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# Förster-Type Energy Transfer as a Probe for Changes in Local Fluctuations of the Protein Matrix†

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ABSTRACT: Much evidence, on both theoretical and experimental sides, indicates the importance of local fluctuations (in energy levels, conformational substates, etc.) of the macromolecular matrix in the biological activity of proteins. We describe here a novel application of the Förster-type energy-transfer process capable of monitoring changes both in local fluctuations and in conformational states of macromolecules. A new energy-transfer parameter, $f$, is defined as an average transfer efficiency, $\langle E \rangle$, normalized by the actual average quantum efficiency of the donor fluorescence, $\langle \phi_D \rangle$. A simple oscillator model (for a one donor–one acceptor system) is presented to show the sensitivity of this parameter to changes in amplitudes of local fluctuations. The different modes of averaging (static, dynamic, and intermediate cases) occurring for a given value of the average transfer rate, $\langle k_t \rangle$, and the experimental requirements as well as limitations of the method are also discussed. The experimental tests were performed on the ribonuclease $T_1$–pyridoxamine 5′-phosphate conjugate (a one donor–one acceptor system) by studying the change of the $f$ parameter with temperature, an environmental parameter expectedly perturbing local fluctuations of proteins. The parameter $f$ increased with increasing temperature as expected on the basis of the oscillator model, suggesting that it really reflects changes of fluctuation amplitudes (significant changes in the orientation factor, $\kappa^2$, as well as in the spectral properties of the fluorophores can be excluded by anisotropy measurements and spectral investigations). Possibilities of the general applicability of the method are also discussed.

The early work of Linderstrom-Lang (Linderstrom-Lang & Schellman, 1959) suggested that protein molecules are not rigid, solidlike entities but rather show conformational fluctuations. Much subsequent research has been concerned with the investigation of protein dynamics. Recent reviews (Careri et al., 1975, 1979; Gurd & Rothgeb, 1979; McCammon & Karplus, 1980; Karplus & McCammon, 1981; Welch et al., 1982) summarize most of the relevant studies.

Naturally, the question arises as to the role of fluctuations in the functional properties of proteins. Heretofore, conventional experimental techniques have yielded little direct evidence thereupon. The well-established hydrogen–deuterium and hydrogen–tritium exchange methods have been applied successfully for some time in studying certain types of conformational motions (Hvidt & Nielsen, 1966; Woodward et al., 1982). For example, this technique was useful in exploring the connection between inhibitor binding and fluctuation of lysozyme (Nakanishi et al., 1973). Fluorescence quenching, a spectroscopic method, was first applied to protein dynamics by Lakowicz & Weber (1973), employing oxygen as quencher. This method was developed further by Eftink & Ghiron (1975), who used acrylamide as the quencher of protein tryptophan fluorescence instead of oxygen. Frauenfelder and co-workers developed numerous techniques for investigating protein fluctuations. Using laser flash photolysis methods, they detected internal protein fluctuations by following the kinetics of ligand rebinding [cf. Staerk & Chance (1969), Austin et al. (1975), and Beece et al. (1980)]. Recently introduced temperature-dependent X-ray crystallographic methods, which give the most accurate procedure for calculating the mean

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displacement of atomic coordinates, are also credited to Frauenfelder and co-workers (Frauenfelder et al., 1979).

Most of these methods have been applied to study the specific connection between ligand binding and protein fluctuation. Matkõ et al. (1980) used acrylamide quenching of tryptophan fluorescence in the enzyme phosphorylase *b* to reveal physical evidence for the interdependency of protein fluctuation and enzymatic function. From a theoretical viewpoint, it seems intuitively obvious that the macromolecular matrix of the enzyme should manifest varying degrees of spatiotemporal structure in its internal motions (Careri, 1974; Lumry & Biltonen, 1969; McCammon & Karplus, 1980). This "structure" must be determined statistically, through energetic and stereochemical coupling among the motions of groups within the protein (Gurd & Rothgeb, 1979; Kemeny, 1974). There are several theories proposing that such ordered fluctuations play a unique role in the regulation and in the activity of enzymes (Somogyi & Damjanovich, 1971, 1975; Damjanovich & Somogyi, 1973, 1978; Careri et al., 1979; Low & Somero, 1975; Gavish & Werber, 1979; Welch et al., 1982).

In the present paper, we introduce a novel application of the Förster-type, singlet–singlet resonance energy transfer. Applying a new parameter, a normalized transfer efficiency, we have followed the changes in protein fluctuations. A similar method has been applied to diffusion phenomena (Stryer et al., 1982). Theoretical and experimental results support the applicability of this parameter as a monitor of changes in conformational states and/or local fluctuations in proteins.

## Theory

Let us consider two fluorescent labels associated with a protein molecule, forming a Förster-type, resonance energy transfer (FRET) donor–acceptor pair. Then, the energy, $h\nu$, of a photon exciting the donor molecule can be transferred to the acceptor molecule by dipole–dipole interaction, and the acceptor will relax through the emission of a photon, $h\nu'$, where $h\nu > h\nu'$. The efficiency (i.e., the probability of the FRET) can be expressed as

$$E = \frac{k_t}{k_t + k_f + k_0} \tag{1}$$

where $k_t$ and $k_f$ are the rate constants for the energy transfer and the donor emission, respectively, while $k_0$ is the sum of the rate constants of all other nonradiative deexcitation processes of the excited donor molecules. The transfer rate constant can be written as

$$k_t = dJn^{-4}k_fR^{-6}\kappa^2 \tag{2}$$

where $d$ is a constant depending on the choice of units and $J$ is the spectral-overlap integral, giving the value of the overlap between the normalized emission spectrum of the donor molecule and the absorption spectrum of the acceptor. Thus

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int F_D(\lambda) \, d\lambda} \tag{3}$$

where $F_D(\lambda)$ is the emission intensity of the donor fluorescence at wavelength $\lambda$ and $\epsilon_A(\lambda)$ is the molar absorbance of the acceptor at the same wavelength. In eq 2, $n$ is the refractive index of the medium between the groups participating in the energy transfer. $R$ is the distance between the two chromophores, while $\kappa^2$ (a measure of the orientation of the electronic transition moments of the emitting state of the donor and the absorbing state of the acceptor) is defined by the relation

$$\kappa^2 = (\cos\theta - 3\cos\omega_1\cos\omega_2)^2 \tag{4}$$

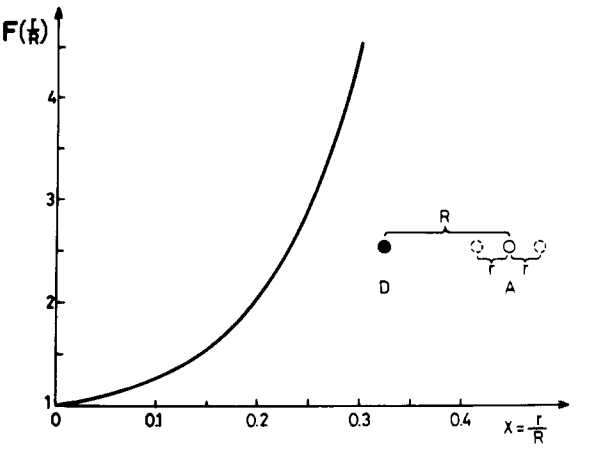


FIGURE 1: Change of $F(r/R)$ with an increase in $r/R$ (i.e., the relative amplitude of the fluctuation). D and A designate the donor and the acceptor, respectively; $R$ is their equilibrium distance, while $r$ is the amplitude of the acceptor vibration.

Here $\theta$, $\omega_1$, and $\omega_2$ define the angles determining the relative orientation of a given donor–acceptor dipole pair (Förster, 1959).

Our principal aim is to characterize the amplitude of the relative motion of the two fluorescent probes as a means of monitoring the relative fluctuation of the different parts of the macromolecule. To this end, we write eq 2 in a simpler form:

$$k_t = Ck_fR^{-6}\kappa^2 \tag{5}$$

Obviously, grouping some of the parameters into the constant $C$ involves the implicit assumption that protein fluctuations leave the values of $J$ and $n$ unchanged.

Labeling of proteins with extrinsic fluorophores cannot be carried out in a perfectly uniform fashion. Even for proteins with intrinsic fluorophores, the homogeneity of the sample is usually not perfect, as evident, for example, in the conformational substates of the protein molecules. Hence, the individual entities of the molecular population will be denoted $k_{ti}$. Therefore, in any experimental study, the determination of $k_t$ will always involve an averaging, as

$$\langle k_t \rangle = Ck_f\langle R^{-6}\kappa^2 \rangle \tag{6}$$

Regarding the averaging involved in eq 6, there are three different cases to be considered (Stryer et al., 1982): (a) the "static" case, when fluctuation is much slower than the transfer rate ($k_t$); (b) the "dynamic" case, when the rate of fluctuation is much faster than the rate of energy transfer; (c) the "intermediate" case, intermediate between (a) and (b) above, when the two processes in question have comparable rates.

In order to show how static fluctuation (i.e., static disorder) affects the rate constant for energy transfer, $k_t$, let us consider the following physical picture (as per case a): We take a one donor–one acceptor system displaying simple (one-dimensional) oscillatory motion about an equilibrium position, defined by the separation distance $R$ (Figure 1). We assume, as a simplification, that the relative orientation of donor–acceptor does not change during the motion; i.e., the value of $\kappa^2$ is constant. (Therefore, $\kappa^2$ can be incorporated into the constant of eq 6.) Suppose the donor–acceptor distance can have the two static values, $R - r$ and $R + r$, with equal probability and the transition from one position to the other requires negligible time (the donor–acceptor "residence time" at a given position is considered much longer than the donor lifetime). Then, the value of $\langle k_t \rangle$ (which will be a kind of static average) can be written as

$$\langle k_t \rangle = (1/2)(k_t^- + k_t^+) \tag{7}$$

where

$$k_t^- = C'k_f(R - r)^{-6} \quad (8)$$

and

$$k_t^+ = C'k_f(R + r)^{-6} \quad (9)$$

(Here, $C'$ contains $\kappa^2$ as well.) Thus, in this static picture, the average $\langle k_t \rangle$ is the result of counting the different subsystems in the population and determining a weighted average, where the weighting factors correspond to the fractional fluorescence intensities of the various subsystems.

Dividing the value of $\langle k_t \rangle$ in eq 7 for the oscillatory system by the value describing the same system in the motionless ("frozen") state (characterized by a constant donor–acceptor distance, $R$), we can define a new parameter as follows:

$$F(r/R) = \frac{\langle k_t(r) \rangle}{\langle k_t(0) \rangle} = \frac{(1/2)C'k_fR^{-6}[(1 - r/R)^{-6} + (1 + r/R)^{-6}]}{C'k_fR^{-6}} = \frac{1}{2}[(1 - r/R)^{-6} + (1 + r/R)^{-6}] \quad (10)$$

Figure 1 shows how $F(r/R)$ changes as $r/R$ varies from 0 to 0.3. Obviously, an increase in the static fluctuation (i.e., an increase in $r$) can result in a dramatic increase in $\langle k_t(r) \rangle$, even if the equilibrium distance ($R$) of the fluorophores remains unchanged.

Regarding cases b and c, it has been shown from both theoretical (Steinberg, 1971; Stryer et al., 1982) and experimental (Elkana et al., 1968; Haas et al., 1978) studies that, when the rate of the relative motions of the donor–acceptor pairs is comparable with (or higher than) that of the energy transfer, the mean value of $k_t$ increases compared to the static picture. This means that in cases b and c the method of averaging used in the static case is not valid. In case b, where the donor–acceptor position might sample all possible values ($R \pm r$) (several times) in an instant of time in the time scale of the transfer, the mean value of $k_t$ reaches the maximal value corresponding to the given distance distribution of the donor–acceptor pairs. In this case, the average value of $k_t$ would not be expected to show a dependence on the frequency of the donor–acceptor oscillation.

In case c, where the transfer rate is comparable with the fluctuation rate, however, one would expect the mean value $\langle k_t \rangle_i$ to be intermediate between the averages of $k_t$ for the static, $\langle k_t \rangle_s$, and the dynamic, $\langle k_t \rangle_d$, cases (see Figure 2).

Hence, for the three different cases (a, b, and c), there are different schemes involved in the averaging of $k_t$ values. One should note that any real (e.g., macromolecular) system will involve, intrinsically, all of these various modalities: thus, any experiment performed on a protein entails all kinds of fluctuations under the usual analytical conditions. Accordingly, the experimentally obtained value of $\langle k_t \rangle$ in eq 6 will necessarily include *all* the modes of averaging superposed. Regarding the role of the relative orientation of the fluctuating donor–acceptor pairs, in a general case the changes in $R$ and $\kappa^2$ usually cannot be described independently, because of the superposition of many different movements occurring within the macromolecule. The problem can be overcome, for example, by using fluorescent lanthanides, since these elements (e.g., terbium and europium) have isotropic (symmetrical three-dimensional) transitions, so that $\kappa^2$ for transfer between them has the unique value of $^2/_3$, equivalent to the rapidly averaged random value under all circumstances (Horrocks et al., 1975). Owing to the limited applicability of such fluorescent labels, one must, in general, deal with $\kappa^2$. However, the selection of a suitable donor–acceptor pair includes the selection of a preferential $\kappa^2$ value as well. Because of the dependence on the inverse sixth power of the actual donor–acceptor distance, the change in $R$ may often dominate the change in $\langle k_t \rangle$, thus reducing the variability to that in $R$ (see Results).

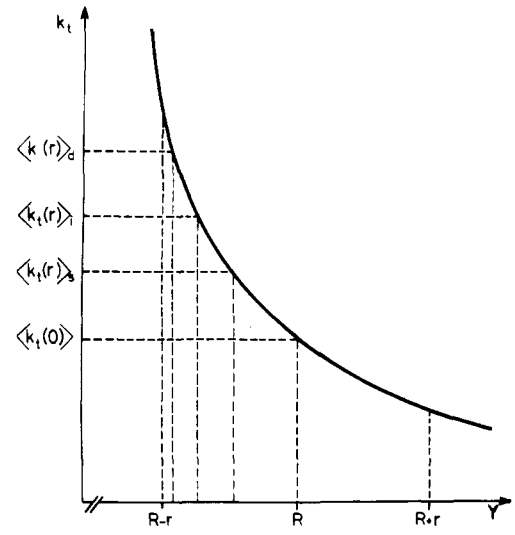


FIGURE 2: Change of $k_t$ with the donor–acceptor distance, $Y$, for a fixed value of $\kappa^2$. $\langle k_t(0) \rangle$ is the value of $k_t$ for the "frozen" system; $\langle k_t(r) \rangle_s$ is the average of $k_t$ for a system oscillating "statically" with an amplitude of $r$; $\langle k_t(r) \rangle_i$ is the average of $k_t$ when the oscillation rate of the system is comparable with the transfer rate, while $\langle k_t(r) \rangle_d$ is the average when the oscillation rate is much faster than the transfer rate (the curve displayed on the figure is a schematic one).

Thus, the mean value, $\langle k_t \rangle$, seems an appropriate parameter for monitoring local fluctuations (of different relaxation times) of a macromolecule under changing environmental conditions.

Having in hand a parameter, i.e., the average value of $k_t$, characterizing local fluctuation in macromolecules, we now concentrate on the question of how to measure it. For a transfer system in a "frozen" state, the answer seems simple. Since the quantum yield of the donor, $\phi_D$, in the presence of the acceptor can be expressed as

$$\phi_D = \frac{k_f}{k_f + k_t + k_0} \quad (11)$$

we introduce the factor $f$ as

$$f = \frac{E}{\phi_D} = \frac{k_t/(k_f + k_t + k_0)}{k_f/(k_f + k_t + k_0)} = \frac{k_t}{k_f} \quad (12)$$

In a real experiment, however, fluctuations result in variability for the values of $k_t$ and $k_0$. Therefore, one can measure only the average values of $E$ and $\phi_D$. In this case

$$f = \frac{\langle E \rangle}{\langle \phi_D \rangle} = \frac{\langle k_{ti}/(k_f + k_{ti} + k_{0i}) \rangle}{\langle k_f/(k_f + k_{ti} + k_{0i}) \rangle} \quad (13)$$

Assuming no change in $k_f$ during the fluctuation, eq 13 can be rearranged as follows:

$$f = \left\langle \frac{k_{ti}}{k_f} \frac{1/(k_f + k_{ti} + k_{0i})}{\langle 1/(k_f + k_{ti} + k_{0i}) \rangle} \right\rangle = \left\langle \frac{k_{ti}}{k_f} \frac{\tau_{Di}}{\langle \tau_D \rangle} \right\rangle \quad (14)$$

where $\tau_{Di}$ is the lifetime of the $i$th donor population, taking a momentary picture of the system, and $\langle \tau_D \rangle$ is the average lifetime, averaged according to all three possible modes described above. With the use of lifetime measurements, the

value of $\langle \tau_D \rangle$ can be expressed as

$$\langle \tau_D \rangle = \frac{\sum_i A_i \tau_{Di}^2}{\sum_i A_i \tau_{Di}} \quad (15)$$

where $A_i$ is the preexponential (amplitude) of the $i$th component of the fluorescence decay curve. Applying eq 6, we have

$$f = C\langle \alpha_i R_i^{-6} \kappa_i^2 \rangle \quad (16)$$

where

$$\alpha_i = \frac{\tau_{Di}}{\langle \tau_D \rangle} \quad (17)$$

Regarding the characteristic features of $\alpha_i$, it is easily seen that

$$\langle \alpha_i \rangle = 1 \quad (18)$$

We must issue a caveat that the parameter $f$ cannot be used, without further assumptions and qualifications, to yield information about the frequency and amplitude distributions of the fluctuations.

However, at this point of discussion, we have to make it clear that the suggested method has the following limitation in the case of static averaging: If the energy-transfer efficiency is higher than 50%, the growing amplitude of the fluctuation is not followed by an increase in the parameter $f$. This is largely due to the specific behavior of the $\alpha_i$ weighting factors in eq 16.

To bring eq 16 into a more useful form, let us assume that

$$k_{ti} << k_f + k_{0i} \quad (19)$$

which means that the rate constant for energy transfer is much less than the composite for all other deactivation processes. One can assess this relationship approximately from the experimentally determined value of the transfer efficiency. In this case, the value of $k_{ti}$ in the expression for $\alpha_i$ can be neglected (i.e., $k_{ti}$ and $\alpha_i$ can be treated as independent variables). Then, eq 14 simplifies, by virtue of eq 18 as follows:

$$f = \left\langle \frac{k_{ti}}{k_f} \right\rangle \left\langle \frac{\tau_{Di}}{\langle \tau_D \rangle} \right\rangle = \left\langle \frac{k_{ti}}{k_f} \right\rangle \quad (20)$$

and eq 16 becomes

$$f = C\langle R_i^{-6} \kappa^2 \rangle \quad (21)$$

From eq 21, one sees that *the change of the parameter f can be related directly to the change of fluctuations, or to a conformational change which alters the static "disorder"* (or fluctuation spectrum) of the donor–acceptor system.

The applicability of the method is broadened, noting that inequality (eq 19) is attainable even for systems having relatively *high* energy-transfer efficiency (i.e., $k_{ti}$ comparable with $k_f + k_{0i}$), *by the use of quencher*. Obviously in the presence of a dynamic quencher, $\tau_{Di}$ becomes

$$\tau_{Di} = \frac{1}{k_f + k_{ti} + k_{0i} + k_{+i}[Q]} \quad (22)$$

where $k_{+i}$ is the diffusion-controlled bimolecular rate constant for the collisions between quencher and the donor molecules, while [Q] is the quencher concentration (Eftink & Ghiron, 1981). In this case, one can modify the assumption used to derive eq 20 and 21 to have

$$k_{ti} << k_f + k_{0i} + k_{+i}[Q] \quad (23)$$

Hence, generally, a change in the value of $f$ indicates a change of fluctuations in a manner corresponding either to an increase of the fluctuation amplitude or to an increase of the frequency of intermediate fluctuation.

Viewing the change of $f$, it may often be more convenient to use another parameter, $f'$, whose definition can be given similarly to that of $f$, except with the use of the fluorescence intensity instead of the quantum yield of the donor (therefore, the parameters $f$ and $f'$ are proportional to each other).

It should be noted that under usual experimental circumstances, every $n$ donor–*one* acceptor system can be treated as a population of $n$ different *one* donor–*one* acceptor pairs. Furthermore, the transfer rates in *one* donor–$n$ acceptor systems can be described as the sum of $n$ independent rates. Consequently, eq 20 and 21 seem to be generally applicable.

## Experimental Procedures

*Materials.* Ribonuclease $T_1$ [EC 3.1.4.8, ribonucleate 3′-guanylo-oligonucleotidohydrolase (cyclizing), Taka-Diastase] (RNase $T_1$) was purchased from Calbiochem and was used without further purification. Pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) were obtained from Sigma. All other chemicals were the best reagent grades available.

*Preparation of PMP-Labeled Ribonuclease $T_1$.* The protein–PMP conjugate was prepared by using sodium borohydride for reduction of the Schiff base formed between PLP and a lysyl side chain of the enzyme as described earlier by Churchich (1965a) for other proteins. The labeling procedure was performed in 50 mM phosphate buffer (pH 7.5), and the conjugate was further purified by repeated dialysis against the same buffer. The extent of the labeling was determined from the absorbance at 325 nm by using $\epsilon_{325} = 8300$ cm$^2$ mol$^{-1}$ for the protein–PMP conjugate (Churchich, 1965a). The protein concentration was determined from the absorbance at 278 nm by using $A_{278nm}^{1cm,0.1\%} = 1.9$ for RNase $T_1$ (Uchida & Egami, 1967).

*Activity Assay.* The activity of the enzyme before and after the labeling procedure was tested by Takahasi's method (Takahashi, 1961), i.e., by measuring the increase in the absorbance at 258 nm when the enzyme was incubated with yeast ribonucleic acid as substrate. In our investigations, the slopes of these steady-state progress curves were used as a measure of enzymic activity.

*Spectroscopic Measurements.* All fluorescence measurements were performed with solutions of RNase $T_1$ and RNase $T_1$–PMP conjugate made in 50 mM phosphate buffer (pH 7.5). The experiments were carried out in a Hitachi Perkin-Elmer MPF-4 spectrophotofluorometer equipped with thermostated cell holder, using quartz cuvettes (1-cm path length).

The protein fluorescence was excited at 295 nm to ensure selective excitation of Trp fluorophores (Sober, 1970) and to decrease the probability of the Tyr → Trp energy transfer (Pongs, 1970). The pyridoxyl fluorescence of the conjugate was excited at 325 nm. The protein and PMP emissions were recorded at 320 and 390 nm, respectively. The absorbance of all samples was kept below 0.1 at the excitation wavelengths to minimize the inner filter effects. Bandwidths of 5 nm were used for both excitation and emission sides. Fluorescence intensities (and spectra) were corrected for the wavelength-dependent fluctuations of the light source output. Fluorescence polarization measurements were performed with a polarization accessory, Hitachi 5013-9. The standard deviation of these measurements was found to be ±0.003 (in polarization units).

The efficiency of energy transfer (from Trp to PMP) in the RNase $T_1$–PMP conjugate was determined from the sensitization of the acceptor fluorescence. When the donor molecules

are intrinsic ones and the acceptor is fluorescent, a simple expression is suitable for the calculation of transfer efficiency (Brand & Witholt, 1967), assuming that the excitation of the acceptor at the donor's excitation wavelength is negligible and the absorbances of the samples at both $\lambda_D$ and $\lambda_A$ are sufficiently low:

$$E = \frac{OD_{\lambda_A}}{OD_{\lambda_D}} \frac{F_A^{\lambda_D}}{F_A^{\lambda_A}}$$

where $OD_{\lambda_A}$ and $OD_{\lambda_D}$ are the optical densities of the protein solutions at the acceptor's and donor's excitation wavelength, respectively, while $F_A^{\lambda_D}$ and $F_A^{\lambda_A}$ are the acceptor's emission intensities measured at the emission maximum upon excitation at $\lambda_D$ and $\lambda_A$ excitation wavelengths, respectively.

For the RNase $T_1$–PMP conjugate, the second requirement was ensured by the choice of experimental conditions, but a further correction of the above expression is required because of a slight but nonnegligible excitability of the acceptor at $\lambda_D$. This was taken into consideration as follows:

$$E = \frac{OD_A^{\lambda_A}}{OD_D^{\lambda_D}} \frac{F_A^{\lambda_D}}{F_A^{\lambda_A}} - \frac{OD_A^{\lambda_D}}{OD_A^{\lambda_A}}$$

where $OD_D^{\lambda_D}$, $OD_A^{\lambda_A}$, and $OD_A^{\lambda_D}$ are the absorbance of the donor at its own excitation wavelength and the acceptor absorbancies at the acceptor's and donor's excitation wavelengths, respectively.

The $OD_A^{\lambda_D}$ values were calculated from the absorbance difference measured on labeled and unlabeled protein samples at the donor's excitation wavelength (295 nm) and at the wavelength of protein absorbance (278 nm) (where the PMP label has no significant absorbance).

Since in the case of these conjugates the protein (Trp) emission band overlaps with the emission spectrum of the acceptor (PMP), the intensity contribution of the donor emission to the measured emission intensity (at the acceptor emission maximum) was also taken into correction in the $F_A^{\lambda_D}$ value.

The $E$ values determined from three independent experimental series showed a percent standard error of about 6%.

Fluorescence lifetime measurements were carried out with a nanosecond impulse fluorometer built in our laboratory by using the technique of time-correlated single photon counting. The samples were excited at 298 nm by using a Pomfret Research Optics Inc. filter (Model 20-3000-1) while the emission was detected at 340 nm by using an ORIEL interference filter (Model 5339). The excitation light source was a $N_2$ flash lamp operating at a rate of 12 kilocycles. The instrument was tested by using quinine disulfate as the lifetime standard (Chen, 1974).

The measured decay curves were fitted to both single- and double-exponential decay curves by using the method of nonlinear least squares (for details, see the legend of Figure 7). Both steady-state and decay measurements on the RNase $T_1$–PMP conjugate were made in the temperature range of 18–40 °C by using thermostated cell holders. The temperature of the samples was kept constant to within ±0.1 °C.

## Results

*Properties of the RNase $T_1$–PMP Conjugate.* Ribonuclease $T_1$ contains *one* tryptophyl side chain and *one* lysyl side chain which is characteristic of the enzyme as compared to other ribonucleases (Takahashi, 1965; Uchida & Egami, 1967; Fukunaga et al., 1982). It makes possible selective fluorescent labeling of the enzyme through specific chemical (covalent) modification of the lysyl residue with PLP (Churchich, 1965a; Means & Feeney, 1971). The extent of labeling was checked after the modification reaction, and the average number of bound PMP molecules per enzyme molecule was found to be 1.

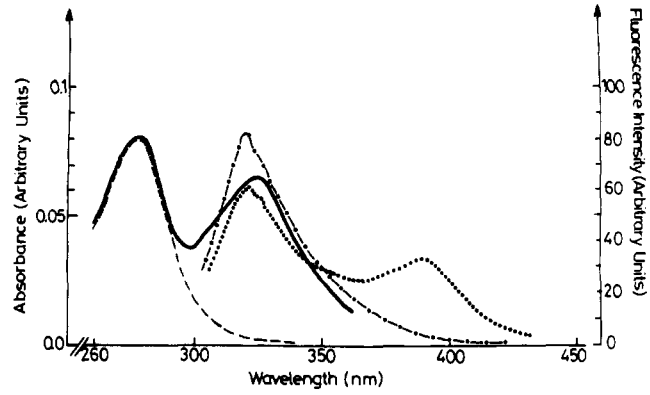


FIGURE 3: (Left curves) Absorption spectra of unlabeled (---) and PMP-labeled (—) RNase $T_1$. The absorbancies were measured at same protein concentration in 50 mM phosphate buffer, pH 7.5, at room temperature. (Right curves) Emission spectra of unlabeled (–·–) and PMP-labeled (···) RNase $T_1$. The fluorescence emission spectra were recorded under the same conditions as the absorption spectra, upon excitation at 295 nm.

The absorption and fluorescence emission spectra of the unlabeled RNase $T_1$ and the labeled RNase $T_1$ are shown in Figure 3. The spectral data indicate the following information: (i) The binding of PLP to the enzyme results in a decrease of the quantum efficiency of the Trp fluorescence, and a new shoulder (centered at 390 nm) appears in the emission spectrum excited at 295 nm. (ii) The binding of PLP does not influence either the position or the fine structure of the Trp emission spectrum. Furthermore, the modification of the Lys-41 residue did not alter significantly the catalytic activity of RNase $T_1$ as proved by the activity assays.

Thus, the RNase $T_1$–PMP conjugate can serve as a good one donor–one acceptor system for the energy-transfer measurements, though due to the relatively short wavelength of the protein emission maximum ($\simeq$ 320 nm) the value of the overlap integral ($J$) is expected to be somewhat lower than that usually observed for a protein–PMP conjugate (Churchich, 1965a).

*Energy-Transfer Studies.* In order to test our theoretical prediction (Figure 1), several experiments were performed to show the usefulness of the parameter $f$ as a measure of the changes of local fluctuations. For this reason, the temperature dependence of $f$ was examined, since an increase in the thermal energy would be expected to increase the amplitude of the fluctuations. To characterize our system, the value of $f'$ ($= \langle E \rangle / \langle F_D \rangle$) was examined.

A change in the parameter $f'$ would characterize changes in local fluctuations until a conformational transition or other major structural change (e.g., unfolding, complete denaturation) occurs. In order to rule out the effect of the latter changes on $f'$, we first studied the temperature dependence of the fluorescence spectra of both the donor (Trp) and the acceptor (PMP) in the RNase $T_1$–PMP conjugate. As Figure 4 demonstrates, there was no detectable spectral shift or change in the fine structure of the emission spectrum resulting from the variation of the temperature. Regarding the similar findings with the absorption spectra, we may assume that the variation in the temperature leaves the value of the overlap integral ($J$) unchanged.

In Figure 5A,B the temperature dependence of the emission intensities of the donor and the acceptor (A) and of the $f'$

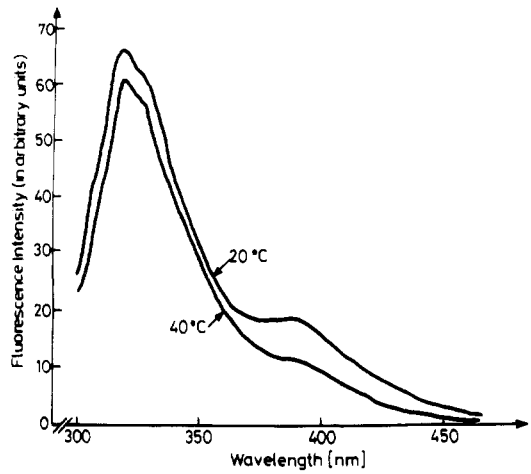


FIGURE 4: Fluorescence emission spectra of the RNase $T_1$–PMP conjugate recorded at different temperatures (indicated on the figure). All the experimental conditions were the same as those in Figure 3.

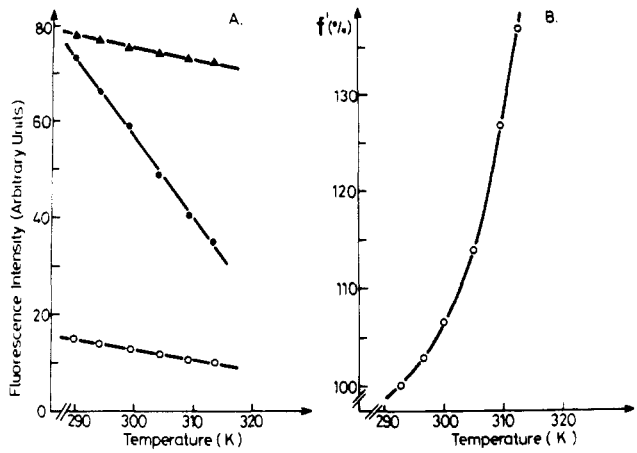


FIGURE 5: (A) Effect of temperature on the donor (Trp) and acceptor (PMP) fluorescence of RNase $T_1$. Tryptophan fluorescence (▲) was excited at 295 nm, and the emission was monitored at 320 nm. PMP fluorescence (●) was excited at 325 nm, and its emission was recorded at 390 nm. The acceptor emission intensity was also recorded by excitation at the donor excitation maximum (○). (B) Percent change of the $f'$ parameter vs. temperature in the RNase $T_1$–PMP conjugate. The $f'$ parameter was determined from the actual average transfer efficiency (calculation described under Experimental Procedures) and the appropriate emission intensity of the donor. (The $f'$ value obtained at 293 K was taken as 100%.)

parameter calculated from the primary data (B) is displayed.

Both donor and acceptor intensities decreased with increasing temperature, presumably due to the increasing efficiency of the temperature-dependent radiationless deactivation process. This decrease (from 18 to 40 °C) proved to be linear and reversible for both donor and acceptor. The latter observations and the lack of spectral changes (Figure 4) refer to the lack of gross conformational transitions of the labeled protein in this temperature range.

To derive parameter $f'$ from the primary data, first the efficiency of energy transfer ($\langle E \rangle$) was examined. From measurements on four independent samples, a transfer efficiency of 0.14 (±0.02) has been found at 25 °C. The transfer efficiency showed a small but definite ($\simeq$10%) increase with increasing temperature (from 20 to 40 °C).

The derived parameter $f'$ as a function of temperature showed a characteristic increase, and its numerical value increased by approximately 40% in the temperature range examined (Figure 5B).

The theoretical analysis is based on the assumption of the dominance of the changes in fluctuation amplitudes over the

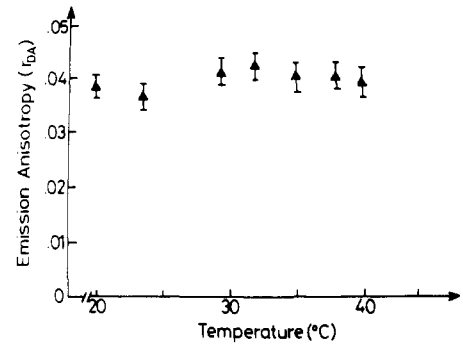


FIGURE 6: Emission anisotropy of the transferred excitation energy in the RNase $T_1$–PMP conjugate as a function of temperature. The emission anisotropy values ($r_{DA}$) were calculated as $r_{DA} = (I_{vv} - GI_{vH})/(I_{vv} + 2GI_{vH})$ recording the emission intensities at 390 nm upon excitation at 295 nm. ($G$ is the constant characteristic of the instrumental setup.) The standard error of the polarization measurements, calculated from three to four independent measurements, is also displayed on the figure.

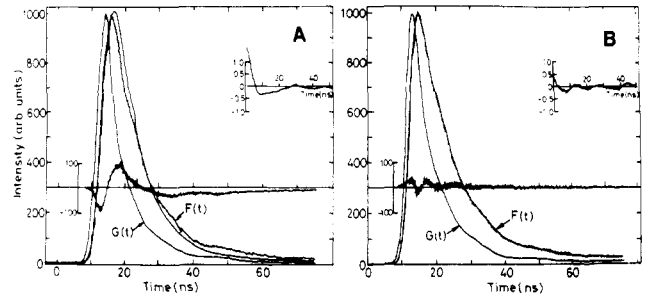


FIGURE 7: Experimental decay curves and results of the analysis for tryptophan fluorescence of the RNase $T_1$–PMP conjugate. The measurements were carried out in 50 mM phosphate buffer, pH 7.5, at 20 °C. Panels A and B show the lamp flash, $G(t)$, the decay curve, $F(t)$, and the best theoretical decay curve. The normalized residuals and the autocorrelation function (Grinvald & Steinberg, 1974) are also shown. The value of the weighted root mean square deviation (WRMS) was calculated as described by Gafni et al. (1975). The timing calibration was 0.1 ns/channel. Preexponents were normalized as $\sum A_i = 1$. (A) Analysis for single-exponential decay. The best fit was achieved with the following parameters: $\tau = 2.91$ ns; WRMS $= 5 \times 10^{-3}$. (B) Analysis for double-exponential decay. The best fit was based on the following decay parameters: $\tau_1 = 1.15$ ns; $\tau_2 = 4.7$ ns; $A_1 = 0.8$; $A_2 = 0.2$; WRMS $= 2.3 \times 10^{-4}$.

changes in $\kappa^2$ with increasing temperature. Beyond the theoretical considerations, however, one should test experimentally the possibility of the changes in $\kappa^2$. Therefore, both donor and acceptor deplarizations as well as the depolarization of the transferred excitation energy have been examined as a function of temperature.

The actual degree of the acceptor fluorescence polarization ($p$) (around 0.11) and the apparent $p_0$ calculated from the thermal Perrin plot by extrapolation to $T/\eta = 0$ (0.28) as well proved to be low relative to the $p_0$ characteristic of this fluorophore ($p_0 = 0.41$; Churchich, 1965b).

On the other hand, the emission anisotropy of the transferred excitation energy ($r_{DA}$) did not show any significant change over the temperature range examined (Figure 6).

The linear representation of the decay data of Trp fluorescence in the RNase $T_1$–PMP conjugate is shown in Figure 7A,B. The experimental decay curves were fitted for single and double exponentials alike. For the single-exponential fit, the deviation and autocorrelation functions indicated some systematic deviations between the experimental data and the calculated function (A). Analysis for double-exponential decay resulted in significant improvement of both the autocorrelation function and the value of the root mean square deviation (see the legend to Figure 7B). (The decay of tryptophan

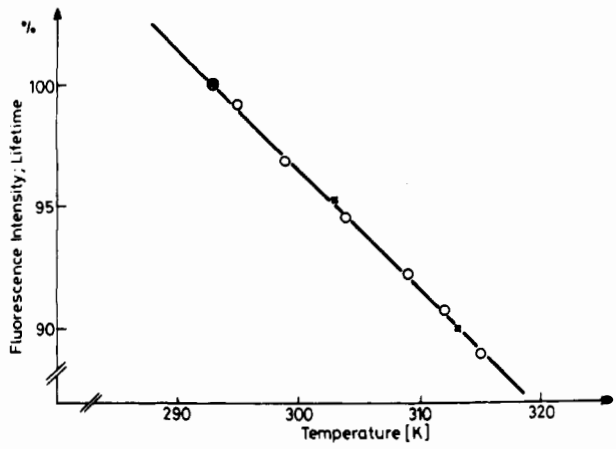


FIGURE 8: Percent change of the donor fluorescence intensity (O) and of the average lifetime (×) in PMP-labeled RNase $T_1$ as a function of temperature. (The lifetime and intensity values measured at 293 K were taken as 100%.)

fluorescence of the unlabeled RNase $T_1$ also proved to be a double-exponential one.) The average lifetime, $\langle\tau_D\rangle$, was calculated according to eq 15 (see Theory). The average lifetime showed a slight tendency to decrease with rising temperature while the $\tau_{Di}/\langle\tau_D\rangle$ ratio for both components proved to be practically unchanged between 20 and 40 °C. In order to obtain experimental evidence about the invariancy of the rate of the radiative transition ($k_f$) with temperature, the temperature dependence of the fluorescence intensity, $\langle F_D\rangle$, and $\langle\tau_D\rangle$ have been examined, especially regarding their correlation (see Figure 8).

## Discussion

A great deal of theoretical work, in addition to accumulating experimental evidence, has recently turned the attention of physicists and chemists, as well as biologists, to molecular dynamics as a focal point in the search for the active mechanisms of biological macromolecules [e.g., see Lipscomb (1981)]. This situation places great importance on the search for experimental methods suitable for monitoring local fluctuations in the macromolecular matrix. Such is the motivation for the elaboration of the experimental parameter $f$ defined under Theory.

Figure 1 shows how $f$ is capable of monitoring the change in local fluctuations occurring inside the macromolecule. On the basis of the data of Figure 1, one sees that if the ratio $r/R$ increases, e.g., from 0.1 to 0.15, the value of $f$ increases by about 25%. This means that, for example, with a 2.0-nm donor–acceptor distance, the fluctuation amplitude should change from 0.2 to 0.3 nm in order to give an increase of 25% in the value of $f$. The quantitative significance of the factor $r/R$ for a globular protein depends on the nature of the interior and on the dynamic behavior of the atomic groupings. It is obvious that in the idealized case of an oscillating spring, the $r/R$ value for a given spanning force is independent of $R$; i.e., the relative "amplitude" remains the same even if $R$ changes. However, in the case of a protein molecule, due to the close packing of the atomic groups inside the protein (Klapper, 1971), the value of $r/R$ might decrease with an increase of $R$. For fluctuations in a protein matrix, one is actually dealing with a superposition of many different changes in atomic positions, energies, and stereochemical arrangements of the different groups. Because of the $R^{-6}$ dependence of $f$ (and also of $f'$), our parameter is more sensitive to the large amplitude fluctuations. Therefore, the aforementioned decrease in $r/R$ with an increase in $R$ might be counterbalanced, at least in some cases, by this property of $f$; i.e., the parameter $f$ is, roughly, equally sensitive to the change in amplitudes of both small and large amplitude fluctuations.

Although because of the sixth power dependence of $k_t$ on $R$ it might be expected that the change in $f$ is dominated by the change in $R$, the effect of the donor–acceptor orientation, expressed by the factor $\kappa^2$, cannot be neglected. The change in $\kappa^2$ can also produce an increase or decrease in $f$ (Dale & Eisinger, 1976). In general, $R_i^{-6}$ and $\kappa_i^2$ cannot be treated as independent variables. However, time-resolved anisotropy measurements should tell us whether the orientational freedoms of the fluorophores have a big change or not; i.e., these methods might indicate or contraindicate the presence of a change in $f$ due to $\kappa^2$.

As shown in Figure 2, the change in the frequency of the fluctuation with unchanged amplitude distribution can also affect the value of $f$ (e.g., change the fluctuation frequency belonging to the dynamic case to another one belonging, e.g., to the static case). It might happen when the viscosity changes, producing a decrease in the fluctuation frequency but leaving the amplitude distribution unchanged. This kind of effect might occur as well through the alteration of $\langle\kappa^2\rangle$, which gives different values for $k_t$ in the dynamic or static limit (Dale et al., 1979). Accordingly, the parameter $f$ is sensitive to the change in amplitude distribution and orientational freedom as well as to the change in the frequency distribution of local fluctuations.

Thus, one can expect that the $f$ value, in an appropriate experimental system (e.g., the one described under Experimental Procedures), will exhibit a detectable increase with increasing temperature within the range where the macromolecule in question shows no gross conformational change. The use of $f'$ ($=\langle E\rangle/\langle F_D\rangle$) instead of $f$ should result in the same tendency, or percent change, because of the proportionality between $f'$ and $f$. The calculation of transfer efficiency and the measurement of $\langle F_D\rangle$ were carried out by using selective wavelength values instead of using whole spectra. Spectral shifts in such cases can alter the values of both $\langle E\rangle$ and $\langle F_D\rangle$, resulting in artifacts. Since there was no detectable spectral shift in the applied temperature range (Figure 4), this possibility could be discounted. However, in order to regard the increase in $f'$ (see Figure 5B) as due to the increased fluctuation amplitude, additional arguments are necessary.

First, the invariancy of the natural lifetime, i.e., that of $k_f$, is an important assumption used for the derivation of the final equations. As shown in Figure 8, the average lifetime and fluorescence intensity values change parallel with each other as the temperature changes, indicating, in agreement with other papers describing a fairly constant value of the natural lifetime under a wide variety of different experimental conditions (De Lauder & Wahl, 1971; Badley & Teale, 1969), that the assumption is satisfied within the specified temperature range (between 20 and 40 °C).

Concerning the contribution of $\kappa^2$ to the observed temperature dependence of $f$, it cannot exactly be excluded. There are, however, experimental observations contraindicating significant changes in $\kappa^2$ in the specified temperature range. One of these is the practically temperature independent low value of the acceptor emission polarization (0.11, compared to the $p_0$ value), which indicates a rather large motional freedom of the acceptor with respect to the protein matrix. At the same time, the fluorescence intensity of the PMP probe decreases by a factor of 2 in the same temperature interval. Assuming that this change corresponds to a similar drop in the lifetime, which is counterbalanced by the change of $T/\eta$

(resulting in no significant change in the observed acceptor anisotropy), one can conclude that PMP rotates more rapidly at higher temperatures. All the above considerations, along with the observed temperature independence of the acceptor emission anisotropy excited through the donor (Figure 6), indicate that the PMP probe can sample all the possible orientations during the lifetime of donor fluorescence ($\simeq$3 ns, which is comparable with that for PMP; Irwin & Churchich, 1971). It means that the PMP motion falls into the dynamic averaging regime over the whole temperature range. In light of the above facts, a significant contribution of $\kappa^2$ to the temperature dependence of $f$ seems to be unlikely.

When the donor fluorescence has multiple decay, one must take into account a possible temperature-dependent redistribution of the subpopulations characterized by different lifetime components. This redistribution may, in itself, produce either an increase or a decrease in $f$. In the case of PMP-labeled RNase $T_1$, the amplitudes of the two components did not change significantly over the temperature interval examined so far. Thus, the effect of this redistribution on the change in $f$ was neglected.

A possible expansion of the protein molecule as well as a change in protein hydration would alter the refractive index, influencing in this way the value of $f$ as well (see eq 2). Direct measurements of refractive indices of protein molecules, however, face several difficulties. The refractive index of the solvent, water, decreases by 0.16% from 290 to 310 K. Assuming a similar change in the refractive index for the protein molecule, the observed increase in $f$ can hardly be attributed to this effect.

Furthermore, the possible expansion of the protein molecule, caused by the increase of temperature, cannot account for the increased value of $f'$. Therefore, the increased donor–acceptor distance, due to the increased average distance between the different groups of the protein molecule, should result in a decreased value of $f'$. In addition, we note that ribonuclease $T_1$ showed no gross conformational change within the specified temperature range.

Consequently, we conclude that the increases measured in $f'$ with temperature (as one of the environmental parameters perturbing protein fluctuations) are due to the increases in the fluctuation amplitudes inside the protein matrix. This gives a unique value to our proposed experimental method in monitoring the change in relative fluctuations of two or more specifically defined points on a macromolecule. However, we emphasize again an important point. Gross conformational or structural changes (e.g., denaturation) might also affect the value of $f$ (or $f'$). (Regarding the effect of conformational changes on $f'$, this might be identified separately in many cases.) Therefore, the method should be characterized as capable of monitoring *both* gross conformational transitions *and* changes in fluctuations inside the macromolecular matrix.

Acknowledgments

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# Effect of Pressure on the Self-Association of Melittin†

Richard B. Thompson‡ and Joseph R. Lakowicz*

ABSTRACT: The effect of increased hydrostatic pressure (1 bar to 1.8 kbar) on the self-association of melittin was measured by using the fluorescence anisotropy of its single tryptophan residue. The degree of self-association was found to decrease with increasing pressure. The volume change ($\Delta V$) for dissociation is surprisingly large. At low pressures, $\Delta V$ for dissociation is near −150 mL/mol. The magnitude of the volume change decreased with increasing pressure, possibly as a result of pressure-induced compression of free volume trapped at the subunit interface region of the tetramer. Overall, the pressure-dependent association of melittin is comparable to that expected for hydrophobic interactions and to that found for micelle formation by detergents.

Melittin is an amphipathic peptide component of bee venom which enhances the venom phospholipase activity and disrupts phospholipid bilayers. It has been shown to interact strongly with phospholipid bilayers, in some cases causing cell lysis (Dufourcq & Faucon, 1977; Mollay & Kriel, 1973; Habermann, 1972, 1980; Sessa et al., 1969). It has thus served as a useful model system for studies of lipid–protein interactions. Melittin exists in two forms in solution, as a monomer and as a tetramer (Talbot et al., 1979). In this report, we describe the effects of increased hydrostatic pressure (1 bar to 2 kbar) on the self-association of melittin.

We measured melittin's self-association by fluorescence spectroscopy. As with other optical methods, it is convenient for observing pressurized samples. Also, it is sensitive enough to be useful at the low protein concentrations where association is incomplete, and the greatest effects of pressure perturbation may be seen (Weber & Drickamer, 1983). Melittin has several properties which make its self-association easily measurable by fluorescence techniques. Each peptide chain contains a single tryptophan residue and no tyrosine residues (Habermann & Jentsch, 1967). Hence, the intrinsic fluorophore population is likely to be homogeneous, and there is no possibility of tyrosine–tryptophan energy transfer. Upon association to form the tetramer, the emission of the protein shifts from 353 to 337 nm and the anisotropy approximately doubles (Talbot et al., 1979; Faucon et al., 1979). The blue shift of the tryptophan emission in the tetramer occurs because the tryptophan residues are shielded from contact with the solvent (Terwilliger & Eisenberg, 1982). The large change in anisotropy occurs because the rotational correlation times of the monomers (1.2 ns) and tetramer (3.8 ns) are substantially different, and because both are comparable to the fluorescence lifetime of the tryptophan residue (≈2.5 ns) (Lakowicz et al., 1983; Georghiou et al., 1981). In addition, the segmental mobility of the tryptophan residue may be less in the tetramer than in the monomer. Thus, we measured the self-association of melittin by measuring the fluorescence anisotropy of its intrinsic tryptophan. Finally, for confirmation, we also measured the self-association of melittin labeled with an extrinsic probe, *N*-methylisatoic anhydride, and obtained similar results.

The recently reported crystal structure is informative with respect to the molecular interactions which influence melittin self-association (Terwilliger & Eisenberg, 1982). The tetrameric structure is not stabilized by either hydrogen bonds or electrostatic interactions. The latter fact is not surprising given that melittin lacks even a single negative charge, but the lack of hydrogen bonding between the subunits clearly makes melittin an unusual case. As Eisenberg and his colleagues have pointed out, the sole interaction that promotes the tetramerization reaction is hydrophobic. Thus, it is similar to the formation of micelles by amphipathic molecules such as detergents. Melittin monomers are grossly similar to such amphipathic molecules, having hydrophobic residues concentrated at one end of the peptide chain and basic residues grouped

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