Published in final edited form as:

Anal Biochem. 2011 March 1; 410(1): 70–76. doi:10.1016/j.ab.2010.11.011.

Applications of Phasor Plots to *in Vitro* Protein Studies

Nicholas G. James^a, Justin A. Ross^a, Martin Štefl^b, and David M. Jameson^{a,*}

^a Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, Biosciences 222, Honolulu, Hawaii 96813

^b J. Heyrovský Institute of Physical Chemistry, v.v.i., Academy of Sciences of the Czech Republic, Department of Biophysical Chemistry, Dolejšková 3, Prague 18223, Czech Republic

Abstract

The previous paper describes the application of the phasor analysis to fluorescence intensity decay data on *in vitro* samples. As detailed in that paper, this method provides researchers with a simple graphical method for viewing lifetime data that can be used to quantify individual components of a mixture as well as to identify excited state reactions. In this report, we extend the use of *in vitro* phasor analysis to intrinsic protein fluorescence. We show how alterations in the excited state properties of tryptophan residues are easily visualized using the phasor method. Specifically, we demonstrate that protein-ligand and protein-protein interactions can result in unique shifts in the location of phasor points, indicative of protein conformational changes. Application of the method to a rapid kinetics experiment is also shown. Finally, we show that the unfolding of lysozyme with either urea or guanidine hydrochloride results in different phasor trajectories, indicative of unique denaturation pathways.

Keywords

Protein fluorescence; lifetimes; kinetics; protein unfolding; protein interactions; ligand binding

Excited-state fluorescence lifetime measurements have become a popular and powerful method for gaining information on the local environment of fluorophores in biomolecular systems such as proteins, membranes or nucleic acids. Excited state intensity decay (lifetime) data are typically obtained using either time-domain or frequency-domain methods [1,2]. In the time-domain, the sample is excited with a short pulse of light and the time evolution of the fluorescence intensity decay is recorded, while in frequency-domain measurements, the sample is typically (but not always) excited with sinusoidally modulated light and the shift in phase and modulation of the emission with respect to the excitation is recorded. Data from each method are essentially equivalent with the frequency-domain data being the Fourier transform of the time-domain data [3]. Traditional methods of extracting decay parameters involve fitting the data to models, such as discrete exponentials or distributions [4], until a “good” fit, as judged by the χ^2 value, is reached. A model-less approach, the Maximum Entropy Method, has also been used to describe excited state decay

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* Correspondence to: David M. Jameson, Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, Biosciences 222, Honolulu, Hawaii 96813 Tel. (808) 956-8332; Fax. (808) 692-1968/(808) 692-1970; djameson@hawaii.edu.

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data in terms of the intensity decay components [5]. For a large number of seemingly simple fluorophore systems, e.g. proteins containing a single tryptophan residue, however, the excited state decay cannot be described by single or even multi-exponential models. In some cases the complexities of the decay may be due to the fact that the fluorophore can sample different molecular environments during the excited state lifetime or to the presence of excited state reactions including resonance energy transfer, dipolar relaxation or transient quenching mechanisms.

Tryptophan residues are the most commonly used intrinsic protein probes and their popularity increased when the method of site directed mutagenesis allowed for removal or insertion of these residues into specific locations in the protein sequence (although we note that tyrosine fluorescence has also been used in the case of proteins lacking tryptophan residues). The sensitivity of tryptophan's fluorescence properties, including its emission maximum, quantum yield and lifetime, to local environments in the protein matrix has been used by a number of researchers over the years to characterize a variety of biological processes [2,6-8]. The use of tryptophan analogs, introduced into proteins using molecular biological methods, has also become increasingly popular in studies of protein fluorescence [9,10]. A complete description of all such applications to protein systems is beyond the scope of this manuscript, however, a few examples include the use of tryptophan fluorescence to monitor ligand interactions [11,12], kinetic rate determinations [13,14] protein-protein, protein-DNA, and protein-RNA interactions [15,16], as well as structural and dynamic features of proteins [17-19] and Förster Resonance Energy Transfer (FRET) [20]. Excited-state characterization of tryptophan, free in solution and incorporated into proteins, has revealed the complex decay parameters for this intrinsic probe [21]. The environmental sensitivity of tryptophan's 1L_A transition [22], the possibility of tryptophan rotamer conformations within the protein matrix [23] and excited-state reactions of tryptophan residues with neighboring amino acid side chains [24] have all been suggested to play a role in the non-exponential behavior of tryptophan decay [2].

Analyzing tryptophan lifetimes using the traditional approaches, including fits to discrete exponentials or continuous distributions, does not always allow an adequate description of the decay process or provide meaningful physical interpretation. Also, many proteins have multiple tryptophan residues and may exhibit energy transfer between these residues, which frustrates attempts to fit the lifetime data to straightforward models. In many cases, however, the goal is simply to utilize the intrinsic protein fluorescence to monitor a process such as ligand binding, unfolding/refolding or to detect a conformational change in a particular region of the protein. In such cases, a less constrained approach may provide a useful tool for monitoring alterations in intrinsic protein decay. The phasor plot offers such an approach – although we note that quantitative information can also be obtained.

We have recently explored the application of the phasor approach to *in vitro* studies and have briefly described its general utility in the characterization of heterogeneous emissions and systems demonstrating dipolar relaxation as well as intrinsic protein fluorescence [25]. In the previous manuscript we discuss the basics of the phasor approach and illustrate its application to heterogeneous mixtures of fluorophores as well as excited state reactions such as dipolar relaxation and FRET. In this manuscript we extend these observations to studies of protein conformations. Specifically, we apply the phasor plot approach to a number of protein related systems, including the effect of pH on tryptophan's lifetime, quenching, the effect of ligand binding, kinetics, protein-protein interactions and protein unfolding pathways.

Materials and Methods

L-Tryptophan and MES were from Fluka (St. Louis, Missouri). N-acetyl-L-tryptophanamide (NATA), fluorescein, human serum albumin (HSA), KI, NaCl, sodium thiolsulfate ($\text{Na}_2\text{S}_2\text{O}_3$), ethanol, CHES and HEPES were obtained from Sigma (St. Louis, Missouri). Lysozyme was obtained from Fisher Scientific (Pittsburgh, Pennsylvania). Thrombin (alpha) and antithrombin III were purchased from Haematologic Technologies Inc. (Essex Junction, Vermont). Sephacryl S-300 resin was obtained from GE Healthcare (Piscataway, New Jersey). Guanidine hydrochloride (GdHCl) was from the Mann Research Laboratories Inc. Urea was purchased from ICN Biomedical (Irvine, California). Dynamin 2 was a generous gift from Dr. Joseph Albanesi and Dr. Barbara Barylko (UT Southwestern Medical School, Dallas, TX). Recombinant human serum transferrin N-lobe, residues 1-337, was a generous gift from Dr. Anne B. Mason (University of Vermont, Burlington, VT).

HSA purification

Many reports have detailed the fact that even 99% pure lyophilized HSA contains up to 10% higher order oligomers, such as dimers, trimers, tetramers, and higher order aggregates formed via disulfides linkage (see, for example, [26]). To isolate the monmeric protein, HSA (20 mg/mL) was loaded onto a 100 mL Sephacryl S-300 column, equilibrated with 20 mM HEPES, pH 7.5. Identification of the oligomeric state and purity of each fraction was determined by non-denaturing native gel electrophoresis (data not shown). The final half of the dominant (monomeric) peak was pooled. HSA concentration was determined using $34,445 \text{ M}^{-1} \text{ cm}^{-1}$ as the molar absorption coefficient at 280 nm [27].

Time Resolved Measurements

Phase and modulation data were obtained using an ISS Chronos Fluorometer using either 280 or 300 nm LEDs as the light source [28]. The bandpass filter FF01-280/20-25 or FF01-295/15-25 (Semrock, Rochester, NY) were used where appropriate with the excitation light and the emission collected through longpass filters (WG315 or WG 320 (Schott)) or a 357/50 nm bandpass filter. Polarizers were set at magic angles to eliminate polarization effects [29]. Reference lifetime standard of NATA at pH 7.5 (2.70 ns at 25°C and 2.95 at 20°C) was used at excitation wavelengths of 280 nm or 300 nm [30]. For those experiments in which temperature was varied, glycogen (0.00 ns) was used as a reference. Absorbance values at the exciting wavelengths were kept below 0.05 to avoid inner filter affects.

For measurements involving acrylamide quenching, small aliquots of an 8 M stock solution were added to the sample cuvette to obtain the desired acrylamide concentration. For KI quenching, NaCl was present to maintain the ionic strength between each measurement. The stock solution of KI (3 M) contained a small amount (10^{-4} M) of $\text{Na}_2\text{S}_2\text{O}_3$ to prevent the formation of I_3^- , which absorbs in the excitation region of tryptophan fluorescence.

Phasor Plots

The theory underlying the phasor approach was presented in the previous paper [31].

Results and Discussion

Tryptophan lifetime/phasor as a function of pH

The sensitivity of tryptophan fluorescence to a number of factors (pH, temperature, polarity of solvent/environment) makes it ideal to monitor alterations in the protein matrix. Such sensitivity has also made interpretation of fluorescence changes in response to specific biological events difficult at best, highlighting the complexity of this fluorescent probe. We examined the excited state properties of L-tryptophan under changing conditions to illustrate

the application of the phasor method for analyzing the heterogeneous lifetime decay of proteins. The phasor plot for L-tryptophan as a function of pH (at 25 MHz) is shown in Fig. 1. As the pH is increased from 6.0 to 11.0, the zwitterion form of tryptophan is converted to the anion form ($pK_a \sim 9.39$); each of these forms has its own unique lifetime and quantum yield [2,32-34]. At pH 9.5 there should be a nearly 1:1 anion to zwitterion molecular ratio, however, one notes that the phasor point corresponding to pH 9.5 is not midway between the high and low pH points. The exact distance of the phasor point along the line joining the starting and ending points on the universal circle depends not only on the relative concentration but also the quantum yields of the species in question (in the case of L-tryptophan, the ratio of the lifetimes and quantum yields of the anion to zwitterion forms is ~ 2.8 [34]). The frequencies utilized will also weight the fractional contributions of the components differently, i.e., the lower frequency phasor points will weight the longer lifetime component while higher frequencies will favor the shorter component [35].

Quenching and temperature studies

Quenching of a protein's intrinsic tryptophan fluorescence, with molecules such as I^- , NO_3^- , Cs^+ , acrylamide and molecular oxygen [17-19], is commonly used to probe the dynamics of protein matrices. Chemical quenchers may provide information on the exposure of a tryptophan residue to the solvent as a consequence of collision of the excited fluorophore with the quencher molecule (dynamic quenching) or, in some cases, by formation of a ground state dark complex (static quenching) [1]. Both steady-state and time-resolved methodologies can be used to obtain quenching information.

In Fig. 2, we examined the effects of quenching molecules on the phasor plot of tryptophan containing samples. NATA was chosen as a model system as it has a single exponential decay under our conditions and the quenching with acrylamide is expected to move the phasor towards shorter lifetimes along the universal circle. The protein lysozyme was used as the photophysics of the tryptophans in this protein have been well investigated [36,37]. Fig. 2 shows the quenching of NATA and lysozyme using the quencher acrylamide. The data was recorded at 89 MHz and 20°C using a 300 nm LED as the excitation source; emission was observed through a WG315 longpass filter. As expected, the position of the phasor points is dependent on the quencher concentration. The lifetime of NATA is a single exponential at each quencher concentration and follows a clockwise trajectory along the universal circle towards $G=1.0$; $S=0$ (i.e. 0 ns). With lysozyme, one notes that the phasor points are all within the universal circle indicating the heterogeneous nature of the lifetime data. Addition of acrylamide results in shorter lifetimes, indicating one or more of the tryptophan residues in lysozyme are sensitive to dynamic quenching, and the subsequent shift in the phasor points in a clockwise direction. Addition of the quencher iodide (data not shown) also shifted the phasor point, following a similar trajectory to shorter lifetimes, albeit to a lesser extent, as with the acrylamide quenching.

Temperature studies are often used to study protein stability and dynamic aspects of the protein matrix [38]. We examined how temperature affects the phasor points for NATA and lysozyme. As shown in Fig. 3, the phasor points corresponding to NATA as a function of temperature remain on the universal circle and move clockwise as the temperature increases, indicating decreased lifetimes. The phasor points for lysozyme also progress clockwise with increasing temperature, however, these points begin to migrate more toward the universal circle with increasing temperature indicating a decrease in the lifetime heterogeneity. This correlation of heterogeneity and temperature is similar to what was observed in human superoxide dismutase, which provided support for treatment of tryptophan decay as a continuous lifetime distribution [38]. At 70°C a change in trajectory occurs, likely indicating a complete thermal unfolding of the protein and a broadening of the distribution of lifetime values. These results are in agreement with the large volume of thermal denaturation

literature on lysozyme [39,40]. Specifically, Morozova et al. [41], along with others, reported that the unfolding takes place in two-steps with the first, reversible step, occurring around $\sim 40^{\circ}\text{C}$ while a second, irreversible step, occurs at $\sim 70^{\circ}\text{C}$.

Protein/ligand and protein/protein interactions

The sensitivity of tryptophan fluorescence to its environment makes it an ideal probe to monitor local and global structural movements and to provide insights into protein conformational changes associated with ligand binding. Using two protein systems, we investigated how phasor plots (derived from the intrinsic protein fluorescence) change during interactions with different ligands. Dynamin 2 is a 100 kDa GTPase associated with the pinching off of the plasma membrane during vesicle fission [42]. Dynamin 2 comprises five domains (an N-terminal GTPase domain, a middle domain, a pleckstrin homology (PH) domain, a GTPase Effector Region (GED) and a proline/arginine rich domain (PRD)) and contains 5 tryptophan residues, 4 of which are in the PH domain, while 1 tryptophan is in the C-terminal PRD domain. Previous work from our lab has shown that the tryptophans in dynamin can transfer energy to a fluorescent nucleotide [43,44]. In Fig. 4A, the phasor points of dynamin alone, GDP bound dynamin and GTP γ S (a slowly hydrolyzable GTP analogue) bound dynamin are presented. The data shown were collected with 300 nm excitation at 84 MHz and 20°C . The addition of the guanine nucleotides clearly causes the phasor point to shift, and the position of the phasor point depends on the precise nucleotide utilized. These results indicate that there is a conformational change around one or more tryptophan residue(s) associated with guanine nucleotide binding and moreover the GDP and GTP γ S ligands elicit different responses indicating that the conformation of the protein matrix is not identical in the two cases. Solomaha and Palfrey [45] using steady-state tryptophan fluorescence and limited proteolysis, concluded that although GDP and GTP bind with similar affinities ($13\text{ }\mu\text{M}$ and $7.1\text{ }\mu\text{M}$, respectively), they produce distinct structural changes, which agrees with our phasor plot data.

HSA, which contains a single tryptophan residue (Trp 214), has been the subject of numerous fluorescence studies, including time-resolved studies, see for example [33,46-51]. HSA is commonly targeted for drug uptake studies and often these drug interactions are studied via changes in tryptophan fluorescence. In Fig. 4B, we show the phasor point, collected using 300 nm excitation at 84 MHz and 25°C , of the intrinsic fluorescence from monomeric HSA. In the presence of furosemide or D-Thyroxine, two drugs known to bind to HSA and to quench the tryptophan fluorescence [11,52], the phasor point shifts clockwise, indicating a shortening of the average lifetime. These shifts in the phasor point indicate that drug binding induces changes in the protein matrix near the tryptophan residue, and also that the conformational state of the protein differs depending on the nature of the drug, likely due to different binding sites (thyroxine has been shown to have multiple sites) on HSA. We also note that the phasor point corresponding to dimeric HSA, isolated from the monomeric protein using size-exclusion chromatography, differs from the monomeric phasor point (seen in Fig. 4B). Hazan et al. [47] also showed that monomeric and dimeric HSA have different excited state lifetime properties. This difference underscores the necessity of isolating monomeric HSA from the aggregates usually found in commercial lyophilized preparations before undertaking a study of its intrinsic tryptophan fluorescence, an obvious step that, surprisingly, is sometimes overlooked.

One may also expect, from the previous sets of experiments, that protein-protein interactions could also be investigated using intrinsic tryptophan fluorescence and phasor plot analysis. For two interacting proteins with distinct average lifetimes, complex formation may be expected to change one or both protein conformations and result in a phasor point that deviates from the “normal” linear combination typically seen for non-interacting mixtures [53]. Fig. 4C shows a set of data (acquired with 280 nm excitation at 43 MHz and 20°C) for

thrombin, anti-thrombin, lysozyme, and mixtures therein. Antithrombin and lysozyme are not predicted to interact and therefore a solution containing the two proteins should produce a phasor point that falls directly on a line between their individual points. This outcome is clearly observed (Fig. 4C) for the phasor plot of a 1:1 (1 μ M) mixture of the two proteins. On the other hand, thrombin/antithrombin is known to form a tight complex [54] and may be expected to produce a distinct phasor point away from the linear combination. The phasor point of the thrombin/antithrombin (1:1 at 1 μ M for each protein) indeed shifts inward away from the line connecting the points corresponding to the two pure proteins, indicating a change in intrinsic fluorescence upon protein interaction. This demonstration shows how phasor plots can provide a facile indication of protein interaction.

Kinetics of Protein-Ligand binding/dissociation

Determination of the rate constants associated with protein-ligand binding/dissociation reactions is a major goal in many studies. Kinetics of such reactions often proceed on a rapid timescale (seconds to a few minutes). Steady-state fluorescence methodologies, such as stopped-flow fluorescence, are frequently preferred over lifetime methods because traditional frequency domain lifetime measurements usually require one or two minutes for reasonable precision (although specialized fast scanning methods are available) [55,56]. The phasor plot method, recorded at a single frequency, is, however, well-suited for rapidly tracking changes in the phase and modulation data. To illustrate this point and validate the phasor method for tracking kinetics of protein-ligand dissociation, the phase and modulation of the intrinsic fluorescence of human serum transferrin (the isolated N-lobe) were recorded over ~300 sec at 80 MHz in pH 6.0 buffer and the presence of a chelator. Human serum transferrin (hTF) is a bilobal glycoprotein that serves as the major transporter of iron in humans (reviewed in [57]). Both lobes, termed the N- and C-lobes, coordinate ferric iron via four amino acid ligands and a synergistic carbonate anion. Numerous studies on hTF have shown that binding of iron quenches the intrinsic tryptophan fluorescence, through radiative and non-radiative means [14,25,58]. Rate constants for iron removal are thus determined by tracking the enhancement in fluorescence emission over time under endosomal like conditions (pH ~ 5.5, ~150-200 mM salt and the presence of a chelator), which takes place within seconds to minutes.

The lifetime of iron-bound and apo hTF N-lobe (which contains three tryptophan residues) has previously been determined [67]. The two discrete lifetime values associated with the hTF N-lobe (~1.4 and ~4.1 ns) undergo minimal change ($\pm$ 0.3 ns) during iron removal. The fractional fluorescence of each lifetime component, however, undergoes large changes (0.66/0.28 for iron-bound and 0.20/0.79 for apo). Based on the theoretical description (described above) the decay during iron release would be predicted to be a line between the iron-bound and apo phasor points. Fig. 5, which shows the phasor plot (taken at 80 MHz and 300 nm excitation) during iron release (black line) and the points for iron-bound (circle) and apo (triangle), clearly demonstrates the predicted linear progression in the phasor point during iron release from the iron-bound point towards the apo phasor point. Sample heterogeneity, which was expected based on the fitting to a discrete exponential model, is seen in the phasor vectors of hTF N-lobe as each point is inside the universal circle. Calculation of the distance at each point over time can provide information regarding excited state changes during iron removal (i.e. one can recover the fractional contribution of each emitting state using standard linear methods). Interestingly, the phasor data during iron release indicates that the change in the excited state properties of the hTF N-lobe occurs in two modes: a faster initial mode (during the first ~ 90 sec) followed by a slower progression towards apo (~90 – 300 sec). These results are similar to those observed following the steady-state emission changes, in which the faster rate was conclusively determined to be iron release and the slower rate assigned to conformational events [14]. Data can be acquired

on a subsecond timescale with the use of a stopped flow attachment, however, such an attachment was not available to us to directly demonstrate more rapid measurements. This kinetic run demonstrates how the phasor method can be utilized for tracking excited-state changes during protein-ligand dissociation, which can complement data obtained using other methods. We also point out that the phasor approach was applied in FLIM by Redford and Clegg [53] to follow the kinetics of mixing in turbulent flow at the microsecond time scale.

Protein Unfolding/Folding Pathways

Protein folding has been intensely investigated, both theoretically and experimentally, for many decades. One of the most popular experimental approaches to protein folding involves examination of the unfolding pathway(s) by means of chemical denaturation [59,60]. Chaotropic agents, such as GdHCl and urea, are commonly used denaturants. Unfolding experiments using such chemical denaturants have one major underlying assumption: that the overall, thermodynamic unfolding of the protein is independent of denaturing agent although the structural changes associated with the change are dependent [61,62]. The molecular mechanism for protein unfolding by GdHCl and urea are still unclear, even though mechanisms have been proposed as early as 1936 [63]. Models describing the direct interaction of the denaturant with the protein and disruption of the hydrophobic interactions via disruption of the hydrogen-bonding network are the commonly accepted mechanisms [64,65]. Recently, Almarza et al. [66] proposed a more detailed model for urea induced protein unfolding in which urea molecules interact with protonated histidines followed by hydrogen bond formation with polar residues leading to hydrophobic collapse of the protein.

As the detailed molecular mechanisms for protein unfolding due to GdHCl and urea are likely different, it would stand to reason that the intrinsic fluorescence properties will be different during denaturation depending on which chaotropic agent is present. Tryptophan fluorescence is sensitive to changes in the local environment and if the structural changes leading to unfolding of the protein are intrinsically different, then the environment around the tryptophan residues could differ depending on the chaotropic agent, resulting in distinct fluorescence properties. The phasor plot of lysozyme with increasing concentrations of GdHCl (0 – 6 M) and urea (0 – 8 M) is shown in Fig 6. Initial additions of either denaturant cause similar changes, yet, by 2 M (squares) the trajectories of the phasor points diverge, with GdHCl shifting the phasor points in a counter-clockwise direction. The final points between GdHCl and urea are dramatically different indicating unique unfolded forms. Similar deviations between unfolding with GdHCl and urea were observed with monomeric HSA (data not shown). It is clear that the phasor plot method has the potential for illustrating different unfolding pathways, as both proteins tested showed significant deviations in phasor point trajectories depending on the denaturant.

Conclusions

In this paper we have described the application of the phasor method to time-resolved studies on intrinsic protein fluorescence. Using this approach, the complex decay of protein fluorescence, either due to multiple emitting tryptophan residues or excited state reactions including tyrosine to tryptophan energy transfer, can be reduced to a single point on a phasor plot. Activities such as ligand or protein binding or protein denaturation, which result in changes in the microenvironment of the tryptophan residue(s) lead to movements of the phasor point. The trajectory of the phasor point in response to a physical or chemical perturbation can be followed to provide insights into the processes under investigation.

Acknowledgments

This work was supported in part by Allergan, Inc and by NIH RO1GM076665. M. Štefl was supported by Ministry of Education of the Czech Republic via grant LC06063 (MS) and by Charles University in Prague via Fond mobility. We thank Dr. Joseph Albanesi and Dr. Barbara Barylko for supplying the dynamin 2. We would also like to thank Dr. Anne B. Mason for supplying the iron-bound human serum transferrin N-lobe.

Abbreviations

NATA	N-acetyl-L-tryptophanamide
HSA	human serum albumin
hTF	human serum transferrin
hTF N-lobe	recombinant N-lobe of human serum transferrin comprising residues 1-337
FRET	Förster Resonance Energy Transfer
GdHCl	Guanidine hydrochloride

References

1. Valeur, B. *Molecular Fluorescence*. Wiley-VCH; Weinheim, Germany: 2002.
2. Ross JA, Jameson DM. Time-resolved methods in biophysics. 8. Frequency domain fluorometry: applications to intrinsic protein fluorescence. *Photochem. Photobiol. Sci* 2008;7:1301–1312. [PubMed: 18958316]
3. Weber G. Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements. *J. Phys. Chem* 1981;85:949–953.
4. Alcalá JR, Gratton E, Prendergast FG. Interpretation of fluorescence decays in proteins using continuous lifetime distributions. *Biophys. J* 1987;51:925–936. [PubMed: 3607213]
5. Brochon JC. Maximum entropy method of data analysis in time-resolved spectroscopy. *Methods Enzymol* 1994;240:262–311. [PubMed: 7823835]
6. Beechem JM, Brand L. Time-resolved fluorescence of proteins. *Annu. Rev. Biochem* 1985;54:43–71. [PubMed: 3896124]
7. Bialik CN, Wolf B, Rachofsky EL, Ross JB, Laws WR. Dynamics of biomolecules: assignment of local motions by fluorescence anisotropy decay. *Biophys. J* 1998;75:2564–2573. [PubMed: 9788952]
8. Vivian JT, Callis PR. Mechanisms of Tryptophan Fluorescence Shifts in Proteins 2001;80:2093–2109.
9. Ross JB, Szabo AG, Hogue CW. Enhancement of protein spectra with tryptophan analogs: fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. *Methods Enzymol* 1997;278:151–190. [PubMed: 9170313]
10. James NG, Byrne SL, Mason AB. Incorporation of 5-hydroxytryptophan into transferrin and its receptor allows assignment of the pH induced changes in intrinsic fluorescence when iron is released. *Biochim. Biophys. Acta* 2009;1794:532–540. [PubMed: 19103311]
11. Voelker JR, Jameson DM, Brater DC. In vitro evidence that urine composition affects the fraction of active furosemide in the nephrotic syndrome. *J. Pharmacol. Exp. Ther* 1989;250:772–778. [PubMed: 2778714]
12. Akyol Z, Bartos JA, Merrill MA, Faga LA, Jaren OR, Shea MA, Hell JW. Apo-calmodulin binds with its C-terminal domain to the N-methyl-D-aspartate receptor NR1 C0 region. *J. Biol. Chem* 2004;279:2166–2175. [PubMed: 14530275]
13. Dunlap CA, Tsai MD. Use of 2-aminopurine and tryptophan fluorescence as probes in kinetic analyses of DNA polymerase beta. *Biochemistry* 2002;41:11226–11235. [PubMed: 12220188]
14. James NG, Berger CL, Byrne SL, Smith VC, MacGillivray RT, Mason AB. Intrinsic fluorescence reports a global conformational change in the N-lobe of human serum transferrin following iron release. *Biochemistry* 2007;46:10603–10611. [PubMed: 17711300]

15. Jameson DM, Gratton E, Eccleston JF. Intrinsic fluorescence of elongation factor Tu in its complexes with GDP and elongation factor Ts. *Biochemistry* 1987;26:3894–3901. [PubMed: 3651421]
16. Bulygin VV, Milgrom YM. Studies of nucleotide binding to the catalytic sites of *Escherichia coli* betaY331W-F1-ATPase using fluorescence quenching. *Proc. Natl. Acad. Sci. USA* 2007;104:4327–4331. [PubMed: 17360523]
17. Lehrer SS. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* 1971;10:3254–3263. [PubMed: 5119250]
18. Eftink MR, Ghiron CA. Dynamics of a protein matrix revealed by fluorescence quenching. *Proc. Natl. Acad. Sci. USA* 1975;72:3290–3294. [PubMed: 810800]
19. Lakowicz JR, Weber G. Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules. *Biochemistry* 1973;12:4161–4170. [PubMed: 4795686]
20. Unruh JR, Kuczera K, Johnson CK. Conformational Heterogeneity of a Leucine Enkephalin Analogue in Aqueous Solution and Sodium Dodecyl Sulfate Micelles: Comparison of Time-Resolved FRET and Molecular Dynamics Simulations. *The Journal of Physical Chemistry B* 2009;113:14381–14392. [PubMed: 19780516]
21. Rayner DM, Szabo AG. Time resolved fluorescence of aqueous tryptophan. *Can. J. Chem* 1978;56:743–745.
22. Callis PR. 1L_a and 1L_b transitions of tryptophan: applications of theory and experimental observations to fluorescence of proteins. *Methods Enzymol* 1997;278:113–150. [PubMed: 9170312]
23. Clayton AH, Sawyer WH. Tryptophan rotamer distributions in amphipathic peptides at a lipid surface. *Biophys. J* 1999;76:3235–3242. [PubMed: 10354448]
24. Chen Y, Barkley MD. Toward understanding tryptophan fluorescence in proteins. *Biochemistry* 1998;37:9976–9982. [PubMed: 9665702]
25. James NG, Stefl M, Ross JA, Jameson DM. Application of Phasor Plots to Analysis of Fluorophore Heterogeneity, Excited State Reactions and Protein Conformations. *Biophys. J* 2010;98:750a–750a.
26. Costantino HR, Shieh L, Klibanov AM, Langer R. Heterogeneity of serum albumin samples with respect to solid-state aggregation via thiol-disulfide interchange -- Implications for sustained release from polymers. *Journal of Controlled Release* 1997;44:255–261.
27. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 1995;4:2411–2423. [PubMed: 8563639]
28. Barbieri B, Terpetschnig E, Jameson DM. Frequency-domain fluorescence spectroscopy using 280-nm and 300-nm light-emitting diodes: measurement of proteins and protein-related fluorophores. *Anal. Biochem* 2005;344:298–300. [PubMed: 15950917]
29. Spencer RD, Weber G. Influence of Brownian Rotations and Energy Transfer upon the Measurements of Fluorescence Lifetime. *The Journal of Chemical Physics* 1970;52:1654–1663.
30. Boens N, Qin W, Basaric N, Hofkens J, Ameloot M, Pouget J, Lefevre JP, Valeur B, Gratton E, vandeVen M, Silva ND Jr, Engelborghs Y, Willaert K, Sillen A, Rumbles G, Phillips D, Visser AJ, van Hoek A, Lakowicz JR, Malak H, Gryczynski I, Szabo AG, Krajcarski DT, Tarnai N, Miura A. Fluorescence lifetime standards for time and frequency domain fluorescence spectroscopy. *Anal. Chem* 2007;79:2137–2149. [PubMed: 17269654]
31. Stefl M, James NG, Ross JA, Jameson DM. Applications of Phasors to In Vitro Time-Resolved Fluorescence Measurements. *Anal. Biochem.* 2010
32. White A. Effect of pH on fluorescence of tryosine, tryptophan and related compounds. *Biochem. J* 1959;71:217–220. [PubMed: 13628557]
33. De Lauder WB, Wahl P. pH dependence of the fluorescence decay of tryptophan. *Biochemistry* 1970;9:2750–2754. [PubMed: 5450239]
34. Jameson DM, Weber G. Resolution of the pH-dependent heterogeneous fluorescence decay of tryptophan by phase and modulation measurements. *The Journal of Physical Chemistry* 1981;85:953–958.

35. Spencer RD, Weber G. Measurement of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorometer. *Ann. N. Y. Acad. Sci* 1969;158:361–376.
36. Imoto T, Forster LS, Rupley JA, Tanaka F. Fluorescence of lysozyme: emissions from tryptophan residues 62 and 108 and energy migration. *Proc. Natl. Acad. Sci. USA* 1972;69:1151–1155. [PubMed: 4504329]
37. Lehrer SS, Fasman GD. Fluorescence of lysozyme and lysozyme substrate complexes. Separation of tryptophan contributions by fluorescence difference methods. *J. Biol. Chem* 1967;242:4644–4651. [PubMed: 6061410]
38. Rosato N, Gratton E, Mei G, Finazzi-Agro A. Fluorescence lifetime distributions in human superoxide dismutase. Effect of temperature and denaturation. *Biophys. J* 1990;58:817–822. [PubMed: 2248991]
39. Segawa S, Nakayama M, Sakane M. Rates of structural fluctuations of lysozyme in the range of thermal unfolding transition. *Biopolymers* 1981;20:1691–1705. [PubMed: 7260336]
40. Jacob J, Krafft C, Welfle K, Welfle H, Saenger W. Melting points of lysozyme and ribonuclease A crystals correlated with protein unfolding: a Raman spectroscopic study. *Acta Crystallogr. D Biol. Crystallogr* 1998;54:74–80. [PubMed: 9761818]
41. Morozova L, Haezebrouck P, Van Cauwelaert F. Stability of equine lysozyme: I. Thermal unfolding behaviour. *Biophys. Chem* 1991;41:185–191. [PubMed: 1773011]
42. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003;422:37–44. [PubMed: 12621426]
43. Binns DD, Helms MK, Barylko B, Davis CT, Jameson DM, Albanesi JP, Eccleston JF. The mechanism of GTP hydrolysis by dynamin II: a transient kinetic study. *Biochemistry* 2000;39:7188–7196. [PubMed: 10852717]
44. Eccleston JF, Binns DD, Davis CT, Albanesi JP, Jameson DM. Oligomerization and kinetic mechanism of the dynamin GTPase. *Eur. Biophys. J* 2002;31:275–282. [PubMed: 12122474]
45. Solomaha E, Palfrey HC. Conformational changes in dynamin on GTP binding and oligomerization reported by intrinsic and extrinsic fluorescence. *Biochem. J* 2005;391:601–611. [PubMed: 15954862]
46. Kasai S, Horie T, Mizuma T, Awazu S. Fluorescence energy transfer study of the relationship between the lone tryptophan residue and drug binding sites in human serum albumin. *Journal of Pharmaceutical Sciences* 1987;76:387–392. [PubMed: 3656100]
47. Hazan G, Haas E, Steinberg IZ. The fluorescence decay of human serum albumin and its subfractions. *Biochim. Biophys. Acta* 1976;434:144–153. [PubMed: 938659]
48. Marzola P, Gratton E. Hydration and protein dynamics: frequency domain fluorescence spectroscopy of proteins in reverse micelles. *The Journal of Physical Chemistry* 1991;95:9488–9495.
49. Lakowicz JR, Gryczynski I. Tryptophan fluorescence intensity and anisotropy decays of human serum albumin resulting from one-photon and two-photon excitation. *Biophys. Chem* 1992;45:1–6. [PubMed: 1467440]
50. Davis DM, McLoskey D, Birch DJ, Gellert PR, Kittlety RS, Swart RM. The fluorescence and circular dichroism of proteins in reverse micelles: application to the photophysics of human serum albumin and N-acetyl-L-tryptophanamide. *Biophys. Chem* 1996;60:63–77. [PubMed: 8679927]
51. Helms MK, Petersen CE, Bhagavan NV, Jameson DM. Time-resolved fluorescence studies on site-directed mutants of human serum albumin. *FEBS Lett* 1997;408:67–70. [PubMed: 9180270]
52. Petersen CE, Ha CE, Jameson DM, Bhagavan NV. Mutations in a specific human serum albumin thyroxine binding site define the structural basis of familial dysalbuminemic hyperthyroxinemia. *J. Biol. Chem* 1996;271:19110–19117. [PubMed: 8702585]
53. Redford GI, Clegg RM. Polar plot for frequency-domain analysis of fluorescence lifetimes. *Journal of Fluorescence* 2005;15:805–815. [PubMed: 16341800]
54. Beeler D, Rosenberg R, Jordan R. Fractionation of low molecular weight heparin species and their interaction with antithrombin. *J. Biol. Chem* 1979;254:2902–2913. [PubMed: 429327]
55. Bilsel O, Yang L, Zitzewitz JA, Beechem JM, Matthews CR. Time-Resolved Fluorescence Anisotropy Study of the Refolding Reaction of the α -Subunit of Tryptophan Synthase Reveals

- Nonmonotonic Behavior of the Rotational Correlation Time. *Biochemistry* 1999;38:4177–4187. [PubMed: 10194334]
56. Feddersen BA, Piston DW, Gratton E. Digital parallel acquisition in frequency domain fluorimetry. *Rev. Sci. Instrum* 1989;60:2929–2936.
57. Mason, AB.; Everse, SJ. Iron Transport by Transferrin.. In: Fuchs, H., editor. *Iron Metabolism and Disease*. Research Signpost; Kerala, India: 2008. p. 83-123.
58. Lehrer SS. Fluorescence and absorption studies of the binding of copper and iron to transferrin. *J. Biol. Chem* 1969;244:3613–3617. [PubMed: 5794228]
59. Dill KA, Ozkan SB, Shell MS, Weikl TR. The protein folding problem. *Annu. Rev. Biophys* 2008;37:289–316. [PubMed: 18573083]
60. Fersht AR. From the first protein structures to our current knowledge of protein folding: delights and scepticisms. *Nat. Rev. Mol. Cell Biol* 2008;9:650–654. [PubMed: 18578032]
61. Pfeil W, Privalov PL. Thermodynamic investigations of proteins. II. Calorimetric study of lysozyme denaturation by guanidine hydrochloride. *Biophys. Chem* 1976;4:33–40. [PubMed: 1247649]
62. Makhatadze GI, Privalov PL. Protein interactions with urea and guanidinium chloride. A calorimetric study. *J. Mol. Biol* 1992;226:491–505. [PubMed: 1322462]
63. Mirsky AE, Pauling L. On the Structure of Native, Denatured, and Coagulated Proteins. *Proc. Natl. Acad. Sci. USA* 1936;22:439–447. [PubMed: 16577722]
64. Simpson RB, Kauzmann W. The Kinetics of Protein Denaturation. I. The Behavior of the Optical Rotation of Ovalbumin in Urea Solutions I. *J. Am. Chem. Soc* 1953;75:5139–5152.
65. Roseman M, Jencks WP. Interactions of urea and other polar compounds in water. *J. Am. Chem. Soc* 1975;97:631–640.
66. Almarza J, Rincon L, Bahsas A, Brito F. Molecular mechanism for the denaturation of proteins by urea. *Biochemistry* 2009;48:7608–7613. [PubMed: 19580327]
67. James NG, Ross JA, Mason AB, Jameson DM. Excited-state lifetime studies of the three tryptophan residues in the N-lobe of human serum transferrin. *Protein Sci* 2010;19:99–110. [PubMed: 19916167]

Fig. 1. Phasor plot of L-tryptophan at various pH values. The black points represent pH 6.0 (circle), pH 9.5 (open triangle), and pH 11.0 (square) data at 25 MHz. The lifetime data were collected with 280 nm excitation at 25°C.

Fig. 2.

NATA (circles) and lysozyme (open triangles) phasor points at 89 MHz, with 300 nm excitation at 20°C, with the addition of various concentrations of acrylamide (0 – 0.2 M with NATA and 0-0.5M with lysozyme).

Fig. 3. Phasor plot, at 70 MHz and 280 nm excitation, of NATA (circles) and lysozyme (open triangles) as a function of increasing temperature.

Fig. 4.

A. Phasor plot for dynamin 2 alone (circle), bound with GDP- (closed triangle), and GTP γ S-bound (open triangle). B. Monomeric HSA (closed circle) plotted with furosemide bound HSA (open triangle) and D-thyroxine bound HSA (closed triangle). Dimeric HSA (open circle), which can be found up to 10% in lyophilized HSA, has a unique decay/phasor point compared to monomeric HSA. C. Phasor plot showing point movements due to protein-protein interaction. Solid lines between antithrombin (circle), lysozyme (closed square) and thrombin (closed triangle) are the projected linear movement of phasor point for non-interacting species. Mixtures of antithrombin/lysozyme (both at 1 μ M, open square) and antithrombin/thrombin (gray triangle 0.5:1, open triangle 1:1) are shown on the plot. The dashed line represents the projected phasor movement for increasing concentrations of antithrombin in the presence of thrombin. Cross-hairs seen in the upper part of each figure represent the statistical error for each phasor point under the expanded phasor plot scale.

Fig. 5.

Phasor plot of iron release from hTF N-lobe. Iron-bound and apo phasor vectors are drawn as circle and triangle, respectively. The change in phasor during iron removal, at pH 6.0 with 4 mM EDTA over 300 seconds, is drawn as a black line. Over the first ~ 90 seconds there is a rapid change in the excited state data followed by a much slower change. Data shown were collected at 80 MHz and 25°C.

Fig. 6.

Phasor point trajectory of lysozyme denaturation as a function of increasing GdHCl (solid line) and urea (dashed line) concentration. Concentrations of 0 M (closed circle), 1 M (open circle), 2 M (open triangle) and 6/8 M (closed triangle) GdHCl and urea, respectively, are highlighted. Data was excited with 280 nm and data plotted were at 70 MHz and 25°C.