Fluorescence energy transfer in the rapid-diffusion limit

(Förster transfer/spectroscopic rulers/terbium/membrane vesicles)

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ABSTRACT Energy transfer is enhanced by translational diffusion of the donor and acceptor [Steinberg, I. Z. & Katchalski, E. (1968) J. Chem. Phys. 48, 2404-2410]. The effect of diffusion on energy transfer depends on $D	au_0/s^2$, in which $D$ is the sum of the diffusion coefficients of the donor and acceptor, $\tau_0$ is the lifetime of the donor in the absence of transfer, and $s$ is the mean distance between donors and acceptors. In most previous studies, $D\tau_0/s^2 < 1$, corresponding to the static limit. We report here steady-state and kinetic fluorescence experiments showing that $D\tau_0/s^2 > 1$, the rapid-diffusion limit, can be attained by using Tb$^{3+}$ chelated to dipicolinate as a long-lived energy donor ($\tau_0 = 3.2$ msec). The concentration of rhodamine B, the energy acceptor, resulting in 50% transfer was 0.07 $\mu$M, which is three orders of magnitude less than the concentration giving 50% transfer in the static limit. The dependence of the transfer efficiency on diffusion coefficients varying from $5 \times 10^{-11}$ to $1.5 \times 10^{-10}$ cm$^2$/sec, spanning the range from the static limit to the rapid-diffusion limit, is in excellent agreement with theory. It is evident that energy donors with millisecond or longer excited state lifetimes can be used to probe translational motions in membranes and other assemblies. Energy transfer in the rapid-diffusion limit is sensitive to the distance of closest approach ($a$) of the donor and acceptor. For a Tb(DPA)$_3$ chelate trapped inside the aqueous space of a membrane vesicle containing eosin phosphatidylethanolamine, $a = 10$ Å. The transverse location of chromophores in model membranes and biological membranes can be determined by this technique.

Fluorescence energy transfer has often been used to measure distances between stationary donors and acceptors (1-4). If diffusion substantially changes the distances between donors and acceptors during the excited state lifetime of the donor, energy transfer can be enhanced, as shown theoretically by Steinberg and Katchalski (5) and verified experimentally by Elkanah et al. (6). Haas et al. (7) have recently determined the diffusion coefficients of the ends of oligopeptide chains in solution by analyzing the effect of diffusion on energy transfer. In the present study, we consider the limiting case in which energy transfer is maximally enhanced by diffusion—i.e., a further increase in the diffusion coefficient has no further effect on the transfer efficiency. Our theoretical analysis of this rapid-diffusion limit shows that these experiments are sensitive to the distance of closest approach between donor and acceptor and that measurements are possible at much lower acceptor concentrations than in the absence of diffusion (the static limit). Calculations using the theory of Steinberg and Katchalski (5) show that, for small molecules in aqueous solution, the rapid-diffusion limit cannot be reached with conventional fluorescent donors having excited state lifetimes in the nanosecond range. This limit can be attained only if the fluorescent donor has a much longer lifetime, in which extensive diffusion can occur.

In order to attain the rapid-diffusion limit and test the theory, we have used as a donor the fluorescent lanthanide ion Tb$^{3+}$, which has a lifetime in the millisecond range (8, 9). Tb$^{3+}$ was chelated to dipicolinic acid (DPA), resulting in a fluorescence enhancement of about $10^4$ (10). This Tb(DPA)$_3$ chelate consists of three molecules of DPA liganded to Tb$^{3+}$ and has a net charge of $-3$ above pH 5 (10). Experiments using Tb(DPA)$_3$ as the donor and either rhodamine B or nitrobenzoxadiazole (NBD) diethanolamine as the acceptor verify the validity of the theory over the entire range from the static limit to the rapid-diffusion limit and demonstrate that the rapid-diffusion limit has been reached for these molecules in aqueous solution at room temperature.

We have also used energy transfer in the rapid-diffusion limit to measure the distance from the aqueous surface to an acceptor chromophore in a membrane. We present a theoretical analysis and report experiments on energy transfer from Tb(DPA)$_3$, trapped in the inner aqueous space of phospholipid vesicles, to acceptors in the membrane. The results indicate that this is a promising technique for measuring the transverse location of chromophores in biological membranes.

THEORY

In Förster’s theory of dipole–dipole energy transfer (1), the rate constant $k_T$ for energy transfer is

$$k_T = k_0(r/R_0)^{-6}$$

[1]

in which $k_0$ is the sum of the rate constants for the decay of the excited donor in the absence of acceptor and $r$ is the distance between the donor and acceptor. $R_0$ is the distance at which the transfer efficiency is 50% for a single donor–acceptor pair, is given by

$$R_0 = (J^2KQn^{-1})^{1/6} \times 9.79 \times 10^{-3}$$

[2]

in which $J$ is the spectral overlap integral (in cm$^3$ M$^{-1}$), $K^2$ is the orientation factor, $Q_0$ is the quantum yield of the donor in the absence of transfer, and $n$ is the refractive index of the medium. The rate of decay of the excited donors is

$$\frac{dn}{dt} = -(k_0 + k_T)n$$

[3]

in which $n$ is the number of excited donors. The efficiency of energy transfer $E$ is

$$E = k_T/(k_T + k_0).$$

[4]

Experimentally, $E$ is determined from the ratio of the quantum yield $Q$ in the presence of acceptor to that in its absence,

$$E = 1 - Q/Q_0,$$

[5]

or from the corresponding ratio of excited state lifetimes,

$$E = 1 - \tau/\tau_0,$$

[6]

in which $\tau = (k_0 + k_T)^{-1}$ is the lifetime of the donor in the

Abbreviations: DPA, dipicolinic acid; Tb(DPA)$_3$, chelate consisting of one terbium ion (Tb$^{3+}$) and three dipicolinate ions (DPA$^{2-}$); NBD, nitrobenzoxadiazole.
presence of acceptor and \( \tau_0 = k_0^{-1} \) is the lifetime in the absence of acceptor.

In an ensemble of stationary donors and acceptors, the decay rate for a single donor is a sum of \( k_f \) terms (Eq. 1) over the acceptors. Only those donors having at least one acceptor within a distance comparable to \( R_0 \) will have an appreciable probability of energy transfer. By contrast, in the presence of translational diffusion, energy transfer is no longer restricted to donors that have an acceptor within a distance at the instant of excitation. Transfer will additionally occur between donors and acceptors that come within a distance of about \( R_0 \) during the donor's lifetime. Specifically, the mean distance diffused by the donor and acceptor relative to each other during \( \tau_0 \) must be comparable to \( s \), the mean intermolecular distance, for diffusion to enhance energy transfer. Because the mean square distance diffused in a time \( t \) is \( 6Dt \) (in three dimensions), in which \( D \) is the diffusion coefficient, diffusion will substantially increase energy transfer if \( 6D\tau_0 \geq s^2 \).

The effect of diffusion on energy transfer has been analyzed theoretically by Steinberg and Katzenelson (5). They derived a partial differential equation for the kinetics of energy transfer (equation 33 in ref. 5), which contains a diffusion term that changes the distance between donors and acceptors during the lifetime of the excited donor. The transfer efficiency calculated by solving this equation numerically (6, 11, 12) depends on \( R_0 \), \( a \), and the concentration of acceptor, as in the absence of diffusion. In addition, the transfer efficiency depends on the product of \( D \) and \( \tau_0 \). The results of these calculations for \( R_0 = 50 \, \text{Å}, \ a = 5 \, \text{Å} \) (a plausible value for the closest approach of two small molecules in solution), and an acceptor concentration of 0.1 mM are shown in Fig. 1, which depicts the dependence of the transfer efficiency on \( D \) for \( \tau_0 = 1 \, \text{msec} \), \( 1 \, \mu\text{sec} \), and \( 1 \, \text{sec} \). The curve for \( \tau_0 = 1 \, \text{msec} \) is shown over the entire range of its variation. For \( D < 10^{-10} \, \text{cm}^2/\text{sec} \), \( E \) approaches a constant minimal value. This is the \textit{static limit}, where \( D\tau_0/s^2 \ll 1 \). For \( D \) between about \( 10^{-10} \) and \( 10^{-9} \, \text{cm}^2/\text{sec} \), \( E \) is sensitive to diffusion. This is the intermediate range, where \( 6D\tau_0/s^2 \approx 1 \). For \( D > 10^{-8} \, \text{cm}^2/\text{sec} \), \( E \) approaches a constant maximal value. This is the \textit{rapid-diffusion limit}, where \( D\tau_0/s^2 \gg 1 \).

Because \( E \) is a function of the product of \( D \) and \( \tau_0 \), the other

\[ E = \frac{1}{N} \sum_{j=1}^{N} E_j \]  

This limit has been treated in detail by Förster (2). By contrast, all donors of an ensemble are identical in the rapid-diffusion limit. The transfer efficiency \( E_r \) for this limit is

\[ E_r = k_r/(k_0 + k_r) \]  

in which \( k_r \), the sum of the rate constants for transfer from the donor to all acceptors, is

\[ k_r = \rho \int \frac{1}{R_0^6} \left( \frac{r}{R_0} \right)^6 d^3r \]

\[ = \rho \int_0^\infty \frac{1}{\tau_0^6} \left( \frac{r}{R_0} \right)^6 4\pi r^2 dr \]

\[ = 4\pi \rho R_0^6/(3\tau_0 a^3) \]

and \( \rho \) is the density of acceptors. An important feature of this equation for the rapid-diffusion limit is the dependence of \( k_r \) on \( a^{-3} \), which is illustrated in Fig. 2A.

Eq. 9 can be applied to other arrangements of donors and acceptors, provided that the diffusion of one or both is rapid. For example, consider the \textit{inset} in Fig. 2B, which depicts a membrane vesicle with a surface density \( \sigma \) of acceptors at radius \( b \) and a solution of donors trapped in the inner aqueous volume. The distance of closest approach between these donors and acceptors is \( a \). Integrating Eq. 9 over the internal volume of the vesicle gives

\[ k_r = \frac{3\pi \rho dbR_0^6}{2(b-a)^3} \int_0^{b-a} h^{-a} h(b-h)^{-4} - (b+h)^{-4} dh \]

The transfer efficiencies calculated from this equation are plotted in Fig. 2B, which shows that the distance of closest approach can be measured over a wide range of values by choosing an appropriate concentration of acceptor.

It should be noted that the above analysis is based on several assumptions. (i) The use of a single \( R_0 \) value in Eqs. 9-12 is valid if all donor–acceptor pairs have the same value of \( R_0 \). (ii) In our experiments, this condition is closely approached because the angular dependence is effectively randomized by the combination of rotational and translational diffusions of the donor during its excited state lifetime. (iii) The rate of energy transfer due to exchange interactions is assumed to be small compared to that of Förster dipole–dipole energy transfer. This assumption is probably valid for donor–acceptor pairs with \( R_0 \) distances of more than about 20 Å. (iv) Spherical symmetry is assumed in deriving Eqs. 11 and 12. Eq. 9 can be used as the starting point for treating nonspherically symmetric systems.
against a calibrated photodiode (United Detector PIN 10-UV).

TbCl₃ (Alfa), dipicolinic acid (Sigma), and rhodamine B (Eastman) were commercial products. NBD diethanolamine was a gift from Richard Hargland (Molecular Probes, Roseville, MN), and eosin phosphatidylethanolamine was from Bernard Fung. Phosphatidylcholine was prepared from egg yolks (13).

Tb³⁺ was chelated to dipicolinate to enhance its fluorescence by a factor of about 10⁶ (10). Solutions containing Tb³⁺ always contained enough DPA to ensure that virtually all of the Tb³⁺ was chelated by DPA. Fluorescent-labeled vesicles containing Tb(DPA)₃ were trapped in their inner aqueous space were prepared by the following procedure (14). Egg phosphatidylcholine and eosin phosphatidylethanolamine were mixed in ethanol solution, at a final phospholipid concentration of 20 mM. A 0.5-ml aliquot of this solution was injected rapidly through a 30-gauge syringe needle into 9.5 ml of a rapidly stirred solution containing 10 mM TbCl₃, 50 mM DPA, and 0.1 M Tris, pH 8.0, at 20°C. This vesicle suspension was centrifuged at 80,000 × g for 15 min to remove large liposomes. External Tb(DPA)₃ was removed by chromatography on Sephadex G-25 (medium).

RESULTS

The emission spectrum of Tb(DPA)₃, the energy donor, extends from about 470 to 640 nm, overlapping the absorption spectra of the three energy acceptors used in this study (Fig. 3). The spectral overlap integrals (J) are 4.58 × 10⁻¹³, 4.51 × 10⁻¹⁴, and 5.13 × 10⁻¹⁴ cm³ M⁻¹ for rhodamine B, eosin phosphatidylethanolamine, and NBD diethanolamine, respectively. The R₀ values for transfer to these acceptors are 65.7, 45.6, and 44.6 A, respectively, assuming K² = 0.67, Q₀ = 1, and n = 1.33.

The emission kinetics of Tb(DPA)₃ in the presence of various concentrations of rhodamine B are shown in Fig. 4. These plots of the logarithm of the fluorescence intensity versus time are linear, indicating that the emission kinetics are characterized by single-exponential decays, as expected for energy transfer in the rapid-diffusion limit. The excited state lifetime decreased from 2.22 nsec in the absence of rhodamine B to 0.12 nsec in

Fig. 2. Effect of the distance of closest approach (a) between the donor and acceptor on the transfer efficiency in the rapid-diffusion limit. R₀ = 50 A in this calculation. (A) Solution of donors and acceptors. The acceptor concentrations are 1.0 (dotted), 0.1 (solid), and 0.01 (dashed) mM. (B) Solution of donors trapped in the inner aqueous space of a membrane vesicle containing a spherical shell of acceptors at radius b = 150 A. The surface densities of acceptor are 0.02 (dotted), 0.002 (solid), and 0.0002 (dashed) per phospholipid (acceptors per 70 A²).

EXPERIMENTAL METHODS

For time-resolved fluorescence measurements, the excitation source used was a pulsed (cavity-dumped) argon-ion laser (Spectra-Physics 171/365). The 514.5 nm line was focused and then doubled with a temperature phase-matched ADP crystal (Inrad 5-1). The second harmonic (257.25 nm) was separated from the fundamental by a dispersing prism and then filtered (Corning 7-54) to remove stray light. The resulting excitation pulse at the sample was 50 nsec wide, illuminating a 1-mm-diameter spot with ~2 × 10⁸ photons per pulse. The repetition rates used varied between 20 Hz and 5 kHz, depending on the lifetime of the sample.

The fluorescence emission at right angles was passed through a UV cut-off filter (Corning 0-53) onto a 56AVP photomultiplier (Amperex) biased for single-photon counting. The anode signal was preamplified and discriminated with a single-channel analyzer (Ortec 4900). The resulting pulses were then counted synchronously with the cavity-dump trigger by using a multichannel scaler (Hewlett-Packard 5400).

System timing and amplitude linearity were calibrated with a frequency counter (Hewlett-Packard 5325A) and a random noise source (dark noise of a 56AVP photomultiplier), respectively. The 50-nsec excitation pulse width and the absence of any significant time jitter produced an instrument response function one channel wide, eliminating the need for deconvolution. The photon counting rate was always less than 10⁴ Hz, resulting in a scalar dead-time correction of less than 1%. The fluorescence lifetime was determined with a least-squares fit of the data to a single-exponential decay plus a constant background.

Steady-state fluorescence was measured with a photon-counting fluorimeter (Spex Fluorolog). Factors used in correcting emission spectra for the wavelength dependence of the detection system were obtained by using a MgO scatterer normalized

Fig. 3. (A) Fluorescence emission spectrum of Tb(DPA)₃, the energy donor. The solution contained 10 μM TbCl₃/0.1 mM DPA/0.1 M Tris buffer, pH 8, at 20°C. The excitation wavelength was 270 nm. (B) Absorption spectra of the energy acceptors: NBD diethanolamine (dotted), eosin phosphatidylethanolamine (solid), and rhodamine B (dashed). The extinction coefficients are in units of M⁻¹ cm⁻¹.
the presence of 10 μM rhodamine B. Transfer efficiencies calculated from these lifetimes according to Eq. 6 are plotted as a function of the concentration of energy acceptor in Fig. 5. These transfer efficiencies agree closely with those obtained from steady-state fluorescence measurements. Also shown in Fig. 5 are the results of steady-state fluorescence measurements of energy transfer from Tb(DPA)3 to NBD diethanolamine. The acceptor concentrations resulting in 50% transfer are 0.67 μM for rhodamine B and 7.1 μM for NBD diethanolamine. The dependence of the transfer efficiency on the concentration of these acceptors is in excellent agreement with the curves (solid lines in Fig. 5) calculated according to Eq. 11 for energy transfer in the rapid-diffusion limit, using \( R_0 = 65.7 \text{ Å} \) and \( a = 5.15 \text{ Å} \) for rhodamine B, and \( R_0 = 44.6 \text{ Å} \) and \( a = 5.2 \text{ Å} \) for NBD diethanolamine.

The strongest evidence that the rapid-diffusion limit was attained in the preceding experiments comes from a study of the dependence of the transfer efficiency on \( D \), the sum of the diffusion coefficients of Tb(DPA)3 and rhodamine B (Fig. 6). \( D \) was varied over seven orders of magnitude by changing the temperature and the concentration of glycerol. The observed transfer efficiencies, ranging from 2 to 94%, are in excellent agreement with the theoretical curve calculated according to equations 11–14 in ref. 5. The diffusion coefficient of 7.6 × 10^{-6} cm^2/sec in H_2O at 20°C determined in this way is close to the value expected (6–8 × 10^{-6} cm^2/sec) for these molecules.

Energy transfer in the rapid-diffusion limit was used to de-
termine the distance of closest approach between Tb(DPA)$_3$ trapped in the inner aqueous volume of a membrane vesicle and the eosin chromophore of eosin phosphatidylethanolamine incorporated into the bilayer. The transfer efficiencies obtained for 0.0006, 0.0011, and 0.003 acceptors per phospholipid were 44, 58, and 82%, respectively. These observed values are compared with theoretical curves calculated for closest approach distances of 5, 10, and 20 Å in Fig. 7. The best fit is given by a closest approach distance of 10 Å, which is plausible because the acceptor chromophore is attached to the headgroup of the phospholipid.

DISCUSSION

We have attained the rapid-diffusion limit in fluorescence energy transfer by using a long-lived energy donor, Tb$^{3+}$ ($\tau = 2.2$ msec). Eu$^{3+}$ ($\tau = 9$) and phosphorescent chromophores such as tryptophan (15) may also be useful in this regard. One of the most striking consequences of the rapid-diffusion limit is that energy transfer occurs at extremely low acceptor concentrations. The transfer efficiency from Tb(DPA)$_3$ to rhodamine B is 50% at a rhodamine B concentration of 0.67 μM, three orders of magnitude less than the concentration required to achieve the same transfer efficiency in the absence of diffusion. At 0.67 μM, the mean distance between acceptors is approximately 1400 Å. In the static limit, the same transfer efficiency would occur when the mean distance between acceptors is about 130 Å. At such low concentrations, possible perturbations caused by the probes are minimized and inner filter effects are negligible, permitting a more direct and accurate measurement of the transfer efficiency than in the slow-diffusion limit.

Probably the most useful feature of the rapid-diffusion limit is the strong dependence of $E$ on the distance of closest approach, $a$, between the donor and acceptor. Distances from a few angstroms to greater than $R_0$ can be determined with precision (Eqs. 11 and 12 and Fig. 2). For example, a solution of long-lifetime donors could be used to measure the distance between the aqueous surface of a protein and an acceptor chromophore in it. This method may be especially valuable in studying membrane proteins. Energy transfer from donors trapped in the inner aqueous space of a membrane vesicle (or alternatively, from donors outside the vesicle) to acceptor chromophores at a fixed distance $a$ from the inside surface provides a direct and sensitive method for the measurement of $a$ (Eq. 12 and Figs. 2B and 7). Our preliminary experiments suggest that the retinal chromophore of rhodopsin is closer to the intradisc surface of the disc membrane than to the extradisc surface. The purple membrane and membranes containing cytochrome oxidase are other examples of chromophoric membrane systems that can be studied in this way.

It is interesting to note that electrostatic interactions will alter the distribution of distances between donors and acceptors and thereby change the transfer efficiency. For example, using Tb(DPA)$_3$ (net charge, −3) as a donor, we have found methylene blue (net charge, +1; $R_0$, 49 Å) to be a more effective energy acceptor than bromphenol blue (net charge, −1; $R_0$, 62 Å), a result opposite that predicted from the $R_0$ values alone. The extent of electrostatic interactions could be ascertained experimentally by varying the ionic strength and by using a series of terbium chelates with different net charges. Alternatively, electrostatic effects can be avoided by using uncharged acceptors, such as rhodamine B and NBD diethanolamine (Fig. 5). The experimentally derived value of 10 Å for the closest approach between Tb(DPA)$_3$ and eosin phosphatidylethanolamine may be somewhat greater than the actual value because both species are negatively charged.

In addition to making it feasible to attain the rapid-diffusion limit, long-lived donors such as Tb$^{3+}$ permit the measurement of small diffusion coefficients. Calculations of the effect of diffusion on energy transfer in two dimensions show that a donor having a millisecond lifetime can be used to measure diffusion coefficients of $10^{-5}$ to $10^{-11}$ cm$^2$/sec, which are characteristic of proteins in membranes and of membrane lipids in the solid phase.

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