DIFFUSION-ENHANCED FLUORESCENCE ENERGY TRANSFER

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INTRODUCTION

Electronic excitation energy can be efficiently transferred by dipole-dipole interaction between suitable chromophores over distances of the order of 50 Å. As proposed by Förster (6), the transfer rate is proportional to the inverse sixth power of the distance between the donor and acceptor (2, 13, 23). This steep distance-dependence enables fluorescence energy transfer to be used as a spectroscopic ruler in the 20 to 80 Å range [for a recent review, see Ref. (22)]. Other transfer mechanisms, such as exchange interactions involving electron overlap, become important at shorter distances when the donor and acceptor are nearly in contact (4).

In most energy transfer studies carried out thus far, the donor-acceptor distance was essentially constant during the lifetime of the excited donor, which was typically a few nanoseconds. A different and potentially
informative facet of energy transfer comes into play when donors with long excited-state lifetimes are used (24). It has been appreciated for over a decade that the rate of energy transfer is markedly enhanced by translational diffusion occurring during the excited-state lifetime of the energy donor (21). A donor-acceptor pair that is too far apart for efficient transfer at the instant of excitation may be brought into close proximity by diffusion while the donor is still excited. Energy transfer is enhanced by diffusion if the distance diffused during the excited-state lifetime of the donor is comparable to or greater than the mean distance between donors and acceptors. The pertinent parameter in determining the effect of diffusion on transfer is \( D\tau_0 / s^2 \), 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. Three cases can be distinguished. The static limit, characteristic of most previous energy transfer studies, corresponds to \( D\tau_0 / s^2 \ll 1 \). Diffusion has no effect on the transfer rate in this limit. The transfer rate becomes responsive to the rate of diffusion as \( D\tau_0 / s^2 \) approaches 1, the intermediate range. Energy transfer can be used under these conditions to monitor translational motions (8). The rapid-diffusion limit corresponds to \( D\tau_0 / s^2 \gg 1 \). This limit can be experimentally attained by using terbium or other fluorescent lanthanides as donors (24). The key property of these donors is that they have millisecond lifetimes. A striking feature of energy transfer in the rapid-diffusion limit is its high sensitivity to the distance of closest approach of diffusing donors and acceptors. This type of energy transfer can reveal the depth of chromophores in proteins and membrane systems.

This review begins with a presentation of the theory of diffusion-enhanced energy transfer. The selection of probes and other facets of the experimental strategy are considered next. The subsequent section deals with experimental tests of the theory, followed by a discussion of the relative contributions of dipole-dipole transfer and exchange interaction transfer. Some recent applications of this new technique in determining the locations of chromophores in proteins and membrane assemblies are then presented.

**THEORY**

**Effect of Diffusion on Transfer Efficiency**

The rate constant \( k_T \) for dipole-dipole energy transfer between a fluorescent donor separated from an acceptor by a distance \( r \) is

\[
k_T = k_0 (r/R_0)^{-6}.
\]
in which \( k_0 \) is the rate constant for emission by the donor in the absence of energy transfer (6). \( R_0 \) (in Å) is given by

\[
R_0 = (JK^2 Q_0 n^{-4})^{1/6} \times 9.79 \times 10^3,
\]

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 energy transfer, and \( n \) is the refractive index [for a review, see Ref. (22)]. Following an exciting light pulse, the donor fluorescence decays exponentially, with a lifetime given by

\[
\tau = (k_0 + k_T)^{-1}.
\]

The transfer efficiency \( (E) \), the fractional decrease in donor fluorescence due to energy transfer, is

\[
E = k_T / (k_T + k_0) = 1 - \tau / \tau_0 = 1 - Q / Q_0,
\]

where \( \tau_0 = 1/k_0 \), and \( Q \) and \( Q_0 \) are the donor quantum yields in the presence and absence of energy transfer.

In an ensemble of stationary donors and acceptors, the decay rate for each donor is a sum of \( k_T \) terms (Equation 1) over the acceptors. Only donors with at least one acceptor within a distance of about \( R_0 \) will have an appreciable probability of energy transfer. In contrast, in the presence of translational diffusion, energy transfer is no longer restricted to donors that have an acceptor within this 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. This effect of diffusion on energy transfer has been lucidly analyzed by Steinberg & Katchalski (21). They derived a partial differential equation for the kinetics of energy transfer which contains a diffusion term that changes the distance between donors and acceptors during the lifetime of the excited donor.

The dependence of the transfer efficiency on the diffusion coefficient in three dimensions, as calculated by Thomas et al (24), is shown in Figure 1. In this calculation, the partial differential equation of Steinberg & Katchalski (21) was converted to a set of finite-difference equations by the Crank-Nicholson algorithm (3, 8, 24) and solved numerically. The method of solution was similar to that of Elkana et al (5), except that donor-acceptor collisions without transfer were allowed. The calculation was markedly accelerated by expressing the differential equation in terms of the variable \( \log r \) instead of \( r \), where \( r \) is the donor-acceptor distance. The calculated transfer efficiency depends on \( R_0 \), the distance of closest approach \( a \) between donor and acceptor, the acceptor concentration, and the donor's excited-state lifetime \( \tau_0 \), as well as on the diffusion coefficient.
$D$ of acceptors relative to donors. As shown in Figure 1, the range of $D$ values that can be measured is proportional to $\tau_0$. Only for donors having lifetimes longer than microseconds is it feasible to measure diffusion coefficients in the range that is typical for biomolecules in aqueous solution ($10^{-7}$ to $10^{-4} \text{ cm}^2/\text{sec}$). The curve for $\tau_0 = 1 \text{ msec}$ is depicted over the entire range of its variation. For $D < 10^{-10} \text{ cm}^2/\text{sec}$, $E$ approaches a constant minimal value (the static limit). For $D$ between about $10^{-10}$ and $10^{-6} \text{ cm}^2/\text{sec}$, $E$ is sensitive to diffusion (the intermediate range). For $D > 10^{-6} \text{ cm}^2/\text{sec}$, $E$ approaches a constant maximal value (the rapid-diffusion limit). A similar relationship between $E$ and $D$ is obtained in two dimensions. For $\tau_0 = 1 \text{ msec}$, $E$ is sensitive to diffusion for values of $D$ ranging from about $10^{-10}$ to $10^{-6} \text{ cm}^2/\text{sec}$. This range can be shifted to lower (or higher) values of $D$ by increasing (or decreasing) the acceptor concentration.

![Diagram](image_url)

*Figure 1* Calculated dependence of the transfer efficiency on the diffusion coefficient in three-dimensions for donor lifetimes $\tau_0$ of 1 nsec, 1 $\mu$sec, 1 msec, and 1 sec. $D$ is the sum of diffusion coefficients of the donor and acceptor. In this calculation, $R_D = 50 \text{ A}$, $\alpha = 5 \text{ A}$, and the acceptor concentration is 0.1 mM.
**Dipole-Dipole Transfer in the Rapid-Diffusion Limit**

In the static limit, donors have different arrangements of acceptors around them and so there is a distribution of values of \( k_T \), which makes the fluorescence decay multi-exponential (7). However, in the rapid-diffusion limit, all donors are equivalent (i.e., have the same \( k_T \) value), and so Equations 2 through 4 again apply. The transfer rate is then calculated by averaging over the space available to the donors (24). The general equation for the rapid-diffusion limit is

\[
k_T/k_0 = \frac{1}{V_D} \iiint_{\text{donor region}} \iiint_{\text{acceptor region}} (r/R_0)^{-6} \rho_A dV_A dV_D
\]

where \( \rho_A \) is the density of acceptors. This equation applies to any case in which rapid diffusion of *either* donors or acceptors makes the donors equivalent. For small molecules in aqueous solution at room temperature \((D \sim 10^{-7} \text{ cm}^2/\text{sec})\), the rapid diffusion limit is attained with donors having lifetimes longer than about a millisecond (Figure 1). Under these conditions, energy transfer is maximally enhanced by diffusion.

In the simplest case, in which uncharged spherical donors and acceptors are together in solution (Figure 2a), Equation 5 becomes

\[
k_T/k_0 = \int_{r_a}^{\infty} (r/R_0)^{-6} \rho_A 4\pi r^2 dr = \frac{4\pi}{3} \rho_A R_0^6 a^{-3}
\]

where \( \rho_A \) is the density of acceptors and \( a \) is the distance of closest approach between the centers of the donor and acceptor (24). In two dimensions (e.g., donors and acceptors free to diffuse laterally in a membrane), the rate constant for transfer is

\[
k_T/k_0 = \int_{a}^{\infty} (r/R_0)^{-6} \rho_A 2\pi r dr = \frac{\pi}{2} \sigma_A R_0^6 a^{-4}
\]

where \( \sigma_A \) is the surface density of acceptors. Thus, the transfer rate is proportional to \( a^{-1} \) in three dimensions and to \( a^{-4} \) in two dimensions. Since \( R_0 \) and the acceptor concentration can be determined, Equations 6 and 7 provide a sensitive means of measuring \( a \).

Suppose that the donor is located at the center of a sphere of a radius \( r_a \) and the acceptor is located a distance \( t \) from the center of a sphere of radius \( r_a \) (Figure 2b). The closest approach of the donor and acceptor is \( a = r_a + r_a - t \). The rate constant for transfer in this system (25) is

\[
k_T/k_0 = \frac{4\pi}{3} \rho_A R_0^6 a^{-3} \left[ 2 - \frac{a}{a + t} \right]^{-3}
\]

Note that this equation is the same as Equation 6 except for the term in
brackets. This relationship can be used to determine the depth of a chromophore beneath the surface of a spherical protein if the radius of the protein is known.

A related case is one in which the acceptor is buried at a depth $h$ beneath a flat surface (Figure 2c). The closest approach distance $a$ is then equal to $r_d + h$, where $r_d$ is the radius of the spherical donor. The transfer rate for this system (15) is

$$k_T/k_0 = \frac{4\pi}{3} \rho_a R_d^3 a^{-3}(1/8).$$

Note that Equation 9 is the same as Equation 6 except for the factor of $1/8$. Thus, the transfer rate to an acceptor buried beneath a flat surface

\[\text{Figure 2} \quad \text{Donor-acceptor geometries in rapid-diffusion limit studies. (a) Spherical donor and acceptor. (b) Spherical donor. Acceptor located away from the center of a sphere. (c) Acceptor buried beneath a flat surface. (d) Acceptor located within a surface protrusion. (e) Membrane vesicle. The donors can be located in the inner aqueous volume of the vesicle or in the external aqueous volume.}\]
is 1/8 that obtained with the same acceptor at the center of a sphere. Equation 9 is a special case of Equation 8 in which \( b \to \infty \).

Also of interest is the case in which a spherical acceptor of radius \( r_a \) extends a distance \( y \) (0 \( \leq y \leq 2r_a \)) above a flat surface (Figure 2d). The closest approach distance \( a \) is \( r_a + r_d \), where \( r_d \) is the radius of the spherical donor. The transfer rate for this system (15) is

\[
k_T/k_0 = \frac{4\pi}{3} \rho_a R_0^6 a^{-3}(1/8)(1 + 3y/a).
\]

The location of a chromophore relative to both surfaces of a membrane can be determined by energy transfer measurements of vesicles in which sidedness is preserved. Suppose that an energy acceptor is located at a distance \( f \) from the external surface and a distance \( g \) from the internal surface of the spherical membrane vesicle (Figure 2e). The closest approach distance to a spherical energy donor of radius \( r_d \) will then be \( r_d + f \) for donors located outside the vesicles and \( r_d + g \) for donors trapped in the inner aqueous volume of the vesicles. The transfer rate for donors located outside the vesicles is in fact given by Equation 8, in which \( \rho_a \) is the total number of acceptors divided by the extravesicular volume and \( b \) is the distance from the center of the vesicle to the spherical shell of acceptors. For donors trapped in the inner aqueous space of the vesicle (25), the transfer rate is

\[
k_T/k_0 = \frac{3\pi b \sigma_a R_0^6}{2(b-a)^3} \left[ \frac{1}{2} \left( (2b-a)^{-2} - a^{-2} \right) + \frac{b}{3} \left( a^{-3} - (2b-a)^{-3} \right) \right]
\]

where \( a \) is the distance of closest approach at the inner surface of the vesicles, \( \sigma_a \) is the surface density of acceptors in the membrane, and \( b \) is the distance from the center of the vesicle to the spherical shell of acceptors. The dependence of the transfer efficiency on the closest approach distance for this system is shown in Figure 3 (24). The vesicle radius has a relatively small effect on the transfer efficiency for radii larger than about 250 Å (25). For example, the transfer efficiency decreases from 50 to 40% in going from a vesicle with a radius of 250 Å to one with a radius of 500 Å (for \( a = 25 \) Å, \( R_0 = 50 \) Å, and \( \sigma_a = 0.01 \) acceptors/phospholipid).

The preceding equations for the rapid-diffusion limit assume that all donor-acceptor pairs have the same \( R_0 \) value. This is in fact so when both donors and acceptors diffuse rapidly in solution, since the orientation factor \( K^2 \) is then 2/3. If the donors rotate rapidly but the acceptors do not (e.g. acceptor chromophores in a membrane protein), then \( K^2 \) is a
function of the angle between the donor-acceptor vector and the acceptor transition moment and can range from $1/3$ to $4/3$. This angular dependence of $k^2$ and hence of $R_0$ can be taken into account in deriving an expression for the transfer rate starting from Equation 5. The assumption that $k^2$ is $2/3$ leads to an error of less than 10% in the estimated closest approach distance (25).

LONG-LIVED FLUORESCENT PROBES

Fluorescent energy donors with lifetimes in the millisecond range are needed for rapid-diffusion limit experiments. Fluorescent lanthanides such as terbium and europium are very suitable in this regard (24). Tb$^{3+}$ emits in the 480 to 630 nm range and Eu$^{3+}$ emits in the 580 to 700 nm range (18) (Figure 4). Both have long excited-state lifetimes because of the forbidden nature of the transition between their ground state and lowest excited state. The absorbance coefficients of these lanthanides are of the order of $0.1 \text{ M}^{-1} \text{ cm}^{-1}$ compared with $10^3$ to $10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for most fluorescent organic chromophores. Consequently, the fluorescence intensity of a solution of Tb$^{3+}$ or Eu$^{3+}$ is orders of magnitude lower than
that of an equimolar solution of a fluorescent organic chromophore. However, the low absorbance of dilute solutions of Tb$^{3+}$ or Eu$^{3+}$ is not a barrier to energy transfer studies. The 488 nm line of an argon-ion laser conveniently excites the $^7F_6 \rightarrow ^5D_4$ transition of Tb$^{3+}$, and the 580 nm output of a rhodamine 6G dye laser excites the $^7F_0 \rightarrow ^5D_0$ transition of Eu$^{3+}$ (10, 18).

Chelates of Tb$^{3+}$ instead of the simple Tb$^{3+}$ cation have been used as donors in rapid-diffusion energy transfer studies for three reasons. First, a chelator with an aromatic ring can sensitize the emission of Tb$^{3+}$.

![Graph](https://via.placeholder.com/150)

**Figure 4** Fluorescence emission spectra of Tb$^{3+}$-EDTA and Eu$^{3+}$-EDTA.
Energy absorbed by an aromatic ring of such a chelate can be efficiently transferred to the adjacent lanthanide ion (30). The absorbance coefficient of an aromatic ring is orders of magnitude higher than that of Tb³⁺. Indeed, the Tb³⁺ fluorescence intensity of some chelates is \( 10^4 \) as large as that of unchelated Tb³⁺. Second, the excited state lifetime of chelated Tb³⁺ is longer than that of unchelated Tb³⁺ because fewer \( H_2O \) molecules are coordinated to the chelate than to the simple Tb³⁺ ion (11). The fluorescence of Tb³⁺ is quenched in proportion to the number of bound \( H_2O \) molecules (11). The longer excited state lifetimes and higher quantum yields (and hence \( R_0 \) values) of Tb³⁺ chelates compared with Tb³⁺ alone make the chelates better donors for rapid-diffusion energy transfer studies. Third, the net charge of a chelate can be varied, whereas that of the lanthanide ion alone is fixed at +3. Chelates with net charges ranging from -3 to +1 have been prepared. As will be discussed shortly, a series of chelates with different net charges can be used to probe electrostatic effects on the spatial distribution of donors and acceptors.

\( \text{Tb}^{3+} \) chelated to three molecules of dipicolinate is a suitable energy donor for rapid-diffusion limit studies (1). This chelate, which has a net charge of -3, can be efficiently excited in the ultraviolet because of the strong pyridyl absorption band (1). Meares and co-workers (15, 32) have synthesized analogs of ethylenediamine-tetraacetic acid (EDTA) that also are valuable chelating agents for energy transfer studies (Figure 5). The terbium chelates of EDTA, HED3A, and BED2A have net charges of -1, 0, and +1, respectively. Benzyl-HED3A (net charge = 0) and other analogs of EDTA bearing aromatic side chains can be synthesized starting from phenylalanine, tyrosine, or tryptophan (32). Chelators bearing reactive groups have been prepared by converting \( \mu \)-nitrophenylalanine to nitrobenzyl-EDTA. The nitro group is reduced to an amine and then treated with thiophosgene, bromoacetyl bromide, or nitrous acid to yield the isothiocyanate, bromoacetamide, or diazonium derivative, respectively. Palmitimidophenyl-EDTA, which is suitable for energy transfer studies of membrane systems, has been synthesized by acylating aminophenyl-EDTA. The versatility of these synthetic procedures makes it feasible to prepare chelating analogs of a wide variety of biomolecules.

**EXPERIMENTAL TESTS OF THE THEORY**

The theory of diffusion-enhanced energy transfer in the intermediate range was first tested by Elkana et al (5). Naphthalene, which has a \( \tau_0 \) of about 100 nsec, served as the energy donor, and anthranilic acid as the
energy acceptor. The diffusion coefficient was varied by using a series of solvents ranging in viscosity from 0.6 cp (methanol) to 1000 cp (glycerol). The observed dependence of the transfer efficiency on $D$ agreed qualitatively with the theory. As can be seen from Figure 1, diffusion is expected to have only a small effect on transfer efficiency for $\tau_0 = 100$ ns and $D = 10^{-5}$ cm$^2$/sec. The rapid-diffusion limit could not be approached in these experiments because the excited-state lifetime of naphthalene was too short. This difficulty was obviated by the subsequent use of terbium dipicolinate as the energy donor. The 2.1 msec lifetime of this chelate enabled Thomas and co-workers (24) to test the theory from the static to the rapid-diffusion limit. In these experiments, rhodamine B was the energy acceptor. The diffusion coefficient was varied over seven orders of magnitude by changing the temperature and concentration of glycerol in glycerol-water mixtures. The observed transfer efficiencies, ranging from 2 to 94%, were in excellent agreement with the theoretical curve (Figure 6). As predicted, the transfer efficiency approached a maximal value when $D$ was greater than about $10^{-6}$ cm$^2$/sec. Thus, the rapid-diffusion limit was attained when terbium dipicolinate was the donor in water at room temperature.
In the static limit or intermediate range, the emission kinetics are expected to be nonexponential because each donor is surrounded by a different distribution of acceptors during its excited-state lifetime (7). In contrast, all donors become equivalent in the rapid-diffusion limit, and so a single value of $k_T$ is expected. This prediction was tested using terbium dipicolinate as the donor and rhodamine B as the acceptor. The emission in fact followed single-exponential decay kinetics (24). In addition, $k_T$ was found to be proportional to the acceptor concentration, as predicted by Equation 6. These experiments also showed that energy transfer in the rapid-diffusion limit occurs at very low acceptor concentrations. The transfer efficiency was 50% when the acceptor concentration was 0.67 μM, three orders of magnitude less than the concentration required to achieve the same transfer efficiency in the absence of diffusion.

The theory has also been tested in a model membrane system in which donors and acceptors diffused in two dimensions (27). The donor was terbium chelated to palmitamidophenyl-EDTA. The acceptor was either

![Graph](image)

*Figure 6* Observed dependence of the transfer efficiency on the diffusion coefficient (6). The donor was terbium dipicolinate and the acceptor was 10 μM rhodamine B in glycerol-water mixtures.
Co$^{3+}$ bound to the same type of chelator or phosphatidylethanolamine labeled in its head group with rhodamine or eosin. The host phospholipid was dipalmitylophosphatidylcholine, which has a phase transition at 42°C. The transfer efficiency increased markedly at this temperature, as predicted by the theory. These measurements also showed that the diffusion coefficient in the fluid phase was about $10^{-7}$ cm$^2$/sec, in agreement with the value measured by other techniques.

Equation 11 for determining closest approach distances to chromophores in membranes was tested using terbium dipicolinate as the donor trapped inside vesicles made of phosphatidylcholine (1). The acceptor was eosin attached covalently to a small proportion of the phospholipid head groups. A transfer efficiency of 58% was observed when only one eosin molecule was present per 1000 phospholipids ($q_A = 0.001$). According to Equation 11, this result shows that the distance of closest approach between terbium dipicolinate and eosin at the inner surface of the vesicle was 10 Å. The same $a$ was obtained at two other values of $q_A$. A closest approach distance of 10 Å indicates that the eosin is near the membrane surface, as expected for a chromophore that is attached to a phospholipid head group.

**EXCHANGE-INTERACTION CONTRIBUTION**

The preceding equations assume that dipole-dipole coupling ( Förster transfer) is dominant in diffusion-enhanced energy transfer and that other mechanisms of energy transfer such as exchange interactions are negligible. However, the observed transfer rates for a variety of donor-acceptor pairs were greater than could be accounted for by dipole-dipole coupling (15, 16). For example, the observed transfer rate from Tb$^{3+}$-HED3A (net charge = 0) to Co$^{3+}$-EDTA (net charge = −1) in solution is $8.35 \times 10^6 c_A$ sec$^{-1}$ (where $c_A$ is the molar concentration of the energy acceptor), whereas the calculated rate for dipole-dipole coupling is $4.23 \times 10^6 c_A$ sec$^{-1}$ [for $a = 8$ Å, a distance derived from space-filling models and X-ray crystallographic data, see Ref. (14, 29)]. A closest approach distance of 3 Å would make the observed and calculated rates equal, but such a value of $a$ is clearly impossible for these chelates. Thus, it seemed likely that most of the observed transfer for this donor-acceptor pair came from exchange interactions arising from overlap of their electron clouds.

At what donor-acceptor separations do exchange interactions become important? Meares et al (16) approached this question by altering the spatial distribution of charged donors and acceptors by varying the ionic strength of the solution because each donor is influenced by a long
Localization of Chromophores in Soluble Proteins

Human serum transferrin, a monomeric 81,400 dalton protein, contains two specific sites (called A and B) for metal ions such as Fe$^{3+}$. Yeh & Meares (31) carried out rapid-limit energy transfer measurements to determine the locations of these sites relative to the surface of the protein. The donor was the electrically neutral Tb$^{3+}$-HED3A chelate and the acceptor was Fe$^{3+}$ bound to either the A or B or both sites of transferrin. The $R_0$ value for this system is 27 Å. The observed transfer rates were an order of magnitude smaller than calculated for sites on the protein surface. Hence, the metal-binding sites of transferrin are buried and so only dipole-dipole transfer need be considered. An analysis of the data indicated that sites A and B are not more than 17 Å away from the surface. The closest approach distance depended on the nature of the anion associated with Fe$^{3+}$. The ferric acceptor became less accessible when (bi)carbonate ion was replaced by the larger oxalate ion, suggesting that the anion lies between the bound metal ion and the solution.

RNA polymerase from Escherichia coli is a complex enzyme composed of five subunits $\alpha_2\beta\beta'\sigma$. The DNA-directed synthesis of RNA by this enzyme is strongly inhibited by the antibiotic rifamycin, which appears to bind to the $\beta$ subunit (9). Affinity-labeling studies indicate, however, that the bound inhibitor is within 10 Å of nearly all the subunits. The rifamycin binding site on RNA polymerase appears to lie in the path of the growing RNA chain, since only di- and trinucleotides may be formed by the enzyme in the presence of rifamycin. Another inhibitor of this enzyme is Cibacron Blue, a dye that binds to the dinucleotide fold of a variety of nucleotide-binding enzymes (28). The binding site for Cibacron Blue is thought to be distinct from that for rifamycin (12). Information concerning the location of these inhibitor sites is clearly of interest in understanding the mechanism of their effect on RNA polymerase. Meares & Rice (15) found that the rate constant for energy transfer from Tb$^{3+}$-Bzl-HED3A or Tb$^{3+}$-HED3A to enzyme-bound rifamycin was an order of magnitude greater than the value calculated for dipole-dipole transfer, assuming that these groups can make direct contact. It is evident that rifamycin is bound so that it is highly accessible to small molecules in solution. Transfer by the exchange mechanism is clearly the dominant process here. In fact, the transfer rate to enzyme-bound rifamycin was only a factor of two less than the transfer rate to free rifamycin in solution, indicating that rifamycin is highly exposed when bound to RNA polymerase. In contrast, there was little energy transfer to enzyme-bound Cibacron Blue, indicating that this dye is quite inaccessible to donors in solution.
Localization of Chromophores in Membranes

The position of the 11-cis retinal chromophore of rhodopsin relative to the intradisc and extradisc membrane surfaces has recently been investigated by Thomas & Stryer (25). The donor in their study was terbium-dipicolinate and the acceptor was 11-cis retinal. Vesicles containing terbium-dipicolinate in their inner aqueous space were prepared by sonicating disc membranes in the presence of this chelate and chromatographing this mixture on a gel filtration column. The sidedness of rhodopsin in these vesicles was the same as in native disc membranes. The transfer efficiency determined from the emission kinetics (Figure 8) was 43%. The closest approach distance was then calculated according to Equation 11. For $R_0 = 46.7$ Å and an average vesicle diameter of 650 Å, an $E$ of 43% corresponds to an $a$ of 22 Å from the inner aqueous space. The distance of closest approach from the outside was then determined by adding terbium-dipicolinate to a suspension of already formed vesicles. According to Equation 8, the observed $E$ of 12% corresponds to an $a$ of 28 Å from the outside. These closest approach distances show that the retinal chromophore is far from both surfaces of the disc membrane.

![Figure 8](image)

Figure 8  Emission kinetics of Tb$^{3+}$-dipicolinate trapped inside retinal disc membrane vesicles (10). The fluorescence intensity (plotted on a logarithmic scale) is shown as a function of time after a 1 μsec exciting light pulse. Unbleached membranes, circles; partially bleached, triangles; completely bleached, squares. The excited-state lifetime increases on bleaching because of the disappearance of 11-cis retinal, the energy acceptor. The observed decay curves are monoexponential, as expected for the rapid-diffusion limit.
Hence, the transverse location of the retinal chromophore is near the center of the hydrophobic core of the disc membrane. These findings imply that the conformational changes induced by photoisomerization are transmitted through a distance of at least 20 Å within the rhodopsin molecule to trigger subsequent events in vision, such as the catalysis of GTP-GDP exchange in transducin, an amplifier protein.

Diffusion-enhanced energy transfer can also be used to study the charge density near an acceptor chromophore. In an experiment of this kind, the effects of the net charge of the donor and of ionic strength on the transfer kinetics are measured. This experimental approach was used in a recent study of the depth of the retinal chromophore in purple membrane sheets from Halobacterium halobium (26). Three terbium chelates served as energy donors: trinegative terbium-dipicolinate \((R_0 = 57 \text{ Å})\), uninnegative \(\text{Tb}^{3+}-\text{Bzl-EDTA}\) \((R_0 = 52 \text{ Å})\), and neutral \(\text{Tb}^{3+}-\text{HED3A}\) \((R_0 = 50\text{ Å})\). At low ionic strength, the transfer efficiencies for the trinegative and uninnegative chelates were much lower than for the neutral chelate. At high ionic strength (\(\sim 2\text{M NaCl}\)), the transfer efficiencies of the charged chelates approached the value of the neutral one, which was independent of the salt concentration. It seems likely that the negatively charged chelates exhibited low transfer efficiencies at low ionic strength because they were repelled by negatively charged groups near the retinal.

CONCLUSIONS

Diffusion-enhanced energy transfer is becoming a valuable technique for elucidating facets of the structure and dynamics of biological macromolecules. The theory for this type of energy transfer has been tested in well-defined systems and shown to be valid. In the intermediate range \((D\tau_0/s^2 \approx 1)\), the transfer rate is sensitive to the magnitude of the diffusion coefficient. In the rapid-diffusion limit \((D\tau_0/s^2 \gg 1)\), the transfer rate is highly responsive to the distance of closest approach of the donor and acceptor. Dipole-dipole coupling ( Förster transfer) is the dominant transfer mechanism until the donor and acceptor are nearly in contact. At a separation of about 11 Å, exchange interactions become important.

Terbium chelates have proven to be choice donors for diffusion-enhanced transfer studies because they have millisecond lifetimes and a small radius (\(\sim 4 \text{ Å}\)). Translational motions corresponding to \(D\) from \(10^{-6}\) to \(10^{-10}\) cm\(^2\)/sec can be monitored with these donors. This range is important for macromolecules in solution and in membranes. Diffusion-enhanced transfer is quite sensitive to small changes in \(D\). This technique
can provide accurate values of $D$ if $R_0$, $a$, and $\tau_0$ are known. Diffusion-enhanced transfer has already provided insight into the dynamics of oligopeptide chains. The folding of proteins might also be fruitfully studied in this way. It will also be interesting to explore motions of swinging arms of multienzyme complexes and other assemblies by diffusion-enhanced transfer. Rapid-diffusion limit measurements are well-suited to determining whether a chromophore at a specific site in a protein is near a surface. This technique can readily be applied if the protein-bound chromophore absorbs at wavelengths longer than 470 nm. Terbium (or europium) chelates can be used as long-lived donors to measure closest approach distances. Small changes in these distances lead to large changes in the transfer rate. For example, the transfer rate due to exchange interactions more than doubles when $a$ changes from 9 to 8 Å. Moreover, the dependence of the transfer rate on the charge of the donor chelate provides information about the net charge of the protein surface. Rapid-limit transfer measurements should be very sensitive to conformational transitions that alter the location of a chromophore relative to the surface of the macromolecule or change the charge distribution in its vicinity. These new aspects of energy transfer spectroscopy complement static-limit measurements, which are best suited to ascertaining the distance between two defined sites.

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