Improved Differentiation Between Luminescence Decay Components by Use of Time-Resolved Optical Activity Measurements and Selective Lifetime Modulation

Joseph A. Schauerte,*‡ Ari Gafni,*‡ and Duncan G. Steel*§
*Institute of Gerontology, Department of Biological Chemistry,‡ and Department of Physics and Electrical Engineering,§ University of Michigan, Ann Arbor, Michigan 48109 USA

ABSTRACT The analysis of luminescence decay experiments from proteins is typically modeled as a combination of independent first-order decay functions. However, Poisson noise in the photon counting experiment limits the ability of this approach to resolve decay components from separate lumiphores with similar lifetimes. To provide further differentiation, we incorporate time-resolved circular polarization of luminescence, an additional independent observable, into the analysis. In the simplest case, for example, each lumiphore's chirality is assumed to be time independent and is determined by the position of the lumiphore with respect to the surrounding chiral environment within the protein. In this paper, we describe the analysis of simultaneously recorded time-resolved luminescence and circularly polarized luminescence data to obtain improved temporal resolution. When combined with selective dynamic luminescence quenching, in a model system comprising a mixture of Tb/transferrin and Tb/conalbumin, we demonstrate resolution between two decay components with a lifetime difference of 7% and a difference in emission anisotropy of 5×10^{-2}. Evidence for the improved discrimination is further demonstrated by the increase in curvature of the $\chi 2$ surface that results from the additional information.

INTRODUCTION

Radiative decay measurements from luminescent centers in proteins are a common and powerful means of studying protein structure, dynamics, and interactions, and hence the approach taken in the analysis of luminescence decay data is of critical importance. The usual approach assumes that the time-dependent emission intensity, $I(t)$, arises from a small number of independent first-order decays and is described by a simple discrete component model given by $I(t) = \Sigma_i \alpha_i \exp(-t \cdot k_i)$. However, typical proteins in solutions may contain multiple lumiphores, as is often the case for the intrinsic emitter tryptophan. Each lumiphore contributes to the observed luminescence decay with a unique but frequently similar lifetime. Nonexponential decay may also characterize the luminescence of single lumiphore-containing proteins, as recent models of proteins in solution show that they form a heterogeneous mixture of interconverting structures (Chen et al., 1991; Alcala, 1994; Schlyer et al., 1994) in which the lifetime of the lumiphore can be different for each structure. Unfortunately, even in a simple two-component system, the ability to accurately resolve two similar lifetimes is limited by the presence of Poisson noise inherent in the photon-counting process.

To better detect and differentiate between different lumiphores characterized by nearly identical decay characteristics, it is necessary to incorporate a physical parameter that distinguishes the different excited states and is independent of the lifetime. One such property in a protein is the excited state chirality, which is the origin of circularly polarized luminescence (CPL) and which has been used extensively to investigate biomolecular structure (Brittain, 1985; Riehl and Richardson, 1993). CPL is complementary to circular dichroism spectroscopy, which probes the ground state and measures the difference between right and left circularly polarized light in the emission. It is characterized by the anisotropy factor

$$g_{em} = \frac{I_l - I_r}{\tfrac{1}{2}(I_l + I_r)} = \frac{4\nu}{\nu_{jk}} \frac{R_{jk}}{|\langle k|\boldsymbol{\mu}|j\rangle|^2}, \quad (1)$$

where $R_{jk} = \mathrm{Im}\{\langle j|\boldsymbol{\mu}|k\rangle\langle k|\mathbf{m}|j\rangle\}$, $\boldsymbol{\mu} = e\Sigma_i \mathbf{r}$, and $\mathbf{m} = e/2mc\Sigma_i \mathbf{r}_i \times \mathbf{p}_i$. By time resolving $g_{em}(t)$, one can in principle assign unique anisotropy factors to lumiphores with similar lifetimes according to the relationship

$$g_{em}(t) = \frac{\sum_i \alpha_i g_{em,i} e^{(-t \cdot k_i)}}{\sum_i \alpha_i e^{(-t \cdot k_i)}}, \quad (2)$$

where it is assumed that each $g_{em,i}$ is time independent for each ith lumiphore (Schauerte et al., 1992) and $k_i = 1/\tau_i$. In this paper we show that by combining luminescence decays with the time-resolved excited-state anisotropy, lumiphores with small differences in both lifetimes and optical activity characteristics can be distinguished. We extend the global analysis approach (Knutson et al., 1983), which exploits the relationship between related luminescence decays to include the time-resolved optical activity, $g_{em}(t)$. Direct association of individual luminescence lifetimes with specific anisotropy factors can be obtained through dynamic luminescence quenching. Using a model system comprising a mixture of Tb^{3+}/transferrin and Tb^{3+}/conalbumin, we demonstrate our ability

Received for publication 22 May 1995 and in final form 17 January 1996.
Address reprint requests to Dr. Duncan G. Steel, Department of Physics, University of Michigan, Randall Laboratory, Ann Arbor, MI 48109. Tel.: 313-764-4469; Fax: 313-763-9694; E-mail: dst@umich.edu.

0006-3495/96/04/1996/05 $2.00

to distinguish lumiphores that differ by as little as 7% in lifetime and have a difference in g_{em} factors of only 5×10^{-2}, a result that is further quantified by examining the enhanced curvature of the χ^2 surface.

MATERIALS AND METHODS

Experimental procedures

Apo bovine transferrin and egg conalbumin were purchased from Sigma (St. Louis, MO) and used without further purification. $TbCl_3*(6H_2O)$ was purchased from Aldrich Chemical (Milwaukee, WI). Proteins were dialyzed in 50 mM Tris:HCl 5 mM sodium bicarbonate at pH 8.5. Time-resolved luminescence decay and time-resolved circularly polarized luminescence (TRCPL) studies were done with an instrument described elsewhere (Knutson et al., 1983). Steady-state CPL measurements used the same system; however, the pulsed laser excitation was replaced with a 100-W mercury source (Oriel, Stamford, CT), and the multichannel scalers and switching logic were replaced with a Stanford Research model 530 lock-in amplifier that was phase locked to the photoelastic modulator.

The two proteins were placed in separate compartments of a tandem compartment cuvette and excited simultaneously by 280-nm light. The combined response was measured at 547.3 nm.

Luminescence decay analysis

Analysis of the luminescence decay data was based on the discrete component model using the standard Marquadt nonlinear least-squares algorithm (Bevington, 1969) with typical photon-counting statistics assumed (luminescence decay data weighted by variance, $\sigma^2 = n$, counts/channel, $g_{em}(t)$ data weighted by $\sigma^2 = 2/n$; Schauerte et al., 1992). Errors reported for lifetimes obtained from the analysis are derived from the error matrix (Bevington, 1969), which, as pointed out by Johnson and Faunt (1992), is limited in reliability, leading to an error value that can depend upon the experiment (for example, it can be due to small systematic errors in the Poisson noise). We report here averaged values for the error matrix and refer the reader to the more robust methods in error estimation (Johnson and Faunt, 1992).

Nevertheless, the experiments show that TR-CPL combined with selective luminescence quenching (lumiphores with different k_q for a quencher) readily enables differentiation between two components with nearly identical lifetimes, where a somewhat arbitrary limit in a more complex system is ultimately set by the quality of the data and of the data analysis procedures.

Additional information is derived from quenching studies that are capable of increasing the difference in the ratio of the two lifetimes (Wasylewski et al., 1988; Schauerte et al., 1994). It is easy to show that in the long time limit for a two-lumiphore system characterized by a long and short decay, that

$$g_{em}(t \rightarrow \infty) = g_l, \tag{3}$$

and at $t = 0$,

$$g_{em}(t = 0) = \frac{g_l + (\alpha_s/\alpha_l)\, g_s}{1 + \alpha_s/\alpha_l}, \tag{4}$$

where α_l and α_s are the preexponential factors for the long and short decay components, respectively, and are determined by the luminescence lifetime data and normalization. Unfortunately, it is usually difficult to obtain sufficient precision to accurately determine the asymptotic behavior. Simple linearization of a biexponential decay at times near $t = 0$ provides a description of the slope of the decay of $g_{em}(t)$ given by

$$\text{Slope} = -\frac{\alpha_s/\alpha_l}{(1 + \alpha_s/\alpha_l)^2}(g_s - g_l)(k_s - k_l). \tag{5}$$

Hence, there is adequate information in the anisotropy data to obtain values for the different $g_{em,i}$'s, given sufficient precision and lifetime differentiation by selective quenching in the luminescence decay measurement. A dynamic quenching experiment will alter the Δk, but not the α's or g's. Therefore, the improved resolution of the lifetimes and the slope of $g_{em}(t)$ in the quenched experiment can be used to extrapolate a more precise value of Δk in the unquenched experiment.

The algorithm applied to the TRCPL to improve lifetime resolution in a two-component model is straightforward. Using differential lifetime quenching (Wasylewski et al., 1988; Schauerte et al., 1994), which relies on distinct quenching rates for lumiphores with similar lifetimes, and assuming that the quenching does not affect either individual g_i values or the prefactors, time-resolved luminescence decay from the quenched system is analyzed in the two-component model to obtain the k_i's and the corresponding α_i's. Then, using Eqs. 4 and 5, values are obtained for g_s and g_l. Time-resolved luminescence data from the unperturbed system are then analyzed to obtain the average inverse lifetime, $|k$, where $|k = \alpha_s k_s + \alpha_l k_l$, subject to $\alpha_s + \alpha_l = 1$. Using the average lifetime and the slope from the TRCPL, it is then possible to obtain the lifetimes in the unperturbed system. If the lumiphores do not have different quenching susceptibilities, then uncertainty will remain in assigning unique values for the α's, k's, and g_{em} factors.

The simultaneous analysis of luminescence decays follows the ideas of Knutsen et al. (1983) and was made with the program Multi (Yamaoka et al., 1981).

RESULTS AND DISCUSSION

To demonstrate the increased resolution among decay components achieved by incorporation of optical activity data into the lifetime analysis procedure, we utilized a model protein system with well-established CPL properties. Tb^{3+} bound to both transferrin and conalbumin has a relatively large CPL signal associated with its 540–550 nm emission band corresponding to the transition of this ion. The luminescence of Tb^{3+} bound to transferrin or conalbumin in buffered aqueous solution decayed monoexponentially with lifetimes of 1250 ± 8 and 1340 ± 9 μs, respectively (their

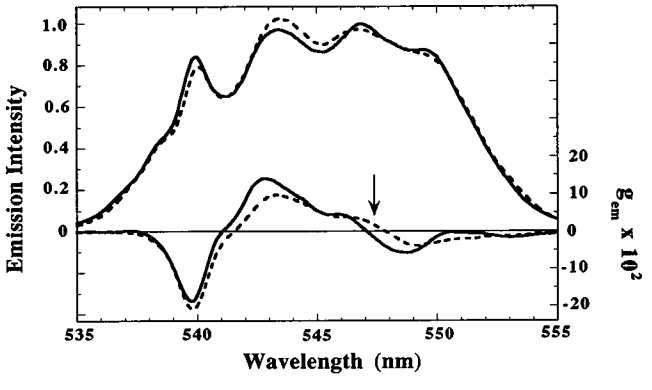

FIGURE 1 The steady state emission (*top*) and optical activity (*bottom*) of 1 equivalent of Tb3+ bound to 10 μM transferrin (– – –) and conalbumin (——) in 50 mM Tris-HCl, 5 mM sodium carbonate at pH 8.5. The proteins were excited at 280 nm. Measurements of TRCPL were performed at 547.3 nm, which is indicated by the arrow.

ratio being 1.07), whereas in a 50/50 mixture of D_2O/H_2O, the Tb^{3+}/conalbumin had a lifetime of 1550 ± 10 μs (i.e., 1.24 times longer than transferrin/Tb^{3+}/H_2O). The steady-state CPL spectra measured with a dispersion of 1.2 nm gave a g_{em} for the different Tb^{3+} sites of the two proteins of −0.0194 ± 0.0005 and +0.0300 ± 0.0005, respectively, at 547.3 nm as shown in Fig. 1. In 50 mM Tris, 5 mM sodium bicarbonate at pH 8.5, the rate of Tb^{3+} dissociation from either protein is very slow (days), and the value of g_{em} does not change during the Tb^{3+} excited-state decay, as demonstrated by independent TRCPL measurements of Tb^{3+}/transferrin and Tb^{3+}/conalbumin. Titration with D_2O did not significantly change the steady-state CPL spectrum of Tb^{3+}/conalbumin.

Fig. 2 *A* shows the time-resolved combined luminescence of aqueous transferrin, conalbumin/Tb^{3+} complexes placed in the two compartments of the tandem cuvette. Fig. 2 *B* shows the decay curve obtained in a similar experiment but with the conalbumin/Tb^{3+} complex dissolved in a 50/50 H_2O/D_2O mixture. The χ^2 surface of the data in the first case (Fig. 2 *A*) is very flat, causing great uncertainty in assigning specific lifetimes in this case, even for a simple

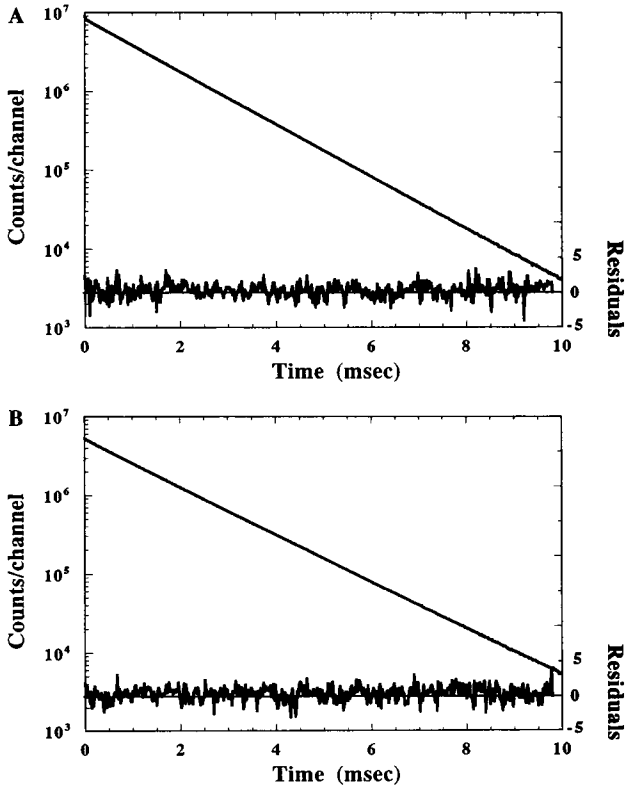

FIGURE 2 Luminescence decays and residuals of an MEM fit of Tb^{3+} bound to transferrin and conalbumin mixtures with lifetime modulated by omission or addition of D_2O. Sample concentrations were adjusted to give equal luminscence intensity to the Tb^{3+} bound to the two proteins. (*A*) With no added D_2O, intrinsic lifetimes of Tb^{3+}-bound transferrin (1250 μs) and conalbumin (1340 μs) differ by 7.2%. (*B*) With 50% D_2O, Tb^{3+} bound to conalbumin had a luminescnece lifetime of 1550 μs, giving a lifetime difference of 24%.

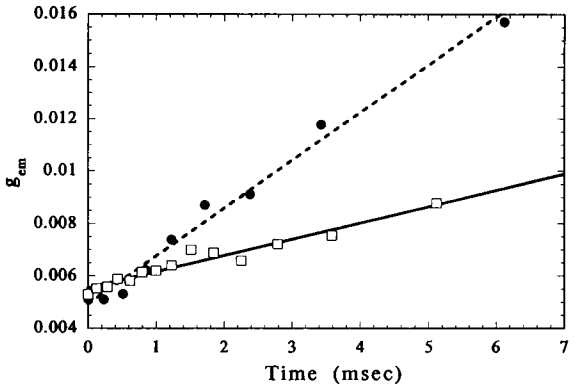

FIGURE 3 The time-resolved g_{em} of the decays is given for the 1.07 (——) and 1.24 (– – –) lifetime ratio decays. The data are summed to provide a minimum of 20 million counts for the left and right channels to provide g_{em} data points with a consistent standard error (3.2×10^{-4}). The slope and intercept for the 1.24 ratio decay are 1.91 (±0.07) s^{-1} and 4.85 × 10^{-3} (±0.15 × 10^{-3}), respectively, compared to the expected values of 1.91 and 5.3 × 10^{-3} that would be observed with the samples measured separately. The 1.07 decay yields a slope and interecept of 0.62 s^{-1} (±0.066) and 5.5 × 10^{-3} (±0.12 × 10^{-3}), respectively, compared to expected values of 0.66 s^{-1} and 5.3 × 10^{-3}.

two-component fit. However, the increased separation between lifetimes in the presence of D_2O (from 7% to 24% difference) is sufficient to form a more well-defined χ^2 surface. The corresponding lifetimes extracted from the data in this case are 1254 ± 12 μs and 1539 ± 10 μs, in excellent agreement with the lifetimes determined separately for each of the complexes. It is useful to note the results of maximum entropy method (MEM) lifetime distribution analysis of the luminescence decays reported here. MEM analysis resulted in unimodal distributions with Gaussian profiles centered at 1293 and 1422 μs (773.4 and 703.2 s^{-1}) with distribution widths 2σ of 6.2% and 15.6% for the decays with the lifetime ratios of 1.07 and 1.24, respectively. Distribution analysis is incapable of uniquely resolving closely spaced lifetimes (Siemiarczuk et al., 1990) but does provide a width to the unimodal distribution to characterize the heterogeneity of the luminescence decays. Although discrete analysis provides specific α's and *k*'s that can adequately fit the data, these values are not unique, inasmuch as the χ^2 surface has no clear minimum, because of the fact that the α's and *k*'s are highly correlated. Therefore the α's and τ's derived from discrete analysis may not necessarily be accurate. Neither discrete nor MEM analysis of closely spaced decays can provide definitive resolution without the inclusion of an additional physical observable.

The observation of a time-dependent CPL (shown in Fig. 3), however, immediately provides additional information beyond that obtained from the analysis of luminescence decay data; namely that the luminescence decay in Fig. 2 clearly is generated by at least two different emitters with different g_{em} values. To obtain additional quantitative information on this system, we now insert the above set of values for α_i and k_i, along with the slope and $t = 0$ intercept

from Fig. 3, into Eqs. 5 and 6 to obtain the value of 0.031 ± 0.002 and −0.021 ± 0.002 for g_s and g_f, respectively, again in excellent agreement with the predetermined g_{em} values for each complex given above. Returning to the unquenched data in Fig. 2, the nonlinear least-squares analysis of the data gives an average lifetime of 1290 ± 6 μs (k = 775 s^{-1}), leading to the 1331 ± 9 μs (k_s = 751.1 s^{-1}) and 1252 ± 10 μs (k_f = 798.9 s^{-1}) lifetimes for the unquenched solution. The lifetime values obtained in this way are within 2% of the ones determined independently from the individual Tb^{3+} complexes.

The improvement in the resolution of closely spaced luminescence species can be shown by describing the difference in the χ^2 surface of a nonlinear least-squares fit in the absence and presence of the constraint imposed by the quenched and time-resolved $g_{em}(t)$ data. Fig. 4 demonstrates the improvement in the uniqueness in the lowest lifetime decay component for a nonlinear least-squares biexponential fit for the unquenched data set. Curve 4 *A* shows an unconstrained fit of the data and demonstrates the lack of uniqueness of the lifetimes. The average lifetime has less than 0.1% variability, but the two lifetimes are not independently unique. Curve 4 *B* demonstrates the improved fit to the unquenched data when it is linked with the quenched data. The improved resolution of the α's and τ's for the quenched data set and the assumption that the α's are linked (global analysis; see Knutsen et al., 1983) provide the improved resolving capability in Curve 4 *B*. Curve 4 *C* includes the information of Curve 4 *B*, along with the additional information that is derived from the $g_{em}(t)$ data. Specifically, the slope of the $g_{em}(t)$ for the quenched data set is associated with an improved Δk, which in conjunction with the improved α's, provides a Δg_{em} for the quenched data set. The assumption that Δg_{em} and the α's are unchanged provides improved Δk, which provides a χ^2 restraint on the lifetime values.

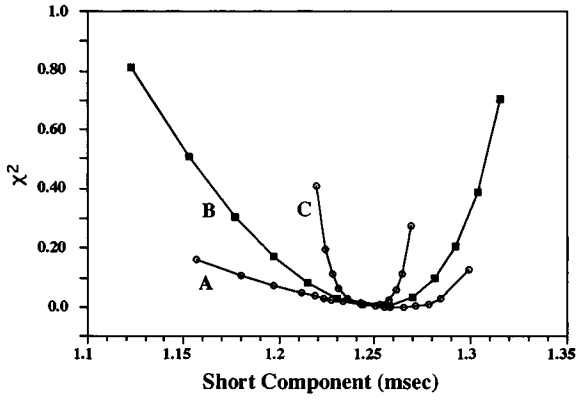

FIGURE 4 Improved discrimination of luminescence decays utilizing the time-resolved optical activity $g_{em}(t)$ is demonstrated by plotting the χ^2 surface for a biexponential fit of the 7% lifetime difference data set. The shortest lifetime in the fit is shown versus the error in the fit. (*Curve A*) Unconstrained fit of the data. (*Curve B*) 7% data set is fit simultaneously (globally) with quenched data to constrain the α's of the 7% data set. (*Curve C*) 7% data set is fit with the constraints of the quenched and $g_{em}(t)$ data (α's constrained by quench data and Δk is fixed at the value derived from the $g_{em}(t)$ data of 53.7 s^{-1}). The preexponential terms and the longer lifetime are allowed to vary to minimize the χ^2.

In summary, we have shown that TRCPL can be used to provide additional information to improve the resolution among the decay components that contribute to nonexponential decay of luminescence. This approach is expected to be particularly useful when time-resolved luminescence is used to probe lumiphores (such as tryptophans) in a large protein where luminescence decay times of each lumiphore may be similar but where the different spatial locations lead to different anisotropy factors. In addition, fluorescence-detected circular dichroism studies could benefit from this analysis in cases where two optically active lumiphores are associated with similar lifetimes. Models describing multiexponential decay may be distinguished, based upon whether the lumiphore exists in different conformations (rotamer model; Chen et al., 1991) or in a single environment but is subjected to an excited-state process (Bajzer and Prendergast, 1993). In each case, the improved resolution of the luminescence decay times leads to an ability to distinguish between models for multiexponential decays.

This research was supported by grant AG09761 from the National Institute on Aging and by grant 90-2093 from the Office of Naval Research.

REFERENCES

Alcala, J. R. 1994. The effect of harmonic conformational trajectories on protein fluorescence, and lifetime distributions. *J. Chem. Phys.* 101: 4578–4584.

Bajzer, Z., and F. G. Prendergast. 1993. A model for multiexponential tryptophan fluorescence intensity decay in proteins. *Biophys. J.* 65: 2313–2323.

Bevington, P. R. 1969. Data Reduction and Error Analysis for the Physical Sciences. McGraw-Hill, New York.

Brittain, H. G. 1985. Excited-state optical activity. *In* Molecular Luminescence Spectroscopy: Methods and Applications. S. G. Schulman, editor. Wiley, New York. 583–620.

Chen, R. F., J. R. Knutson, H. Ziffer, and D. Porter. 1991. Fluorescence of tryptophan dipeptides: Correlations with the rotamer model. *Biochemistry.* 30:5184–5195.

Johnson, M. L., and L. M. Faunt. 1992. Parameter Estimation by Least-Square Methods. *In* Numerical Computer Methods. L. Brand and M. L. Johnson, editors. Academic Press, San Diego. 1–37.

Knutson, J. R., J. M. Beechem, and L. Brand. 1983. Simultaneous analysis of multiple fluorescence decay curves: a global approach. *Chem. Phys. Lett.* 102:501–507.

Riehl, J. P., and F. S. Richardson. 1993. Circularly polarized luminescence. *In* Mettallobiochemistry. Part C. Spectroscopic and Physical Methods for Probing Metal Ion Environments in Metalloenzymes and Metalloproteins. J. F. Riordan and B. L. Vallee, editors. Academic Press, San Diego. 539–553.

Schauerte, J. A., B. D. Schlyer, D. G. Steel, and A. Gafni. 1994. Increased resolution among phosphorescence decay components of LADH by the use of quenching. *In* Time-Resolved Laser Spectroscopy in Biochemistry IV. J. R. Lakowicz, editor. *Proc. SPIE.* 2137:564–569.

Schauerte, J. A., B. D. Schlyer, D. G. Steel, and A. Gafni. 1995. Nanosecond time-resolved circular polarization of fluorescence: a study of NADH bound to horse liver alcohol dehydrogenase. *Proc. Natl. Acad. Sci. USA.* 92:569–573.

Schauerte, J. A., D. G. Steel, and A. Gafni. 1992. Time-resolved circularly polarized protein phosphorescence. *Proc. Natl. Acad. Sci. USA.* 89: 10154–10158.

Schlyer, B. D., J. A. Schauerte, D. G. Steel, and A. Gafni. 1994. Time-resolved room temperature protein phosphorescence: nonexponential decay from single emitting tryptophans. *Biophys. J.* 67:1192–1202.

Siemiarczuk, A., B. D. Wagner, and W. R. Ware. 1990. Comparison of the maximum entropy, and exponential series methods for the recovery of distributions of the lifetimes from fluorescence lifetime data. *J. Phys. Chem.* 94:1661–1666.

Wasylewski, Z., H. Koloczek, and A. Wasniowska. 1988. Fluorescence-quenching-resolved spectroscopy of proteins. *Eur. J. Biochem.* 172: 719–724.

Yamaoka, K., Y. Tanigawara, T. Nakagawa, and T. Uno. 1981. A pharmokinetic analysis program (Multi) for microcomputer. *J. Pharm. Dyn.* 4:879–885.