

Time-resolved methods in biophysics. 8. Frequency domain fluorometry: applications to intrinsic protein fluorescence†

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Time-resolved fluorescence spectroscopy is an indispensable tool in the chemical, physical and biological sciences for the study of fast kinetic processes in the subpicosecond to microsecond time scale. This review focuses on the development and modern implementation of the frequency domain approach to time-resolved fluorescence. Both intensity decay (lifetime) and anisotropy decay (dynamic polarization) will be considered and their application to intrinsic protein fluorescence will be highlighted. In particular we shall discuss the photophysics of the aromatic amino acids, tryptophan, tyrosine and phenylalanine, which are responsible for intrinsic protein fluorescence. This discussion will be illustrated with examples of frequency domain studies on several protein systems.

Introduction

George Gabriel Stokes was the first to understand that the phenomenon originally termed “dispersive reflection” resulted from absorption of light followed by emission. In his seminal paper published in 1852 he also renamed this phenomenon “fluorescence”, specifically he wrote “I am almost inclined to coin a word and call the phenomenon ‘fluorescence’ from flourspar as the analogous term opalescence is derived from the name of a mineral”.¹ Stokes was certainly aware of the phenomenon known now as phosphorescence and that a time interval existed between absorption of light and emission of phosphorescence (which had in fact been remarked upon by Galileo!) but could

not distinguish any such time delay between absorption and fluorescence. Interestingly he stated “I have not attempted to determine whether any appreciable duration could be made out by means of a revolving mirror.” The first serious attempt to determine the lifetime of the fluorescence process was reported by R. W. Wood in 1921² who tried an ingenious arrangement involving shooting a jet of anthracene at high pressure from a narrow nozzle while illuminating the tip of the nozzle to see if one could measure the length of the emitted light in the fast flowing stream and hence ascertain the duration of the emission. Given the spatial resolution (0.1 mm) and the flow speed (230 m s⁻¹) available, Wood was only able to state that the fluorescence lifetime must be less than 2 300 000⁻¹ s, about ~435 ns. Accurate fluorescence lifetime measurements were first achieved by Enrique Gaviola^{3,4} using the Kerr effect to sinusoidally modulate the exciting light (the fact Gaviola named his phase instrument a “fluorometer” is why that name is usually used for lifetime apparatus while the term “fluorimeter” is reserved for steady-state instruments). Gaviola reported lifetimes of 4.5 ns for uranin (sodium fluorescein) and 2.0 ns for rhodamine B, in reasonable agreement with modern

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values. Needless to say, in the eight decades following these seminal efforts enormous progress has been made in time-resolved instrumentation.

Frequency domain methods

Intensity decays – lifetimes

Time-resolved fluorescence spectroscopy has proven to be an indispensable tool in the chemical, physical and biological sciences for the study of fast kinetic processes in the subpicosecond to microsecond time scale. The relevance to biophysics lies in the information such kinetic details provide concerning the dynamics of macromolecular assemblies, *i.e.* proteins, membranes and nucleic acids. It is important to realize that fluorescence techniques, with the exception of Förster Resonance Energy Transfer (FRET), do not *per se* provide direct information on biomolecular structure. Rather these methods provide information on the dynamics of biomolecules on the nanosecond time scale and on the accessibility of fluorescent moieties to solvent. Fluorescent molecules serve as “molecular stopwatches” which start with the absorption of light and stop with the emission process. To fully understand the molecular events being observed, such as biomolecular dynamics, FRET or interactions with quencher molecules, accurate determinations of the excited state lifetime must be obtained. These lifetimes are usually measured by either frequency domain or time domain methods. In principle the information content of these two approaches are very similar and are related *via* Fourier transforms. This review will be restricted primarily to the frequency domain approach.

In the frequency domain approach the exciting light is typically modulated sinusoidally at high frequencies. Due to the persistence of the excited state, fluorophores subjected to such sinusoidal excitation will give rise to a modulated emission, shifted in phase relative to the exciting light as depicted in Fig. 1.

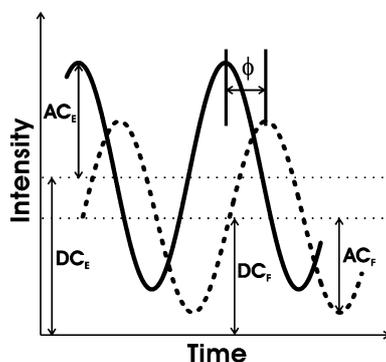


Fig. 1 Relative phase shift (ϕ) and modulation of the excitation (subscript E) and emission (subscript F) light in frequency domain fluorescence spectroscopy. AC and DC indicate the amplitude and offset of the respective waves.

In such a scheme, Dushinsky⁵ first demonstrated that:

$$F(t) = F_0 [1 + M_F \sin(\omega t + \phi)] \quad (1)$$

where $F(t)$ and F_0 are the fluorescence intensities at time t and time zero, respectively, ϕ is the phase delay between the excitation and emission, and ω is the angular modulation frequency, equal to $2\pi f$, where f is the linear modulation frequency. M_F is the modulation

of the fluorescence, as defined below. This relationship signifies that measurement of the phase delay, ϕ , forms the basis of one measurement of the lifetime, known as the *phase lifetime*, τ_p . In particular:

$$\tan \phi = \omega \tau_p \quad (2)$$

The ratio of the AC to DC components of the excitation (M_E) and the emission (M_F) are given by:

$$M_E = \left(\frac{AC_E}{DC_E} \right) \text{ and } M_F = \left(\frac{AC_F}{DC_F} \right) \quad (3)$$

The relative values of the emission and excitation modulations are thus:

$$M = \frac{(M_F)}{(M_E)} \quad (4)$$

The lifetime can be determined from these modulation values from:

$$M = \frac{1}{\sqrt{1 + (\omega \tau_M)^2}} \quad (5)$$

This lifetime is known as the *modulation lifetime*, τ_M . One can also describe the waveforms *via* the relations:

$$\phi = \tan^{-1}(S/G) \quad (6)$$

and

$$M = (S^2 + G^2)^{1/2} \quad (7)$$

where

$$S = \sum_i f_i M_i \sin \phi_i \quad (8)$$

$$G = \sum_i f_i M_i \cos \phi_i \quad (9)$$

The values of ϕ_i and M_i for each component are given by eqn (2) and (5), respectively; f_i is the fractional intensity of the i^{th} component ($\sum f_i = 1$). The functions S and G are the sine and cosine transforms of the impulse response.⁶ If $I(t)$ represents the free decay after excitation then:

$$S = \frac{\int_0^\infty I(t) \sin(\omega t) dt}{\int_0^\infty I(t) dt} \quad (10)$$

$$G = \frac{\int_0^\infty I(t) \cos(\omega t) dt}{\int_0^\infty I(t) dt} \quad (11)$$

These equations provide the basis for the mathematical equivalence between the harmonic (frequency domain) and impulse (time domain) response methods. Differences between the lifetime determined by phase (τ_p) and that determined by modulation (τ_M) and the frequency dependence of these values form the basis of the methods used to analyze for lifetime heterogeneity, *i.e.*, the component lifetimes and their relative amplitudes. We note that practitioners of frequency domain fluorometry typically

describe the contributions of various components to the emission in terms of fractional intensities, f_i , which strictly correspond to the contribution of the i^{th} component to the photocurrent. Practitioners of time domain methods, on the other hand, typically refer to the pre-exponential terms, α_i , associated with each lifetime component; these terms are related to the actual number of fluorescing species being observed. The relationship between the f and α functions, assuming that the quantum yields of the emitting species are proportional to their lifetimes, is given by the expression:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (12)$$

During the four decades after Gaviola's seminal work, several groups described phase fluorometers which introduced different approaches to light modulation and detection of the fluorescence. Notable advances were made by Szymanoski,⁷ Maercks,⁸ Bailey and Rollafson,⁹ Birks and Little,¹⁰ Schmillen,¹¹ Ravilious *et al.*,¹² Bauer and Rozwadowski,¹³ and Birks and Dyson.¹⁴ A significant advance in the field of frequency domain fluorometry came with the development of the cross-correlation fluorometer of Spencer and Weber¹⁵ which used a Debye–Sears ultrasonics tank to generate the sinusoidal light. In this instrument white light from a xenon arc lamp could be passed through the Debye–Sears tank (filled with a 19% solution of ethanol in water to minimize temperature effects) which, due to the standing wave of solution densities, gave rise to light modulation at the entrance of the excitation monochromator. This instrument could operate at two modulation frequencies, namely 14.2 and 24.4 MHz, although switching between these frequencies was not a trivial operation (DMJ personal observation). An important feature of this instrument was the use of heterodyning *i.e.*, cross-correlation, which transferred the phase and modulation values from the high frequency regime to a low frequency regime. The heterodyning was accomplished by modulating the voltage at one of the photomultiplier dynodes at a frequency corresponding to the sum of the light modulation frequency plus a small additional frequency. In the case of the Spencer–Weber instrument the cross-correlation frequency was 36 Hz.

The company SLM (the initials came from the names Richard Spencer, David Laker and George Mitchell—all of whom were associated with Gregorio Weber at the time the company was started) introduced the first commercial phase and modulation fluorometer in the early 1970s, using cross-correlation and a Debye–Sears light modulator, which operated at two frequencies, 10 MHz and 30 MHz. This design was soon followed by a three frequency instrument which operated at 6, 18 and 30 MHz. Phase fluorometers offering truly variable—and many—light modulation frequencies appeared a decade or so after the Spencer–Weber instrument. Haar and Hauser¹⁶ described a multifrequency phase fluorometer in 1978, and in 1983 Gratton and Limkeman¹⁷ described a multifrequency phase and modulation fluorometer using cross-correlation. The original Gratton–Limkeman instrument used laser sources for excitation (originally either helium–cadmium or argon-ion) and was soon (by 1984) implemented commercially by the company ISS, Inc (ISS, Inc, Champaign, IL) to allow utilization of a xenon arc lamp. The Gratton design used a

Pockels cell, a voltage-controlled waveplate, for light modulation. Specifically, the plane of polarization of the light entering the Pockels cell can be rotated by application of voltage and hence the frequency of this voltage determines the light modulation frequency. For a comparison of the SLM and Gratton frequency domain instruments see Jameson *et al.*¹⁸

Another innovation in frequency domain fluorometry came about in 1984 when Gratton *et al.*,¹⁹ described a frequency domain instrument set up at the ADONE synchrotron radiation source in Frascati, Italy which utilized the harmonic content of the pulsed light source to generate a set of modulation frequencies. When the ADONE electron storage ring was operated in the single bunch mode the light pulses were approximately 346 ns apart and the shape of the pulse was approximately Gaussian with a half-width of about 2 ns. The corresponding frequency transform consisted of a set of harmonic frequencies spaced 2.886 MHz apart. This frequency set (a comb function) had a Gaussian envelope with a half-width of ~500 MHz. Although this original implementation with synchrotron radiation was used with single bunch operation of the storage ring to achieve a low base frequency, the same concept was later used at the Aladdin storage ring at the University of Wisconsin by reducing the intensity of one of the 15 electron bunches thus essentially creating a “hole” which propagated at 3.377 MHz, which also generated a complete set of harmonic frequencies but without the disadvantage of greatly reducing the overall light from the storage ring by restricting operation to single bunch mode. Soon after the Frascati instrument was built, the harmonic content approach was implemented on a mode-locked argon-ion laser/frequency doubled dye laser system which provided light in the 280–310 nm range. Amplitude modulation of the pulse train permitted quasi-continuous modulation of the frequency from a few hertz up to gigahertz.²⁰ Although laser sources did not have the virtually unlimited wavelength range and tunability of synchrotron radiation sources, they had the great advantage of accessibility and relatively low cost. Modern laser sources, *e.g.*, mode-locked femtosecond lasers such as Titanium:Sapphire systems, are now widely used as excitation sources for both frequency domain and time domain lifetime measurements (see for example²¹).

More recently LEDs and laser diodes have been used in frequency domain measurements and these systems are commercially available. Barbieri *et al.*,²² for example have reported the use of UV emitting LEDs (280 nm and 300 nm) with frequency domain measurements to study intrinsic protein fluorescence. These LEDs allow direct modulation of the excitation source by sinusoidally modulating the input voltage of the LED. These light sources can in principle be modulated to higher frequencies than available using a Pockels cell (see, for example, <http://www.iss.com/> for technical information and measurement examples).

When working with samples of biological origins one often encounters background fluorescence which is not negligible compared to the sample signal. In the early days of phase fluorometry such background fluorescence presented a problem since there was no easy way to subtract the background signal from the sample signal, whereas background subtraction was simple to implement in the time-domain approach. Reinhart *et al.*,²³ however, developed a simple background subtraction method for frequency domain data based on determination and subtraction of the phasor associated with the background from the sample

phasor. Another technical consideration for either time-domain or frequency domain lifetime determinations is the use of “magic angle” polarizers to eliminate the effect of polarized emission on the lifetime.²⁴ One such case would be vertical polarization for excitation and observation of the emission through a polarizer oriented at 55° from the vertical.

Anisotropy decay – rotations of fluorophores

In addition to providing fluorescence intensity decay data, time-resolved fluorescence can provide important information on rotational modalities of fluorophores.²⁵ In the time-domain this technique is called time-decay anisotropy while in the frequency domain it is often known as dynamic polarization. In principle the information content of the two approaches is identical. In the frequency domain approach the data consists of measurements of the phase delay between the parallel and perpendicular components of the emission as well as the relative modulation of the AC components of the parallel and perpendicular emissions. The changes in the phase delay and relative modulation are due to the rotation of the excited fluorophore during the excited state lifetime, which changes the orientation of the emission dipole. For a single rotating spherical component, the relations between the observed phase and modulation data and the rotational parameters, derived by Weber,⁶ are:

$$\Delta\phi = \tan^{-1} \left[\frac{18\omega r_o R}{(k^2 + \omega^2)(1 + r_o - 2r_o^2) + 6R(6R + 2k + kr_o)} \right] \quad (13)$$

$$Y^2 = \frac{\left((1 - r_o + 6R)^2 + (1 - r_o)^2 \omega^2 \right)}{[(1 + 2r_o)k + 6R]^2 + (1 + 2r_o)^2 \omega^2} \quad (14)$$

where $\Delta\phi$ is the phase difference between perpendicular and parallel components, Y the modulation ratio of the perpendicular and parallel AC components, ω the angular modulation frequency, r_o the limiting anisotropy (which is the anisotropy observed for the fluorophore in the absence of rotation),²⁶ k the radiative rate constant ($1/\tau$), and R the rotational diffusion coefficient, which is equal to twice the reciprocal of the rotational relaxation time. We should note that the rotational relaxation time (ρ), which is the time required for a species to rotate through an angle equal to the arccos of $1/e$ ($\sim 68.42^\circ$), is equal to three times the rotational correlation time.

Dynamic polarization allows one to resolve rotational components just as one can resolve intensity decay components in lifetime measurements. Fig. 2a depicts differential phase data for three rotational species, namely 3 ns, 30 ns and 300 ns (rotational relaxation times). The associated modulation data are shown in Fig. 2b. In cases where the fluorophore is attached non-covalently to a macromolecule there may be little or no motion of the fluorophore other than that due to the overall, “global” motion of the macromolecule. This lack of “local” probe mobility is due to the fact that a non-covalently bound fluorophore must be attached to a macromolecule at several points in order to have appreciable binding affinity. In some cases a fluorophore may be attached to a macromolecule but capable of “local” mobility independent of the “global” rotation of the larger particle. Such may be the case if a probe is attached to a macromolecule *via* a covalent linkage, *e.g.*, a

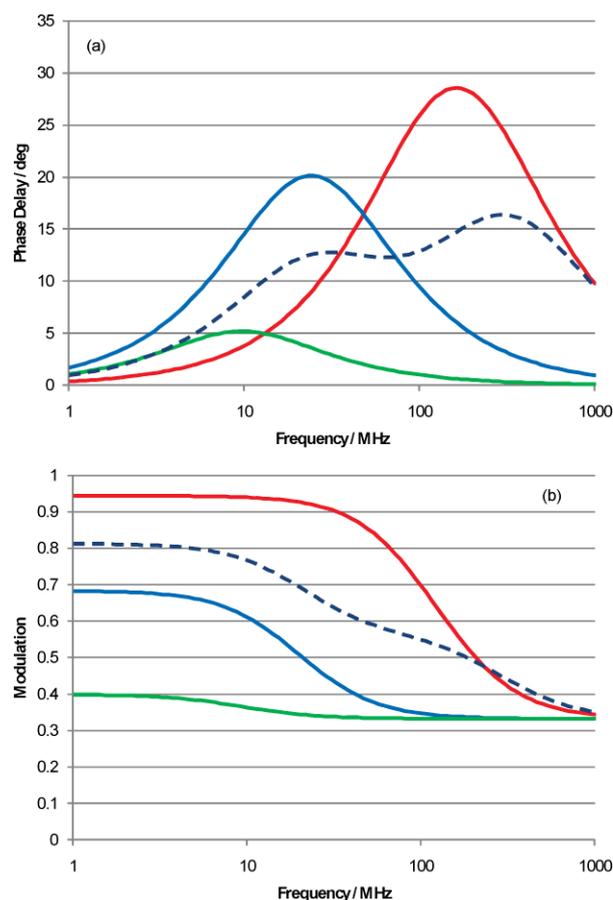


Fig. 2 Differential phase data (a) and modulation ratio (b) for an isotropic rotator with 3 ns (solid red), 30 ns (solid blue) and 300 ns (solid green) rotational relaxation times. The dashed blue line in the phase and modulation curves correspond to the case of two rotational relaxation times, namely 30 ns and 1.5 ns, with associated anisotropies of 0.2 for each component. In each case a fluorescence lifetime of 20 ns was used and colinear excitation and emission dipoles (*i.e.*, limiting anisotropies of 0.4) were assumed.

probe attached to an amine or sulfhydryl group in a protein. Also, tryptophan residues in proteins can have considerable “local” mobility due to the rotation of the indole moiety and dynamic aspects of the polypeptide chain which can experience mobility separate from the overall rotation of the entire protein. Fig. 2 also illustrates the effect of a second, faster rotational component, *e.g.* a “local” mobility component, on dynamic polarization plots. Specifically, a fast, 1.5 ns rotational relaxation time component is shown added to a 30 ns “global” rotational relaxation time component; in this case the anisotropy associated with each component is equal to 0.2.

Data analysis – lifetime components

In 1981 Weber solved the formidable mathematical problem of recovering lifetime components based on knowledge of the phase and modulation lifetime values at 2 or 3 frequencies. This analytical solution²⁷ allowed one to extract two lifetime components extremely rapidly from the data set and was first applied to the case of tryptophan lifetimes as a function of pH.²⁸ The availability of multiple frequencies (*e.g.* from the Gratton

design) immediately raised an interesting issue as regards data analysis. Namely, it was discovered that the exact analytical solution (Weber's Algorithm), which was rigorously correct for resolving N-components given N-modulation frequencies, was not practical for more than three frequencies.²⁹ The reason for the difficulties was the fact that the equations used in Weber's Algorithm involved a weighting of the moments of a distribution which had the effect of requiring increasingly higher precision as the number of modulation frequencies increased. For example, in the case of two frequencies, precision of 100 ps was sufficient for most cases whereas a precision of 5 ps was required to resolve three components from a set of three frequencies. For these reasons a non-linear least squares approach to fitting multi-frequency data was developed.²⁹ It is important to note that in this approach the phase and modulation data must be fit—not the phase and modulation lifetimes. The quality of the data fit is then judged by the reduced chi-squared value (χ^2) which is given by:

$$\chi^2 = \sum [(P_c - P_m)/\sigma_p]^2 + [(M_c - M_m)/\sigma_M]^2 / (2n - f - 1) \quad (15)$$

where P and M refer to phase and modulation data, respectively, the subscripts c and m refer to calculated and measured values, and σ_p and σ_M refer to the standard deviations of each phase and modulation measurement, respectively. n is the number of modulation frequencies and f is the number of free parameters. As an aside, we note that the precision of modern commercial phase and modulation instruments is generally around 0.2° of phase angle and 0.004 modulation values (or better) and these values are typically used in the chi-squared calculations. "Good" fits between the data and the model (*i.e.*, single exponential, two-exponential, *etc.*) typically are characterized by chi-squared values around 1. A typical set of phase and modulation data as well as a residual plot (which gives rise to the chi-squared) is shown in Fig. 3 for a solution of tryptophan at different pH values.

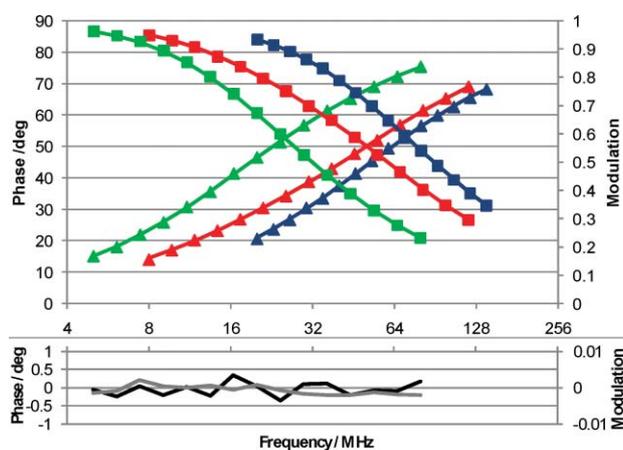


Fig. 3 Phase (triangles) and modulation (squares) of tryptophan in solution at pH 6.5 (blue), 8.9 (red) and 10.3 (green). Solid lines indicate fit to experimental data points. For each of the pH values, the data is fit to two discrete lifetime components of 3.1 ns and 9.0 ns with relative proportions of 99% and 0%, 68% and 31%, and 6% and 94% for pH 6.5, 8.9 and 10.3 respectively. Additionally there was a small <1% scattering component for each. Inset below indicates residuals of fit of phase (black) and modulation (grey) of tryptophan at pH 10.3. The χ^2 of the fit was 0.49.

In addition to decay analysis using discrete exponential decay models, one may also choose to fit the data to distribution models.

In this case, it is assumed that the excited state decay characteristics of the emitting species actually results from a distribution of decay rates. We note that distributions arise not just from distributions of discrete lifetime components but rather are due to the overall kinetic scheme of the system. This approach was developed for intrinsic protein fluorescence by Alcalá *et al.*,^{30,31} as discussed later.

Data analysis – rotational components

Rotational data, as well as intensity decay data, are typically analyzed using non-linear least squares methods. For the simplest cases the faster motions are usually assigned as "local" motions and the slower rates are termed "global" motions. For example, "local" versus "global" tryptophan mobility was demonstrated using frequency domain fluorometry for the case of Elongation Factor Tu (a single tryptophan protein; 43 kDa) by itself and in a heterodimer with Elongation Factor Ts (a protein with no tryptophan residues; 31 kDa).³² The dynamic polarization curves for this system (Fig. 4) show that the tryptophan residue in EF-Tu has limited local mobility, a fact evident from the clear decrease in the $\Delta\phi$ data after the maximum is reached. In the complex with EF-Ts, however, this tryptophan residue is able to move more readily, as evidenced by the increasing value of $\Delta\phi$ at higher frequencies. The "global" rotational rate of the TuTs complex increased relative to that of EF-Tu alone; specifically the "global" rotational relaxation times of TuTs and EF-Tu were 84 ns and 63 ns, respectively. We note that the relative orientations of the absorption and emission dipoles of the tryptophan moiety with respect to the rotational axes of the protein system will affect the observed "global" rotational rate.

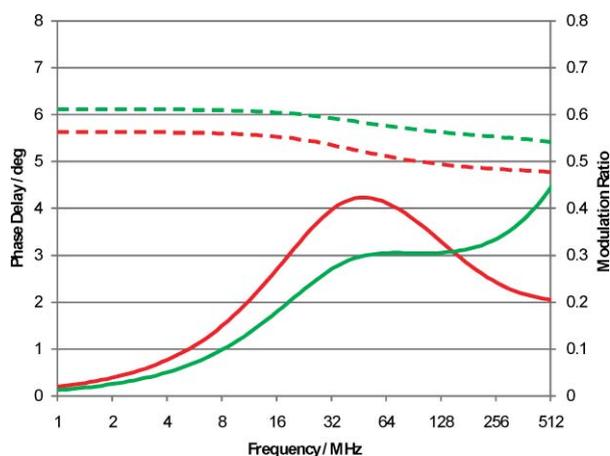


Fig. 4 Differential phase data (solid) and modulation ratio (dashed) EF-Tu-GDP (red) ($\tau_1 = 4.8$ ns, $\tau_2 = 0.31$ ns, $f_1 = 0.79$, $\rho_1 = 63$ ns, $r_1 = 0.23$, $\rho_2 = 1.5$ ns, $r_2 = 0.05$) and EF-Tu-EF-Ts (green) ($\tau_1 = 4.6$ ns, $\tau_2 = 0.23$ ns, $f_1 = 0.82$, $\rho_1 = 84$ ns, $r_1 = 0.19$, $\rho_2 = 0.3$ ns, $r_2 = 0.12$). Adapted from.³² Reproduced with permission from *Biochemistry*, 1987, **26**, 3894–3901. © 1987 American Chemical Society.

One must realize, though, that these assignments, *i.e.*, "global" versus "local", are merely a shorthand way of summarizing the system. In the case of highly dynamic biomolecules, such as proteins, a hierarchy of mobilities may exist and analysis in terms of 2 or 3 discrete rotational rates may be an oversimplification. In such cases one may be justified in analyzing the rotational data

in terms of distributions of rates, in much the same way in which one may analyze intensity decay data in terms of distributions of lifetimes. For example, Gryczynski *et al.*,³³ analyzed frequency domain dynamic polarization data of several single tryptophan peptides and proteins using distributions of rotational rates based on both Lorentzian and Gaussian functions. In cases of multiple lifetime components and multiple rotational rates one must also face the difficulty of assignment of lifetimes to specific rotational components. For example, one may assign all of a protein linked fluorophore's lifetime components to both the global and local rotations of the protein, a so-called "non-associative" model or one may assign one lifetime component to one rotational modality and another lifetime component to a different rotational modality, a so-called "associative" model. Associative models are clearly appropriate when the system contains two distinct fluorophores, such as a free and bound population and when the lifetime properties change upon binding. Clearly such specific models should be based on additional knowledge of the system.

Both frequency and time domain techniques have taken advantage of the "global analysis" method.³⁴ This approach allows one to simultaneously analyze multiple data sets and to link various parameters across these data sets. For example, in systems wherein the decay kinetics have a known relationship between samples, such as binding isotherms, one can affect a more robust solution by linking appropriate parameters. One can also subject the system to various alterations, such as physical (temperature or pressure), chemical (solvent variations, pH, *etc.*) or biochemical (ligands), and facilitate investigate which spectroscopic parameters are influenced by the alteration. An example of the application of frequency domain lifetimes and global analysis applied to the binding of ethidium bromide to transfer RNA is given by Tramier *et al.*³⁵ Global analysis software is available from the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu).

Another approach to analysis of time-resolved data is the Maximum Entropy Method (MEM) which uses "model-less" fits of the lifetime data to reveal the underlying decay kinetics.^{36,37} An insightful analysis of the application of MEM to both frequency domain and time domain data has been written by Brochon.³⁶ In the intervening years since this seminal work, however, very few frequency domain studies using MEM have appeared. Perhaps this dearth is due to the fact that the commercial software for MEM analysis of frequency domain data is still expensive and not normally provided by the instrument vendors with their software packages. In any event, the choice of whether to use direct model-fitting approaches or the MEM method is more of a philosophical choice since some researchers prefer to evaluate their data against a particular model whereas others prefer to avoid the constraints of models altogether. To date, the vast majority of MEM applications have used time-domain data.

Intrinsic protein fluorescence

Fluorescence studies of proteins usually rely on either (i) intrinsic protein fluorescence, (ii) fluorophores associated non-covalently with the protein, *e.g.*, 8-anilino-1-naphthalenesulfonic acid (ANS), porphyrins, NADH or FAD, or (iii) fluorophores covalently attached to particular amino acid residues, such as the ϵ -amino of lysine or the sulfhydryl of cysteine. Additionally, fluorescent proteins based on the original sequence of the Green

Fluorescent Protein isolated from the *Aequorea Victoria* jellyfish³⁸ have assumed a central role in cell and molecular biology—but the vast majority of such studies are not focused on understanding the photophysics of the protein/chromophore system. Each type of approach has its advantages and disadvantages, but this review shall focus on the use of intrinsic protein fluorescence.

The fluorescence excitation and emission spectra of the aromatic amino acids, namely, tryptophan, tyrosine and phenylalanine were reported by Teale and Weber in 1957³⁹ and this seminal work was followed by a series of papers by these investigators on protein fluorescence. Absorption and corrected emission spectra for tryptophan, tyrosine and phenylalanine are shown in Fig. 5. The transfer of excitation energy from tyrosine to tryptophan in proteins was also demonstrated during this time.^{40,41} Although Weber had speculated in 1961 that the lifetime of tryptophan in solutions or in proteins would be around 2.5–4 ns and 1–3 ns, respectively,⁴² based on considerations of oscillator strength, quantum yields and polarization measurements, instrumentation capable of measuring tryptophan lifetimes did not appear until the mid-1960s. The first direct determination of the fluorescence lifetime of tryptophan, free in solution and in proteins, appears to be due to Chen *et al.*,⁴³ These authors pointed out, however, that after they submitted their manuscript they became aware of work

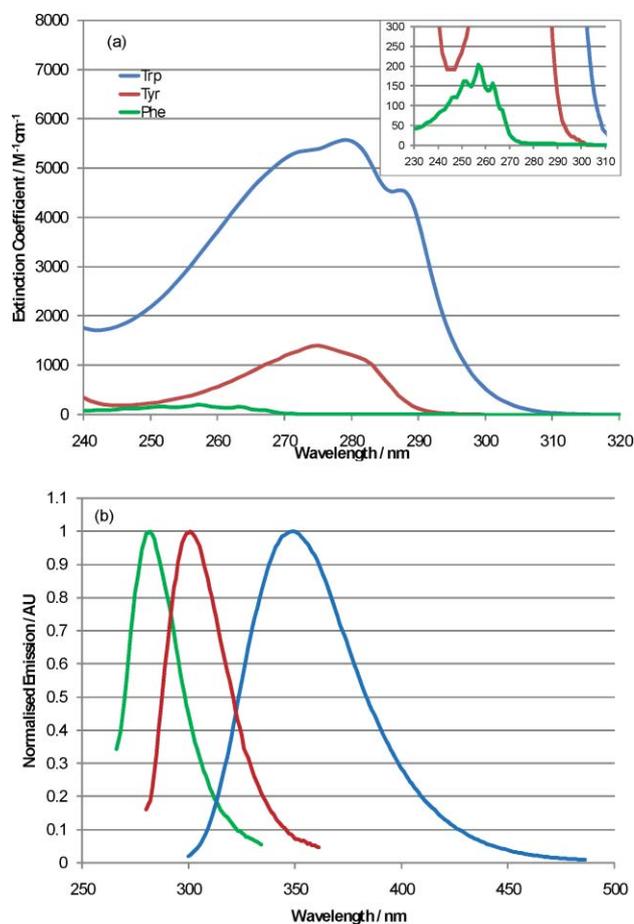


Fig. 5 (a) Absorption and (b) normalised corrected emission spectra of tryptophan (blue), tyrosine (red) and phenylalanine (green) in phosphate buffer, pH 7.1.

by Konev on the lifetime of intrinsic protein fluorescence.‡ Chen *et al.*,⁴³ reported lifetimes for tryptophan and various proteins in the range of 2–5 ns, quite reasonable results given the time-domain instrumentation available at that time. The first frequency domain measurements of protein fluorescence appear to have been carried out by Badley and Teale;⁴⁴ their values were in general agreement with Chen *et al.*⁴³ In the decades following these pioneering studies, the intrinsic fluorescence, including lifetime measurements, of a great many proteins has been investigated.

In the last two decades, the development of site-directed mutagenesis has led to a renewed interest in protein fluorescence. Using modern methods of molecular biology researchers are now able to add or remove tryptophan residues in almost any protein. In this way it has proved possible in many instances to place a tryptophan reporter residue in a particular location of a protein to monitor a suspected conformational change upon ligand binding or some other type of biochemical intervention. Additionally, as described in more detail later, site-directed mutagenesis has been used to alter the amino acid residues near a tryptophan moiety to study the effect of such alterations on that residue's photophysical properties.

Absorption of UV light by the aromatic amino acids is due to two major absorption bands, ¹L_a and ¹L_b (using the Platt nomenclature). The absorption maximum of the lower energy band occurs near 280 nm, 275 nm and 257 nm for tryptophan, tyrosine and phenylalanine respectively. Recent studies have indicated that the fluorescing state for tryptophan in proteins is the solvent sensitive ¹L_a state.^{45,46} In tyrosine the ¹L_b band has the lower energy while the higher energy ¹L_a band absorbs near 223 nm.⁴⁷

Tryptophan fluorescence

The fluorescence lifetime and the number of lifetime components of tryptophan in aqueous solution are strongly dependent on the pH of the solution.^{28,48} (We note that Audrey White first published the pH dependence of tryptophan fluorescence intensity in 1959⁴⁹). Fig. 6 shows the variation of the phase lifetime value (at 10 MHz modulation frequency) as a function of pH. At neutral pH, tryptophan exhibits two lifetime components, one major component of ~3 ns with an emission maximum at 350 nm and one minor component of ~0.5 ns with an emission maximum near 335 nm.^{50,51} At higher pHs, when the amino group becomes protonated, the 3 ns component gives way to a longer component near 8.7 ns. As the pH is raised through the pK_a of the amino group, the relative contribution of the 3 ns or 8.7 ns component follows the proportion of the protonated or deprotonated form.^{28,48} Fig. 3 shows phase and modulation data of tryptophan in aqueous solution at pH values of 6.5, 8.9 and 10.3. The data in Fig. 3 can be analyzed in terms of discrete exponential decays and fractional contributions to the total intensity; the results are given in the figure legend and demonstrate the change in the lifetime of tryptophan upon protonation/deprotonation of the amino group.

‡ We note that much early work on intrinsic protein fluorescence was carried out in the former Soviet Union by researchers such as S. V. Konev and E. A. Burstein but since much of this work was published in Russian it remains difficult for us to access—we apologize if our brief review appears to overlook these valuable contributions.

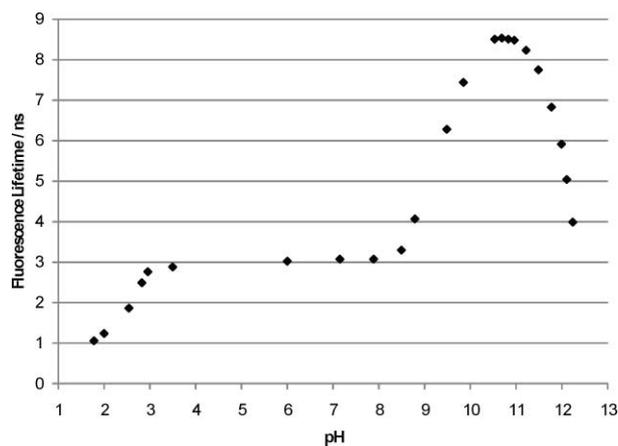


Fig. 6 Phase lifetime of tryptophan in solution as a function of pH measured at 10 MHz (modified from²⁸). Note the change of lifetime as the amino group becomes deprotonated as the pH is increased through the pK_a (9.4). Reproduced with permission from *J. Phys. Chem.*, 1981, **85**, 953–958. © 1981 American Chemical Society.

Factors affecting tryptophan lifetimes in proteins

The emission maxima of tryptophan in proteins ranges from 308 nm to 352 nm,^{52,53} the excited state lifetimes range from several picoseconds to nearly 10 ns^{47,53} and the quantum yields from near zero to around 0.35.^{52,54} *N*-acetyl-L-tryptophanamide (NATA) is typically used in solution as an analog of a tryptophan residue within a protein. NATA emission decays with a single exponential of ~3.0 ns at 20 °C. The lifetime of tryptophan in proteins is affected by many processes which enhance the non-radiative decay rate, including solvent quenching, excited state proton transfer, excited state electron transfer, intersystem crossing and temperature. Excited state proton transfer may be characterized by three different mechanisms depending on the pH. At both acidic (<3) or alkaline (>11) pH, fluorescence is quenched due to acid catalyzed protonation of the indole ring or base-catalysed deprotonation of the indole amine (Fig. 6). The fluorescence can be quenched at intermediate pH by proton exchange with the C2, C4 and C7 carbons of the indole. Another important factor affecting the lifetime of tryptophan residues in proteins is the nature of the amino acid residues surrounding the indole rings. The mechanism of quenching by lysine and tyrosine is attributed to excited state proton transfer, while cysteine and histidine are thought to form ground state complexes with tryptophan.⁵⁵ Glutamine, asparagine, glutamic and aspartic acid, and even the peptide bond can quench by excited state electron transfer.^{55,56} In addition to these residues, solvent (*e.g.* water) molecules can quench the fluorescence. This quenching can be modulated by the proximity of the indole rings to ammonium groups.^{56,57}

It has been postulated that the biexponential decay of tryptophan at neutral pH (discussed above) is due to three possible 'rotamers' of the indole rings about the C_α–C_β bond within tryptophan.⁵¹ These three rotamers have been labeled I, II and III (Fig. 7). Rotamer I is the preferred orientation due to the proximity of the positive charge on the α-ammonium group. Rotamer II is the least energetically favorable as a result of the electrostatic repulsion between the deprotonated carboxylic acid group and the π-electron

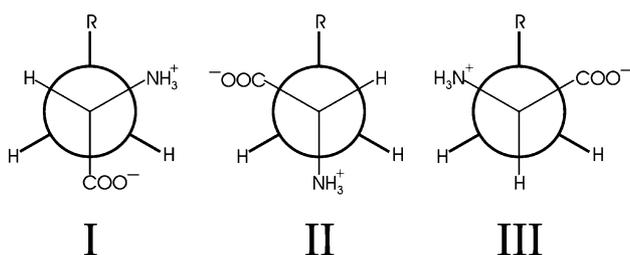


Fig. 7 Newman projections about the C_{α} - C_{β} bond indicating the rotational isomers of the aromatic amino acids. R is indole for tryptophan, phenol for tyrosine and benzene for phenylalanine. For tryptophan the relative rotamer populations are [I] \gg [II] $>$ [III] while for tyrosine and phenylalanine the rotamer populations are [I] $>$ [II] \approx [III].

cloud of the indole rings. While rotamer III is more energetically favorable than II, it may suffer from steric hindrance from the close proximity of the C_{α} .⁵¹ This model has been labeled the *classical rotamer model*. The biexponential behavior of tryptophan has also been attributed to solvent equilibrated excited states, with transitions denoted as 1L_a and 1L_b . The 1L_a transition exhibits a high sensitivity to the local environment due to the larger change in the dipole compared to the ground state. The relative orientation of the 1L_a and 1L_b states was first determined by Yamamoto *et al.*,⁵⁸ in the angular dependence of the absorbance of UV light in oriented crystals of indole. This orientation was later confirmed from the excitation polarization spectrum of indole and tryptophan by Valeur and Weber⁵⁹ who also resolved the excitation spectrum of each transition.

The classical rotamer model has been proposed to account for the lifetime heterogeneity seen in single tryptophan proteins (see for example⁶⁰). The basic concept is that ground state rotamers exist for tryptophan residues in the protein matrix, that these exist on time scales long compared to the fluorescent lifetime, and that each rotamer orientation subjects the indole moiety to a different environment which can in turn give rise to a different excited state lifetime. This model has been advanced/superseded by an explanation which in part combines aspects of each. Namely that while the indole of tryptophan can exist in different ‘rotamer’ conformations, there are many substates which each interact with the amino acid residues of the local environment in a slightly different manner. These nearly isoenergetic substates are due to the interaction of the indole in the ground state and the excited state, with the excited state interactions not only resulting in quenching but also interactions between the excited state dipole and the local charge environment. Clayton *et al.*,⁶¹ showed that the lifetime components of tryptophan in various positions within an α -helix in a polypeptide agreed well with the relative proportions of the rotamers of the indole rings. Additionally they concluded that the effect of sequence position was secondary to the unordered conformation of the peptide. In regards to these models, Xu *et al.*,⁶² made an excellent, succinct observation which we quote, namely: “The popularity of shorthand terms “rotamer model” and “solvent relaxation” should not restrict either model unnecessarily. In addition to explicit rotamers of the tryptophan side chain, one must recognize that *microconformational* states of proteins with different local environments of the indole ring constitute a source of ground-state heterogeneity (*i.e.*, in proteins, we should discuss “conformers” instead of “rotamers”). Regarding solvent

relaxation, motions of *either* the protein matrix or solvent water near the indole ring can produce complex decay, and they are strongly coupled.” The approach to tryptophan lifetime heterogeneity developed by Alcalá *et al.*,^{30,31} takes the viewpoint that continuous lifetime distributions (such as Lorentzian or Gaussian distributions) are more appropriate than discrete exponentials to describe excited state decays in proteins. In this model, the physical basis for the distributions resides in the interconversion between conformations, each characterized by a quasi-continuum of energy substates, which place the tryptophan residue in different environments. The observed lifetime heterogeneity, in this approach, is thus a function of the interconversion rates and hence assignment of a discrete lifetime component to a particular protein conformation may be an oversimplification.

Single tryptophan proteins

The excited state intensity decay properties of many single tryptophan proteins have been studied using both time and frequency domain methods. Human serum albumin (HSA) is, however, probably the most studied single tryptophan protein. The fluorescence lifetime of the single tryptophan, residue 214, in HSA has been analyzed in terms of 2–3 discrete lifetimes^{48,63–69} or as a (Lorentzian or Gaussian) distribution.^{70,71} Even though all of these lifetime measurements were carried out under somewhat different conditions (pH, temperature, ionic strength, excitation and emission wavelengths) they all agree that the lifetime is heterogeneous and that the average lifetime of tryptophan 214 is around 6 ns. The interpretation of the excited state heterogeneity differs, however, between laboratories.

Helms *et al.*,⁷¹ studied the excited state properties of tryptophan 214 in HSA while using site-directed mutagenesis to change amino acid residues near the tryptophan. Specifically, they changed residue 218, normally arginine, into either methionine or histidine (HSA with histidine in position 218 occurs in the population and is responsible for a condition known as familial dysalbuminemic hyperthyroxinemia). The His218 and Met218 mutants exhibited lifetimes best fit to Lorentzian distributions centered at 4.23 ns and 6.08 ns, respectively. The widths of these distributions were significantly less than for wildtype HSA. These narrower distributions are particularly interesting since Gratton and coworkers^{30,31,72} have demonstrated that the widths of such lifetime distributions in cases of single-tryptophan proteins can be related to the motion of the fluorophore, namely decreasing as mobility increases. Dynamic polarization data on the HSA mutants in fact indicated an increased rate of local mobility for tryptophan in the His218 and Met218 mutants compared to wildtype HSA.

An interesting study of several single tryptophan proteins was carried out using the frequency domain method by Mei *et al.*,⁷³ who evaluated a new, nonsymmetrical Lorentzian function containing three free parameters, namely the center, left and right widths (as compared to the traditional symmetrical function which had only two parameters, the center and the width). Another detailed study of a single tryptophan containing protein, human superoxide dismutase, was reported by Silva *et al.*,⁷² who used frequency domain fluorometry to obtain lifetime and anisotropy decay data on this protein in an 80% glycerol-water mixture over a wide temperature range (–30 °C to +50 °C).

Their results were consistent with the existence of conformational substates.

Multi-tryptophan proteins

When more than one tryptophan is present, the assignment of multiple lifetime components to a particular residue is problematic at best. For example, it may be that several of the tryptophans have very similar lifetimes, as is the case for the large GTPase dynamin. Even though dynamin has 5 tryptophan residues per 100 kDa subunit, the excited state intensity decay can be well fit to one relatively narrow Lorentzian distribution as shown in Fig. 8. Since most naturally occurring proteins have multiple tryptophan residues, the question often arises of whether one can resolve the individual components. The question of lifetime resolvability arises in both time and frequency domain measurements. In both cases, of course, a fundamental consideration is the precision of the data. In the time correlated single photon counting approach one can improve the precision of the data by accumulating more photons. In the frequency domain one can also average the analog signal at each frequency to reduce the standard deviations of the phase angle and modulation ratio. For most cases, as mentioned above, the average values of these standard deviations are usually lower than 0.2° of phase and 0.004 for the modulation ratio. Assuming values of 0.2° and 0.004, however, Gratton *et al.*,²⁵ calculated the resolvability of two lifetime components in a mixture containing equal fractions of each component. In this case one finds that lifetime components should differ by a factor of around two in order to be clearly resolved. As the lifetime values converge, or as the fractional contribution from one component decreases, higher precision is required for resolution. In many cases, however, one component is known and then resolution of the second component is easier; such may be the case, for example, when one is studying ligand binding to a biomolecule or cases involving FRET. A good example of resolvability of lifetime components is given by horse liver alcohol dehydrogenase, a homodimeric protein which has two tryptophan residues, W314 and W15, in each 40 kDa subunit. W314 is buried in the protein

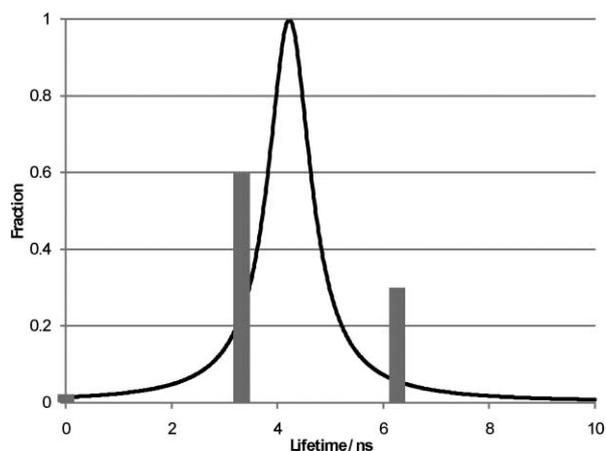


Fig. 8 Comparison of fluorescence lifetimes of dynamin to a Lorentzian distribution and three discrete components. Fits are: Lorentzian (centre = 4.16 ns, width = 0.99 ns, 1% scattering, $\chi^2 = 0.38$); discrete (3.31 ns (60%), 6.25 (38%), 2% scattering, $\chi^2 = 0.55$).

interior and has a lifetime of 3.6–3.8 ns while W15 is exposed to the solvent and has a lifetime of 6.9–7.2 ns.^{74,75}

The advent of site-directed mutagenesis has allowed researchers to remove tryptophan residues in multi-tryptophan proteins to construct single tryptophan proteins. Many such studies have been reviewed by Ross *et al.*,⁵² who point out that while replacement of one tryptophan residue may not affect the stability or conformation of a protein, replacement of multiple tryptophan residues may have an unfavorable cumulative effect on the protein's structure. As an example of this approach, Helms *et al.*,⁸⁴ carried out steady-state and frequency domain fluorescence studies on the single and double tryptophan mutants of the enzyme fructose 6-kinase, 2 kinase fructose 2,6 bisphosphatase. The wildtype enzyme is a homodimer with four tryptophan residues per subunit and each possible single and double tryptophan mutant was constructed. These studies demonstrated the presence of energy transfer between two particular tryptophan residues which indicated their proximity in the protein matrix.

Tyrosine fluorescence

In addition to tryptophan, tyrosine may also be used as an intrinsic fluorescent probe within proteins. However, due to the much lower extinction coefficient of tyrosine compared to tryptophan (Fig. 5), fluorescence emission from tyrosine is typically inundated by that from tryptophan. For this reason, tyrosine fluorescence is generally only used for proteins which do not contain tryptophan. The fluorescence lifetime of tyrosine in aqueous solution is a single exponential around 3.3 to 3.8 ns^{47,76} depending on the pH and measurement conditions. The multi-exponential decay of tyrosine derivatives in solution under some conditions has also been attributed to a rotamer model, analogous to the case of tryptophan. For example, *N*-acetyl-L-tyrosinamide exhibits a dual exponential decay with one component at 0.4–0.5 ns and another at 1.2–1.9 ns again depending on measurement conditions. However, the case is more complex for tyrosine within a protein. As with the indole of tryptophan, the fluorescence lifetime from the phenol group of tyrosine may vary depending on its proximity to other amino acid residues and solvent accessibility. The phenolic hydroxyl group may be deprotonated forming tyrosinate, resulting in a shift of the absorption peak to 294 nm and of the emission peak to near 340 nm. Thus an emission peak at 340 nm may actually not be due to tryptophan but could indicate the presence of tyrosinate.⁷⁷ The pK_a of the phenolic hydroxyl varies substantially whether it is in the ground state ($pK_a \approx 10.3$) or the first excited singlet state ($pK_a \approx 4.2$).⁷⁷ The difference between the pK_a of the ground and excited state (and hence their ionization potential) can lead to ionization during the fluorescence lifetime of the excited state, *i.e.* excited state proton transfer if a suitable proton acceptor is present.⁴⁷ Ferreira *et al.*,⁷⁶ presented a detailed application of frequency domain fluorometry to bovine erythrocyte Cu, Zn superoxide dismutase, a homodimeric protein containing a single tyrosine residue per subunit but no tryptophan residues. In this study, lifetime data was obtained over a temperature range of 8 °C to 45 °C, and results were analyzed using various distribution models.

Energy transfer from tyrosine to tryptophan, first described by Gregorio Weber,⁴⁰ has also been used to study protein conformation and dynamics. This energy transfer can be monitored

through the increase of the apparent lifetime associated with the tryptophan residue or by the depolarization of the tryptophan emission excited *via* energy transfer from tyrosine residues, relative to direct tryptophan excitation. In cases where energy transfer from tyrosine to tryptophan is undesirable, excitation at 300 nm can be used to excite the tryptophan directly.

Phenylalanine fluorescence

Although phenylalanine is fluorescent, it is not commonly used in intrinsic fluorescence studies of proteins, due mainly to its low extinction coefficient (Fig. 5) coupled with its low quantum yield especially compared to the other aromatic amino acids. Typically fluorescence from phenylalanine is only detectable within proteins that do not have any tryptophan or tyrosine residues as the emission from these residues dominates the protein fluorescence. The fluorescence lifetime of phenylalanine displays a single exponential decay near 7.5 ns in aqueous solution.^{78,79} While some preliminary evidence exists for rotamers of phenylalanine, a comprehensive study has not yet been conducted. Ross *et al.*,⁵² discuss in detail recent intrinsic protein fluorescence studies which utilized phenylalanine emission.

Effective brightness of the aromatic amino acids

The relative utility of the three aromatic amino acids as regard studies of intrinsic protein fluorescence may be considered from the viewpoint of their “effective brightness.” In this case, effective brightness may be defined as the product of the extinction coefficient and the quantum yield, and is a measure of how many photons will be emitted for a given incident illumination power. Comparing the effective brightness of the three amino acids further illustrates why phenylalanine is useful for fluorescence studies of protein generally only in the absence of tryptophan or tyrosine residues (Table 1).

Lifetime microscopy

We should point out that frequency domain methods have been applied to microscopy longer than any other time-resolved fluorescence technique. Specifically, in 1959 Venetta⁸² described the construction and operation of a phase fluorometer coupled to a microscope. Using a frequency of 5.8 MHz (in part chosen due to the availability of FM transformers in televisions which could be salvaged for his work), Venetta was able to measure a lifetime of 2.7 ns for proflavin bound to the nuclei of tumor cells. Modern versions of this approach include Fluorescence Lifetime Imaging

Microscopy (FLIM). The early FLIM instruments utilizing the frequency domain method typically operated at single frequencies, which could be modified depending on the system under investigation, and often utilized Weber's Algorithm to analyze data since it provided a very rapid analytical solution. In these cases, of course, the method worked best if only two lifetime components were present and when one component was known (a condition approximated in some cases of FRET). One of the most recent innovations in FLIM utilizing frequency domain methods involves the use of “phasor” analysis, an approach which enormously simplifies analysis of the complex systems encountered in FLIM studies on living cells. Readers with a sustaining interest in this topic are referred to the recent publication in this area from the Gratton lab.²¹

Summation

Frequency domain methods have stood the test of time as more than 8 decades have passed since the original phase measurements of Gaviola. Although this overview has focused on applications to protein fluorescence it should be clear to all readers that the method has had—and continues to have—wide applications to many areas of biochemistry, biology, chemistry, physics, biomedical engineering, biotechnology and material science. As regards the application of frequency domain—or for that matter any time-resolved method—to protein fluorescence we wish to emphasize that the most useful aspects of the technique are undoubtedly to detect and quantify conformational alterations in protein matrices as a consequence of physical (temperature, pressure), chemical (solvent composition, denaturant, pH) or biochemical (ligand binding, protein–protein, protein–membrane, protein–nucleic acid) interactions. As pointed out earlier, the precise photophysical origins of the excited state decay kinetics of tryptophan residues in proteins is still a field of active research. During the last decade or so there has been renewed interest in intrinsic protein fluorescence due to the development of site-directed mutagenesis and the increasing interest in protein folding—partly due to interest in protein production in the biotechnology field and partly due to the recognition of diseases caused by accumulation of misfolded proteins. Intrinsic protein fluorescence offers a unique method to study protein conformation of these systems without the necessity of introducing extrinsic probes into the system, for example as in the case of misfolded Alzheimer tau aggregates.⁸³ These imperatives will continue to give impetus to optical spectroscopy on proteins—and fluorescence methods promise to be central to this endeavour.

Experimental

L-tryptophan (Fluka), L-phenylalanine (Calbiochem, CA), and L-tyrosine (Sigma) were dissolved in 20 mM K₂HPO₄–KH₂PO₄ buffers at pH 7.1 to concentrations which resulted in an absorbance of less than 0.05 at the excitation wavelength used. *N*-acetyl-L-tryptophanamide (United States Biochemical Corporation, OH) dissolved in phosphate buffer pH 7.1 was used as a lifetime reference. Recombinant human dynamin 1 was provided by Professor Joseph Albanesi and Dr Barbara Barylko from UT Southwestern Medical School (Dallas).

Table 1 Extinction coefficients, quantum yields and effective brightness of the three aromatic amino acids. Values listed are average values from the literature and as noted above the quantum yields can change greatly in proteins

	$\epsilon_{\text{max}}/M^{-1} \text{ cm}^{-1}$ ($\lambda_{\text{abs,max}}/\text{nm}$)	Quantum yield	Effective brightness at $\lambda_{\text{abs,max}}$	Effective brightness at 280 nm
Tryptophan	5579 (279) ⁸⁰	0.13 ⁸¹	725	722
Tyrosine	1405 (275) ⁸⁰	0.14 ⁸¹	197	170
Phenylalanine	195 (258) ⁸⁰	0.024 ⁸¹	5	0.11

Absorbance measurements were conducted on a Shimadzu UV-2401PC absorption spectrometer (Shimadzu Corp., CA). Steady state fluorescence measurements were conducted on an ISS PC1 (ISS Inc., Champaign, IL) steady state fluorimeter using a Xenon lamp as the excitation source and with excitation and emission slit widths of 8 nm and the emission polarizer vertically oriented. Emission spectra were corrected for instrument response parameters. Frequency domain time-resolved spectroscopy was conducted on an ISS Chronos Fluorometer using Marconi 2022A frequency synthesizers and 280 nm or 300 nm LEDs as the excitation light source. Modulation frequencies were chosen such that the phase delay stayed within the range of 15° to 75°. All fluorescence measurements were made in a 10 × 4 mm quartz cuvettes (Starna Cells Inc., CA).

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