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TIME-RESOLVED FLUORESCENCE IN STUDIES OF PROTEIN STRUCTURE AND DYNAMICS

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INTRODUCTION

Fluorescence spectroscopy continues to develop as a useful technique for understanding the complex relationships between structure and function of proteins and other biological macromolecules. Fluorescence spectroscopy has several advantages, including sensitivity (only a small amount of sample is needed), the ability to examine processes over a wide time range (picoseconds to seconds), the ability to work under "physiological" conditions, and the ability to measure many different physical parameters (quantum yield, lifetime, emission energies, anisotropy) that can contribute to the understanding of the system.

Time-resolved fluorescence studies involve the investigation of the rate of loss of either the first excited singlet electronic state (intensity decay) or an induced polarization of the excited state (anisotropy decay). Protein structural fluctuations can perturb either the intensity or anisotropy decay; these perturbations may be related to local events near or involving the probe (relatively fast), to "global" processes involving the entire protein (relatively slow), or to both. Since the lifetime of the excited state for most probes used to study proteins is subnanosecond to tens of nanoseconds, protein dynamics which occur on this time scale can be examined.

Fluorescent probes of protein structure and dynamics include the intrinsic aromatic amino acids tryptophan, tyrosine, and phenylalanine, and extrinsic chromophores that can be added to the system. Extrinsic probes can either be covalently attached to the protein through a free amino or sulfhydryl group, or linked through noncovalent interactions as, for example, a fluorescent substrate analogue binding to the active site of an enzyme. This diverse set of probes for protein structure and dynamics studies necessitates diverse spectral and temporal requirements for the excitation source. Synchrotron radiation is capable of providing many of these requirements. Several protein systems have been examined using time-resolved fluorescence techniques by taking advantage of synchrotron radiation.

Intensity Decay

Most of the planar aromatic molecules used as probes of proteins can be promoted into their first excited singlet electronic state within an energy range between the near ultraviolet and the near infrared. The absorption of the photon, creating the excited state from the ground state, occurs in less than a femtosecond. Vibrational relaxation processes occurring on the picosecond time scale force the chromophore to attain the lowest vibrational level of the first excited singlet state. It is from this energy level that most planar aromatic molecules will undergo fluorescence in competition with all the nonradiative processes. The deactivation of the excited state (A*) can be expressed in terms of the fluorescence rate constant, k_F, and a combined rate constant for all the nonradiative events, k_{nr}, by the equation

$$-d[A^*]/dt = (k_F + k_{nr})\,[A^*]. \qquad (1)$$

On integrating equation 1, the fluorescence intensity decay should follow the simple exponential function

$$I(t) \approx \exp(-t/\tau), \qquad (2)$$

where τ is the lifetime of the excited state and equals the reciprocal of the sum of the two rate constants. This expression holds only if the probe is in a noninteracting environment like a vacuum.

Fluorescent molecules in an interacting system are not likely to have simple photophysics. Complicating processes can occur either in the ground state or the excited state. In the ground state, even though the molecule is chemically pure, interactions with itself, other solutes, or the solvent can result in more than one environment for the probe. This ground-state heterogeneity will lead to more than one excited state; these different excited states may have different fluorescence and nonradiative rate constants, and this will complicate the analysis of the intensity decay function.

In the excited state, the probe is essentially a different chemical entity. The dipole moment of the excited state is different than that of the ground state, and this change can induce photochemistry and/or interactions with the environment. Depending on the relative rates of these processes, the overall kinetic scheme describing the decay of the excited state could become more complex. If the interactions are faster than the resolution of the experiment, then the effect is averaged and a single exponential decay is expected. If the processes are much slower than the fluorescence and nonradiative rates, then fluorescence will have occurred before those events could affect the kinetic scheme. But if the interactions are on the same time scale as the excited-state lifetime, then excited-state reactions are occurring and the kinetic expression for the loss of the excited species can become very complex.

In most cases of complex kinetic decay mechanisms, the overall intensity decay can be expressed by the sum of many first order events, i.e.

$$I(t) = \sum_i \alpha_i \exp(-t/\tau i), \qquad (3)$$

where α's are the pre-exponential weighting factors, or amplitudes. Other kinetic mechanisms can yield different complex rate expressions; for example, a diffusion-dependent system will require a square root of time term.

Anisotropy Decay

The polarization of an excited state, created by the absorption of polarized light, can be lost through rotation of the excited molecule, provided that the rotation occurs on the same time scale or faster than the decay of the excited state. Relationships have been derived relating the dimensions of the rotating unit, sweeping out a volume, to the time dependence of the decay of the initial polarized excited state (Rigler and Ehrenberg, 1973). A symmetrical molecule rotating as a sphere will exhibit only one emission anisotropy decay time constant. If the molecule is an ellipsoid of revolution, theory predicts five rotational correlation times containing the three diffusion constants. In practice, however, only three of the five are "obtainable" since two of the correlation parameters are essentially degenerate with the others (Small and Isenberg, 1977). The preceeding description assumed that the rotating fluorophore itself is a rigid body. A protein probe, either intrinsic or extrinsic and either covalent or noncovalent, could have depolarizing motions other than those caused by the rotation of the protein. The fluorophore may have rotational motion about its linkage independent of the protein. The portion (domain) of the polypeptide chain where the fluorophore is linked may also have independent motion (segmental flexibility). Thus the "simple" case of three exponentials to describe the decay of the emission anisotropy for an ellipsoid of revolution can be complicated by other motions within the protein. For the specific case of a mobile fluorescent probe covalently attached to a spherical protein, the decay of the emission anisotropy is the sum of three exponential terms (Rigler and Ehrenberg, 1973), where one term represents the global motion of the entire unit and the other two terms are related to motions involving the entire unit and the mobile probe.

The anisotropy decay, r(t), is typically evaluated by the sum of exponentials

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j). \qquad (4)$$

The ϕ_j terms must then be related to the rotational correlation times of the system. The pre-exponential β terms depend on trigonometric functions of the angles between the absorption and emission dipoles and a rotational symmetry axis (Rigler and Ehrenberg, 1973). The sum of the β terms equals the initial anisotropy of the system, r_o, before any depolarizing motions can occur.

Instrumentation

Our ability to probe biological systems through time-resolved fluorescence techniques has advanced rapidly over the recent years. Advances have come in both instrumentation and data analysis capabilities. Two conceptually different, but complementary methods have been developed and are used extensively. One method works in the time domain and is known as the pulse technique (Badea and Brand, 1979). The other method, known as the phase/modulation technique, works in the frequency domain (Gratton et al., 1984a).

Time Domain. Pulse fluorometers operate by accumulating a histogram of the probability of the decay of the excited state as a function of time. This operation is accomplished by measuring the time between the detection of a short pulse of light used to excite the sample and the detection of the photon emitted by fluorescence. One count is added to a multichannel memory device at the channel that corresponds to the measured time interval. This process is performed under single photon-counting conditions, and repeated thousands of times to collect a decay curve as outlined in Fig. 1.

The pulse of exciting light should be a true delta function with respect to the temporal resolution required by the experiment. In many cases, this can be achieved using current laser technology. The detection electronics, particularily the photomultiplier tube, of the pulse fluorometer, however, establish an instrument response function that can "broaden" a few picosecond-wide delta function into a measured distribution on the order of several hundreds of picoseconds up to more than a nanosecond. The collected decay curve, unfortunately, is therefore convolved with the instrument response function. Although recent advances in photomultiplier technology have significantly reduced the instrument response function, the temporal resolution required to resolve events with rate constants of 10^8 S^{-1} or faster will still result in an appreciable instrument response function.

To obtain the parameters describing the decay, the collected decay curve must be analyzed by curve fitting techniques. Several mathematical algorithms have been employed to perform this "deconvolution," including nonlinear least squares, method of moments, and LaPlace and Fourier transformations (Badea and Brand, 1979). Fig. 2 demonstrates the use of the nonlinear least squares technique to resolve the sum of two exponentials where the lifetimes are short and only separated by a factor of two. The ability to resolve lifetimes on the order of a 10% difference has recently been achieved through the use of "global" data analysis, where many decay curves collected as a function of an independent variable such as wavelength, pH, or temperature are analyzed together for common parameters (Knutson et al., 1983). The ability to resolve complex kinetic decay mechanisms has also been improved by the introduction of the linked-function analysis approach where specific decay parameters can be restrained within certain limits based on information obtained from another physical measurement (Ross et

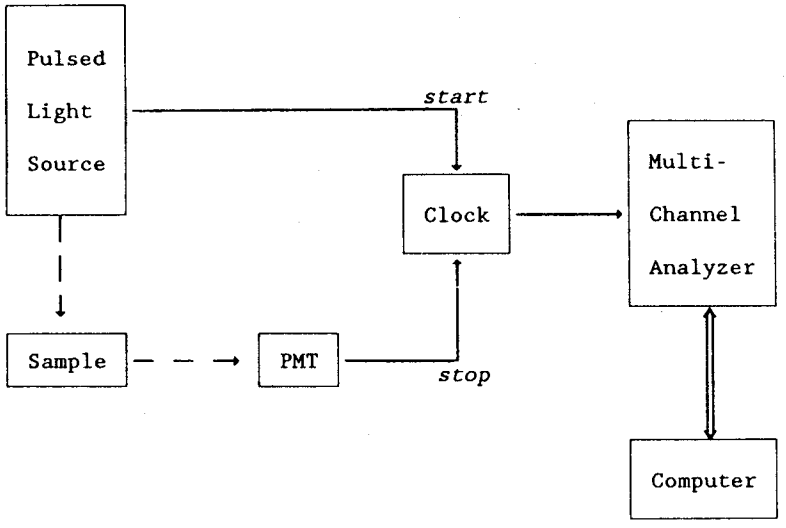

Fig. 1. Block diagram of a pulse fluorometer. Broken arrows represent light while solid arrows represent electrical signals. The start signal indicates when the light source has pulsed and the stop signal from the photomultiplier tube (PMT) is generated by the detection of a photon through fluorescence of the sample.

al., 1986b). By combining the linked-function approach with the global method, previously unresolvable systems, such as tryptophan fluorescence from a protein, can begin to be unraveled.

With the pulse method, time-resolved emission anisotropy can be determined by collecting decay curves through a polarizer oriented parallel and perpendicular to vertically polarized excitation light, hence obtaining I_p and I_s respectively. Eq. 5 defines r(t), where D(t) denotes the

$$r(t) = \{[I_p(t) - I_s(t)]/[I_p(t) + 2I_s(t)]\} = D(t)/S(t) \qquad (5)$$

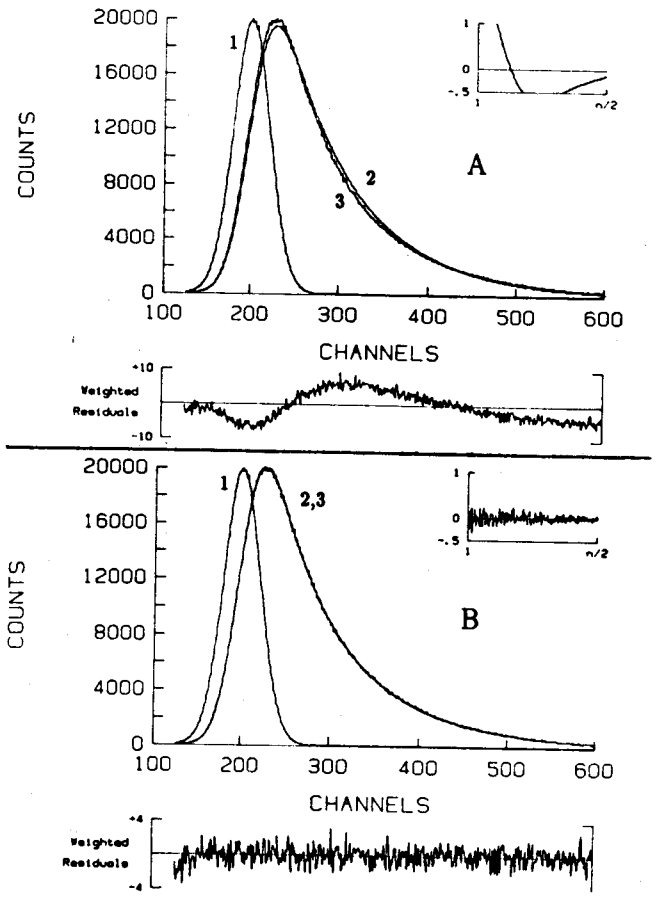

Fig. 2. Analysis of a simulated fluorescence decay curve. Curve 1 is the instrument response function, curve 2 is the calculated fit, and curve 3 is the decay curve. The weighted residuals are displayed below and the autocorrelation function of the residuals is in the inset. The decay was generated with τ_1 = 1.0 ns, τ_2 = 2.0 ns, $\alpha_1 = \alpha_2$, and "a" timing calibration of 20 ps/channel. Panel A: a fit to a single exponential. Panel B: a fit to a sum of two exponentials; the recovered parameters were within 5% of those used to generate the decay.

difference curve and S(t) is the sum curve which gives the total intensity decay. The parameters for r(t), as given in equation 4, can be determined by constructing S(t), obtaining its decay parameters using equation 3, and then iterating r(t) parameters, using the S(t) values, to fit D(t). With the recent advent of global data analysis, it is more precise to analyze the parallel and perpendicular decays together, without constructing D(t). As shown in equations 6 and 7, these decays depend both on the

$$I_p(t) = 1/3 \{S(t) [1 + 2r(t)]\} \quad (6)$$

$$I_s(t) = 1/3 \{S(t) [1 - r(t)]\} \quad (7)$$

total intensity as well as the anisotropy decay. Again by knowing S(t) parameters, r(t) parameters can be iterated to fit both polarized decays.

Frequency Domain. In frequency domain fluorescence spectroscopy, the fluorescent molecules are excited by a light source with an intensity sinusoidally modulated at high angular frequency, ω, typically in the megahertz range. If the fluorophore is characterized by a single exponential decay time, τ, then the emitted light will be sinusoidally modulated at the same frequency but delayed in phase and demodulated with respect to the excitation (Fig. 3). Equations 8 and 9 give the relationships for the phase shift Φ and relative modulation M,

$$\tan \Phi = \omega\tau \quad (8)$$

$$M = \{(ac/dc)_{EM} / (ac/dc)_{EX}\} = [1 + (\omega\tau)^2]^{-1/2}. \quad (9)$$

For a homogeneous emitting population the phase (τ^P) and modulation (τ^M) lifetimes will be equal and independent of the modulating frequency. If the emission is multiexponential or nonexponential, then a composite sinusoidal emission waveform results with frequency ω and a phase delay and de-

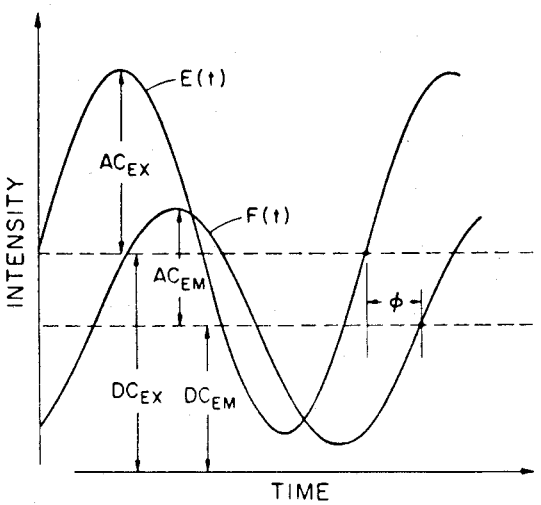

Fig. 3. Schematic representation of the excitation E(t) and fluorescence F(t) waveforms. Fluorescence is delayed by an angle Φ and demodulated with respect to the excitation.

modulation given by equations 10 and 11, where S and G are defined in equations 12 and 13,

$$\Phi = \tan^{-1}(S/G) \tag{10}$$

$$M = (S^2 + G^2)^{-1/2} \tag{11}$$

$$S = \sum_i f_i M_i \sin\Phi_i \tag{12}$$

$$G = \sum_i f_i M_i \cos\phi_i . \tag{13}$$

In equations 12 and 13 ϕ_i, M_i, and f_i are the phase delay, demodulation, and fractional intensity associated with each individually emitting species ($\sum f_i = 1$). In such a system, $\tau^P \neq \tau^M$ and both τ^P and τ^M are frequency dependent. The functions S and G are the sine and cosine transforms of the impulse response as shown in equations 14 and 15 (Weber, 1981).

$$S = \frac{\int_0^\infty I(t) \sin(\omega\tau)\, dt}{\int_0^\infty I(t)\, dt} \tag{14}$$

$$G = \frac{\int_0^\infty I(t) \cos(\omega\tau)\, dt}{\int_0^\infty I(t)\, dt} \tag{15}$$

These equations form the basis for the mathematical equivalence between the time domain and the frequency domain approaches. Consequently, the parameters describing the decay of the fluorescence (equation 3) are obtained by regression algorithms to fit the phase and modulation data as a function of ω. This fitting procedure can be enhanced by taking data as a function of an independent variable and analyzing in a global manner as was done in the pulse technique (Beechem et al., 1983).

Dynamic polarization measurements are the frequency domain equivalent of anisotropy decay measurements. In this method, the fluorescent solution is illuminated by intensity modulated light which is polarized parallel to the vertical laboratory axis. The phase delay, $\Delta\Phi$, between the parallel and perpendicular polarization components of the emission can then be directly determined as well as the ratio of their AC components, Y. Equations 16 and 17 can be derived for an isotropic rotator, where r

$$\Delta\Phi = \tan^{-1}\{(3\omega rR)/[(k^2 + \omega^2)(1 + r - 2r^2) + R(R + 2k + kr)]\} \tag{16}$$

$$Y^2 = \{[k + 6R/(1 - r)]^2 + \omega^2\}/\{[k + 6R/(1 + 2r)]^2 + \omega^2\} \tag{17}$$

is the limiting anisotropy, R is the rotational diffusion coefficient, and k is the radiative decay rate (Gratton et al., 1984a). Examples of simulated dynamic polarization curves are shown in Fig. 4.

The relatively recent appearance of true multifrequency phase and modulation fluorometers, developed in the laboratory of Enrico Gratton (Gratton and Limkeman, 1983), has enormously extended the scope and power of this method. A true multifrequency instrument provides facile selection of arbitrary modulation frequencies over a wide frequency range. Two of the more significant advances in phase fluorometry were the application of cross-correlation techniques (Spencer and Weber, 1969) and the use of electro-optic devices (Pockels cells) for light modulation.

Excitation Light Sources. The pulse technique requires that the exciting light come from a pulsed source. Recently, utilization of intrinsically modulated sources such as synchrotron radiation and mode-locked lasers to collect phase and modulation data has affected a union of the impulse and harmonic response approaches. This union has stemmed from the need to resolve all of the parameters involved in a complex kinetic scheme or to accurately recover events occurring with rates of 10^8 s^{-1} or faster. This goal requires that frequency domain instruments be able to provide modulation frequencies into the gigahertz range, which is presently impossible through electro-optic devices.

High repetition pulsed light sources, such as synchrotron radiation, were suggested as possible light sources for multifrequency phase and modulation fluorometry in an analogous manner to the traditional sinusoidally modulated source (Gratton and Lopez-Delgado, 1980; Munro, 1983; Munro and Schwentner, 1983). As shown in Fig. 5 for the temporal structure of the ADONE storage ring at the National Laboratory in Frascati, Italy, the pulsed nature of the light source corresponds to a set of equally spaced frequencies separated by the inverse of the time between the pulses (Gratton et al., 1984b). This frequency set has a Gaussian envelope (because the synchrotron pulse is Gaussian) with a half width the reciprocal of the half width of the pulse duration. Consequently, the shorter the pulse the higher the frequency distribution will extend into the gigahertz region.

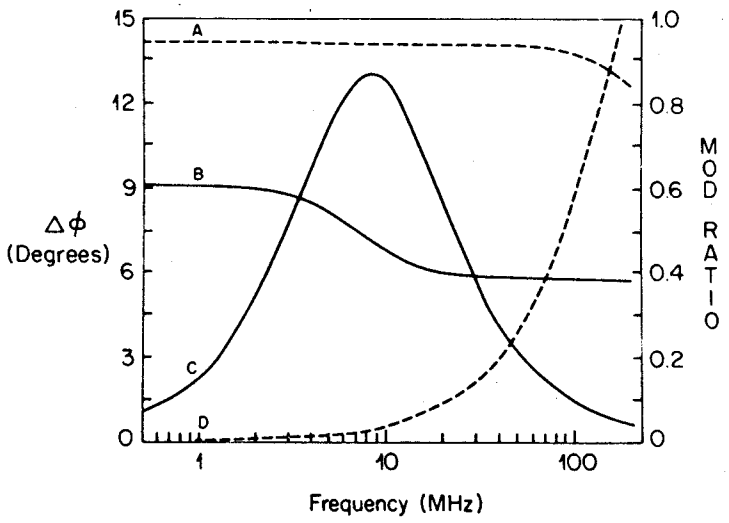

Fig. 4. Simulated dynamic polarization results for two specific cases. Solid lines denote $\Delta\Phi$ and dashed lines denote Y, or the modulation ratio. Curves B and C were generated with τ = 26 ns and the Debye rotational relaxation time ρ = 87 ns. Curves A and D were generated with τ = 1.9 ns and ρ = 0.54 ns.

An ideal light source for both a pulse and a phase/modulation fluorometer would have: 1) easy selection of a 1 nm-wide band of a continuous wavelength distribution from 200 to 800 nm; 2) high photon flux at all wavelengths; 3) a pulse width less than 10 ps and 4) polarized light. The two instruments have different repetition rate requirements, however. The pulse instrument would be benefitted by having a light source with a selectable pulse rate up to 25 MHz, while the phase/modulation instrument would be best operated at a fixed frequency near 1 MHz to obtain optimal harmonic content.

Synchrotron radiation from storage rings can provide many of these qualifications for an ideal light source for either type of fluorescence instrument. The light is continuous over the desired energy range, it is intense, and it is highly polarized. While the pulse width varies from machine to machine, and many are not as short as desired, this need is not as critical as the others. Many machines also do not have an optimal time structure, but experiments can usually be performed. Also, different electron bunch fill patterns can often be accomodated in the storage ring if a particular repetition rate is required.

PROTEINS, TIME-RESOLVED FLUORESCENCE, AND SYNCHROTRONS

The major advantage that synchrotrons have as the excitation source for time-resolved fluorescence studies on proteins is the ability to excite in the ultraviolet. Consequently, the work on proteins using synchrotron radiation has concentrated on the intrinsic fluorescence of the aromatic amino acids. Even with this advantage over other light sources, only a few studies have been done due to the fact that the intensity decay

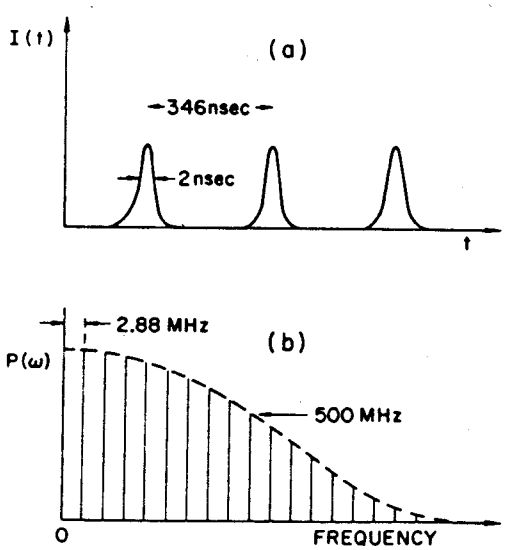

Fig. 5. Panel (a) gives a schematic representation of the light pulses emitted by the ADONE storage ring operating in single bunch mode. Panel (b) gives the power spectrum of the pulse train in panel (a).

characteristics of tryptophan and tyrosine are still not fully understood. The problem is explaining the causes of the multi-exponential decay behaviour of the free amino acids, simple amino acid analogues, and proteins with either a single tryptophan or tyrosine (Beechem and Brand, 1985). Without a complete understanding of the underlying photophysics, it will be difficult to use a change in a decay parameter to explain a property of a protein.

One of the first studies of intrinsic fluorescence of proteins using synchrotron radiation was carried out on hemoglobin and its isolated α and β chains (Alpert and Lopez-Delgado, 1976). The intrinsic fluorescence of these proteins is very weak due to very efficient energy transfer to the heme moiety. The measurements, in the time domain, were facilitated by the high intensity and repetition rate of the ACO storage ring in Orsay, France. A static quenching mechanism was implied by the data, since the decrease observed in the quantum yield between apohemoglobin and hemoglobin was not corroborated by a equivalent decrease in the "average" lifetime.

The excellent deep ultraviolet advantages of synchrotron radiation were utilized in a study of the excitation wavelength dependence of tryptophan's fluorescence lifetime. The pulse instruments at both the ACO storage ring and the SPEAR facility at Standford were used (Alpert et al., 1979; Jameson and Alpert, 1979). The major 3.1 ns lifetime at 20° C and pH 7 was demonstrated to be invariant with excitation between 220 to 320 nm, confirming that fluorescence stems from the lowest energy level of the first excited singlet state independent of the initial excited state.

The SPEAR facility was also used for some pioneering work on protein dynamics using time-resolved fluorescence anisotropy. Several proteins containing a single tryptophan were examined, and it was shown that the tryptophan residue exhibited different degrees of rotational freedom with respect to the rest of the protein (Munro et al., 1979). The single rotational correlation time of 9.9 ns at 20° C for the tryptophan residue in the 20,000 dalton Staphylococcus aureus nuclease B demonstrates that it has essentially no independent mobility. Two rotation times of 0.09 and 1.26 ns were seen for the tryptophan residue in the bovine basic A1 myelin protein (18,000 daltons) suggesting a very unhindered residue. Intermediate of these two examples was the single tryptophan residue of human serum albumin. At 8° C, it showed no independent motion and a single rotational correlation time consistent with rotation of the protein. But at 43° C, a second time of 0.14 ns was seen indicating an increase in mobility of the tryptophan residue with temperature.

Tyrosine to tryptophan resonance energy transfer in bovine serum albumin was studied using the phase/modulation instrument constructed at the ADONE storage ring at Frascati, Italy (Gratton et al., 1984b). Energy transfer was detected by examining the $\tau^P \neq \tau^M$ inequality, particularly the fact that $\tau^P > \tau^M$ with 270 nm excitation which is an indication of an excited-state reaction. Using a fixed modulation frequency, a "transfer excitation spectrum" was recorded by monitoring the phase lifetime as a function of the excitation wavelength. This spectrum was quite similar to the absorption profile of tyrosine, with a maximum near 270 nm (Antonangeli et al., 1983).

The pulse instrument at the SRS facility in Daresbury, England, has been used to examine the time-dependent emission anisotropy of the single tyrosine residue in the peptide hormone angiotensin (Munro et al., 1985). Two rotational correlation times were reported; the shorter time of 0.3 ns when compared to the 3.7 ns component probably indicates some independent motion by the tyrosine residue with respect to the rest of the hormone.

The pulse instrument at ACO has recently been used for two different protein systems. As might be expected, the intensity decay of the seven tryptophans in wheat germ hexokinase LI is complex (Merola and Brochon, 1986). On the other hand, the anisotropy decay appears to be very simple with a single rotational correlation time relating to the entire protein. The two tryptophans in aspartate transcarbamylase from Escherichia coli were also found to have complex decay kinetics (Royer et al., 1987). Two lifetimes, however were found to contribute over 95% of the total fluorescence; by assuming one lifetime for each tryptophan residue, the effects on various portions of the protein were examined upon binding of a substrate analogue and nucleotide effector molecules.

In an attempt to understand the complex fluorescence decay kinetics of tyrosine, a systematic study was conducted using the pulse instrument at the NSLS at Brookhaven National Laboratory in New York (Laws and Sutherland, 1986). Researchers examined phenol, straight-chained phenol analogues, tyrosine, and tyrosine derivatized at the α-amino and α-carboxyl groups as a function of pH below neutrality (Laws et al., 1986). Only in tyrosine and its analogues were multi-exponential kinetics observed that could not be explained by the number of ionic species in solution or the possibility of excited-state proton transfer. The kinetics could be explained in terms of the rotamer model (Gauduchon and Wahl, 1978), where ground-state heterogeneity exists due to the different environments seen by the three configurations about the C^{α}-C^{β} bond. The analysis of the data was possible through the use of the linked-function approach (Ross et al., 1986b); ground-state rotamer populations calculated from H-NMR spectra were linked to the amplitudes during iteration. If the rotamer model is the correct interpretation of the kinetic mechanism, then an upper limit of 10^8 s^{-1} must be placed on the rate of interconversion for the rotamers about the C^{α}-C^{β} bond. These studies were extended to oxytocin, a small peptide hormone containing a single tyrosine residue (Ross et al., 1986a). The rotamer model was able to explain the complex decay behaviour, including the requirement that one of the tyrosyl rotamers be statically quenched by an interaction with the internal disulfide bond of the hormone. These tyrosine studies were facilitated by synchrotron radiation because it helped overcome the low extinction coefficient and quantum yield of tyrosine as well as the problem of Raman light scattering.

The ADONE phase/modulation instrument has recently been used for studies on two different protein systems. Preliminary lifetime determinations were done on the single tryptophan in the protein biosynthesis elongation factor Tu of prokaryotes (Jameson et al., 1987). The fluorescence decay kinetics were shown to be complex, but the tryptophan residue could be dynamically quenched by added acrylamide. The fluorescence decay characteristics of the single tryptophan of the iron storage protein ferritin is also complex (Rosato et al, 1987). A difference was found in the pattern of the decay parameters between the individual subunit and the assembled apoprotein. Since the tryptophan is near the subunit-subunit interface, this probably indicates an altered environment for the tryptophan residue upon assembly. Furthermore, while the addition of iron to the apoprotein quenched the intrinsic fluorescence, it was a nonlinear process indicating the presence of both static and dynamic quenching of the tryptophan induced by the binding of iron ions.

With the abilities of the technology of genetic engineering, it should be possible to probe specific domains of a protein by introducing a single tryptophan or tyrosine residue and then examining it by time-resolved fluorescence. A similar approach has been taken in the study of lactate dehydrogenase from Bacillus stearothermophilus using the SRS pulse instrument (Waldman et al., 1987). The native protein contains three tryptophan residues, and its fluorescence decay could be fit by the sum of three

exponentials. Site-directed mutagenesis was used to make two mutant proteins, replacing either one or two of the tryptophans with tyrosine. The mutant enzymes are fully active with near normal physical properties and substrate affinities. With one tryptophan removed, the fluorescence decay was double exponential and with two of the three residues replaced the decay was single exponential. Thus in this protein system, it appears that the individual tryptophans do not have complex kinetics but are single exponentials, and that this then allows lifetimes to be assigned to individual residues.

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