# Resolution of Molecular Dynamics by Time-Resolved Fluorescence Anisotropy: Verification of Two Kinetic Models

**Nicholas P. Vyleta, Abram L. Coley, and William R. Laws***

*Department of Chemistry, University of Montana, Missoula, Montana 59812*

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Two of nine possible kinetic models have been experimentally validated for the general scheme that describes a system with two fluorescent species and two depolarizing processes. These kinetic models define different associations between the emitting species and the possible depolarizing processes. Association model 1 is defined as one species exhibiting the shorter fluorescence lifetime and experiencing only the fastest depolarizing rotation, and the longer-lifetime second species undergoing only the slower rotation. For model 2, one species exhibits the shorter fluorescence lifetime and experiences only the slower rotation and the longer-lifetime second species undergoes only the faster rotation. To confirm model 1, we studied a binary solution of indole and $\beta$-cyclodextrin. Model 2 was verified using a binary solution of melatonin and rat F102W parvalbumin. In each case, determination of the correct association model and recovery of appropriate intensity and anisotropy decay parameters required global analysis of related data sets. The indole system required $\beta$-cyclodextrin concentration as the independent variable, while emission wavelength was employed for the melatonin/parvalbumin system. To demonstrate further the capabilities of this analysis and kinetic approach, it was shown that the melatonin/parvalbumin system could be converted to a model 1 association by the addition of a quenching agent.

## Introduction

Time-resolved fluorescence anisotropy experiments yield kinetic information about size, shape, and local dynamics properties for a macromolecule. A system that consists of fluorophores in different environments, or different kinds of fluorophores, will yield complex kinetic information because the fluorophores are likely to have different excited-state lifetimes and be subject to different depolarizing processes. Analysis of the anisotropy decay kinetics for such a system is not straightforward, especially without a priori knowledge of the appropriate kinetic model, because the analysis involves resolution of multiple, highly correlated kinetic parameters.

Data analysis methods in which multiple depolarizing processes may be differentially associated with multiple fluorophores should provide more accurate and detailed kinetic information about a biological molecule. For example, if two fluorophores are on the same macromolecule, both will be depolarized by the macromolecular rotation(s). However, when there are different fluorophore environments, only one fluorophore might undergo a separate depolarizing process consisting of segmental and/or local motions. Therefore, to understand local dynamics properties of that molecule it is important to be able to associate a depolarizing process with the specific fluorophore(s). In the simple but relevant system of two emitting species that may undergo two depolarizing processes, nine different kinetic models are possible.[1] Each model relates a species with fluorescence lifetime (tau) to a depolarizing process with rotational correlation time (phi), and thus represents a different tau-phi association scheme. An analysis method has been developed and shown to be successful in recovering not only the correct parameters but also the corresponding kinetic models possible for two-lifetime, two-correlation time systems.[1] This analysis method demonstrated that these systems could be evaluated properly by collecting multiple anisotropy data sets as a function of an independent variable, and analyzing them simultaneously with common variables. Dynamic properties of a tryptophan residue in a collagen-like triple helix have been evaluated by this analysis method.[2] Two distinct fluorescence lifetimes were recovered in the collagen-based system, consistent with two populated indole side chain rotamers for the tryptophan residue, and the results of the fluorescence anisotropy decay analysis indicated that only one undergoes a fast depolarizing process representative of local dynamic motions.

To test directly the robustness of this analysis approach for recovering accurately the dynamic properties of macromolecular systems, it is desirable to demonstrate accurate parameter and correct kinetic model recovery from experimental data using well-characterized systems for each of the possible kinetic models. In this report, we present experimental results for two systems, each with two fluorescence lifetimes and two rotational correlation times, which should have different tau-phi associations. The first system consists of a binary mixture of indole and $\beta$-cyclodextrin, which forms a 1:1 complex in solution. The free and bound forms of indole have different fluorescence lifetimes,[3] and are expected to exhibit different rotational correlation times. In this system, the fluorescence of free indole has the shorter lifetime, and is depolarized by very rapid rotations leading to a short correlation time. Bound indole has the longer fluorescence lifetime, and should be depolarized at a slower rate, leading to a longer correlation time, due to the larger size of the complex. This system, therefore, demonstrates a specific short-short/long-long tau-phi association model.

A different association scheme is achieved with a binary solution of melatonin and the mutant rat protein F102W parvalbumin. Rat F102W parvalbumin is a $Ca^{2+}$-binding,

* Corresponding author. Tel: 406-243-4107. Fax: 406-243-4227. E-mail: bill.laws@umontana.edu.

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spherical, 11.8 kDa molecule containing a single tryptophan residue,[4] which is completely shielded from water and exhibits a fluorescence lifetime near four nanoseconds.[4,5] A rotational correlation time on the order of five nanoseconds has been reported for this protein.[5] Melatonin is a tryptophan derivative with a fluorescence lifetime of approximately six nanoseconds. Being a much smaller molecule than parvalbumin, melatonin is expected to exhibit a much faster rotational correlation time. Thus, in this system, the species with the shorter fluorescence lifetime is associated with the longer correlation time, and the species with the longer fluorescence lifetime is associated with the shorter correlation time. To demonstrate further the ability of this analysis approach to associate distinct fluorescent species with specific depolarizing processes, a quencher was added to convert the short-long/long-short tau-phi associations expected for the melatonin/parvalbumin system into short-short/long-long associations. This change in tau-phi association is achieved by the decrease in the melatonin fluorescence lifetime.

## Materials and Methods

**Sample Preparation.** All standard chemicals were reagent grade. $\beta$-cyclodextrin ($\beta$-CD, Sigma, 18F-340) was purified by recrystalization in deionized water. Solutions of indole (Aldrich, 14818CG) and $\beta$-CD were each made in deionized water. An indole concentration of $5 \times 10^{-5}$ M was used for both the steady-state and time-resolved experiments. Concentrations of $\beta$-CD ranging from $2.5 \times 10^{-4}$ to $9 \times 10^{-3}$ M were used.

Rat F102W parvalbumin was a generous gift from Dr. Arthur Szabo at Wilfrid Laurier University. Purification protocols were previously described for the rat protein.[4] Rat parvalbumin was prepared in a 0.01 M HEPES, 0.14 M NaCl, $5 \times 10^{-3}$ M $CaCl_2$, pH 7.5 buffer. The concentration of the protein was determined by absorbance at 280 nm using an extinction coefficient of 5500 $M^{-1}$ $cm^{-1}$.[5] Melatonin (ACROS, B-2440) was prepared to a final concentration of $1 \times 10^{-5}$ M in the same buffer.

**Spectroscopy.** Absorption measurements were obtained with a Hitachi U-3210 spectrometer, and steady-state fluorescence experiments were performed with an SLM-4800 fluorometer converted to a single-photon counting instrument.

Time-resolved fluorescence decay curves were obtained by time-correlated, single-photon counting using an instrument previously described.[5] Samples maintained at 20 °C were excited at 295 nm with vertically polarized light. Emission was collected through a polarizer set at the magic (M), vertical (V), or horizontal (H) positions, and emission wavelengths were selected by a SpectroPro-150 monochromator (Acton Research Corp., Acton, MA) with a band-pass of 10 nm. For both experimental systems, decays were collected into 8000 channels at a timing calibration of 6 ps/channel. Decay curves were collected to a minimum of 40 000 peak counts, whereas instrument response functions (IRF), obtained by light scattering, were collected to a minimum of 100 000 peak counts. Blanks were not required. Decay curves collected at each polarizer angle (M, V, and H), along with IRFs at each angle, constitute an anisotropy dataset for a specific set of experimental parameters.

**Anisotropy Decay Data Analysis.** Anisotropy datasets were analyzed simultaneously (globally) to recover the intensity and anisotropy decay parameters.[1,6] Decays were analyzed by a reconvolution procedure[7] using nonlinear least-squares regression.[8] Anisotropy datasets collected as a function of an independent variable were also analyzed globally,[9,10] making selected iterated parameters common to all datasets. Joint support plane confidence intervals were calculated for all iterated parameters by an approximation method.[11]

**TABLE 1: Tau-Phi Associations for a Two Tau, Two Phi System**

| | tau-phi associations | | |
|---|---|---|---|
| model | $\tau_1$ | $\tau_2$ | $\beta_{ij} \neq 0$[a] |
| 0 | $\phi_1, \phi_2$ | $\phi_1, \phi_2$ | $\beta_{11}, \beta_{12}, \beta_{21}, \beta_{22}$ |
| 1 | $\phi_1$ | $\phi_2$ | $\beta_{11}, \beta_{22}$ |
| 2 | $\phi_2$ | $\phi_1$ | $\beta_{12}, \beta_{21}$ |
| 3 | $\phi_1, \phi_2$ | | $\beta_{11}, \beta_{12}$ |
| 4 | | $\phi_1, \phi_2$ | $\beta_{21}, \beta_{22}$ |
| 5 | $\phi_1, \phi_2$ | $\phi_1$ | $\beta_{11}, \beta_{12}, \beta_{21}$ |
| 6 | $\phi_1, \phi_2$ | $\phi_2$ | $\beta_{11}, \beta_{12}, \beta_{22}$ |
| 7 | $\phi_2$ | $\phi_1, \phi_2$ | $\beta_{12}, \beta_{21}, \beta_{22}$ |
| 8 | $\phi_1$ | $\phi_1, \phi_2$ | $\beta_{11}, \beta_{21}, \beta_{22}$ |

[a] $\beta_{ij} \neq 0$ signifies that the $i^{th}$ fluorophore is depolarized by the process leading to the $j^{th}$ rotational correlation time.

A system of two independent fluorophores, each exhibiting first-order decay kinetics resulting in an overall decay with two resolvable fluorescence lifetimes, and two depolarizing motions, providing two distinct rotational correlation times, can be described by nine unique kinetic models.[1] The tau-phi associations for these kinetic models are outlined in Table 1. In general, the anisotropy decay of the $i^{th}$ fluorophore can be described by the expression:[1,12]

$$r_i(t) = \sum_{j=1}^{m} \beta_{ij} e^{-t/\phi_j} \qquad (1)$$

The preexponential $\beta_{ij}$ factors depend on the directions of both the absorption and emission transition dipoles, and therefore will generally differ for each fluorophore. A $\beta_{ij}$ term is the degree to which the $i^{th}$ fluorophore is depolarized by the process(es) giving rise to the $j^{th}$ correlation time. The sum of the $\beta_{ij}$ terms for a fluorophore over all correlation times ($j = 1$ to $m$) is the limiting anisotropy $r_{0i}$ for that fluorophore, and is subject to the theoretical range of $-0.2 \leq r_{0i} \leq 0.4$.

As noted for the two-by-two kinetic system in Table 1, certain models can have one or two $\beta_{ij}$ terms equal to zero indicating a specific process does not depolarize a specific fluorescent species. For example, models 3 and 4 have only one emitting species being depolarized. This could occur if one species is in solution, and the second species is immobilized on a surface. If the species in solution experiences both local and global rotational motions, two correlation times will be observed. However, the surface-immobilized species will not exhibit these depolarizing motions.

Initial criteria for accepting or rejecting analyses according to each kinetic model were based on the minimum reduced chi-square, the weighted residuals,[8] and the autocorrelation of residuals for the M, V, and H decays of each dataset. Most analyses met these statistical fitting criteria. Consequently, the recovered iterated parameters were then compared with an acceptable range of values derived from known properties of the molecules employed for these studies. For example, when an indole-based molecule, such as melatonin or the Trp residue in the parvalbumin, is excited with 295-nm light, $r_{0i}$ must be positive and all $\beta_{ij}$ terms must be $\geq 0$.[13] Therefore, recovery of negative $\beta$ values, as well as $r_{0i}$ terms greater than 0.4, were grounds for rejection of that kinetic model. In addition, limits were applied for acceptable phi terms. For the indole/$\beta$-CD system, the rotational correlation time for $\beta$-CD is estimated to be between 0.5 and 1 ns. Therefore, any phi values >1 ns recovered for this system were grounds for model rejection. For the other system, parvalbumin has a rotational correlation time near 5 ns. Consequently, phi values >10 ns recovered for this

system suggested an improper model. It should be noted that the expected intensity decay parameters generally were recovered regardless of the kinetic model. By contrast, recovery of appropriate phi and beta terms for the anisotropy decay requires the appropriate kinetic model.

## Results

The nine possible models for the associations between two lifetimes and two correlation times are outlined in Table 1. The indole/$\beta$-CD system was designed to provide an experimental test of the model 1 kinetic scheme. Because it had been shown that model discrimination required a global analysis of several datasets collected as a function of an independent variable that yielded variation in the relative contribution of each emitting species,[1] and because it had been shown that free and bound indole have slightly different emission spectra,[3] we initially obtained fluorescence anisotropy decay datasets for a solution of indole and $\beta$-CD as a function of emission wavelength. Datasets were collected every 10 nm between 310 and 390 nm. Concentrations were set to have the two indole species (free and complexed) contribute approximately equal fluorescence intensities at the median emission wavelength of 350 nm. Analysis of the anisotropy decay data according to the expected kinetic model (model 1) yielded acceptable intensity and anisotropy decay parameters. Analyses according to models 0, 5, 6, 7, and 8 could be rejected because of unacceptable beta and phi values. However, analyses according to models 2, 3, and 4 yielded acceptable statistical fits and parameter values. We reasoned that the difference between the emission spectra of free and bound indole (2−3 nm) is too small to provide sufficient discrimination against all kinetic models under these experimental conditions.

To distinguish between the nine possible models in Table 1, we then designed an experiment in which the ratio of free versus bound indole in solution was varied. This was accomplished by keeping the total indole concentration constant while varying the $\beta$-CD concentration. Having different $\beta$-CD concentrations changes the proportion of indole in the complexed state, and thus significantly alters the contribution of free and bound indole to the total fluorescence of each sample. An estimated association constant ($K_a$) of 200 $M^{-1}$, based on values at different temperatures,[3] was used to calculate initial $\beta$-CD concentrations.

Analyses were initially performed by applying all nine association models to the individual anisotropy decay datasets collected as a function of $\beta$-CD concentration. As expected from previous studies,[1] analysis of an individual dataset was incapable of discriminating the different models. To recover acceptable anisotropy parameters and distinguish between models, it was necessary to implement global analysis. Analysis of all datasets simultaneously by all nine kinetic models yielded model 1 as the only model to both fit the data *and* recover acceptable lifetime and anisotropy parameters. A wide range of contribution ratios between the species was necessary for the resolution of the proper kinetic model.

As shown in Table 2, model 1 yielded one fluorescence lifetime of 4.65 ns, as expected for free indole,[3] associated with a rotational correlation time near 40 ps, and another lifetime of 6.23 ns, as expected for bound indole,[3] associated with a longer correlation time of 0.55 ns. In this system, the fluorescent species with the shorter of the two lifetimes (indole free in solution) is depolarized faster than the species with the longer lifetime (indole/$\beta$-CD complex). Table 3 shows a variation in contribution to fluorescence by free and bound indole, as represented by the amplitude terms for the intensity decay, which was

**TABLE 2: Indole/$\beta$-CD System Parameters**

| | common parameters[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| model | $\tau_1$ (ns) | $\tau_2$ (ns) | $\phi_1$ (ns) | $\phi_2$ (ns) | $\beta_{11}$ | $\beta_{12}$ | $\beta_{21}$ | $\beta_{22}$ |
| 1 | 4.65 | 6.23 | 0.044 | 0.55 | 0.168 | 0 | 0 | 0.060 |
| 2 | 4.66 | 6.26 | 0.46 | 0.056 | 0 | 0.284 | 0.186 | 0 |
| 6 | 4.65 | 6.19 | 0.059 | 0.66 | 0.205 | -0.001 | 0 | 0.039 |
| 0 | 4.66 | 6.22 | 0.21 | 1.95 | 0.003 | 0.004 | 0.074 | 0.008 |
| 3 | 4.68 | 6.27 | 2.31 | 0.20 | 0.007 | 0.033 | 0 | 0 |
| 4 | 4.64 | 6.21 | 0.20 | 1.57 | 0 | 0 | 0.076 | 0.014 |
| 5 | 4.60 | 6.13 | 0.36 | 4.21 | 0.001 | 0.004 | 0.053 | 0 |
| 7 | 4.64 | 6.19 | 0.19 | 1.64 | 0 | 0.005 | 0.081 | 0.009 |
| 8 | 4.57 | 6.10 | 0.14 | 1.30 | 0.029 | 0 | 0.069 | 0.017 |

[a] Values are averages from two experiments. Model 1 parameter confidence intervals[11] comparable to two standard deviations are < ±0.01 ns for both $\tau_1$ and $\tau_2$, ±0.006 ns for $\phi_1$, ±0.07 ns for $\phi_2$, ±0.074 for $\beta_{11}$, and ±0.012 for $\beta_{22}$.

**TABLE 3: Amplitudes ($\alpha_i$) Recovered by Kinetic Model 1 for the Indole/$\beta$-CD System**

| | $\beta$-CD concentration (mM) [a] | | | | | | |
|---|---|---|---|---|---|---|---|
| $A^b$ | 9 | 4.5 | 2.2 | 1.1 | 0.5 | 0.25 | 0 |
| $a_1$ | 0.39 | 0.50 | 0.64 | 0.78 | 0.87 | 0.92 | 1 |
| $a_2$ | 0.61 | 0.50 | 0.36 | 0.22 | 0.13 | 0.08 | 0 |

[a] Values are averages from two experiments; indole concentration was $5 \times 10^{-5}$ M. [b] $\alpha_1$ and $\alpha_2$, which correspond with indole free in solution ($\tau_1$) and the indole/$\beta$-CD complex ($\tau_2$), respectively, are normalized to sum to 1. Confidence intervals[11] comparable to two standard deviations are < ±0.01 for both $\alpha_1$ and $\alpha_2$.

sufficient for the resolution of the proper kinetic model. A brief evaluation of the amplitude terms in Table 3 shows that the estimated $K_a$ was adequate for our needs. Although not required to resolve the kinetic mechanism, attempts were made to increase the amount of complex but were hindered by $\beta$-CD solubility.

When data analysis was performed according to kinetic models 2 and 6, as shown in Table 2, both analyses returned values depicting a model 1 relationship. Model 2, a short-long/long-short tau/phi association scheme, reversed its phi values upon iteration, returning two lifetimes of 4.66 and 6.26 ns associated with two correlation times of 0.056 and 0.46 ns, respectively. Model 6 designates that the short lifetime is associated with both the long and short correlation times (Table 1). In the model 6 analysis, the appropriate beta term associating the short lifetime with the longer correlation time ($\beta_{12}$) iterated to zero, as expected if the appropriate model is in fact model 1. Analyses by all other kinetic models in Table 1 yielded unacceptable results. As shown in Table 2, models 0, 3, 4, 5, 7, and 8 can be eliminated based on rotational correlation time criteria. Models 0, 5, and 7 can also be rejected because the $\beta_{11}$ and $\beta_{12}$ terms are too small.

Emission spectra (Figure 1) for melatonin and rat F102W parvalbumin reveal significant wavelength separation between maximum emission intensities for the two species. Therefore, we collected anisotropy datasets as a function of emission wavelength. For the melatonin/rat F102W parvalbumin solution, datasets were collected every 20 nm between 320 and 400 nm. Again, analysis of an individual dataset was incapable of discriminating the different models. Therefore, these datasets were grouped with datasets for the individual species, and global analyses were performed by each of the nine possible kinetic models. Acceptable parameters were recovered for model 2 only. As seen in Table 4, this model yielded lifetimes of 4.08 and 5.41 ns associated with correlation times of 4.97 ns and ~40 ps, respectively. These results are consistent with a short-long/long-short association scheme.

**TABLE 4: Melatonin/Rat F102W Parvalbumin System Parameters**

| | | common parameters[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $[I^-]$ (M) | model | $\tau_1$ (ns) | $\tau_2$ (ns) | $\phi_1$ (ns) | $\phi_2$ (ns) | $\beta_{11}$ | $\beta_{12}$ | $\beta_{21}$ | $\beta_{22}$ |
| 0 | 2 | 4.08 | 5.41 | 0.039 | 4.97 | 0 | 0.204 | 0.233 | 0 |
| 0.061 | 1 | 2.37 | 4.18 | 0.071 | 4.91 | 0.105 | 0 | 0 | 0.235 |

[a] Values are averages from two experiments. Model 2 ($[I^-] = 0$ M) parameter confidence intervals[11] comparable to two standard deviations are $< \pm 0.01$ for both $\alpha_1$ and $\alpha_2$ (not given), $< \pm 0.01$ ns for both $\tau_1$ and $\tau_2$, $\pm 0.009$ ns for $\phi_1$, $\pm 0.16$ ns for $\phi_2$, $\pm 0.003$ for $\beta_{12}$, and $\pm 0.11$ for $\beta_{21}$.

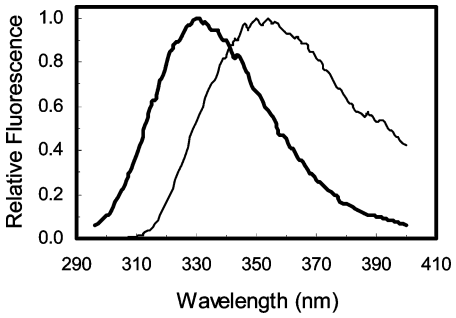


**Figure 1.** Uncorrected, peak normalized fluorescence emission spectra for rat F102W parvalbumin (—) and melatonin (−). Excitation was at 295 nm, and monochromator band-passes were 8 nm.

The Trp residue in rat F102W parvalbumin is known to reside in a nonpolar environment buried inside the protein and, therefore, to be insensitive to addition of quenching agents.[4] Consequently, only the lifetime of melatonin should be affected by addition of a quencher to the melatonin and rat F102W parvalbumin binary solution. Provided enough quencher is added to decrease melatonin's fluorescence lifetime to a value that is resolvable and shorter than parvalbumin's fluorescence lifetime, this system should be converted from the model 2 to model 1 kinetic scheme. A KI concentration of 0.06 M is expected to quench the fluorescence lifetime of melatonin to about 2 ns, based on the Stern−Volmer expression: $\tau_0/\tau = 1 + k_q\tau_0[Q]$, where $\tau_0$ and $\tau$ are the lifetimes in the absence and presence of quencher, respectively, and assuming a value near $5 \times 10^9$ $M^{-1}s^{-1}$ for $k_q$, the quenching rate constant. Therefore, $3.2 \times 10^{-5}$ L of 1.0 M KI was added to $5 \times 10^{-4}$ L of the melatonin/parvalbumin solution. As before, datasets were collected as a function of emission wavelength and analyzed globally by each of the nine kinetic models. Acceptable parameters were recovered only from an analysis by model 1. Table 4 shows that the ∼4 ns lifetime expected for rat F102W parvalbumin was maintained and that a second lifetime of 2.4 ns was recovered, consistent with a decrease of more than a factor of 2 in the melatonin fluorescence lifetime. This decrease indicates a $k_q$ value of $3.9 \times 10^9$ $M^{-1}$ $s^{-1}$, which is typical for the diffusion of small molecules. The model 1 analysis also reported that the ∼5 ns rotational correlation time was associated with only the 4 ns lifetime of rat F102W parvalbumin, and that the short correlation time was only associated with the 2.4 ns lifetime of the quenched melatonin species. Thus, with addition of iodide ion, a short-short/long-long tau-phi association scheme was generated, like that of the indole and $\beta$-CD system.

## Discussion

Time-resolved fluorescence anisotropy experiments can be used to determine the size, shape, and local dynamics properties of a macromolecular system. The ability to recover the correct kinetic model and accurate kinetic parameters from these experiments is crucial for utilizing this information. Results from these experiments demonstrate that it is possible to correlate specific kinetic phenomena with individual fluorescent species involving two lifetimes and two correlation times. As this is a two-by-two system, nine kinetic models are possible (Table 1) that differ by specific tau-phi associations.[1] Previously, it was demonstrated that kinetic models 6 and 7 uniquely associate the intensity decay parameters of tryptophan in liver alcohol dehydrogenase[1] and synthetic collagen-like polypeptides,[2] respectively, with appropriate anisotropy decay parameters. Here, we demonstrate with the binary indole/$\beta$-CD and melatonin/rat F102W parvalbumin systems that appropriate associations also can be made uniquely for kinetic models 1 and 2, respectively.

It was anticipated that the indole/$\beta$-CD system could be used to test kinetic model 1 because the free and bound indole species have different fluorescence lifetimes and molecular volumes that will result in different correlation times. Our results confirm this hypothesis, as only a model 1 association is able to provide an acceptable analysis based on the criteria given in Materials and Methods. Global analyses based on two other kinetic models yielded acceptable fitting statistics, but the recovered parameters were those expected for a model 1 association. Another important point to note from this system is that emission wavelength did not provide a satisfactory independent variable to distinguish between the nine kinetic models. However, the appropriate kinetic model could be identified by variation of the $\beta$-CD concentration, which provided different ratios of free to bound indole, resulting in a large range of amplitude terms for the intensity decays. Global analysis of this series of anisotropy datasets provided the means to resolve a specific kinetic association, model 1, which is an accurate physical description of the system. This emphasizes the importance of achieving separation between relative intensities of each species in this kind of analysis.

The melatonin and rat F102W parvalbumin binary system was designed to test the model 2 association scheme. The analysis results demonstrated that only kinetic model 2 provided an acceptable fit of the datasets collected as a function of emission wavelength. By application of this analysis approach to this system, we show that rotational correlation times differing by more than 2 orders of magnitude can be easily recovered, even when the lifetime terms differ by no more than ∼30%. This system has two important features that allow further evaluation of the two-by-two kinetic analysis approach. First, the species with the longer lifetime (melatonin) has the shorter correlation time. Second, only melatonin fluorescence is affected by addition of a solute quencher because the Trp residue in the rat F102W parvalbumin is completely buried within the interior of the protein.[4] Consequently, addition of a solute quencher should convert this system to one that is best described by model 1 associations. The results confirmed this hypothesis: the fluorescence lifetime of the parvalbumin Trp residue (4.1 ns) remained associated with only the 5 ns correlation time ascribed to parvalbumin, and the lifetime shortened by iodide quenching was only associated with the correlation time expected for melatonin free in solution. This experimental system thus proved suitable for demonstrating a particular association model as well as the ability to change (and monitor that change) an association scheme by altering experimental conditions.

The indole/$\beta$-CD system, used to test model 1, could also be analyzed successfully by models 2 and 6, but with results indicating a model 1 association. It is interesting that models 2 and 6 did not yield acceptable parameters for the quenched melatonin/parvalbumin system, which also demonstrates the model 1 association. A possible reason being explored for the difference in the analyses results for the two systems involves the relative magnitudes of the two rotational correlation times which differ by one and 2 orders of magnitude for the indole/$\beta$-CD and melatonin/parvalbumin systems, respectively (Tables 2 and 4).

In summary, time-resolved fluorescence anisotropy experiments were conducted on species with known fluorescence lifetimes and known or well-estimated rotational correlation times to evaluate whether global analyses could resolve uniquely two of nine possible lifetime-correlation time association models for a two-lifetime, two-correlation time system. It is desirable to examine well-defined experimental systems to evaluate each of the nine possible association schemes for a two-by-two kinetic system. Because models 3, 4, 5, and 8 represent physically unlikely scenarios, it may be difficult to find an experimental system to test them. With the demonstration here of both models 1 and 2, and previous resolution of models 6 and 7,[1,2] the two-by-two kinetic system has been well established. To expand this kinetic approach to the resolution of more complex systems, analysis of systems with either two lifetimes and three correlation times or three lifetimes and two correlation times should also be explored. However, because the number of possible association schemes increases dramatically as the number of lifetimes and/or depolarizing processes increases,[1] resolution of unique kinetic models becomes more difficult. Criteria for accepting or rejecting an analysis may need to be more rigorous for these more complex matrices, and additional experimental protocols may be required to help resolve the appropriate kinetic model.[12]

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## References and Notes

(1) Bialik, C. N.; Wolf, B.; Rachofsky, E. L.; Ross, J. B. A.; Laws, W. R. *Biophys. J.* **1998**, *75*, 2564.

(2) Simon-Lukasik, K. V.; Persikov, A.; Brodsky, B.; Ramshaw, J. A.; Laws, W. R.; Ross, J. B. A.; Ludescher, R. D. *Biophys. J.* **2003**, *84*, 501.

(3) Orstan, A.; Ross, J. B. A. *J. Phys. Chem.* **1987**, *91*, 2739.

(4) Pauls, T. L.; Durussel, I.; Cox, J. A.; Clark, I. D.; Szabo, A. G.; Gagne, S. M.; Sykes, B. D.; Berchtold, M. W. *J. Biol. Chem*. **1993**, *268*, 20897.

(5) Feinstein, E.; Deikus, G.; Rusinova, E.; Rachofsky, E. L.; Ross, J. B. A.; Laws, W. R. *Biophys. J.* **2003**, *84*, 599.

(6) Waxman, E.; Laws, W. R.; Laue, T. M.; Nemerson, Y.; Ross, J. B. A. *Biochemistry* **1993**, *32*, 3005.

(7) Grinvald, A.; Steinberg, I. Z. *Anal. Biochem.* **1974**, *59*, 583.

(8) Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences*; McGraw-Hill: New York, 1969.

(9) Knutson, J. R.; Beechem, J. M.; Brand, L. *Chem. Phys. Lett*. **1983**, *102*, 501.

(10) Beechem, J. M.; Knutson, J. R.; Ross, J. B. A.; Turner, B. W.; Brand, L. *Biochemistry* **1983**, *22*, 6054.

(11) Straume, M.; Frasier-Cadoret, S. G.; Johnson, M. L. In *Topics in Fluorescence Spectroscopy*; Lakowicz, J. R., ed., Plenum: New York, 1991; Vol. 2, 177−240.

(12) Rachofsky, E. L.; Laws, W. R. *Methods Enzymol*. **2000**, *321*, 216.

(13) Valeur, B.; Weber, G. *Photochem. Photobiol*. **1977**, *25*, 441.