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# The effect of a distribution of separations upon intramolecular distances in biopolymers, as determined by radiationless energy transfer

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If a fluorescent donor group and a nonfluorescent acceptor group are incorporated into a biopolymer, so that radiationless energy transfer occurs between the two groups, the apparent separation of the groups, as determined by energy transfer, will be influenced by the existence of a distribution of separations. This might arise from the presence of significant localized flexibility at the sites of attachment of the two groups, or from internal flexibility involving the biopolymer itself. If a Gaussian form is assumed for the distribution of separations of the donor and acceptor groups, the efficiency of transfer is dependent upon the width of the distribution, as well as the average distance between the groups. Significant differences may thereby arise between the true average separation and the separation computed from transfer efficiencies by the usual procedures. The deviations are different for transfer efficiencies computed from quantum yields and from decay times. They become more important with increasing width of the distribution of separations and increasing efficiency of transfer. In general, if a distribution of separations is present, the average separation is most reliably computed by procedures which take into account the effects of this distribution.

## 1. Introduction

Radiationless energy transfer, employing a fluorescent donor group and an absorbing acceptor group, has become a popular method for measuring the distance separating such groups in biopolymers [1–3]. Because of the dependence of transfer efficiency upon the sixth power of the separation of the groups, this technique possesses high sensitivity and, in principle, permits the determination of intramolecular separations with high precision.

The method is most readily adapted to the case where (1) the mutual orientation of the transition dipoles of donor and acceptor groups is random and (2) the separation of the two groups is a fixed and well-defined quantity. In actuality, the rigorous fulfillment of both conditions is probably exceptional. The existence of potential errors arising from a nonrandom orientation of donor and acceptor groups is well-recognized and methods have been developed to correct for these [4,5]. In practice, the existence of localized mobility of the probes often minimizes uncertainties arising from this source.

Considerably less attention has been paid to the effect upon the apparent donor-acceptor separation of a continuous distribution of separations, although such a distribution is probably of common occurrence. Even if the labeled biopolymer is itself rigid the existence of substantial probe mobilities will result in an effective range of separations; this is especially the case if one, or both, of the probes is linked to the biopolymer by an elongated attaching arm. Superimposed upon the effects of probe mobility will be those of any internal flexibility of the biopolymer itself. The occurrence of such flexibility in many proteins and nucleic acids is now well-established [6–8].

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In recent years, methods have been developed for the direct determination of distance distributions employing time- and frequency-domain measurements [9–11]. These procedures require relatively sophisticated equipment and data analysis. More commonly, what is desired is the average, or most probable, separation, which is usually estimated from a single determination of the transfer efficiency, as obtained from measurements of the quantum yield or average fluorescence decay time in the absence and presence of acceptor [1–3].

The questions to be considered here are under what conditions measurements of this kind can be expected to yield accurate values of the average separation and how potential errors arising from a distribution of separations may be estimated.

## 2. Theory

In the most general case, the intensity decay of a fluorophore linked to a biopolymer, in the absence of acceptor, is given by:

$$I_F(t) = \sum_i \alpha_{Fi} e^{-t/\tau_{Fi}} \tag{1}$$

where $I_F(t)$ is the intensity of the fluorophore as a function of time, $t$, whereas $\alpha_{Fi}$ and $\tau_{Fi}$ represent the amplitude and decay time, respectively, of the $i$-th decay mode.

The average decay time is given by:

$$\langle \tau_F \rangle = \sum_i \alpha_{Fi}\tau_{Fi}^2 / \sum_i \alpha_{Fi}\tau_{Fi} \tag{2}$$

In the presence of an acceptor group characterized by a distance, $R_0$, for 50% transfer efficiency, the expression for the time dependence of intensity, for a constant separation, $r$, of donor and acceptor groups [1–3] is:

$$\begin{aligned} I_{FA}(t) &= \sum_i \alpha_{Fi} \exp\left\{ -t/\tau_{Fi} - (t/\tau_{Fi})(R_0/r)^6 \right\} \\ &= \sum_i \alpha_{Fi} \exp\left\{ -(t/\tau_{Fi})\left[1 + (R_0/r)^6\right] \right\} \end{aligned} \tag{3}$$

$R_0$, which depends solely on the properties of the donor and acceptor groups, has a value equal to $[\{9000(\ln 10)K^2\phi_D/128\pi^4 N n^4\}\int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda]^{1/6}$ where $K^2$ is an orientation factor (equal to 2/3 for random orientation), $\phi_D$ the donor quantum yield, $N$ Avogadro's number, $n$ refractive index, and $F_D(\lambda)$ and $\epsilon_A(\lambda)$ the normalized donor emission spectrum and the acceptor absorption spectrum, respectively, as a function of wavelength, $\lambda$ [1–3]. In this case, the ratio of the average intensities in the presence, $I_{FA}$, and absence, $I_F$, of acceptor becomes:

$$\begin{aligned} \frac{I_{FA}}{I_F} &= \frac{\int_0^\infty \sum_i \alpha_{Fi} \exp\left\{ -(t/\tau_{Fi})\left[1 + (R_0/r)^6\right] \right\} dt}{\int_0^\infty \sum_i \alpha_{Fi} \exp(-t/\tau_{Fi})\, dt} \\ &= 1/\left[1 + (R_0/r)^6\right] \end{aligned} \tag{4}$$

The expression for the ratio of average decay times in the presence, $\langle \tau_{FA} \rangle$, and absence, $\langle \tau_F \rangle$, of acceptor is:

$$\begin{aligned} \frac{\langle \tau_{FA} \rangle}{\langle \tau_F \rangle} &= \left\{ \sum_i \alpha_{Fi}\tau_{Fi}^2 \left[1 + (R_0/r)^6\right]^{-2} \right. \\ &\quad \left. \times \left[ \sum_i \alpha_{Fi}\tau_{Fi} \left[1 + (R_0/r)^6\right]^{-1} \right]^{-1} \right\} \\ &\quad \times \left[ \sum_i \alpha_{Fi}\tau_{Fi}^2 / \sum_i \alpha_{Fi}\tau_{Fi} \right]^{-1} \\ &= 1/\left[1 + (R_0/r)^6\right] \end{aligned} \tag{5}$$

For the case of a single constant separation between donor and acceptor, the ratios of intensity and average decay time in the presence and absence of acceptor are equivalent and simply related to the separation.

If, however, there exists a continuous distribution of distances between donor and acceptor groups, eqs. 4 and 5 must be replaced by:

$$\begin{aligned} \frac{I_{FA}}{I_F} &= \int_0^\infty \int_0^\infty \sum_i \alpha_{Fi} \exp\left\{ -(t/\tau_{Fi}) \right. \\ &\quad \left. \times \left[1 + (R_0/r)^6\right] \right\} P_r\, dr\, dt \\ &\quad \times \left[ \int_0^\infty \sum_i \alpha_{Fi} \exp(-t/\tau_{Fi})\, dt \right]^{-1} \end{aligned} \tag{6}$$

and

$$\frac{\langle\tau_{FA}\rangle}{\langle\tau_F\rangle} = \int_0^\infty \sum_i \alpha_{Fi}\tau_{Fi}^2\left[1+(R_0/r)^6\right]^{-2} P_r\,dr \times\left\{\int_0^\infty \sum_i \alpha_{Fi}\tau_{Fi}\left[1+(R_0/r)^6\right]^{-1} P_r\,dr\right\}^{-1} \times\left[\sum_i \alpha_{Fi}\tau_{Fi}^2/\sum_i \alpha_{Fi}\tau_{Fi}\right]^{-1} \quad (7)$$

where $P_r$ is the probability distribution function for the separation.

Eqs. 6 and 7 simplify to

$$\frac{I_{FA}}{I_F} = \int_0^\infty \frac{P_r\,dr}{1+(R_0/r)^6} \quad (8)$$

and

$$\frac{\langle\tau_{FA}\rangle}{\langle\tau_F\rangle} = \frac{\int_0^\infty\left[1+(R_0/r)^6\right]^{-2} P_r\,dr}{\int_0^\infty\left[1+(R_0/r)^6\right]^{-1} P_r\,dr} \quad (9)$$

It should be stressed that the above assumes that only a single and constant value of $R_0$ is associated with all of the decay modes.

The form of the distribution function is usually unknown. However, in the absence of exact knowledge, it is reasonable to represent it by a Gaussian function of the form [10–12]

$$P_r = \frac{1}{\sigma'\sqrt{2\pi}}\exp\left(-\left\{\frac{r-\bar{r}}{\sqrt{2}\,\sigma'}\right\}^2\right) \quad (10)$$

where $\bar{r}$ is the average separation and $\sigma'$ the standard deviation. It is convenient to replace $\sigma'$, $r$ and $\bar{r}$ by the reduced quantities

$$u = r/R_0$$
$$\bar{u} = \bar{r}/R_0$$
$$\sigma = \sigma'/R_0$$

In terms of these quantities, eqs. 8 and 9 become

$$\frac{I_{FA}}{I_F} = \frac{1}{\sigma\sqrt{2\pi}}\int_0^\infty (1+u^{-6})^{-1} \times\exp\left(-\left\{\frac{u-\bar{u}}{\sqrt{2}\,\sigma}\right\}^2\right) du \quad (11)$$

$$\frac{\langle\tau_{FA}\rangle}{\langle\tau_F\rangle} = \frac{\int_0^\infty (1+u^{-6})^{-2}\exp\left(-\left\{\frac{u-\bar{u}}{\sqrt{2}\,\sigma}\right\}^2\right) du}{\int_0^\infty (1+u^{-6})^{-1}\exp\left(-\left\{\frac{u-\bar{u}}{\sqrt{2}\,\sigma}\right\}^2\right) du} \quad (12)$$

These equations may be put in simpler form by introducing the substitution

$$z = (u-\bar{u})/\sqrt{2}\,\sigma \quad (13)$$

We then have

$$\frac{I_{FA}}{I_F} = \frac{1}{\sqrt{\pi}}\int_{z^*}^\infty\left\{1+(\bar{u}+\sqrt{2}\,\sigma z)^{-6}\right\}^{-1} e^{-z^2}\,dz \quad (14)$$

$$\frac{\langle\tau_{FA}\rangle}{\langle\tau_F\rangle} = \frac{\int_{z^*}^\infty\left\{1+(\bar{u}+\sqrt{2}\,\sigma z)^{-6}\right\}^{-2} e^{-z^2}\,dz}{\int_{z^*}^\infty\left\{1+(\bar{u}+\sqrt{2}\,\sigma z)^{-6}\right\}^{-1} e^{-z^2}\,dz} \quad (15)$$

where $z^* = -\bar{u}/\sqrt{2}\,\sigma$. If $\sigma < 0.5$ the lower limit of the above integrals may be replaced by $-\infty$ with negligible error. The integrals in eqs. 14 and 15 may be evaluated numerically.

It should be noted that, in eq. 14, the ratio $I_{FA}/I_F$ may usually be replaced by $\Sigma_i\alpha_{Fi}\tau_{Fi}/\Sigma_i\alpha_{Fi}$, to which it is equivalent unless conformations exist for which the donor is totally quenched. The latter ratio, like $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$, may be evaluated from dynamic fluorescence measurements.

## 3. Calculations

Computations of the ratios of fluorescence intensities and of average decay times for the donor in the presence and absence of acceptor, according to eqs. 4 and 5, were made using a program written for the Apple II computer. The integrals in eqs. 4 and 5 were evaluated numerically, using trapezoidal integration.

A program (GRIDLS), written for the IBM XT computer, was developed to identify the values of $\bar{u}$ and $\sigma$ which simultaneously satisfy eqs. 4 and 5 so as to minimize the difference between observed

and computed values of $I_{FA}/I_F$ and $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$. The program utilizes an iterative least-squares search [13].

## 4. Results and discussion

Figs. 1 and 2 indicate the dependence of $I_{FA}/I_F$ and of $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$, respectively, upon $\bar{u}$ and $\sigma$. In both cases the dependence is substantial. The two ratios become equal only as $\sigma$ approaches 0, corresponding to an infinitely sharp distribution. The inequality of the two ratios, with $\langle\tau_{FA}\rangle/\langle\tau_F\rangle > I_{FA}/I_F$, is indicative of a distribution of separations.

In practice, both intensities and average decay times have been used to compute transfer efficiencies and apparent separations. In the case of intensities, deviations of $I_{FA}/I_F$ from the value expected for the average separation, as a result of the distribution of separations, are minimal for $\bar{u}=1$ ($\bar{r}=R_0$) and become progressively more severe the more $\bar{r}$ differs from $R_0$, or $\bar{u}$ differs from 1 (fig. 1). For values of $\bar{u}>1$ ($\bar{r}>R_0$) the effect of the distance distribution is to reduce the apparent value of $\bar{r}$, while for values of $\bar{u}<1$, the reverse is true.

In the case of average decay times, the values of $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$ show a pronounced dependence upon $\sigma$ even when $\bar{u}=1$. The effect of a distribution of separations is here always to increase the ratio of decay times and to increase the apparent value of $\bar{r}$.

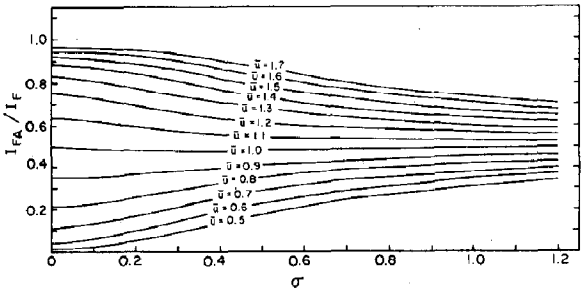


Fig. 1. Dependence of the ratio of donor emission intensities in the presence ($I_{FA}$) and absence ($I_F$) of acceptor upon the standard deviation of the Gaussian distribution of separations for a series of values of the average separation.

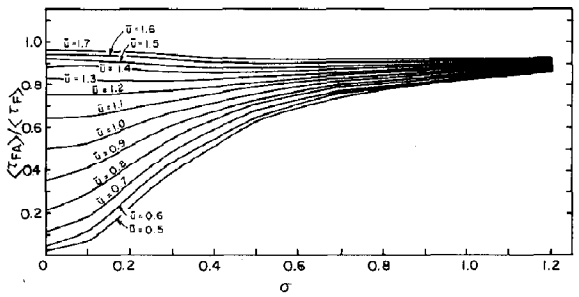


Fig. 2. Dependence of the ratio of average donor decay times in the presence ($\langle\tau_{FA}\rangle$) and absence ($\langle\tau_F\rangle$) of acceptor upon the standard deviation of the Gaussian distribution of separations for a series of values of the average separation.

If the separation of donor and acceptor groups, as determined by radiationless energy transfer, is to correspond to the average, or most probable, distance, as is usually assumed, then the effects of a distribution of separations must be taken into account. If only static measurements of fluorescence intensity are available, then errors from this source may be minimized by choosing a donor probe such that the efficiency of transfer is close to 50%. The use of average decay times to determine transfer efficiency is generally less satisfactory and tends to overestimate distances if a distribution of separations is present.

Combined static and dynamic data are currently available only for donor-acceptor pairs in synthetic polymers. We shall take as our example the set of naphthalene derivatives described by Gryczynski et al. [12], in which dansyl is the acceptor group. The donor model compound is octanoyl-1-methylnaphthylamide (NMN); the donor-acceptor pair-containing compound is dansyl-undecanoyl-undecanoyl-1-methylnaphthylamide (NU2D). Using the procedures described in section 3 and taking the data from ref. 12, the results shown in table 1 are obtained. The values of $r$ and $\sigma'$ thereby computed are in reasonable agreement with those obtained via the more elaborate frequency-domain procedures described elsewhere [10].

The available information as to distance distribution in native proteins is as yet too limited to provide a reliable estimate of the distribution widths to be expected in practice. A recent

Table 1

Computation of distance distribution for model systems

Solvent: propylene glycol (−5 °C) for NMN and NU2D; 0.4 M KCl, 5 mM Tris, 1 mM EGTA, pH 7.5 (5 °C) for troponin I (TnI). The respective wavelengths of excitation and emission were 295 and 340 nm. Data are taken from refs. 11 and 12. IE-TnI, troponin I with Cys 133 labeled with *N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine.

| Compound | $R_0$ (Å) | $I_{FA}/I_F$ | $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$ | $\bar{r}$ (Å) | $\sigma'$ (Å) | $r_{fd}$ [a] | $\sigma'_{fd}$ [a] | $r_s$ [b] |
|---|---|---|---|---|---|---|---|---|
| NMN | 23.45 | – | – | – | – | – | – | – |
| NU2D | 23.45 | 0.248 | 0.49 | 17.8±0.1 | 6.3±0.2 | 18.1 | 6.1 | 19.6 |
| TnI | 18.40 | – | – | – | – | – | – | – |
| IE-TnI | 18.40 | 0.750 | 0.82 | 24.1±0.1 | 5.5±0.1 | 24.3 | 4.9 | 20.1 |

[a] Values computed from frequency-domain measurements.
[b] Values of separation computed from static measurements of fluorescence intensity.

frequency-domain determination of the distribution of separations between Trp 158 and Cys 133 for troponin I yielded a value of 0.5 for the ratio of half-width to mean separation, corresponding to a value of $\sigma$ of 0.3 for the donor-acceptor pair used [11]. Table 1 compares the frequency-domain values for $\bar{r}$ and $\sigma'$ with those computed from combined static and dynamic data; the agreement is excellent. If static intensity measurements were employed, the error in computed apparent separation which could be introduced by this value of $\sigma$ would range from negligible ($\bar{u} = 1$) to 40% ($\bar{u} = 0.5$). If lifetime measurements were employed, errors of 30% for $\bar{u} = 1$ and of over 80% for $\bar{u} = 0.5$ would be encountered.

It would be expected that any significant unfolding of the protein would be accompanied by a major increase in distribution width. This proved to be the case for troponin I (TnI), the half-width of whose distribution of separations increased over 4-fold upon unfolding by guanidine hydrochloride [11]. Under these conditions the uncertainties associated with the determination of separation by single measurements of relative quantum yield or average decay time become unacceptably large. Native proteins showing a significant degree of internal flexibility represent an intermediate case.

In practice, perhaps the simplest criterion for the presence of a single, well-defined distance between donor and acceptor is the equivalence of $I_{FA}/I_F$ and $\langle\tau_{FA}\rangle/\langle\tau_F\rangle$. A major difference between the two ratios is suggestive of a distribution of distances. An alternative criterion is the invariance of $\bar{r}$ to donor quantum yield, as varied by quenching [12]. A systematic variation of $\bar{r}$ is indicative of a range of separations. When a range of separations exists, the determination of the average, or most probable, separation from a single ratio of intensities or average decay times is subject to ambiguities, which become more serious with increasing broadness of the distribution. Under these conditions the most reliable procedure is to determine the average separation using the available procedures for computing the distribution of separations [10–12].

The preceding discussion has not considered the effects of any distribution of mutual orientations of donor and acceptor. These would be superimposed upon those of the distribution of separations. However, if the localized motions of the probes, which tend to randomize their mutual orientation, are rapid in comparison with the time decay of fluorescence, then only an average orientation will be sensed by the system. If these motions are of substantial amplitude, this orientation will be effectively random. However, it should be stressed that this remains an unevaluated factor for most biopolymer systems.

A second approximation has been to assume only a single, constant value of $R_0$. If the donor decay in the absence of acceptor is strictly monoexponential, this does not present a problem. However, if more than one decay mode is present for the donor, this is equivalent to assuming the

same rate of transfer for all decay modes. Any failure of this assumption would tend to accentuate the effects of a distribution of separations.

Several workers have considered the migration of excitation energy through radiationless transfer in flexible polymer coils [14–17]. Although these studies do not address the question of the effect of such flexibility upon overall transfer efficiencies as determined by static and dynamic methods, they establish that such flexibility has a major influence upon the time dependence of the population of initially excited groups.

Finally, considerations of the above kind are not necessarily confined to fluorescence, but may also be a factor in any measurement of distance involving dipole-dipole interactions, with the accompanying pronounced dependence upon separation.

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