

INTRAMOLECULAR DYNAMICS OF CHAIN MOLECULES MONITORED BY FLUCTUATIONS IN EFFICIENCY OF EXCITATION ENERGY TRANSFER

A Theoretical Study

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ABSTRACT The fluorescence quantum yield of a polymer molecule to which an energy donor chromophore and an energy acceptor chromophore are attached depends on the distance between the donor and acceptor chromophores. If this distance fluctuates with time, the fluorescence intensity is expected to fluctuate as well, and the time course of the intensity fluctuations will be correlated with the time course of the changes in the interchromophore distance. The intensity fluctuations are experimentally measurable if the number of illuminated molecules is small. A theoretical treatment of such fluorescence intensity fluctuations is presented in terms of a parameter that describes the polymer chain dynamics. Computer simulations were performed to illustrate the dependence of the autocorrelation function of the intensity fluctuations on the polymer chain conformation, the interchromophore energy transfer properties, and the macromolecular dynamics. These simulations demonstrate that the intensity fluctuations due to nonradiative energy transfer between chromophores attached to polymer chains can be large enough to be experimentally useful in the study of intramolecular dynamics of macromolecules.

INTRODUCTION

Various important properties of polymeric substances depend on the dynamics of the macromolecules that constitute them. Thus, the intramolecular chain dynamics are expected to affect the kinetics, and possibly the pattern, of the folding of biological macromolecules, the rheological properties of polymers and polymer solutions, and the facility of conformational interconversions of polymer chains. The problem of chain dynamics can be addressed at different levels: by studying local dynamics involving a small number of bonds along the main chain or side chains, or by studying the dynamics of global changes in the macromolecular conformation. Although the local and global dynamics are physically interrelated, there is a useful distinction between the two because they are investigated by different experimental techniques. The present study is primarily concerned with the global dynamics of chain molecules, as manifested by the thermal Brownian

motion of the molecular ends relative to one another in a given molecule.

The distance between the molecular ends of a polymer chain can be monitored by measuring the energy transfer between an energy donor and an energy acceptor attached to the chain ends, as was proposed and demonstrated by Stryer and Haugland (1). To be useful for studying the distances of the dimensions of macromolecules, the donor and acceptor chromophores attached to the polymer chain should exchange their energy by the long-range nonradiative mechanism, often referred to as the Förster mechanism, which may lead to measurable energy transfer over distances exceeding 50 Å. This type of energy transfer requires the fluorescence spectrum of the donor to overlap the absorption spectrum of the acceptor. The efficiency of energy transfer by this mechanism is a well-defined function of the distance between the donor and the acceptor, and is thus used to measure this distance. The probability, $n_{A \rightarrow B}$, of energy transfer from the donor A to the acceptor B is given by (2)

$$n_{A \rightarrow B} = \frac{9,000(\ln 10)\kappa^2\eta_0}{128\pi^5 n^4 N r^6 \tau} \int_0^\infty \frac{f(\bar{\nu})\epsilon(\bar{\nu})}{\bar{\nu}^4} d\bar{\nu} = \frac{1}{\tau} \left(\frac{R_0}{r} \right)^6, \quad (1)$$

A preliminary report on part of this work was presented at the 15th Jerusalem Symposium on Quantum Chemistry and Biochemistry: Intramolecular Dynamics (18).

where η_0 is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, N is Avogadro's number, r is the distance between the donor and acceptor chromophores, τ is the lifetime of the donor in the absence of the acceptor, $f(\bar{\nu})d\bar{\nu}$ is the normalized fluorescence intensity of the donor in the wavenumber range $\bar{\nu}$ to $\bar{\nu} + d\bar{\nu}$, $\epsilon(\bar{\nu})$ is the absorption coefficient of the acceptor at the wavenumber $\bar{\nu}$, and R_0 , as defined by Eq. 1, is the distance between A and B when there is 50% efficiency of energy transfer. κ^2 is a factor that expresses the orientational dependence of the probability of energy transfer. It has been shown (3) that the orientational dependence of the transfer probability can be made weak or insignificant by choosing donor and acceptor chromophores that exhibit low-limiting polarization properties for the electronic transitions involved in the transfer process. This will be assumed to be the case in the present study.

The end-to-end distance in flexible chain molecules is not a single value; the efficiency of energy transfer from donor to acceptor measured for an ensemble of such molecules is thus an average quantity from which it is not possible to evaluate $f(r)$, the distribution function of end-to-end distances. It is possible, however, to reconstruct $f(r)$ if one measures the fluorescence decay kinetics of the donor instead of the efficiency of energy transfer (4-6). If the conditions are such that the end-to-end distance does not change during the lifetime of the donor excited state, the donor fluorescence decays monoexponentially with a time constant of $(1/\tau) (1 + R_0^6/r^6)$ for every subpopulation of molecules having a donor-acceptor separation r . In response to an extremely short excitation pulse, the decay kinetics $I(t)$ for the entire population of molecules is thus given by

$$I(t) = k \int_0^\infty f(r) \exp - [(t/\tau) (1 + R_0^6/r^6)] dr, \quad (2)$$

where k is a proportionality factor. Obviously, $I(t)$ contains information about $f(r)$. To obtain this information, methods have been presented for the analysis of $I(t)$ (4-5).

Eq. 2 does not apply to cases in which the end-to-end distance of the chain molecules changes during the lifetime of the donor excited state because in this equation it is assumed that the only processes responsible for the change in population of excited molecules of a given end-to-end distance are spontaneous decay of the excited state and energy transfer. Any Brownian motion of the chain ends would enhance the rate of decay of $I(t)$ relative to that indicated by Eq. 2. This happens because the distribution of end-to-end distances of the molecules that have an excited donor starts to deviate from the equilibrium distribution, $f(r)$, as time increases after the excitation pulse, because molecules with a small r value have a faster decay of excitation than molecules with a large r value. If permitted, the subsequent rearrangement towards the equilibrium distribution by diffusion of the molecular ends would enhance the efficiency of energy transfer. Obviously,

ly, this enhancement in efficiency contains information about the Brownian motion of the chain ends relative to one another. The analysis of the fluorescence decay kinetics of donor chromophores in chain molecules that carry donor-acceptor pairs has been used as a method for the study of the intramolecular dynamics of chain molecules (6,7).

Because of the steep dependence of the efficiency of energy transfer on the donor-acceptor separation, r , changes in r by Brownian motion are readily reflected in the decay kinetics of the donor fluorescence. However, this technique is limited to cases in which the Brownian motion is not negligible during the donor fluorescence lifetime. Thus many problems of interest, such as chain dynamics in highly viscous solvents or in biological membranes, as well as slow structural fluctuations in folded biopolymers, are not approachable by the above method. Therefore, it may be valuable to extend the application of energy transfer to the study of slow molecular movements. As will be shown below, this can be accomplished by a different approach, which involves analyzing the fluorescence intensity fluctuations from an ensemble of a small number of chain molecules that carry donor-acceptor pairs. In the following sections, the theoretical background for this approach will be presented, as well as a method to analyze experiments concerned with fluorescence intensity fluctuations in systems involving energy transfer, which will yield information on the macromolecular dynamics. Note that the study of fluorescence intensity fluctuations from an ensemble of a small number of fluorophores is experimentally feasible (8-10).

BASIC CONSIDERATIONS

The idea underlying the method for studying macromolecular dynamics by fluctuations of the fluorescence intensity of donor-acceptor pairs is as follows. When a collection of chain molecules, in which each molecule carries a donor-acceptor pair, is excited by a light beam of steady intensity, the emitted fluorescence intensity of the donor chromophores is the sum of the intensities of the donors of the individual illuminated molecules, each of which is characterized at any given moment by its end-to-end distance. For an infinitely large collection of molecules, the observed fluorescence intensity is the value expected for the equilibrium end-to-end distribution of distances and does not vary with time. However, when a small number of molecules is illuminated by the excitation beam, their end-to-end distribution function may deviate from the equilibrium distribution; the degree of deviation varies with time, with concomitant fluctuations in the fluorescence intensity about the average value. The magnitude of these fluctuations relative to the time average intensity would obviously become larger as the sample size became smaller. The time scale on which the fluctuations occur would be related to the time scale on which the internal motions of

the chain molecules take place. The temporal behavior of the intensity fluctuations may thus yield information about the kinetics of the intramolecular dynamics of the macromolecules.

The time scale of intensity fluctuations is conveniently expressed by the time dependence of the autocorrelation function, $AC(\tau)$, of the fluorescence intensity, defined by:

$$AC(\tau) = (1/T) \int_0^T I(t) \cdot I(t + \tau) dt, \quad (3)$$

where $I(t)$ and $I(t + \tau)$ are the fluorescence intensities at times t and $t + \tau$, respectively. For long integration intervals T , $AC(\tau)$ averages out intensity variations in time that are completely random, but retains information about processes that change systematically with time. Note that analysis of fluorescence intensity fluctuations has been previously applied to a few other problems (8–17).

For a collection of molecules in which each molecule exhibits fluctuations in fluorescence intensity that are not correlated with those of the other molecules, there is a simple relationship between the autocorrelation function $AC(\tau)(n)$ of the intensity $I(t)(n)$ emitted by the collection of molecules (n in number) and the autocorrelation function $AC(\tau)$ of the intensity $I(t)$ emitted by a single molecule. This is a useful relationship because it is not experimentally feasible to study a single molecule, whereas theoretically, it is convenient to investigate the autocorrelation function of the fluorescence intensity emitted by a single molecule. The relationship is derived as follows:

$$I(t)(n) = \sum_{i=1}^n I_i(t), \quad (4)$$

where $I_i(t)$ is the intensity emitted at time t by the i th molecule. $AC(\tau)(n)$ is thus given by

$$\begin{aligned} AC(\tau)(n) &= \frac{1}{T} \int_0^T I(t)(n) \cdot I(t + \tau)(n) dt \\ &= \frac{1}{T} \int_0^T \sum_{i=1}^n I_i(t) \sum_{j=1}^n I_j(t + \tau) dt \\ &= \frac{1}{T} \int_0^T \sum_{i=1}^n I_i(t) \cdot I_i(t + \tau) dt \\ &\quad + \frac{1}{T} \int_0^T \sum_{i=1}^n \sum_{\substack{j=1 \\ i \neq j}}^n I_i(t) \cdot I_j(t + \tau) dt. \end{aligned} \quad (5)$$

For a collection of identical molecules the first term in Eq. 5 equals $n \cdot AC(\tau)$. The second term in this equation equals $n(n - 1)\langle I \rangle^2$ ($\langle I \rangle$ is the average intensity of a single molecule) because it is assumed that the intensities of the various molecules are not mutually correlated. Thus

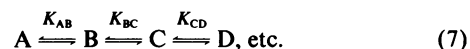
$$AC(\tau)(n) = n AC(\tau) + n(n - 1)\langle I \rangle^2. \quad (6)$$

Because the autocorrelation function of the fluorescence intensity of n molecules is readily deduced from that of a single molecule by use of Eq. 6, subsequent calculations

will refer to the analysis of the fluorescence intensity fluctuations of a single molecule. The ultimate aim is to analyze $AC(\tau)$ for the case involving intramolecular energy transfer in terms of macromolecular dynamics. To clarify the approach, $AC(\tau)$ for a molecule undergoing many reactions will be discussed first.¹

INTENSITY FLUCTUATIONS OF THE FLUORESCENCE OF A MOLECULE UNDERGOING MULTIPLE ISOMERIZATION REACTIONS

Let us consider a molecule that reversibly isomerizes into a series of different forms:



K_{AB} , K_{BC} , K_{CD} , etc. represent the corresponding equilibrium constants. The scheme represented in Eq. 7 does not necessarily imply that kinetically A can be converted into C only via B; in principle all possible isomerization paths may occur with their corresponding rate constants. Generally, the transitions between the various states do not have to obey first-order kinetics and the solutions of the kinetics behavior may require numerical analysis. The fluorescence intensity of the molecule will obviously fluctuate if the various isomers have different quantum yields. For a given intensity of the excitation light beam, let I_A , I_B , I_C , etc., be the intensity of the fluorescence emitted by the isomers represented by the corresponding subscripts. Given the kinetic behavior that controls the system described by Eq. 7, one can evaluate the probability of finding a given molecule in any isomeric form j at time $t + \tau$ if the molecule started out at time t in any given form i (where i and j designate any of the forms in which the molecule can be). Let $c_{ij}(\tau)$ designate this probability. Note that the function $c_{ij}(\tau)$ is actually the concentration of the isomeric form j that is obtained after a time interval τ , starting out with unit concentration of isomeric form i . At various times t , the molecule will be in one of the isomeric forms described in Eq. 7. Let us denote by F_A , F_B , F_C , etc., the fraction of time the molecule will be found in the isomeric form represented by the corresponding subscript. The quantities F_A , F_B , F_C , etc., are given by

$$F_A = 1/\sigma, F_B = K_{AB}/\sigma, F_C = K_{AB}K_{BC}/\sigma, \text{ etc.}, \quad (8)$$

where

$$\sigma = 1 + K_{AB} + K_{AB}K_{BC} + \dots \quad (9)$$

The time coordinate in the integral in Eq. 3 that serves to

¹One may also define an autocorrelation function $g(\tau)$ (see reference 12) in terms of the residuals $\delta I = I - \langle I \rangle$: $g(\tau) = 1/T \int_0^T \delta I(t) \cdot \delta I(t + \tau) dt$. It can be readily shown that $g(\tau) = AC(\tau) - \langle I \rangle^2$ and that $g(\tau)$ for n molecules, $g(\tau)(n)$ is related to that of a single molecule, $g(\tau)(1)$, by the following expression: $g(\tau)(n) = n G(\tau)(1)$.

evaluate $AC(\tau)$ can be subdivided into groups of intervals, each group being characterized by the isomeric form in which the molecule exists at time t . The autocorrelation function $AC(\tau)$ can thus be evaluated by summing the contributions from $AC_A(\tau)$, $AC_B(\tau)$, $AC_C(\tau)$, etc., each of which is obtained by integrating $I(t) \cdot I(t + \tau)$ over the intervals at which the molecule is in the form A, B, C, etc., respectively, at time t . Note that if the molecule starts out at time t in one isomeric form i , it may or may not be in that form at time $t + \tau$. In fact, it has the probability $c_{ii}(\tau)$ of being in this state at time $t + \tau$ and a probability $c_{ij}(\tau)$ of being in state j at time $t + \tau$. $AC_A(\tau)$, $AC_B(\tau)$, etc. may thus be expressed as follows:

$$\begin{aligned} AC_A(\tau) &= F_A I_A [I_A c_{AA}(\tau) + I_B c_{AB}(\tau) + I_C c_{AC}(\tau) + \dots] \\ AC_B(\tau) &= F_B I_B [I_A c_{BA}(\tau) + I_B c_{BB}(\tau) + I_C c_{BC}(\tau) + \dots] \\ AC_i(\tau) &= F_i I_i [I_A c_{iA}(\tau) + I_B c_{iB}(\tau) + I_C c_{iC}(\tau) + \dots] \\ &= F_i I_i \sum_j I_j c_{ij}(\tau). \quad (10) \end{aligned}$$

The autocorrelation function of the intensities is obtained by summation of all the $AC_i(\tau)$ contributions:

$$AC(\tau) = \sum_i AC_i(\tau) = \sum_i F_i I_i \sum_j I_j c_{ij}(\tau). \quad (11)$$

Eq. 11 expresses the time dependence of the autocorrelation function of the fluorescence intensities, $AC(\tau)$, in terms of the time evolutions $c_{ij}(\tau)$ of species j when one starts out with species i . The inverse process, i.e., the evaluation of the various functions $c_{ij}(\tau)$ from a single function $AC(\tau)$, is, however, not feasible unless simplifying circumstances prevail; e.g., when the kinetic behavior of the various processes can be linearized, when the number of reactions is small, and when additional information is available about the system. Some aspects of these problems have been treated by Elson and Magde (12), but they will not be pursued here because they are not required for the treatment of the intramolecular dynamics of chain molecules. It will be shown in the Appendix that much new information can be obtained about the system if the various species in Eq. 7 have different emission or excitation spectra (or both).

FLUORESCENCE INTENSITY FLUCTUATIONS OF ENERGY DONOR-ACCEPTOR PAIRS ATTACHED TO CHAIN MOLECULES

The above treatment, which expresses the autocorrelation function of the fluorescence intensity of a molecule undergoing isomerization reactions in terms of the kinetic behavior of the system, can be readily extended to the study of chain dynamics by intramolecular nonradiative energy transfer. The two cases become analogous if one views a chain with a given distance r between the donor and acceptor chromophores as an isomer of the chain, character-

ized by a fluorescence intensity that is proportional to $r^6/(r^6 + R_0^6)$. The following points should, however, be considered. (a) In contrast to the reactions described by Eq. 7, in the case of intramolecular energy transfer, there is a continuum of states for the polymeric molecules, where each state is defined by the distance r . (b) The transition from one state to another is not governed by mass-action laws, but rather by rules that describe Brownian motion. These aspects of intramolecular chain dynamics and energy transfer do not, however, require new conceptual approaches to analyze the fluorescence intensity fluctuations of a donor chromophore attached to a chain molecule that carries an energy acceptor somewhere else along the chain.

The analysis may be carried out as follows. Let us start with a molecule whose end-to-end distance is ρ at $t = 0$. (For simplicity, we assume that the donor and acceptor chromophores are attached to the chain ends, but the following argument is not restricted to such cases.) Let $N(\rho, r, \tau)dr$ denote the probability of finding this molecule at time τ with an end-to-end distance in the range of r to $r + dr$. $N(\rho, r, \tau)$ is obtained by solving the equation that governs the behavior of $N(r, \tau)$ subject to the initial conditions $N(r, 0) = \delta(\rho - r)$, where $N(r, \tau)dr$ is the fraction of molecules in an ensemble of polymer chains that have an end-to-end distance r at time τ . The change of $N(r, \tau)$ with time may be expressed as follows:

$$\frac{\partial N(r, \tau)}{\partial \tau} = -\frac{\partial}{\partial r} (4\pi r^2 J). \quad (12)$$

J designates the diffusional flux per unit area for one set of ends of the chains through the surface of a sphere of radius r , whose center is the origin of the coordinate system; the other ends of the chains are conceptually assumed to be at the center of the sphere. Obviously, the chain ends do not undergo free diffusion relative to one another because the internal restrictions in the chain molecules generate driving forces on the chain ends that are a function of r . These forces are responsible for the shape of the end-to-end distribution function of distances between the chain ends at equilibrium, $N_0(r)$, characteristic for each chain type. In other words, the standard chemical potential $\mu_0(r)$ of a population of molecules of end-to-end distance r is a function of r . Assuming that the average rate of change of r with time depends linearly on the gradient of the chemical potential $\mu(r, \tau)$ with respect to r , one obtains:

$$J = -\frac{1}{f} \frac{\partial \mu(r, \tau)}{\partial r} \cdot \frac{N(r, \tau)}{4\pi r^2}, \quad (13)$$

where f is a frictional coefficient. The chemical potential $\mu(r, \tau)$ may be expressed in terms of $\mu_0(r)$ and $N(r, \tau)$ as follows:

$$\mu(r, \tau) = \mu_0(r, \tau) + RT \ln [N(r, \tau)/4\pi r^2]. \quad (14)$$

Combining Eqs. 13 and 14 and the relation $J = 0$ at

equilibrium leads to

$$\mu_0(r) = -RT \ln [N_0(r)/4\pi r^2]. \quad (15)$$

The differential equation that governs the evolution of $N(r, \tau)$ with time is finally obtained by combining Eqs. 12–15:

$$\frac{\partial \bar{N}(r, \tau)}{\partial \tau} = D \frac{1}{N_0(r)} \cdot \frac{\partial}{\partial r} \left[N_0(r) \cdot \frac{\partial \bar{N}(r, \tau)}{\partial r} \right], \quad (16)$$

where $\bar{N}(r, \tau) = N(r, \tau)/N_0(r)$, and where $D = RT/f$ is the diffusion coefficient of one set of chain ends relative to the other (assumed to be independent of r , see reference 6). As stated above, to obtain the required function $N(\rho, r, \tau)$, Eq. 16 has to be solved subject to the initial conditions, $N(r, 0) = \delta(r - \rho)$. Note that there is only one adjustable parameter, D , in the solution of Eq. 16 because the equilibrium end-to-end distribution function $N_0(r)$ can be obtained independently by measuring the fluorescence decay kinetics of the donor under conditions where the internal Brownian motion is negligibly small during the lifetime of the donor excited state (4, 5).

As stated above, the problem of evaluating the autocorrelation function of the fluorescence intensity fluctuations of donor-acceptor pairs attached to flexible chain molecules is analogous to that of the isomers depicted in Eq. 7, if each molecule of an end-to-end separation r is viewed as an isomer characterized by a well-defined quantum yield. One may thus derive the appropriate expressions for the autocorrelation functions by arguments that are similar to those put forward in the derivation of Eqs. 10 and 11. In this derivation, $N_0(\rho)d\rho$ is analogous to F_i ; $N(\rho, r, \tau)dr$, to c_{ij} ; $\rho^6/(\rho^6 + R_0^6)$, to I_i ; and $r^6/(r^6 + R_0^6)$, to I_j . If we define $AC_\rho(\tau)d\rho$ as the molecule's contribution to the autocorrelation function when it starts out with an end-to-end distance in the range of ρ to $\rho + d\rho$, we obtain

$$AC_\rho(\tau)d\rho = N_0(\rho)d\rho \frac{\rho^6}{\rho^6 + R_0^6} \cdot \int_0^{R_{\max}} N(\rho, r, \tau) \frac{r^6}{r^6 + R_0^6} dr, \quad (17)$$

and

$$AC(\tau) = \int_0^{\rho_{\max}} AC_\rho(\tau)d\rho = \int_0^{\rho_{\max}} N_0(\rho) \frac{\rho^6}{\rho^6 + R_0^6} \int_0^{r_{\max}} N(\rho, r, \tau) \frac{r^6}{r^6 + R_0^6} dr d\rho. \quad (18)$$

Note that in terms of the dynamics of the chain molecules, the interpretation of $AC(\tau)$ in Eq. 18 should be straightforward. This is because (a) the dynamics has been characterized by a single adjustable parameter, D , (b) the fluorescence intensities are relatively simple, well-defined functions of the distance between the donor and acceptor chromophores, and (c) the equilibrium distribution function of distances between the donor and acceptor chromo-

phores can be obtained by independent experiments, as has been previously demonstrated. (4,5).

To illustrate the expected time course of the autocorrelation function $AC(\tau)$, computer simulations were carried out for a series of oligomer chain molecules. The end-to-end equilibrium distribution functions, $N_0(\rho)$, of these oligomers were previously measured by fluorescence decay techniques (5). The repeating unit in these compounds was N^5 -(2-hydroxyethyl)-L-glutaminy, and the number of units per chain varied from four to nine. The chains were tagged with a naphthalene chromophore at one and a dansyl chromophore at the other end, which served as energy donor and acceptor, respectively. In the simulations, different values of R_0 and D were used. Fig. 1 illustrates the effects of varying D on the shape of the autocorrelation function. In this figure, $AC(\tau)$ was evaluated for the eight-residue oligomer, assuming $R_0 = 25 \text{ \AA}$. Note that changing the value of D affects the time course of $AC(\tau)$ only by stretching or contracting the function along the τ -coordinate; otherwise, the function remains the same. This may actually be deduced by inspection of Eq. 16 because this equation becomes independent of D if one changes the time variable τ to $D\tau$.

The change of $AC(\tau)$ that occurs with change of $N_0(\tau)$ is illustrated in Fig. 2, in which the simulated $AC(\tau)$ is plotted for oligomers containing 4–8 repeating units. The end-to-end distribution functions used are those obtained previously (see reference 6, Fig. 11 and reference 18, Fig. 1). R_0 and D were assumed to be 20 \AA and $10^{-7} \text{ cm}^2/\text{s}$, respectively. Note that the shape of $AC(\tau)$ is significantly affected by the shape of the end-to-end distribution function. The change in $AC(\tau)$ that occurs with change in R_0 is illustrated in Fig. 3; this simulation relates to an oligopeptide of five repeating units and a diffusion coefficient of $10^{-7} \text{ cm}^2/\text{s}$. Obviously $AC(\tau)$ is quite sensitive to changes in R_0 . Note, however, that although $AC(\tau)$ depends on R_0 and $N_0(\tau)$, in addition to depending on D , this fact does not

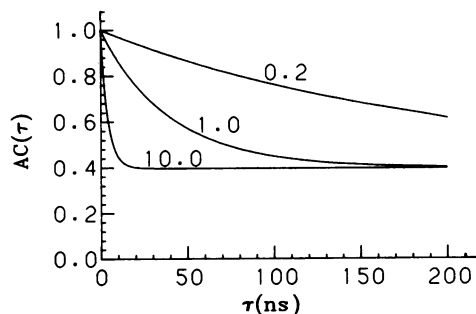


FIGURE 1 A simulation of $AC(\tau)$ of the donor fluorescence calculated for different diffusion coefficients of one molecular end relative to the other end. The calculations were performed for a single molecule of an eight-residue oligomer. The end-to-end distance distribution function for this oligomer was taken from Fig. 11 in reference 6. R_0 was assumed to be 25 \AA . The numbers marking the different curves are the assumed values for the end-to-end diffusion coefficients, in units of $10^{-7} \text{ cm}^2/\text{s}$. $AC(\tau)$ is given in arbitrary units. $AC(0)$ was set to 1.0.

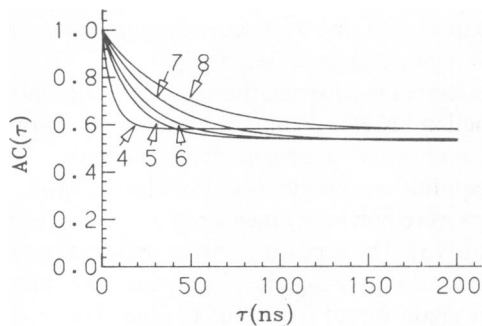


FIGURE 2 A simulation of $AC(\tau)$ of the donor fluorescence calculated for a single molecule of different obligomers whose end-to-end distances distribution functions were taken from Fig. 11 in reference 6 and Table II in reference 5. R_0 and D were assumed to be 20 Å and 10^{-7} cm²/s, respectively. The number marking each curve gives the number of N^5 -(2-hydroxyethyl)-L-glutaminyl residues in each oligomer. $AC(\tau)$ is given in arbitrary units and was set to unity at zero time.

complicate the evaluation of D from measurements of $AC(\tau)$ because R_0 and $N_0(\tau)$ can be obtained by independent measurements. To increase the accuracy of the data analysis, one is interested in obtaining a large difference between $AC(\tau = 0)$ and $AC(\tau = \infty)$. Fig. 3 shows that this can be controlled, within limits, by proper choice of the value of R_0 , because this parameter depends on the spectral properties of the donor and acceptor chromophores chosen. Note that Figs. 1–3 relate to $AC(\tau)$ of single molecules; the values for n molecules can be obtained by using Eq. 5. The difference between $AC(\tau = 0)$ and $AC(\tau = \infty)$ diminishes as n is increased.

Finally, analysis of the time course of $AC(\tau) - AC(\tau = \infty)$ in the simulations performed indicates that it is close to monoexponential decay. Thus, the shape of $AC(\tau)$ cannot serve as a diagnostic tool that distinguishes between intensity fluctuations that originate from conformational fluctuations of the type discussed above and intensity

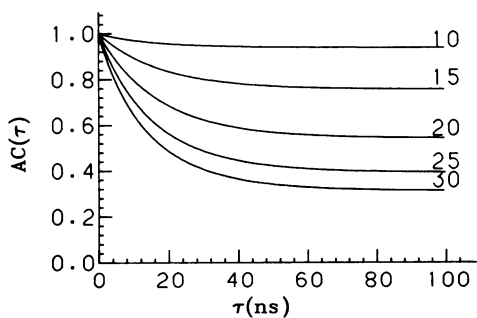


FIGURE 3 A simulation of $AC(\tau)$ calculated for different R_0 values. The calculations were performed for a single molecule of the oligomer consisting of five N^5 -(2-hydroxyethyl)-L-glutaminyl residues. The structure of the peptide and its end-to-end distance distribution function were given in Fig. 11 of reference 6. The diffusion coefficient of one molecular end relative to the other was assumed to be 10^{-7} cm²/s. The numbers marking the different curves designate the assumed values of R_0 in ångströms. $AC(0)$ was set to 1.0.

fluctuations due to simple reactions that obey the mass action law. As in similar cases, one has to rely on independent information or to invoke acceptable assumptions when one proposes a model to describe the system studied.

DISCUSSION

If a flexible macromolecule carries a pair of chromophores between which resonance energy transfer can occur, the efficiency of energy transfer varies with the inverse sixth power of the interchromophore distance. The fluorescence quantum yield of the donor will thus depend on the macromolecular conformation and will fluctuate on the same time scale as that on which the macromolecule changes conformation. The efficiency of resonance energy transfer also depends on the orientation of the transition dipole moments of the donor and the acceptor chromophores relative to one another and relative to the vector joining them. Thus, the fluorescence intensity of the donor or acceptor will also generally depend on the Brownian rotatory motion of the chromophores. It has been shown, however, that the angular dependence of the efficiency of energy transfer can be attenuated or even reduced to a negligible level by choosing chromophores that exhibit low polarization in their absorption or emission at the pertinent electronic transitions (3). In the present study this is assumed to be the case; thus the fluctuations in the fluorescence intensity reflect predominantly or exclusively the fluctuations in the distance between the donor and acceptor chromophores.

The relationship between the molecular dynamics and the intensity fluctuations has been quantitatively evaluated above. Note that the arguments we presented concerning the intensity fluctuations of the donor fluorescence apply equally well to the sensitized fluorescence of the acceptor when only minor, straightforward modifications that are related to the expressions for the intensity of the sensitized fluorescence are inserted in Eqs. 17 and 18. As has been demonstrated by the computer simulations described, the magnitude of $AC(\tau)$ changes significantly on the τ scale, which demonstrates that the analysis of $AC(\tau)$ in terms of the macromolecular dynamics is feasible in principle.

Although not easy, measurement of the fluctuations of the fluorescence intensity from a small number of illuminated molecules has been accomplished in a few laboratories. Probably the most serious experimental problem is the bleaching of the chromophores that occurs because of the high intensity of the excitation beam which is required to obtain sufficient fluorescence intensity from the small number of illuminated molecules. The effect of the bleaching process on the measured autocorrelation function can be readily evaluated when the bleaching is a first-order reaction with respect to the chromophore concentration, with a rate constant that is of the same order of magnitude for all isomers. Under such circumstances the various

concentration functions $c_{ij}(\tau)$ should be replaced by the functions $c_{ij}(\tau) \exp - (k_b \tau)$, where k_b is the bleaching rate constant (which will generally depend on the intensity of the excitation light). Substituting this term into Eq. 11 leads to an expression for the autocorrelation function $AC^b(\tau)$ for the case when bleaching occurs

$$AC^b(\tau) = AC(\tau) \exp(-k_b \tau). \quad (19)$$

It is desirable for k_b to be as small as possible, and in any case for the bleaching reaction to be not much faster than the other reactions under study. These objectives can be accomplished by lowering the intensity of the excitation light at the expense of the signal-to-noise ratio for a given time of measurement.

To extend the time of measurement, one may periodically select fresh sample volumes to be illuminated, and sum up the results, or instead, one may measure from a steady constant stream of solution that flows past the cross-section of the illuminating beam. The effect of the latter procedure on the shape of the autocorrelation function can be readily evaluated. Let us treat a model case of constant flow velocity v across a light beam, which has an intensity profile that has a square shape. Thus, for a single molecule flowing past the beam, the modified autocorrelation function $AC'(\tau)$ is given by

$$AC'(\tau) = \frac{1}{T} \int_{T'}^{T'+(\ell/v-\tau)} I(t) \cdot I(t + \tau) dt, \quad (20)$$

where T is the duration of the experiment, T' is the time at which the molecule enters the light beam, ℓ/v is duration of its stay in the light beam, and $\ell/v - \tau$ is the duration of the time interval at which we obtain nonzero contributions to $AC'(\tau)$. Comparing Eq. 20 with Eq. 3 yields the following relation between $AC'(\tau)$ and $AC(\tau)$,

$$AC'(\tau) = \frac{\ell/v - \tau}{T} AC(\tau) \quad (21)$$

for cases when $\ell/v - \tau > 0$. No information can be obtained about $AC(\tau)$ when $\ell/v - \tau \leq 0$ because $AC'(\tau) = 0$. This relation limits the permissible range for the velocity of flow, v , for a given τ of interest. If we define V_i as the illuminated volume, then the total volume, V_T , of solution passing through the light beam in the time interval T is given by

$$V_T = \frac{T}{\ell/v} V_i. \quad (22)$$

Let there be N molecules in this volume. Obviously, the positions and dynamic behavior of the various molecules are not correlated; thus we may use Eq. 5 for the evaluation of the autocorrelation function of intensities of the N

molecules:

$$\begin{aligned} AC'(\tau)(N) &= N AC'(\tau) + N(N-1) \langle I \rangle^2 \\ &= N \frac{\ell/v - \tau}{T} AC(\tau) + N(N-1) \left\langle I_c \cdot \frac{\ell/v}{T} \right\rangle^2 \\ &= N \frac{\ell/v}{T} \cdot \frac{\ell/v - \tau}{\ell/v} AC(\tau) \\ &\quad + N(N-1) \left(\frac{\ell/v}{T} \right)^2 \langle I_c \rangle^2 \\ &= N \frac{V_i}{V_T} \frac{\ell/v - \tau}{\ell/v} AC(\tau) + N(N-1) \left(\frac{V_i}{V_T} \right)^2 \langle I_c \rangle^2 \\ &= n \frac{\ell/v - \tau}{\ell/v} AC(\tau) + n \left(n - \frac{V_i}{V_T} \right) \langle I_c \rangle^2, \quad (23) \end{aligned}$$

where $\langle I_c \rangle$ is the average intensity of a molecule that is continuously illuminated by the excitation light beam and n is the average number of molecules in the light beam. Eq. 23 permits the evaluation of the autocorrelation function of a single molecule, $AC(\tau)$, if $AC'(\tau)(N)$ is measured in a flow experiment.

In principle, one should add to the autocorrelation function the effect of translational diffusion of the fluorescing chromophores in and out of the excitation beam. In fact, fluctuations of fluorescence intensity were used in the pioneering work of Elson, Webb, and Magde (8,12,14-16) to measure translational diffusion coefficients. Note, however, that the time scale of $AC(\tau)$ due to translational diffusion depends on the dimensions of the cross section of the incident beam, whereas $AC(\tau)$ due to the flexibility of the polymer chains originating in energy transfer is not dependent on the dimensions of the incident beam because the intramolecular Brownian motion is limited to the dimensions of the chain molecules, which are far smaller than the dimensions of the incident beam. Thus, the contributions of the two processes to $AC(\tau)$ can be readily separated, although the intramolecular diffusion coefficient may be an order of magnitude smaller than the diffusion coefficient of the entire molecule (5).

The method described to study the dynamics of macromolecules by analysis of fluorescence fluctuations requires that the number of molecules illuminated at any given instant be small. Whereas this requirement imposes experimental difficulties (which, as mentioned, can be overcome), many systems that are candidates for this kind of study may not be able to be studied as large numbers of molecules anyway. One example is when macromolecules are embedded in, or associated with, cell membranes. Although we have implied that the donor and acceptor chromophores are attached to the ends of chain molecules and we have referred to the end-to-end distances of the molecules, the method described may be applied to other cases in which the chromophores are attached to other sites

of interest in the macromolecule. Finally, fluctuations of fluorescence intensity of donor-acceptor pairs attached to macromolecules may not only be used to study the dynamics of flexible random coil chains, but also to follow fluctuations in conformation of ordered structures. Thus, we may have a way to study conformational fluctuations in globular proteins. Of course, under such circumstances the model that implies that there is relative diffusional motion of the chromophores (which underlies the analysis that led to Eq. 18) is not applicable. In each case studied, a model that is plausible for the system under consideration should be selected.

APPENDIX

In a system undergoing a series of reactions described by Eq. 7, the analysis of fluorescence intensity fluctuations can be extended if the various species have different emission or absorption spectra (or both) and if the fluorescence intensity fluctuations are measured simultaneously at pairs of wavelengths. Because we assumed the various species exhibit different intensities at different wavelengths, the results at one wavelength accentuate the contributions of some species relative to those at other wavelengths. As will be shown below, the cross correlations of the intensities at pairs of wavelengths can be readily analyzed to yield the various time dependent concentrations $c_{ij}(\tau)$. If the system described by Eq. 7 contains n species, we shall assume that the fluctuations are measured at n wavelengths and that the time sequences at pairs of wavelengths λ_k and λ_l are compared by cross correlations designated $CC^{\lambda_k, \lambda_l}$. By an analogous treatment to that leading to Eq. 11 one obtains

$$CC^{\lambda_k, \lambda_l}(\tau) = \sum_i F_i I_i^{\lambda_k} \sum_j I_j^{\lambda_l} c_{ij}(\tau), \quad (\text{A1})$$

where $I_i^{\lambda_k}$ and $I_j^{\lambda_l}$ are the intensities of species i at the k th wavelength, λ_k , and species j at the l th wavelength, λ_l , respectively. From the matrix of $CC^{\lambda_k, \lambda_l}(\tau)$ at any given τ , one can readily deduce the matrix $c_{ij}(\tau)$ at this value of τ by standard techniques. Eq. A1 can be rewritten as follows:

$$\begin{aligned} \underline{CC} &= \underline{I} \cdot \underline{F} \cdot \underline{c} \cdot \underline{I}^T, & (\text{A2}) \\ \underline{CC} &= \begin{pmatrix} CC^{\lambda_1, \lambda_1} & CC^{\lambda_1, \lambda_2} & \dots & CC^{\lambda_1, \lambda_n} \\ CC^{\lambda_2, \lambda_1} & CC^{\lambda_2, \lambda_2} & \dots & CC^{\lambda_2, \lambda_n} \\ \vdots & \vdots & \ddots & \vdots \\ CC^{\lambda_n, \lambda_1} & CC^{\lambda_n, \lambda_2} & \dots & CC^{\lambda_n, \lambda_n} \end{pmatrix}, \\ \underline{I} &= \begin{pmatrix} I_1^{\lambda_1} & I_2^{\lambda_1} & \dots & I_n^{\lambda_1} \\ I_1^{\lambda_2} & I_2^{\lambda_2} & \dots & I_n^{\lambda_2} \\ \vdots & \vdots & \ddots & \vdots \\ I_1^{\lambda_n} & I_2^{\lambda_n} & \dots & I_n^{\lambda_n} \end{pmatrix}, \\ \underline{F} &= \begin{pmatrix} F_1 & 0 & \dots & 0 \\ 0 & F_2 & \dots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & \dots & F_n \end{pmatrix}, \end{aligned}$$

$$\underline{c} = \begin{pmatrix} c_{11} & c_{12} & \dots & c_{1n} \\ c_{21} & c_{22} & \dots & c_{2n} \\ \vdots & \vdots & \ddots & \vdots \\ c_{n1} & c_{n2} & \dots & c_{nn} \end{pmatrix},$$

and \tilde{I} is the transpose of I . The matrix c may thus be evaluated from \underline{CC} by

$$\underline{c} = \underline{F}^{-1} (\underline{I}^T)^{-1} \underline{CC} \tilde{I}^{-1}. \quad (\text{A3})$$

Thus, the concentration of the various species at any time τ can be evaluated from the matrix of values of the various cross-correlation functions at τ without resorting to a model that describes the various kinetic steps. In principle, this is advantageous when fluctuations are analyzed because fluctuations are most pronounced in systems that have a small number of molecules. For such systems, linearization procedures that assume small deviations from equilibrium values may thus be inapplicable. In conclusion, note that $CC^{\lambda_k, \lambda_l}(\tau)(n)$ for n molecules is related to $CC^{\lambda_k, \lambda_l}(\tau)$ of a single molecule by the relation:

$$CC^{\lambda_k, \lambda_l}(\tau)(n) = n CC^{\lambda_k, \lambda_l}(\tau) + n(n-1) \langle I^{\lambda_k} \rangle \langle I^{\lambda_l} \rangle, \quad (\text{A4})$$

where $\langle I^{\lambda_k} \rangle$ and $\langle I^{\lambda_l} \rangle$ are the average intensities at wavelengths λ_k and λ_l , respectively. Eq. A4 is derived by the same procedure as Eq. 5.

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