. These data were reanalyzed with continuous and discrete distributions, respectively. Both models were also applied in the analyses of lifetime data obtained from experiments. The acceptor fluorescence was measured in the presence and absence of a donor group. Here we studied energy transfer from Trp (donor) to BODIPY (acceptor) in the ribosomal protein S6 obtained from *Thermus thermophilus*. The structure of S6 is illustrated in Figure 1a.

In addition, the fluorescence relaxation of interacting chromophores that undergo partial donor−donor energy migration (PDDEM) was monitored. PDDEM is a recently developed method for measuring distances within a pair of chemically identical, but photophysically nonidentical, fluorophores.[17,18] Photophysical nonidentity of fluorescent groups bound to different positions in a biomacromolecule (e.g., a protein) may be caused either by different physicochemical properties (e.g., pH, polarity, etc.) or induced by adding quenchers (Isaksson, M. et al. **2003**, submitted). Here we have analyzed PDDEM

* Author to whom correspondence may be addressed. E-mail: lennart.johansson@chem.umu.se. Tel.: +46-90-786 5149. Fax: +46-90-786 7779.

10.1021/jp031096x CCC: $27.50
Published on Web 02/06/2004

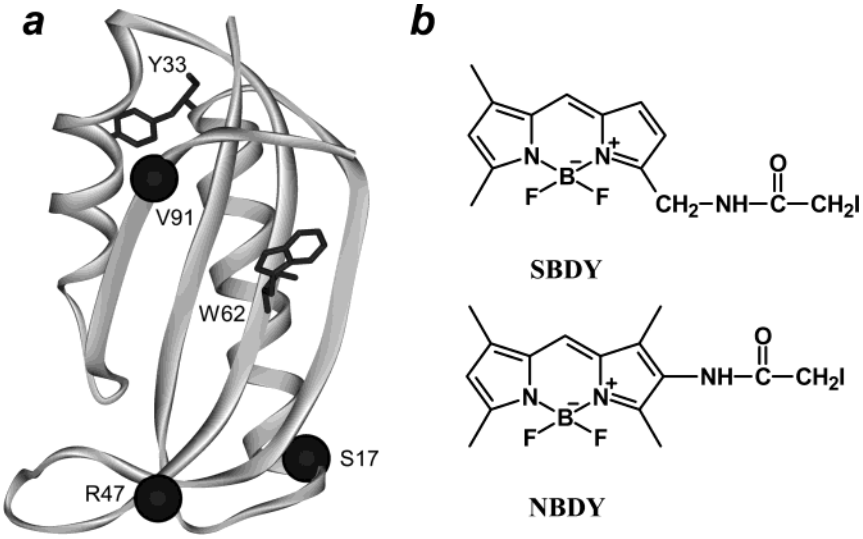

Figure 1. (a) Schematic structure of ribosomal protein S6.[36] The mutated positions are illustrated as filled black circles. The distances between tryptophan and BODIPY were measured for V91C, Y33W/W62Y/S17C, Y33W/W62Y/R47C, and Y33W/W62Y/V91C mutants (Olofsson, M. et al. **2003**, unpublished data). (b) The chemical structures of sufhydryl-specific BODIPY derivatives *N*-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,-4a-diaza-*s*-indacene- 2-yl)iodoacetamide (NBDY) and *N*-(4,4-difluoro-5,7-dimethyl- 4-bora-3a,4a-diaza-*s*-indacene-3-yl)methyl) iodoacetamide (SBDY).

data obtained for two different BODIPY derivatives (Figure 1b) specifically incorporated in mutant forms of plasminogen activator inhibitor type 2 (PAI-2) and exposed to the quencher potassium iodide.

Theory

For a continuous distribution of lifetimes, the fluorescence relaxation is given by (see, e.g., ref 19)

$$F(t) = \int_0^\infty a(\tau) \exp(-t/\tau)\, d\tau \qquad (1)$$

In discrete form, eq 1 reads

$$F(t) = \sum_i a(\tau_i) \exp(-t/\tau_i) \qquad (2)$$

where $a(\tau_i)$ denote pre-exponential factors defined for a lifetime grid $\{\tau_i\}$. In this study, the total number of lifetimes was about 200.

To minimize the number of floating parameters in the analyses, the shape of the distribution $a(\tau_i)$ can be chosen a priori. We selected a Gaussian distribution, which was also used previously by Lakowicz et al.[11,20]

$$a(\tau_i) = \frac{1}{(2\pi)^{1/2}\sigma} \exp\left(\frac{-(\tau_i - \bar{\tau})^2}{2\sigma^2}\right) \qquad (3)$$

In eq 3, $\bar{\tau}$ is the average fluorescence lifetime and σ is the standard deviation. The full width at half-maximum of the distribution is fwhm $= 2\sigma(2 \ln 2)^{1/2} \approx 2.355\sigma$. A sum of 2−3 functions such as eq 3 was typically needed to fit the experimental data.

The lifetime distribution can also be reconstructed using the maximum entropy method (MEM).[10,21−23] The MEM selects the distribution that maximizes the function

$$Q = \nu S - \chi^2 \qquad (4)$$

where S is an entropy-like function[21,22]

$$S = \sum_i \left[a(\tau_i) - m(\tau_i) - a(\tau_i) \log \frac{a(\tau_i)}{m(\tau_i)} \right] \qquad (5)$$

and ν is a constant. The role of ν is described elsewhere.[23] In particular, the case of $\nu = 0$ is referred to as the exponential series method.[19,24] In eq 5, $m(\tau_i)$ is the initial model for the distribution $a(\tau_i)$.[10,22,23] Provided that the values of τ_i are spaced logarithmically and no a priori knowledge of the system is available, one should use $m(\tau_i) = 1/\tau_i$.[10] For the analysis, we used an algorithm proposed by Vinogradov and Wilson.[25] However, we maximized Q in the following representation

$$Q = [\mathbf{g}^0 - 0.5\nu(\mathbf{L} - \mathbf{1})]^t\mathbf{a} - \frac{1}{2}\mathbf{a}^t[\mathbf{H} + \nu\Delta]\mathbf{a} \qquad (6)$$

In eq 6, Δ is a diagonal matrix, $\Delta_{ii} = 1/a(\tau_i)$, and **a** (denoted by **p** in ref 25) is a vector of $a(\tau_i)$ values. $\mathbf{g}^0$, **L**, and **H** in eq 6 are constant matrixes defined as elsewhere.[25] Equation 6 is a quadratic approximation to Q (eq 4), provided S is given by eq 5.

The lifetime distributions in the presence of PDDEM were calculated as follows. To start the fluorescence decays of two donors D_α and D_β (denoted by $F_\alpha(t)$ and $F_\beta(t)$, respectively) were analyzed as described above. The analyses provide the distributions of lifetimes $a(\tau_i)$ and $b(\tau_j)$ in absence of energy transfer. Then the fluorescence decays were calculated for all possible combinations of the lifetimes using the PDDEM model

$$F_{\alpha\beta}(t) = \sum_i \sum_j a(\tau_i)b(\tau_j)F(t,\tau_i,\tau_j,\omega_{\alpha\beta},\omega_{\beta\alpha}) \qquad (7)$$

In eq 7, $\omega_{\alpha\beta}$ and $\omega_{\beta\alpha}$ denote $D_\alpha \rightarrow D_\beta$ and $D_\beta \rightarrow D_\alpha$ energy migration rates, respectively. The function $F(t,\tau_i,\tau_j,\omega_{\alpha\beta},\omega_{\beta\alpha})$ denotes the fluorescence relaxation of the coupled $D_\alpha \leftrightarrow D_\beta$ system for which $F_\alpha(t) = \exp(-t/\tau_i)$ and $F_\beta(t) = \exp(-t/\tau_j)$. $F(t,\tau_i,\tau_j,\omega_{\alpha\beta},\omega_{\beta\alpha})$ was calculated as described elsewhere.[17,18] For the experimental systems considered in this work, we assumed $\omega_{\alpha\beta} = \omega_{\beta\alpha}$,[26] which simplifies the analysis (Isaksson, M. et al. **2003**, submitted). The lifetime components of $F_{\alpha\beta}(t)$ were sorted according to the lifetimes, and the distribution was transformed to initial τ-grid so that

$$F_{\alpha\beta}(t) = \sum_i c(\tau_i) \exp(-t/\tau_i) \quad (8)$$

The fluorescence relaxation of donor or acceptor groups in the presence of energy transfer (denoted by $D \rightarrow$ A and D $\rightarrow A$, respectively) was calculated in the same way, when using the different models[1,2,27,28] to calculate $F(t,\tau_i,\tau_j,\omega_{\alpha\beta},\omega_{\beta\alpha})$ (cf. eq 7).

In accordance with Förster's theory,[29] the rates in eq 7 ($\omega = \omega_{\alpha\beta} = \omega_{\beta\alpha}$) are

$$\omega = \frac{3\langle\kappa^2\rangle}{2\tau}\left(\frac{R_0}{R}\right)^6 \quad (9)$$

In eq 9, τ stands for the fluorescence lifetime of the donor, $\langle\kappa^2\rangle$ is the square of the angular part of dipole−dipole interaction, and R is the distance between the interacting molecules. The Förster radius R_0 is defined by

$$R_0 = \left(\frac{9000 \ln 10(^2/_3)\Phi J}{128\pi^5 n^4 N_A}\right)^{1/6} \quad (10)$$

In eq 10, N_A, n, Φ, and J denote the Avogadro constant, the refractive index, the quantum yield of the donor, and the overlap integral, respectively.

We here assumed that the rates $\omega_{\alpha\beta}$ and $\omega_{\beta\alpha}$ are the same for the whole ensembles of D_α and D_β, respectively. This approximation is not the only one possible,[2] but this assumption leads to the exact expression for a nonexponential decay caused by a dynamic quenching process ($\Phi \propto \tau$ in eqs 9−10).[30,17]

Methods

The synthetic TCSPC data were generated as described by Chowdhury et al.[31] The decay curves contained 25 000 counts in the peak maximum. The excitation pulse was taken to be Gaussian with the fwhm $= 0.1\tau_0$. For simplicity, the radiative lifetime τ_0 was chosen the same for all fluorescent species in the synthetic "experiments". The time resolution was $0.005\tau_0$/channel.

The distances (R) were obtained by fitting eqs 7−10 to the fluorescence decay of the coupled systems $D \rightarrow$ A, D $\rightarrow A$ or $D_\alpha \leftrightarrow D_\beta$ by using a common deconvolution procedure.[32] With the exception of the MEM analyses, all data sets were analyzed globally.[33] The fluorescence decays of the donor in the absence and in the presence of the acceptor, respectively, were analyzed simultaneously for the $D \rightarrow$ A system. Three data sets corresponding to the individual fluorescence relaxations and for the coupled system were analyzed for the D $\rightarrow A$ and the $D_\alpha \leftrightarrow D_\beta$ systems. The synthetic data were analyzed in the same way as the experimental data. The quality of the global fitting was judged by standard χ^2 and Durbin−Watson parameters as well as by the weighted residuals and autocorrelation function graphs.

SPC measurements were performed on a PRA 3000 system (Photophysical Research Association Inc., Ontario, Canada). The excitation source for BODIPY was a Nanoled-01 pulsed diode, operating at 800 kHz (IBH, Glasgow, Scotland). A flash lamp (510C, PRA) was used to excite Trp fluorescence. The lamp was filled with deuterium and was operated at ca. 30 kHz. Lifetime data were collected with an emission polarizer set to the "magic angle" (54.7°) relative to the excitation polarizer. At least 10 000 counts were collected in the maximum peak. The resolution was 50 ps/channel, and the fluorescence decay was collected over 1024 channels.

Results and Discussion

Even fluorophores that are exceptionally insensitive to their microenvironment, such as BODIPY,[34] often exhibit a complex fluorescence relaxation when linked to a protein.[35] In absence of coupling, the fluorescence relaxation of Trp and BODIPY is nonexponential in the proteins ribosomal protein S6 (Olofsson, M. et al. **2003**, unpublished data) and PAI-2 (Isaksson, M. et al. **2003**, submitted).

Very often the complex relaxation is modeled as a sum of discrete exponential decays.[2] However a continuous distribution of the fluorescence lifetimes often describes the data equally well.[12,19,11,10,22] Moreover, it was shown that various distributions could be described by a sum of two−four exponentials with good statistics according to known tests.[12] Therefore none of these models can be preferred unless additional information is available. Hence it is not surprising that our recent data (Olofsson, M. et al. **2003**, unpublished data; Isaksson, M. et al. **2003**, submitted) can also be described in terms of continuous distributions of lifetimes. The single Trp residue in ribosomal protein S6 from *Thermus thermophilus* exhibits biexponential fluorescence kinetics ($a_1 = 0.61$, $\tau_1 = 5.2$ ns, $a_2 = 0.39$, $\tau_2 = 7.6$ ns), but a distribution of lifetimes describes the data equally well. A similar pattern was observed for NBDY- and SBDY-labeled PAI-2. The chemical structures of NBDY and SBDY are displayed in Figure 1b.

Synthetic Experiments. We generated TCSPC-like data, assuming that the "true" fluorescence relaxation is described by a distribution of lifetimes. The synthetic data were then reanalyzed using the double-exponential relaxation model as well as the continuous lifetime distribution model (eqs 7−10).

***D* → A System.** At first we considered the donor−acceptor system, for which the fluorescence decay of the donor is measured (i.e., $D \rightarrow$ A). The only floating parameter in the analysis was the distance R. Figure 2a shows the initial lifetime distribution of the donor and the calculated distribution for the $D \rightarrow$ A system ($R = R_0$). The ratios of obtained distances R to the values assumed in simulations (R_{true}) are presented in Figure 2d. Except for the very short distances, the results are almost invariant to the model used to fit the D's fluorescence relaxation (Figure 2d).

In fact, the above example does not demonstrate that the double-exponential model works that well for *any* distribution of lifetimes. The difference is likely bigger for broader distributions, but on the other hand, one would probably need more exponentials to fit broader distribution of lifetimes. Thus in the following we chose the maximal distribution widths for which the double-exponential model was still acceptable. In other words, the double-exponential model gave poor fits for any broader distributions. To find the desired fwhm, we applied the condition

$$\chi^2_{2-exp} \approx 1 + 1/(N)^{1/2} \quad (11)$$

where N is a number of data points (channels). From a series of simulations, we found that at 25 000 counts in the peak maximum, two exponentials were sufficient to fit distributions for which fwhm $\approx 0.85\bar{\tau}$, for $\bar{\tau} = 0.3\tau_0$, $0.5\tau_0$, or $0.8\tau_0$. The condition (eq 11) is somewhat arbitrary, but its exact shape seems to be less important due to the rapid growth of χ^2 as a function of the distribution width at fwhm $\geq 0.8\bar{\tau}$. The fwhm used in simulations was therefore $0.25\tau_0$, which is about 0.83 of the shorter amplitude average lifetime ($\bar{\tau}_D$ or $\bar{\tau}_\beta$, parts b and c of Figure 2, respectively). We expect that for these distributions the difference between the two methods of analysis should be the most considerable because more exponentials should be used to fit broader distributions.

D → *A* System. The fluorescence decay of the acceptor in a donor−acceptor system (D $\rightarrow A$) also contains information about

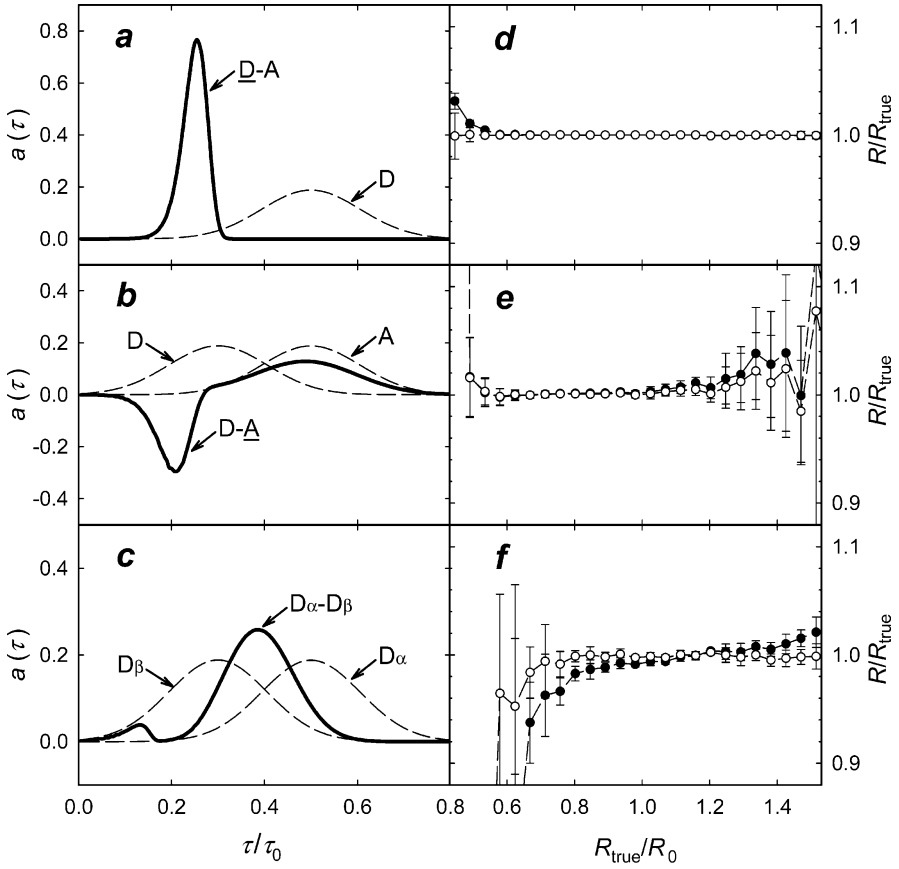

Figure 2. (a, b, c) Distributions of lifetimes for $D \rightarrow$ A, D $\rightarrow A$, and $D_\alpha \leftrightarrow D_\beta$ systems, respectively. The lifetime distributions in absence (dashed lines) and in the presence of energy transfer ($R = R_0$, solid bold lines) are shown. (d, e, f) For $D \rightarrow$ A, D $\rightarrow A$, and $D_\alpha \leftrightarrow D_\beta$ systems, the ratios between the distance (R) obtained to the true distance (R_{true}) are presented. The distances were obtained from synthetic data assuming a continuous distribution of lifetimes and analyzed for such a distribution model (○) as well as the multiexponential relaxation model (●). The values are based on 10 independent simulations, and the error bars indicate random scatter of the results.

the energy-transfer rate.[27,28] A common problem with measuring the acceptor fluorescence is the direct excitation of some fraction of the acceptor molecules.[2] It can be taken into account by introducing the probability of the direct acceptor excitation as an extra floating parameter in the analysis.

The distributions of acceptor lifetimes in the absence and in the presence of energy transfer are shown in Figure 2b. Figure 2e shows the ratio R/R_{true}, for which the distance values were obtained from the acceptor fluorescence decays. At long distances, the double-exponential model yields slightly longer distances. However, the deviation is rather small as compared to random scatter of R values (Figure 2e).

$D_\alpha \leftrightarrow D_\beta$ System. Distance measurements obtained by using the PDDEM were also modeled. It was then assumed that $\omega_{\alpha\beta} = \omega_{\beta\alpha}$, whereas only the distribution of lifetimes for D_α and D_β differ. The difference between the amplitude average lifetimes ($\bar{\tau}_\beta = 0.6\bar{\tau}_\alpha$) was about that required for accurate distance measurements[17] (Figure 2c). Analogous to the $D \rightarrow$ A and D $\rightarrow A$ systems, differences between the distances obtained using two different models are minor over the range of distances where stable results are expected (Figure 2f).

The D $\rightarrow A$ and $D_\alpha \leftrightarrow D_\beta$ data were also analyzed using triple-exponential fittings to D and D_β fluorescence relaxation, respectively. The obtained distances were almost identical to those obtained using the distributions of lifetimes (data not shown).

Experimental Data. The BODIPY fluorophore forms a donor−acceptor pair with tryptophan (Olofsson, M. et al. **2003**, unpublished data). Recently we have found that distances between tryptophan residues and BODIPY could be obtained from BODIPY fluorescence decay curves (Olofsson, M. et al. **2003**, unpublished data) (Figure 3a, D $\rightarrow A$ system). The lifetime distributions of Trp and BODIPY within the Y33W/W62Y/S17C-S6 mutants are shown in parts e and f of Figure 3. In the analysis, the R and the probability of direct excitation of BODIPY were treated as floating parameters. More details of the analysis procedure will be given elsewhere (Olofsson, M. et al. **2003**, unpublished data).

The distances calculated using the lifetime distribution model are presented in Table 1. The distribution of lifetimes calculated for the Y33W/W62Y/S17C-S6 mutant and obtained distance $R = 25.3$ Å (Table 1) is shown in Figure 3e. As expected, the distribution exhibits a negative peak at about $1/\tau = 1/\tau_D + \omega$ and a positive peak at $1/\tau \approx 1/\tau_A$. As compared to multiexponential analyses, only minor deviation of R values (within 0.5%) was found for all mutants. The difference in R values was also negligible as compared to other errors (Table 1). Fitting statistics was also almost the same for both methods (parts b and c of Figure 3).

The D $\rightarrow A$ data were also analyzed using the MEM approach. First, the distributions of lifetimes of tryptophan and BODIPY in absence of energy transfer were analyzed using eqs 4 and 5. The distribution of BODIPY lifetimes in the coupled system, calculated using eq 7, is shown in Figure 3f. The fitting procedure yields distances very similar to those obtained using other models (Table 1). The deviations from the multiexponential model are slightly larger for the MEM method (Table 1). This might be explained by the fact that data were not analyzed

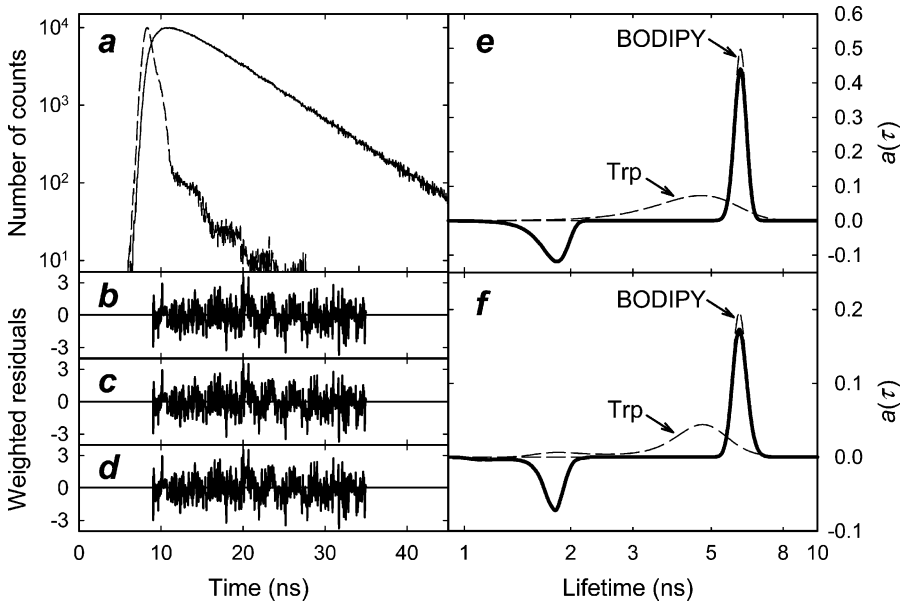

Figure 3. (a) Fluorescence decay of BODIPY excited through energy transfer from Trp within the Y33W/W62Y/S17C-S6 mutant. The response function is also shown (dashed line). (b, c, d) Weighted residuals obtained in the analyses using the multiexponential relaxation model, the Gaussian distribution of lifetimes, and the MEM approach, respectively. (e, f) Lifetime distributions of Trp, BODIPY (dashed lines), and Trp-BODIPY (bold solid lines), calculated assuming Gaussian (e) and MEM (f) distributions of lifetimes.

TABLE 1: Distances (*R*) between the Center of Masses of Trp and SBDY Groups in Different Mutants of Ribosomal Protein S6 and between the SBDY and NBDY Groups, Respectively, in Mutant Forms of PAI-2[a]

mutant	method	*R* (Å), multiexponential model	*R* (Å), Gaussian distribution of lifetimes	*R* (Å), MEM reconstruction	$\delta_{Gaussian}$[b]	δ_{MEM}[c]
SBDY-S17C-S6	D → *A*	26.6 ± 0.2	26.6 ± 0.2	26.5 ± 0.2	+0.04%	−0.31%
SBDY-Y33W/W62Y/S17C-S6	D → *A*	25.5 ± 0.4	25.5 ± 0.4	25.6 ± 0.3	+0.08%	+0.20%
SBDY-Y33W/W62Y/V91C-S6	D → *A*	24.3 ± 0.8	24.3 ± 0.8	24.3 ± 0.6	−0.20%	+0.00%
SBDY-Y33W/W62Y/R47C-S6	D → *A*	30.3 ± 0.5	30.3 ± 0.5	30.5 ± 0.4	−0.06%	+0.50%
NBDY-p79/p171 PAI-2	$D_\alpha \leftrightarrow D_\beta$	51.7 ± 1.8	52.1 ± 2.0	52.1 ± 1.9	+0.85%	+0.81%
SBDY-p79/p171 PAI-2	$D_\alpha \leftrightarrow D_\beta$	55.6 ± 1.0	55.8 ± 0.9	55.6 ± 1.0	+0.34%	+0.07%

[a] The distances were obtained using various models of the nonexponential photophysics' decay. For details, see text. [b] Relative difference between the distances obtained using Gaussian lifetime distribution model and multiexponential model. [c] Relative difference between the distances obtained using MEM distribution model and multiexponential model.

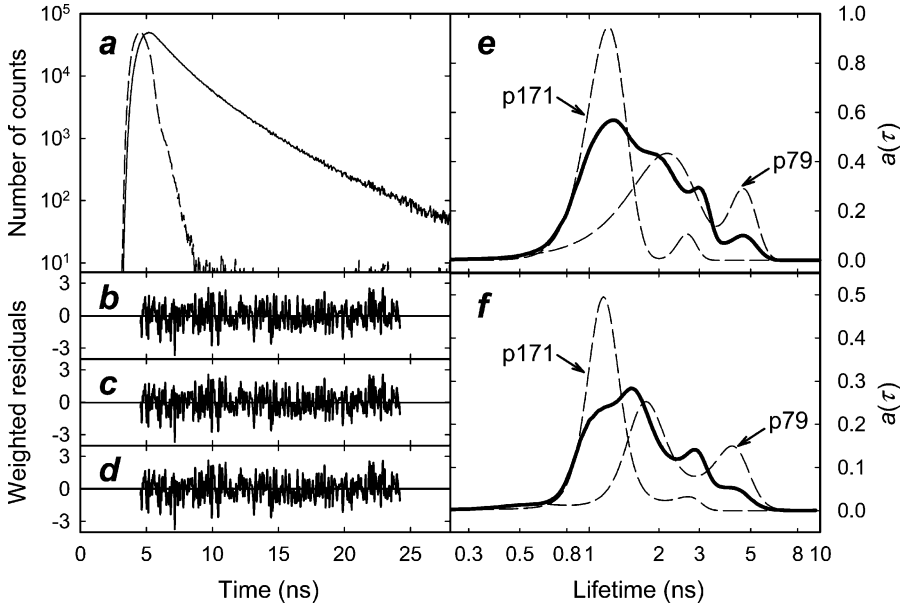

Figure 4. (a) Fluorescence decay curve obtained for the NBDY-labeled p79/p171 PAI-2 mutant. The response function is also shown (dashed line). (b, c, d) Weighted residuals obtained using the multiexponential relaxation model, the Gaussian distribution of lifetimes, and the MEM approach, respectively. (e, f) The calculated distributions of lifetimes of NBDY-p79, NBDY-p171 (dashed lines), and NBDY-p79/p171 (bold solid lines), when assuming the Gaussian (e) and the MEM (f) distributions of lifetimes.

in a global manner.[33] We used $\nu = 0.001$, 0.01, and 0.1 in eq 4. Although the optimal value of ν can be determined,[23] we are here more interested in testing various distributions consistent with lowest χ^2 values. While substantially different distributions were obtained when using different ν values (typically less structured at high ν), only a minor and nonsystematic difference in R values was observed (data not shown).

The PDDEM data on the PAI-2 protein were also reanalyzed (Isaksson, M. et al. **2003**, submitted). The BODIPY derivatives linked to different cysteine residues are quenched differently by iodide. Indeed we find that the distributions of lifetimes do depend on the positions of PAI-2, as shown in parts e and f of Figure 4. Furthermore, eqs 7−10 were used to calculate the lifetime distributions in the presence of energy migration (parts e and f of Figure 4). The distance R and the labeling efficiencies were considered as floating parameters in the analyses (Isaksson, M. et al. **2003**, submitted). The distances obtained are summarized in Table 1. The values found when using the discrete exponential model are also shown. For all mutants, the difference between methods of analysis was within 1% (Table 1). On average, the distribution model provides slightly shorter distances, which is consistent with the TCSPC simulations (Figure 2f, $R \leq R_0 \leq 1.2R_0$). The weighted residuals plots for all methods of analysis are almost indistinguishable (parts b, c, and d of Figure 4) as well as the fitting statistics.

Conclusions

Synthetically generated and experimental data show that nonexponential fluorescence relaxation of single fluorescent probes in proteins can be accurately modeled by discrete and continuous distributions of lifetimes as well as in a MEM approach. From measurement of the fluorescence relaxation of acceptors within donor−acceptor pairs, the transfer rates and D−A distances determined exhibit little or negligible dependence on the model used to describe the fluorescence relaxation. The influence of model is also negligible for distances obtained from PDDEM experiments. In these experiments, one analyzes the fluorescence of donor−donor pairs for which the donors have different excited-state relaxation.

Acknowledgment. This work is supported by grants from the Swedish Research Council and the Kempe Foundations.

References and Notes

(1) Van der Meer, B. W.; Coker, G., III.; Chen, S.-Y. S. *Resonance Energy Transfer: Theory and Data*; VCH Publishers: New York, 1994.

(2) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.

(3) Karolin, J.; Johansson, L. B.-Å. *Trends Phys. Chem.* **1997**, *6*, 171−185.

(4) Bergström, F.; Hägglöf, P.; Karolin, J.; Ny, T.; Johansson, L. B.-Å. *Proc. Natl. Acad. Sci.* **1999**, *96*, 12477−12481.

(5) Karolin, J.; Fa, M.; Wilczynska, M.; Ny, T.; Johansson, L. B.-Å. *Biophys. J.* **1998**, *74*, 11−21.

(6) Fa, M.; Bergström, F.; Hägglöf, P.; Wilczynska, M.; Johansson, L. B.-Å.; Ny, T. *Structure* **2000**, *8*, 397−405.

(7) Runnels, L. W.; Scarlata, S. F. *Biophys. J.* **1995**, *69*, 1569−83.

(8) Mikhalyov, I.; Bogen, S.-T.; Johansson, L. B.-Å. *Spectrochim. Acta Part A* **2001**, *57*, 1839−1845.

(9) Bogen, S.-T.; Karolin, J.; Molotkovsky, J. G.; Johansson, L. B.-Å. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 2435−2440.

(10) Livesey, A. K.; Brochon, J. C. *Biophys. J.* **1987**, *52*, 693−706.

(11) Lakowicz, J. R.; Cherek, H.; Gryczynski, I.; Joshi, N.; Johnson, M. L. *Biophys. Chem.* **1987**, *28*, 35−50.

(12) James, D. R.; Ware, W. R. *Chem. Phys. Lett.* **1985**, *120*, 455−459.

(13) Wagner, B. D.; Ware, W. R. *J. Phys. Chem.* **1990**, *94*, 3489−3494.

(14) Liu, G. *Macromolecules* **1993**, *26*, 1144−1151.

(15) Rolinski, O.; Birch, D. J. S. *J. Chem. Phys.* **2000**, *112*, 8923−8933.

(16) Lakshmikanth, G. S.; Sridevi, K.; Krishnamoorthy, G.; Ungaonkar, J. B. *Nat. Struct. Biol.* **2001**, *8*, 799−804.

(17) Kalinin, S. V.; Molotkovsky, J. G.; Johansson, L. B.-Å. *Spectrochim. Acta, Part A* **2002**, *58*, 1087−1097.

(18) Kalinin, S.; Molotkovsky, J. G.; Johansson, L. B.-Å. *J. Phys. Chem. B* **2003**, *107*, 3318−3324.

(19) James, D. R.; Ware, W. R. *Chem. Phys. Lett.* **1986**, *126*, 7−11.

(20) Gryczynski, I.; Johnson, M. L.; Lakowicz, J. R. *Biophys. Chem.* **1994**, *52*, 1−13.

(21) Livesey, A. K.; Skilling, J. *Acta Crystallogr. B* **1985**, *A41*, 113−122.

(22) Brochon, J. C. *Methods Enzymol.* **1994**, *240*, 262−311.

(23) Gull, S. F.; Skilling, J. *Quantified Maximum Entropy: MemSys5 Users' Manual*, 1.2 ed.; Maximum Entropy Data Consultants Ltd., Suffolk, U.K.: Suffolk, 1999.

(24) Siemiarczuk, A.; Wagner, B. D.; Ware, W. R. *J. Phys. Chem.* **1990**, *94*, 1661−1666.

(25) Vinogradov, S. A.; Wilson, D. F. *Appl. Spectrosc.* **2000**, *54*, 849−855.

(26) Isaksson, M.; Kalinin, S.; Lobov, S.; Ny, T.; Johansson, L. B.-Å. *J. Fluoresc.* **2003**, *13*, 379−384.

(27) Birks, J. B. *Photophysics of Aromatic Molecules*; John Wiley & Sons, Ltd: London, 1970.

(28) Kulinski, T.; Visser, A. J. W. G.; O'Kane, A.; Lee, J. *Biochemistry* **1987**, *26*, 540−549.

(29) Förster, T. *Ann. Phys.* **1948**, *2*, 55−75.

(30) Albaugh, S.; Steiner, R. F. *J. Phys. Chem.* **1989**, *93*, 8013−8016.

(31) Chowdhury, F. N.; Kolber, Z. S.; Barkley, M. D. *Rev. Sci. Instrum.* **1991**, *62*, 47.

(32) O'Connor, D. V.; Phillips, D. *Time-correlated Single Photon Counting*; Academic Press: London, 1984.

(33) Knutson, J. R.; Beechem, J. M.; Brand, L. *Chem. Phys. Lett.* **1983**, *102*, 501−507.

(34) Bergström, F.; Mikhalyov, I.; Hägglöf, P.; Wortmann, R.; Ny, T.; Johansson, L. B.-Å. *J. Am. Chem. Soc.* **2002**, *124*, 196−204.

(35) Karolin, J.; Johansson, L. B.-Å.; Strandberg, L.; Ny, T. *J. Am. Chem. Soc.* **1994**, *116*, 7801−7806.

(36) Lindahl, M.; Svensson, L. A.; Liljas, A.; Sedelnikova, S. E.; Eliseikina, I. A.; Fomenkova, N. P.; Nevskaya, N.; Nikonov, S. V.; Garber, M. B.; Muranova, T. A. *EMBO J.* **1994**, *13*, 1249−1254.