Fluorescence Lifetime Data Analysis Using Simplex Searching and Simulated Annealing

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Simulated annealing, a nonlinear parameter estimation method, is used to address the problem of bieponential decay law determinations. With a laboratory-constructed fluorometer using a nitrogen-pumped dye laser as a source of very short light pulses and a photomultiplier tube whose output is sampled by a boxcar averager, the distribution of noise in the data may not be assumed to be normal and the noise level may be relatively high. Perhaps for these reasons the traditional means of data analysis sometimes fails. Implemented on a powerful laboratory workstation, simulated annealing yields reasonable estimates of decay laws, which may be further refined by simplex searching. This hybrid data analysis method has been applied to the determination of bieponential decay laws of systems of dansylated amino acids and bovine serum albumin.

INTRODUCTION

Among the instrumental analyses commonly applied to fluorescent systems are determinations of excitation and emission spectra and the fluorescent lifetime or lifetimes. Such characterizations can yield information on the chemical environment of a fluorescent species. Fluorescence lifetimes are typically in the range of 100 ps to 50 ns, a scale which presents a particular set of challenges. Lifetime determinations can be considered to be either time-domain or frequency-domain measurements. Although the advent of lasers in spectroscopy has brought frequency-domain techniques to prominence, much interest remains in time-domain fluorescence lifetime measurements.

Time-domain measurements typically involve the observation of the decay of fluorescence intensity from a system following a brief exciting pulse of light. Two popular forms of this general type of experiment are time-correlated photon counting (TCPC) and oscilloscopic sampling. While the latter is conceptually simpler, the former is apparently the more reliable in practice. Oscilloscopic sampling is more applicable to the time-correlated photon-counting technique that has been more successful.

This paper reports the development of a data analysis method for time-domain fluorescence lifetime measurements.

The time-dependent fluorescence, $F(t)$, following excitation by a brief pulse of scattered or fluoresced light and by the finite width of the pulse of exciting light. Fortunately, these two effects can be addressed concurrently. Of course, it may occur that the lifetime of the species under consideration is long with respect to these other concerns and the decaying luminescence can be observed at a sufficient time after the exciting pulse so that departure from ideal behavior is negligible. This is often the case for phosphorescence, but for shorter fluorescence lifetimes, the observed decay curves are often influenced by instrumental factors to such an extent as to require correction or allowance in the data analysis method.

The time-dependent fluorescence, $F(t)$, following excitation of a sample with this instrument is given by

$$F(t) = \int_0^\infty P(x)f(t - x)\,dx$$

where $f(t)$ is the true decay law, generally a mono- or multieponential, and $P(t)$ is the response of the instrument itself to a pulse of exciting light. If excitation were by an infinitely narrow pulse and the instrument responded infinitely fast, $P(t)$ would be a $\delta$ pulse and $f(t)$ could be observed directly. In practice, one observes $F(t)$ and $P(t)$ separately as fluoresced and scattered light, respectively, and arrives at the decay law $f(t)$ by suitable analysis. The most common analysis of such data uses nonlinear least-squares fitting by Marquardt's method.

The author's interest in the development of fluorescence lifetime instrumentation is the investigation of the effect of the proximity of binding proteins on the fluorescence decay of drug molecules or their analogues. During the course of constructing the fluorometer for these experiments, it became apparent that the popular means of data analysis, most commonly applied to the time-correlated photon-counting experiment, was not particularly well-suited to this apparatus. This paper reports the development of a data analysis technique that has been more successful.

Simplex searching has enjoyed recent popularity in analytical chemistry, both for optimization of experimental conditions and as a means of finding extrema of complicated functions of several variables. A problem with simplex searching, however, is that if more than one extreme exists, the simplex will find just one extreme, not necessarily the most extreme. A parameter estimation method that is resistant to becoming trapped by such local extrema is simulated annealing. Based on an algorithm by Metropolis et al., the method has found renewed interest not only for parameter estimation in model fitting but also in combinitorial optimization problems such as circuit layout design.

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mounted directly to the monochromator. Neutral density filters et al.19 and housed in a laboratory-constructed aluminum case the monochromator by a photomultiplier tube (Model 1P28, RCA chromator. Fluoresced or scattered light is observed through chromator (Model DH-BOA, Instruments SA, Inc., Metuchen, NJ); fluoresced light from an analyte or scattered light form the pulse of exciting light with a full width at half-maximum (fwhm) in Figure 1. The nitrogen-pumped dye laser (Models LNl00 and LN102, respectively, Photochemical Research Associates, now in VLSI chips.13 The use of simulated annealing in analytical chemistry is also becoming more widespread.14-17 A variable step-size algorithm has also been developed,17 which was not used in this work. The use of this and related techniques in analytical chemistry is the subject of a recent report in this journal.18

**EXPERIMENTAL SECTION**

The fluorescence lifetime determination apparatus is shown in Figure 1. The nitrogen-pumped dye laser (Models LN100 and LN102, respectively, Photochemical Research Associates, now Laser Photonics, Orlando, FL) provides the sample cell with a pulse of exciting light with a full width at half-maximum (fwhm) of 200 ps. The sample cell holder is attached directly to a monochromator (Model DH-20A, Instruments SA, Inc., Metuchen, NJ); fluoresced light from an analyte or scattered light form the solvent matrix is selected by the wavelength control on the monochromator. Fluoresced or scattered light is observed through the monochromator by a photomultiplier tube (Model 1P28, RCA Corp., Lancaster, PA) mounted on a base constructed after Harris et al.19 and housed in a laboratory-constructed aluminum case mounted directly to the monochromator. Neutral density filters

are inserted in the light path to allow adjustment of signal intensity to a reasonable level and, within a series of data sets, adjustment of the signals' peaks to approximately equal values.

Output from the PMT is interrogated by a boxcar averager through a gated integrator (Models 4420 and 4422, respectively, EG&G Princeton Applied Research Corp., Princeton, NJ) whose integration time is typically set at 2 ns. The boxcar is triggered by a photodiode assembly (Model LP141, Molecron Corp., Sunnyvale, CA) on which is incident a small fraction of the exciting beam, provided by a glass microscope slide positioned in the path of the exciting beam at 45°. The time base on the boxcar is not initiated until 40 ns after the trigger pulse, which requires that the signal from the PMT be delayed by passage through an appropriate length (~40 ft.) of RG141 coaxial cable. The PMT, its power supply, and the boxcar averager are all enclosed in a laboratory-constructed Faraday cage to shield them from radio-frequency interference, which presented a severe problem otherwise and presumably emanated from the spark-gap-excited nitrogen laser.

Setting of the boxcar parameters and control of data acquisition is performed by an IBM PS/2 Model 25 microcomputer; the boxcar averager is interfaced to the computer over a GPIB connection (National Instruments, Austin, TX). The Model 25 computer is connected via Ethernet to an IBM RT PC; all forms of data analysis are done with the RT system.

The data analysis methods outlined in this paper are written in the C programming language. The programs for both non-linear least-squares analysis by Marquardt’s method and simplex searching incorporate functions found in ref 20. The simplex searching function was easily modified to allow the imposition of bounds on the parameters. The simulated annealing algorithm was developed from an outline given by Bohachevsky et al.11

Dansyl-l-proline and dansylamide were used as received (Sigma Chemical Co., St. Louis, MO). Bovine serum albumin (BSA), Fraction V (Sigma), was used without further purification. No fluorescence from the protein or from associated impurities, such as fatty acids, was observed with this fluorometer at the wavelengths used. A stock protein solution was made up to approximately 100 μM in 1 mM phosphate buffer at neutral pH. Solutions of the dansyl derivatives in the same buffer were made up to 100 μM with various quantities of the protein solution.

The general procedure for determining the fluorescence decay law with this instrument is to first acquire the system’s response to scattered light, P(t) in eq 1, with a BSA solution in the sample cell and the dye laser’s monochromator and the emission monochromator at the same wavelength setting. Then the observed fluorescence, F(t), is acquired with a solution of a dansyl derivative and BSA in the sample cell and the emission monochromator set to some higher wavelength. Both data sets are then used with one of the data analysis methods to determine R(t).

**RESULTS AND DISCUSSION**

For the case of a monoexponential decay

\[ I(t) = A e^{-t/\tau} \]  \hspace{1cm} (2)

where \( \tau \) is the fluorescence lifetime and \( A \) is a preexponential factor related to the number of fluorescing molecules, eq 1 becomes

\[ F(t) = A \int P(x) e^{-x(t-x)/\tau} \ dx \]  \hspace{1cm} (3)

Since \( F(t) \) and \( P(t) \) are determined at discrete times, the integration can be replaced by a summation

\[ F_{cal}(t) = A \sum_{j} P(t_j) e^{-t_j(t-x)/\tau} \]  \hspace{1cm} (4)

Numerous methods exist to solve for \( \tau \), the lifetime. Indeed, since it is \( \tau \) which is of interest for a monoexponential decay and \( A \) exists as a vertical scaling factor between the observed

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course of fluorescence, \( F_{\text{obs}}(t) \), and the convolution integral, one can simply guess values for \( \tau \), compute \( F_{\text{calc}}(t) \) from eq 4 and compare \( F_{\text{obs}}(t) \) and \( F_{\text{calc}}(t) \). It is generally agreed that the \( \chi^2 \) merit function is an acceptable goodness-of-fit indicator; here

\[
\chi^2 = \sum \frac{(F_{\text{obs}}(t) - F_{\text{calc}}(t))^2}{\sigma_i}
\]

where \( \sigma_i \) is the standard deviation associated with \( F_{\text{obs}}(t) \).

While minimizing \( \chi^2 \) should provide the best fit between a given \( F(t) \) and \( P(t) \) and is extremely useful in guiding automated searches, the \( \chi^2 \) value simply identifies the value which gives the closest fit to the data, but does not necessarily indicate whether the calculated lifetime is reasonable for the observed data. A less ambiguous indicator of the quality of the fit is the autocorrelation function, defined for the present case by Grinvald and Steinberg as

\[
C(t_j) = \frac{1}{n} \sum_{i=1}^{n} \sqrt{w_i \Delta_i \sqrt{w_{i+j}}} \frac{1}{n} \sum_{i=1}^{n} w_i \Delta_i
\]

where \( \Delta_i = F_{\text{obs}}(t_i) - F_{\text{calc}}(t_i), w_i \) is the weight assigned to \( \Delta_i \) (typically \( w_i = 1/\sigma_i^2 \)), \( n \) is the number of points in the fluorescence data set, and \( m \) is chosen for each \( j \) so that \( i + j \leq n \); typically \( C(t_j) \) is calculated for \( j \) from 0 to \( n/2 \).

An acceptable procedure for determining monoeponential lifetimes, then, consists of systematically varying \( \tau \) in eq 4 to produce the lowest \( \chi^2 \) and then verifying with the autocorrelation that the lifetime is reasonable and explains the relationship of \( F(t) \) and \( P(t) \).

For a biexponential decay that follows the decay law

\[
I(t_j) = \alpha_1 e^{-t_j/\tau_1} + \alpha_2 e^{-t_j/\tau_2}
\]

or

\[
I(t_j) = \alpha_1 e^{-t_j/\tau_1} + (1 - \alpha_1) e^{-t_j/\tau_2}
\]

(assuming \( \alpha_1 + \alpha_2 = 1 \), which is permissible since the \( a \)'s indicate the relative degree of contribution of each component while the absolute intensity is adjusted by scaling the calculated fluorescence response to the observed), finding the parameters \( \alpha_1, \tau_1, \) and \( \tau_2 \) from

\[
F_{\text{calc}}(t_j) = \sum_{j=1}^{i} P(t_j) I(t_i - t_j)
\]

or

\[
F_{\text{calc}}(t_j) = \sum_{j=1}^{i} P(t_j) [\alpha_1 e^{-(t_i-\tau_1)/\tau_1} + (1 - \alpha_1) e^{-(t_i-\tau_2)/\tau_2}]
\]

is a problem in nonlinear parameter estimation and is complicated in general by the high degree of correlation among the parameters and in this case in particular by the relatively large amount of noise in the data. The former complication requires that systematic guessing at one parameter (in the monoeponential case) be abandoned in favor of a computerized parameter estimation algorithm for the three parameters.

**Nonlinear Least-Squares Fitting.** Most literature procedures involving curve simulation use nonlinear least-squares routines based on Marquardt's algorithm. The source code for the Marquardt method was modified from that given in ref 20. The algorithm requires that the partial derivatives of \( F_{\text{calc}}(t) \) with respect to all the parameters be evaluated; much of the computational burden rests with this requirement.

As mentioned previously, the most popular data acquisition method currently reported in the literature is time-correlated photon counting. In this method, noise in the data is distributed normally for large numbers of counts. The Marquardt fitting algorithm works well on simulated data sets such as these where the variance of a given point is equal to the data value at that point. However, on real data sets collected with this instrument, where the noise in the data is not from counting error but is dominated by the extreme pulse-to-pulse variation of the laser, the Marquardt method often converges to unreasonable parameter values. Although the \( \sigma_i \)'s cannot be assumed to be equal to the inverse of the square root of the corresponding data value, as is done in photon-counting experiments, because each plotted point represents the average of 64 acquired points and all data points are retained on disk, one can calculate the standard deviation at each point directly. Whether the lack of success here is due to the degree or the distribution of the noise is not clear.

**Simplex Searching.** The curve simulation routine was also incorporated into a simplex search program; the curve simulation routine returned an indicator of goodness-of-fit. The rms of both the autocorrelation and the residuals has been used, and both give similar results. In the work reported here, the rms of the autocorrelation is used as the goodness-of-fit indicator. This value tends to zero as the fit to one data set progresses, but the magnitude, compared among different data sets, is not meaningful, since the simulated curve is fit to normalized observed data. To initiate a search, the user provides the program with some guesses at complete sets of parameters; the number of guesses required is one more than the number of adjustable parameters in the proposed decay law. In the case of a biexponential decay, four guesses are required; these are the vertices of the starting simplex. The optimization control was provided by the amoeba(1) function of ref 20. Optimization ceases when the goodness-of-fit indicator can no longer be improved by a given tolerance through generating new simplexes.

Figure 2 shows the simplex-generated fit to fluorescence observed at 560 nm from a solution of 10 \( \mu \)M dansylamide and 100 \( \mu \)M BSA when excited at 360 nm. No monoexponential decay law could be fit well to the data. The biexponential law obtained was \( I(t) = 0.46 e^{-t/22} + 0.54 e^{-t/32.9} \); the shorter lifetime is associated with free dansylamide in solution, while the longer is due to dansylamide bound to the protein. Figures 2 and 3 are similar to the plots of Grinvald and Steinberg. Here the central plotted data are the observed fluorescence decay (unconnected points) and the simulated curve (solid line) from the convolution of the impulse response function of the instrument with the assumed decay law. The ordinate is unlabeled because the observed data set is normalized and the simulated curve is fit to it, so the absolute magnitude of the plots is lost. Below these curves the point-by-point difference between the observed and the calculated fluorescent decays are plotted; also the range and rms of the residuals is displayed. This plot is useful for visual inspection for systematic deviations of the calculated data from the observed data. The autocorrelation is plotted in the upper right corner of the figures. The abscissa of the autocorrelation plot is the index \( j \) of the autocorrelation function defined by eq 6. Note that the first point of the autocorrelation is always fit directly to the data. By comparing the two methods, the autocorrelation should rapidly approach zero and itself show no systematic variation.

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Assumed decay law: \( \tau_1 = 2.55 \text{ nsec (0.46)} \) \( \tau_2 = 25.90 \text{ nsec (0.54)} \)

\[
\begin{align*}
\text{range of autocorrelation:} & \quad 1.00000, -0.21491 \\
\text{rms of autocorrelation:} & \quad 0.08001 \\
\text{range of residuals:} & \quad -0.00276, 0.00447 \\
\text{rms of residuals:} & \quad 0.00089
\end{align*}
\]

Figure 2. Curve simulation plot of fluorescence from 10 \( \mu M \) dansylamide and 100 \( \mu M \) BSA (excitation at 386 nm, emission at 560 nm).

The searching algorithm allows the imposition of bounds on parameters. One may, for example, have a good idea of the lifetime of one or both components and so restrict the searching algorithm to a range of lifetime values. Also, negative preexponential parameters have no physical significance; it is pointless to allow the searching routine to explore, for eq 10, parameter space where \( a_1 < 0 \) or \( a_1 > 1 \). Again, simplexes are easily guided away from these regions.

The simplex algorithm as implemented here essentially searches the hypersurface defined by eqs 5 and 10 for the point \((a_1, \tau_1, \tau_2)\) in parameter space at which \( x^2 \) is at a minimum. The goal is to find the global minimum, but the simplex algorithm, always seeking to minimize \( x^2 \), allows itself to be trapped in local minima, unable to escape and continue its search for the global minimum.

**Simulated Annealing.** A parameter estimation method that is resistant to becoming trapped by local extrema is simulated annealing. The usefulness of simulated annealing arises from its willingness to accept a "bad" move in parameter space (a move in which \( x^2 \) increases) and so extricate itself from a local minimum in further search of a possible global minimum.

The notation of Bohachevsky et al.\textsuperscript{11} is convenient for this application; one selects an arbitrary starting point in parameter space \( x_0 = [a_{1,0}, \tau_{1,0}, \tau_{2,0}]^T \)\textsuperscript{12} and calculates \( x_0^2 = x^2(x_0) \), using eqs 10 and 5. Then one generates a unit vector with random direction and adds a predefined fraction of it to \( x_0 \). In the literature method this fraction is a scalar \( \Delta r \), but in this case since the absolute values of lifetimes in nanoseconds (e.g., 1–30) are much different from those of the preexponential factors (always between 0 and 1), \( \Delta r \) can be replaced with a vector \( \mathbf{r} \) whose components take into account the magnitudes of the different parameters (for example, while preexponential factors should be varied in steps no larger than 0.05, using this step size for a lifetime of about 20 ns would be very tedious and may not permit reasonably rapid exploration in the \( \tau_2 \) domain). The new vector is called \( x' \). If \( x' \) is out of bounds (e.g., a negative exponential), this \( x' \) can be rejected and a new one generated as above. For an acceptable \( x' \), one assigns \( x_1^2 = x^2(x') \) and \( \Delta x^2 = x'^2 - x_0^2 \). If \( x_1^2 < x_0^2 \), the step has been favorable and one sets \( x_0 = x' \) and repeats after generating a new \( x' \). This is similar to other search strategies, always accepting beneficial moves in parameter space.

The annealing algorithm differs by defining a probability

\[
p = e^{-\beta x^2(\Delta x)^2}
\]

if \( x_1^2 > x_0^2 \), a situation which results from going "uphill", or by considering a detrimental move of parameters. Since the three terms \( \beta, x_0^2, \text{and} \Delta x^2 \) are all positive, \( p \) is between 0 and 1. The probability \( p \) is compared with a random number from a uniform distribution between 0 and 1; if \( p \) is greater than this random number, the detrimental step is accepted and one sets \( x_0 = x' \), \( x_0^2 = x'^2 \) and repeats after generating a new \( x' \). This is similar to other search strategies, always accepting beneficial moves in parameter space.

The constant \( \beta \) is chosen to regulate the percentage of detrimental steps accepted; as recommended by Bohachevsky et al.,\textsuperscript{11} this percentage was kept between 50% and 90% by empirically setting \( \beta \), typically between 2 and 4. The constant \( g \) is a negative number, typically −0.5 or −1.0, and makes the probability of accepting a detrimental step tend to zero as the global minimum is approached.
This is the great advantage of the simulated annealing method; also, it is easy to constrain the parameters to particular ranges. A disadvantage of our algorithm is that variable step sizes have not been implemented, which would allow larger steps to be taken farther away from the global minimum and, as $x_0$ decreases and finally becomes stable (presumably near its minimum), take smaller steps to "fine-tune" the final parameters.

Combination of Simplex Searching and Simulated Annealing. In an effort to find the final parameters more accurately, it is possible to combine simulated annealing with a version of simplex searching that allows upper and lower bounds on all parameters. This takes advantage of the benefits of both types of analysis. The simulated annealing procedure can come close to the true global minimum in parameter space, while the simplex search, now begun near the global minimum, can search for the global minimum without fear of falling into a local minimum. In practice, for a biexponential analysis, the results from four runs of the annealing program can be used as the vertices of the starting simplex; also these results should provide one with a good idea of the constraints to impose on each parameter.

Table I shows the results of this approach applied to one pair of fluorescent and scattered light data sets, for 10 µM dansyl-L-proline and 5 µM BSA. The top part of Table I lists results from four runs of the simulated annealing program. The bottom part of Table I lists results from the simplex searching program, using the parameter sets from the simulated annealing runs as the starting simplex. Given a fixed step size, our simulated annealing algorithm is not in general able to find the parameters exactly that give the best goodness-of-fit indicator. However, the simplex searching program does find the best parameters and can give estimates of the errors of the fitted parameters.

### Table I. Comparison of Biexponential Fluorescence Decay Parameter Estimation by Simulated Annealing and Simplex Searching

<table>
<thead>
<tr>
<th>method</th>
<th>$\tau_1$, ns</th>
<th>$\sigma_1$</th>
<th>$\tau_2$, ns</th>
<th>$\sigma_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>simulated annealing</td>
<td>2.726</td>
<td>0.874</td>
<td>24.510</td>
<td>0.128</td>
</tr>
<tr>
<td>(four runs)</td>
<td>2.543</td>
<td>0.869</td>
<td>21.740</td>
<td>0.141</td>
</tr>
<tr>
<td></td>
<td>2.588</td>
<td>0.864</td>
<td>22.726</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>2.711</td>
<td>0.872</td>
<td>23.998</td>
<td>0.126</td>
</tr>
<tr>
<td>simplex searchingb</td>
<td>2.61</td>
<td>0.864</td>
<td>22.6</td>
<td>0.136</td>
</tr>
<tr>
<td>error estimates</td>
<td>0.04</td>
<td>0.004</td>
<td>0.7</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Fluorescence system is 10 µM dansyl-L-proline and 5 µM BSA excited at 366 nm with emission monitored at 575 nm. Parameter estimates from the four simulated annealing runs are used as the starting vertices for simplex searching.

Estimation of Errors in Parameters. The method of Phillips and Eyring was used to provide estimates of the errors in the fitted parameters. With this method a quadratic approximation to the error surface is developed, which allows calculation of an error matrix that is related to the covariance matrix. Standard deviations of the fitted parameters are calculated from the diagonal elements of the error matrix.

Dansylproline and BSA Binding. As an example of the usefulness of this data analysis technique, results from an attempt to show that fluorescence lifetime data can be used

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Table I. Relative Fluorescence from Free and Bound Dansyl-L-proline When Titrated with BSA

<table>
<thead>
<tr>
<th>[BSA], µM</th>
<th>α1, ns</th>
<th>2.67 (0.02)</th>
<th>0.39 (0.001)</th>
<th>2.26 (0.02)</th>
<th>3.07 (0.001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.71 (0.02)</td>
<td>0.984 (0.001)</td>
<td>24.2 (0.8)</td>
<td>0.016 (0.001)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.90 (0.02)</td>
<td>0.958 (0.001)</td>
<td>24.1 (0.2)</td>
<td>0.042 (0.001)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.61 (0.04)</td>
<td>0.924 (0.004)</td>
<td>23.6 (0.7)</td>
<td>0.136 (0.004)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.43 (0.08)</td>
<td>0.745 (0.002)</td>
<td>24.2 (0.3)</td>
<td>0.255 (0.002)</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Biexponential Decay Laws from Titration of Dansyl-L-proline with BSA

| [BSA], µM | τ1, ns | α1 | 2.71 (0.02) | 0.984 (0.001) | 24.2 (0.8) | 0.016 (0.001) | α2 |
|-----------|--------|----|-------------|--------------|-------------|--------------|
| 1         | 2.71 (0.02) | 0.984 (0.001) | 24.2 (0.8) | 0.016 (0.001) |            |
| 2         | 2.90 (0.02) | 0.958 (0.001) | 24.1 (0.2) | 0.042 (0.001) |            |
| 5         | 2.61 (0.04) | 0.924 (0.004) | 23.6 (0.7) | 0.136 (0.004) |            |
| 10        | 2.43 (0.08) | 0.745 (0.002) | 24.2 (0.3) | 0.255 (0.002) |            |

α1 and τ1 are associated with free dansyl-L-proline; α2 and τ2, with ligand bound to protein. Parameter error estimates are indicated in parentheses.

to semiquantitatively examine ligand–protein interactions are presented. The fluorescence intensity from dansylated amino acids increases dramatically in the presence of serum albumins. The work of Sudlow, Birkett, and Wade demonstrated that fluorescence intensity measurements of such systems could be used to determine binding parameters of both the dansylated ligands and their nonfluorescent competitors. Little work has been reported, however, that makes use of the change in fluorescence lifetimes of the dansyl derivatives on binding to serum albumins. However, Thomas et al. reported that dansylamide bound to human serum albumin exhibited a fluorescence lifetime of 23 ns, a 10-fold increase over that of the free form.28

Table II shows biexponential decay law parameters and error estimates obtained with the simulated annealing/simplex searching analysis from fluorescence decays from 10 µM dansyl-L-proline in 1 mM phosphate buffer with various concentrations of BSA. Excitation was at 366 nm, with emission monitored at 575 nm. A representative plot of a decay law estimation for a BSA concentration of 5 µM is shown in Figure 3. The total number of photons fluoresced from the ith species is αiτi, which is proportional to the number of excited molecules of the ith species and, thus, through the fluorescence yield φi, to the number of molecules of the ith species present. Thus for this system, α1τ1 is related to the concentration of dansyl-L-proline free in solution and α2τ2, to that of dansyl-L-proline bound to BSA. Because the fluorescence yields are not the same (φ2 is in fact much greater than φ1), direct comparisons between α1τ1 and α2τ2 are not quantitative. However, Table III shows the trends expected for titration of dansyl-L-proline with BSA; namely, for a constant amount of dansyl-L-proline, as the concentration of BSA increases, the amount of free dansyl-L-proline decreases while the amount bound to BSA increases. If α1 and α2 were known it might be possible to more quantitatively describe this binding, which suggests a method for elucidating binding parameters without separating the bound and free fractions.

**CONCLUSION**

Simulated annealing and simplex searching have been applied in series to determine the parameters of biexponential fluorescence decays. In essence, simulated annealing is used to provide the starting vertices for simplex searching, thus minimizing the risk of simplex searching being trapped in a local minimum in parameter space. This hybrid method has been found to be very useful in the analysis of biexponential decays, in spite of the relatively high level of noise in the data.

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