Protein Fluorescence Decay: Discrete Components or Distribution of Lifetimes? Really no Way Out of the Dilemma?

Armelle Vix and Hans Lami
Laboratoire de Physique, Centre de Recherches Pharmaceutiques, Université Louis Pasteur, 67401 Illkirch Cedex, France

ABSTRACT A new methodology of fluorescence decay analysis by iterative reconvolution is presented. It is based on the recent finding that the statistics of single-photon time-correlated data are best described by a compound Poisson law and requires the recording of a sample of at least 20 decays. Application of multivariate statistical methods to the analysis of the recovered decay parameters results in improved accuracy and better estimation of the uncertainties of mono- and multiexponential decays. If it is, of course, not possible to distinguish unambiguously between discrete components and a continuous distribution of lifetimes, it is, however, possible to determine a higher limit of the width of such a distribution should it be present. With our methodology, the presence of a distribution of lifetimes with a width of ~20% of its center value inevitably leads to a failure in the deconvolution procedure, a fact of crucial importance in protein conformational studies, for example.

INTRODUCTION

Considerable progress has been made recently (Siemiarczuk et al., 1990; Bajzer et al., 1990) in developing and testing the resolving power of decay-curve analysis methods. However, they are all based on the fundamental assumption of a Poisson distribution for the single-photon data. In a previous paper (Lami and Piémont, 1992), the statistical properties of single-photon time-correlated (SPTC) data, obtained with pulsed laser instruments, were critically investigated and it appears that the counts per channel (CPC) are strongly correlated, the probability law being a compound Poisson distribution. This is an intrinsic property of SPTC data-collecting instruments, as a test on the experimental setups of three other laboratories has shown (see details below). To account for this fact an iterative reconvolution procedure based on the use of the variances obtained from a sample of 30 decays was proposed. The present work is an attempt to explore its possibilities and limitations. At the core of this procedure lies the fact that the outcome of any deconvolution is a random event and should be treated accordingly. Since several, say N, decays have to be recorded to estimate the variances, why not use these data to carry out N deconvolutions and analyze statistically the N sets of decay parameters obtained? As we will see below this first permits the determination of the confidence intervals of the decay parameters and, more importantly, allows the unambiguous detection of a continuous distribution of lifetimes whose width amounts to 20% of its center value. The existence of such distributions in protein fluorescence decay is presently a much debated point. Distribution of lifetimes, indicative of conformational freedom, has been suggested (Bajzer and Prendergast, 1993) but never clearly proven.

The outline of this article is the following. After describing our equipment and methods, we discuss the major problem of deconvolution based on the χ^2 test, which is the correct estimation of the weighting factors, i.e., the variances. Then, a statistical method for determining the confidence intervals of the decay parameters from a sample of 20 decays is described, and its resolving power on multicomponent decays evaluated. Finally, its discriminating power between discrete components and a distribution of lifetimes is tested. In this study we exclusively used data obtained by adding recorded simple decay curves of known decay time and amplitude, because the usual simulated data lack the compound Poisson law fluctuations and are therefore useless.

MATERIALS AND METHODS

The fluorescence lifetimes were measured by the SPTC method using a rhodamine 6G dye laser as excitation source. The main part of the experimental setup has been fully described in Lami and Piémont (1992). The detection part was modified in the following manner: a microchannel plate photomultiplier tube (PMT) R3809U (Hamamatsu, Hamamatsu City, Japan) coupled to a model 6954 pulse pre-amplifier (voltage gain, 50; bandwidth, 1.8 GHz; Phillips, Mahwah, NJ) delivered the single-photon pulses. Both start and stop signals were obtained with a fast discriminator (model 454, Tennelec, Oak Ridge, TN) operating in constant-fraction mode. The instrumental response function (IRF) was recorded with a polished aluminum reflector. Its full width at half maximum (FWHM) is 50 ps. During the deconvolution procedure the IRF was allowed to time-shift relative to the decay curve. As a comparison between the position of the maximum of the IRF obtained with the reflector and with the Raman effect on water revealed, this shift is mainly due to a color effect of the PMT and/or the monochromator and amounts to a shift of ~0.8 ps/nm in our case. The main characteristics of the instrument and the measurement procedure were the following: excitation pulse width, ≤2 ps (measured with an autocorrelator); repetition rate, 800 KHz; excitation wavelength, 289 nm; maximum data accumulation rate, 5 KHz, because higher rates create interference effects in the Tennelec discriminator or the PMT; channel widths, 26.5, 53, or 106 ps; number of channels, 512 or 1024. The background was negligible, <1 CPC. Care must be taken when the channel width exceeds the FWHM of the IRF: a systematic error arising from the quantification of the IRF leads

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Address reprint requests to Dr. Hans Lami, Laboratoire de Physique, Centre de Recherches Pharmaceutiques, URA 491 CNRS, Université Louis Pasteur, BP 24, 67401 Illkirch Cedex, France. Tel.: 33-88-676900; Fax: 33-88-674011; E-mail: h1@phypha2.u-strasbg.fr.

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to the appearance of a purely artifactual ultrashort, low-weight (decay time, several ps; weight, ≈1%) component among the recovered parameters.

During the last part of this work we used a Ti:sapphire (Tsunami, Spectra-Physics, Mountain View, CA) laser as the excitation source and a fast Hamamatsu photodiode (type S 5533) to detect the start signal. The FWHM of the IRF dropped then to ~40 ps.

The compound Poisson law was also found to describe the fluctuations of the SPTC data stemming from three other experimental setups, whose main characteristics are the following.

1) Excitation source: Ti:sapphire laser (Tsunami, Spectra-Physics); detector: Hamamatsu microchannel plate photomultiplier; use: protein fluorescence studies; location: J. C. Brochon, Groupe de Biofluorescence, URA 1131, CNRS, Orsay, France.

2) Excitation source: nuclear particles; detector: XP2020 photomultiplier; use: positron lifetime spectroscopy; location: G. Duplatre, Chimie Nucléaire, Centre de Recherches Nucléaires IN2P3-CNRS, Strasbourg-Cronenbourg, France.

3) Excitation source: Rd 6G dye laser (Spectra-Physics); detector: XP2020 photomultiplier; use: photophysical studies; location: J. A. Miehe and F. Heisel, Groupe d'Optique Appliquée, Centre de Recherches Nucléaires, IN2P3-CNRS, Strasbourg-Cronenbourg, France.

The fluorescent samples were aerated or degassed (freeze-thaw cycles technique) solutions of *p*-terphenyl (PT); 2,5-diphenyloxazole (PPO); 2,5-diphenyloxadiazole (PPD); 1-naphthtyl 2-phenyloxazole (α-NPO) (all from Nuclear Enterprises, Edinburgh, U.K.) indazole (IAZ) (Aldrich, Beerse, Belgium) indole (IND) and 2,3-dimethylindole (DMI) (vacuum sublimated; Sigma Chemical Co., St. Louis, MO) in cyclohexane, pentane, ethanol (all MERCK, UVASOL, Darmstadt, Germany) or ultrapure water (Millipore Q, St-Quentin, France) of ~0.1 optical density.

During the measurements on IAZ and DMI solutions, the temperature of the optical cell was controlled within ±0.05°C by a circulating water bath (Haake F3, Karlsruhe, Germany).

The iterative reconvolution was performed using a Marquardt nonlinear least-squares procedure, the integration of the convolution integral being carried out according to the trapezoidal rule. For details see Lami and Piémont (1992).

The normality of the weighted residuals appearing in the χ_r^2 procedure was examined using the Shapiro-Wilk test (SAS Instutute, UNIVARIATE procedure). A value of $W \simeq 0.9$ was usually found when the fitting procedure was successful, a lower value ($W \simeq 0.8$) in the opposite case. The histogram and the normal probability plot of the weighted residuals resulting from a successful fitting of a biexponential decay are displayed in Fig. 1. Clearly the distribution is only approximately normal.

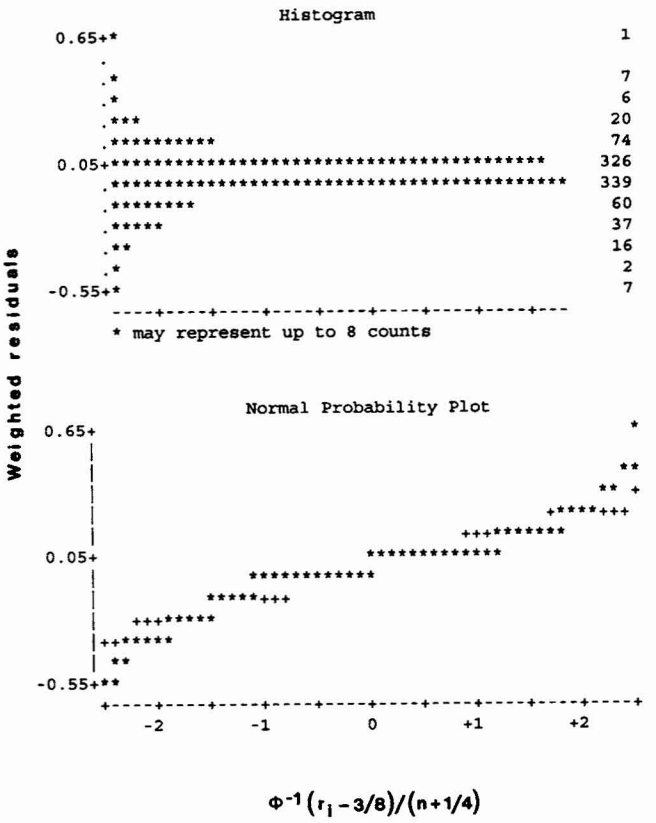

FIGURE 1 Histogram and normal probability plot of the weighted residuals obtained when successfully fitting a biexponential decay. Asterisks mark the data values, the plus signs provide a reference straight line that is drawn using the sample mean and standard deviation. Φ^{-1} represents the inverse of the standard normal distribution function, r_i and n the rank and number of data values, respectively.

ESTIMATION OF THE χ^2 WEIGHTING FACTORS

In the conventional iterative deconvolution procedure a Poisson noise is assumed, and the weighting factors appearing in the χ^2 test are the inverse of the theoretical number of counts in the considered channel. A previous investigation has shown that this assumption is not valid (Lami and Piémont, 1992). In fact, the CPCs are strongly correlated for a number of channels mostly situated in the rising part of the decay curve, and consequently the variances largely exceed the CPCs in this domain (see Fig. 2, *a* and *b*). If we take into account the correlation between the CPCs, the χ^2 expression takes the following form: let the *p*-dimensional line vector

$$\mathbf{x}^e = [\mathbf{x}_1^e, \cdots, \mathbf{x}_p^e]'$$

represent the CPCs of a decay curve measurement stored in p channels and $\mathbf{x}^t$ the vector of the corresponding theoretical values. Then

$$\chi^2 = (\mathbf{x}^e - \mathbf{x}^t)'\mathbf{S}^{-1}(\mathbf{x}^e - \mathbf{x}^t)$$

where **S** represents the sample covariance matrix. Now **S** is always singular, which impedes its inversion and hence its use in the χ^2 calculation. Use of the generalized inverse only leads to aberrant results. To overcome this problem we proposed (Lami and Piémont, 1992) to use the inverses of the diagonal elements of **S** as weighting factors in the χ^2, and this worked rather well with our old experimental setup based on the use of a Philips XP 2020-Q PMT (FWHM of the response function: ≈700 ps). Since the new experimental setup displays a much better time resolution, the so-estimated variances depend strongly on the sample size, as can be seen in Fig. 2 *b*. Hence, it was not surprising to discover that the reduced χ^2 values (χ_r^2) obtained with the variances derived from a sample of 20 decays always remained greater than 1.2 for monoexponential decays. The variances obviously had been underestimated, so the diagonal elements of **S** were multiplied by a corrective factor f_c in order to achieve χ_r^2 values in the range $1 \pm 3 \cdot \sigma$, (σ being the standard deviation of the χ_r^2 law), for a series of 20 monoexponential decays of a standard fluorophore. The standard deviation σ is given by the relation

$$\sigma = (2/\nu)^{1/2}$$

where ν represents the number of degrees of freedom, i.e.,

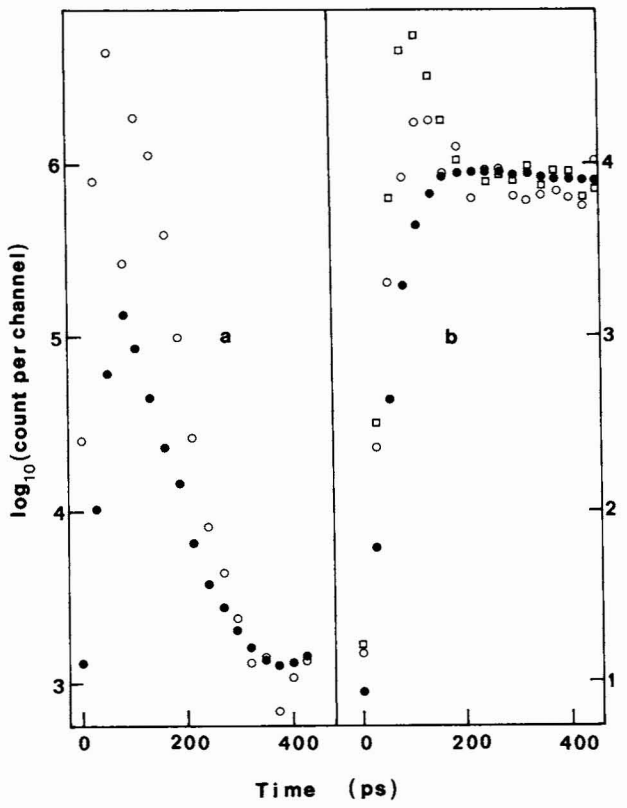

FIGURE 2 (*a*) (●) Instrumental response function obtained at 289 nm with an A1 reflector. (○). Variances calculated from a sample of 20 measurements. (*b*) (●). Fluorescence decay of an aerated solution of PT in cyclohexane. (○, □). Variances calculated from samples of 20 or 200 decays, respectively. Channel width, 25.6 ps.

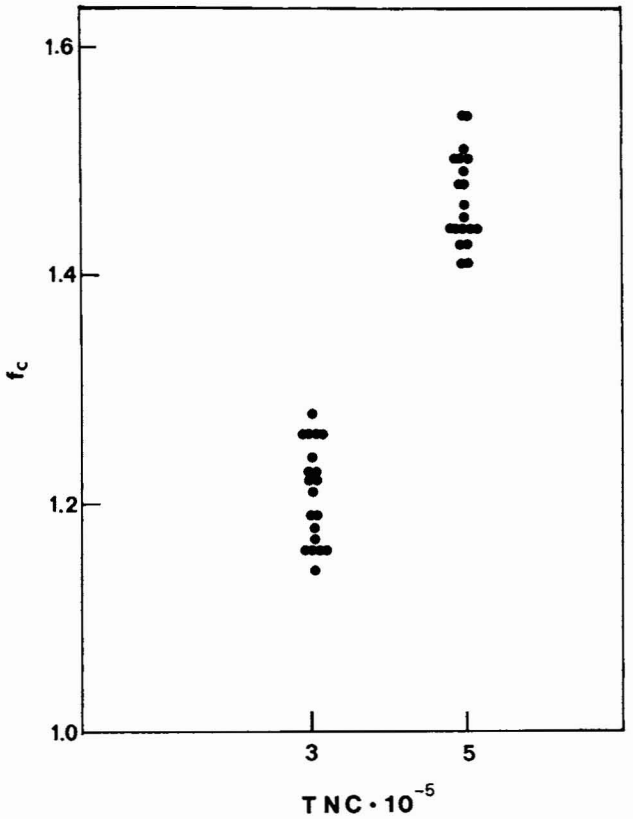

FIGURE 3 Values of the corrective factor f_c vs. *TNC*. The f_c values were obtained from 20 samples of 20 monoexponential decays of a standard fluorophore (IAZ), chosen at random from a recording of 100 decays.

the number of channels minus the number of decay parameters. The value of f_c was found to be independent of the nature of the fluorophore but slightly dependent on the total number of counts, TNC, according to the approximate linear relation

$$f_c = 1 + \beta \cdot \text{TNC}$$

where β represents a random variable. During an experiment the value of f_c fluctuates with a standard deviation of 3.5% of the mean value as illustrated in Fig. 3, which displays the f_c values obtained from 20 samples of 20 monoexponential decays of a standard fluorophore (IAZ) chosen at random from a recording of 100 decays. Fortunately, this slight uncertainty in the value to be adopted during the deconvolution procedure has only negligible consequences on its outcome. More serious are the fluctuations of the f_c value on a day-to-day basis: variations of 25% may easily be observed. To eliminate this source of error, before each use of the instrumental setup f_c was carefully determined with a monoexponential decaying fluorophore. Only if this last step is respected can reproducible results be obtained.

The abovementioned formula simply reflects the inability of the sample covariance matrix to estimate correctly the increase of the variances with the *TNC*, a characteristic property of the compound Poisson law (Feller, 1943). Underlying this corrective procedure is the implicit assumption that the shape of the variance-versus-channel curve as given by the diagonal elements of **S** is correct, whereas the magnitude of the variances is underestimated. It should be noted that any instability in the instrument has two immediate effects: a broadening of the response function and, what at first sight seems paradoxical, a better estimate of the variances, i.e., the corrective factor f_c drops to 1. The f_c factor is also very sensitive to the presence of scattered excitation light, originating from the Raman effect of the solvent, e.g. The value of f_c immediately drops to <1. Because an IRF with its steep rising and falling parts is added to the decay curve, the variances are heavily increased in the concerned channels, thus leading to an underestimation of the χ^2 weighting factors. One should also be aware of the fact that problems due to multiple reflections between the fluorescence source and the detector, or with the electronic equipment, can easily be "corrected" by manipulating the value of f_c. Of course in doing this no correct conclusions can be drawn from the accumulated data and therefore this should be strictly avoided. Finally, it is of some interest to note that the value of f_c is independent of the sample size, i.e., the number N of recorded decays, for $N >$ 7–8 in the case of a single-exponential decay, and for $N > 15$ in the case of a multiexponential decay. Operating below these N values leads to a rapid decrease of the number of accepted χ_r^2 values.

CONFIDENCE INTERVALS FOR THE DECAY PARAMETERS

So far the 20 decays of a sample have been used to estimate the weighting factors of the χ^2 test but they can also serve to estimate the confidence intervals of the retrieved decay parameters. To do this, let us consider the decay parameters, i.e., the decay times τ_i and pre-exponential terms α_i, recovered from a given measurement as a p-dimensional random variable $\mathbf{x}$ defined as a row vector:

$$\mathbf{x}' = [\tau_i \cdots \tau_{p/2}, \alpha_1 \cdots \alpha_{p/2}] = [\mathbf{x}_i \cdots \mathbf{x}_p]$$

whose elements are continuous unidimensional random variables. To a very good approximation, they are correlated random variables, a property that has been proven by a test on a sample of a 100 monoexponential decays measured with a degassed solution of PT in ethanol. The Shapiro-Wilk test of normality yielded $W = 0.962$ for τ_1 and $W = 0.974$ for α_1, whereas the correlation coefficient between τ_1 and α_1 was found to be -0.978 (results obtained with the SAS UNIVARIATE and CORR procedures).

Within this formalism the estimates of the true values of the decay parameters (μ_i) are the sample means ($\bar{x}_i$), the confidence intervals are given by the inequality (Morrison, 1967):

$$\bar{x}_i - \sqrt{1/N \cdot S_{ii}}T_{\alpha;p,N-p} \le \mu_i \le \bar{x}_i + \sqrt{1/N \cdot S_{ii}}T_{\alpha;p,N-p}$$

where S_{ii} represents the ith diagonal element of the sample covariance matrix defined as

$$\mathbf{S} = 1/(N-1) \sum_{h=1}^{N} (x_h - \bar{x}) \cdot (x_h - \bar{x})'$$

and T^2, Hotelling's statistic, with

$$T^2_{\alpha;p,N-p} = (N-1)p/(N-p) \cdot F_{\alpha;p,N-p} \qquad (1)$$

where $F_{\alpha;p,N-p}$ denotes the 100-α percentage point of the F distribution. In this way we obtain much better estimates of the decay parameters and, most importantly, we take fully into account the uncertainty of a measurement.

To complete the procedure we only retained those results of the N deconvolutions that yielded χ^2_r values of $1 \pm 3 \cdot \sigma$. Normally all N deconvolution results are retained, but in some multicomponent decays this was not the case and the confidence intervals increased accordingly as can be easily deduced from Eq. 1.

Finally, the whole deconvolution procedure described above was tested by recording the decays for a number of simple monoexponential fluorophores under the following conditions: sample size $N = 20$, TNC $= 4 \cdot 10^5$, recording of the IRF with the same TNC, data accumulation rate 5 kHz.

The results are displayed in Table 1. The confidence intervals at the 5% level are quite small, but this is rather misleading given that a dispersion of 1% is actually observed on a day-to-day basis. We then proceeded to test the method on multicomponent decays, which were generated by accumulating the different decays separately and then adding the corresponding CPCs to a TNC of $4 \cdot 10^5$ for two components and $6 \cdot 10^5$ for three components. This procedure avoids all problems arising from energy transfer between the different chromophores while retaining the compound Poisson law structure of the statistical fluctuations. The results displayed in Table 2 reveal that two components of the same weight can still be resolved if the ratio τ_1/τ_2 exceeds 1.35, but it should be noted that only 7 out of 20 deconvolutions yielded an acceptable χ^2_r value. Moreover, the component fractions are poorly recovered, the corresponding confidence intervals remaining misleadingly small. Such a poor score (7/20) always indicates, as we will see below, the presence of components with close-lying decay times. For a three-component decay the method is still reasonably accurate with an average error in recovered parameters of between 5 and 10%, the decay time ratios being ≥ 1.9. Since the confidence intervals are rather large, indicating a great dispersion of the results, it is illusory to use the data from an individual measurement to obtain a reliable result. Recording of a sample of N decays must be the rule rather than the exception for studies of complex photophysical or biological systems where multiexponential decays are commonplace.

TABLE 1 Monoexponential decays

No.	Solute	Solvent	Fluorescence lifetime (ps)
1	TP*	Cyclohexane	985 ± 2.3
2	TP‡	"	926 ± 1.1
3	PPD*	"	1107 ± 2.7
4	PPO*	"	1323 ± 2.3
5	α-NPO‡	"	1747 ± 2.6
6	IND*	Pentane	7697 ± 30

*Deaerated.
‡Aerated.

TABLE 2 Multiexponential decays generated from decays in Table 1

Decay numbers		Decay parameters* True	Decay parameters* Recovered	Relative error of mean values %	Number of successful deconvolutions
1+4	τ_1	985	1014 ± 65	2.9	7/20
	τ_2	1323	1363 ± 72	3.0	
	f_1	50	60.9 ± 0.5	21.8	
	f_2	50	39.1 ± 8.8	−21.8	
2+5	τ_1	926	898 ± 57	−3.0	20/20
	τ_2	1747	1715 ± 87.8	−1.8	
	f_1	50	46 ± 5.3	−8.0	
	f_2	50	54 ± 10.7	8.0	
1+6	τ_1	985	960 ± 24.9	−2.5	20/20
	τ_2	7697	7945 ± 291	3.2	
	f_1	50	49.3 ± 0.3	−1.4	
	f_2	50	50.7 ± 2.1	1.4	
2+5+6	τ_1	926	827 ± 147	10.7	20/20
	τ_2	1747	1672 ± 360	−4.3	
	τ_3	7697	8144 ± 868	5.8	
	f_1	33.3	24 ± 10	−28.0	
	f_2	33.3	42 ± 20	26.0	
	f_3	33.3	34 ± 5.7	2.0	

*f: decay-component fraction in %; τ: fluorescence lifetime in ps.

CONTINUOUS DISTRIBUTIONS OF LIFETIMES

Continuous distributions of lifetime can be easily simulated if one disposes of a series of single exponential decay curves with close-lying lifetimes. During this work, we used to this purpose the single exponential decays of an aqueous aerated solution of IAZ measured at different temperatures. In this manner, as can be seen in Fig. 4, the lifetime can be varied quasicontinuously between 3.37 and 2.34 ns. To obtain still larger differences in lifetimes, we also used an aqueous aerated solution of DMI at 18°C (τ = 4.54 ns) and 30°C (τ = 2.60 ns) or of IND at 21°C (τ = 4.29 ns). We then performed a file summation to a *TNC* of $5 \cdot 10^5$ of two monoexponential decays with the same weight and lifetimes τ_1 and τ_2, and tried to deconvolute the data successively using a one- or two-exponential function. The results are plotted in Fig. 5 as the number of "accepted" deconvolutions versus the factor $d = (\tau_1 - \tau_2)/\tau_1$, τ_1 being the longest lifetime. Obviously, a d value of ~0.4 ($\tau_1/\tau_2 \simeq 1.7$) is necessary to obtain full success (20/20 acceptable χ_r^2 values) with a two-exponential function and no success with a one-exponential function. Similar results were already obtained by Ware and coworkers (Siemiarczuk et al., 1990) with the maximum entropy method (MEM). For values of d between 0.15 and 0.4 the number of successful deconvolutions with one- or two-exponential functions sharply decreases. Finally, if $d \leq 0.15$ the two components could no longer be distinguished because a one-exponential function always yielded a 20/20 score.

A similar behavior was found in the case of discrete distributions of 10 equally spaced lifetimes with amplitudes determined by the functional forms displayed in Fig. 6: uniform, single gaussian, or double gaussian. The distributions were obtained by file summation of 10 monoexponential decays of IAZ solutions measured at different temperatures. Table 3 displays the results obtained with two series of distributions denoted A and B and characterized by maximum

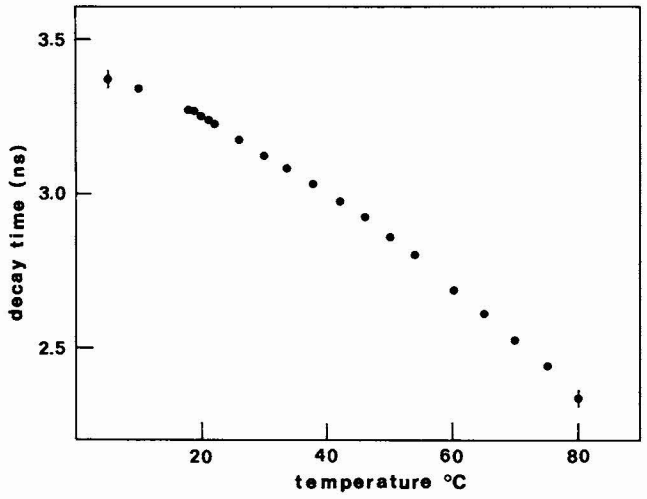

FIGURE 4 Fluorescence decay time of a solution of IAZ in aerated water, vs. temperature.

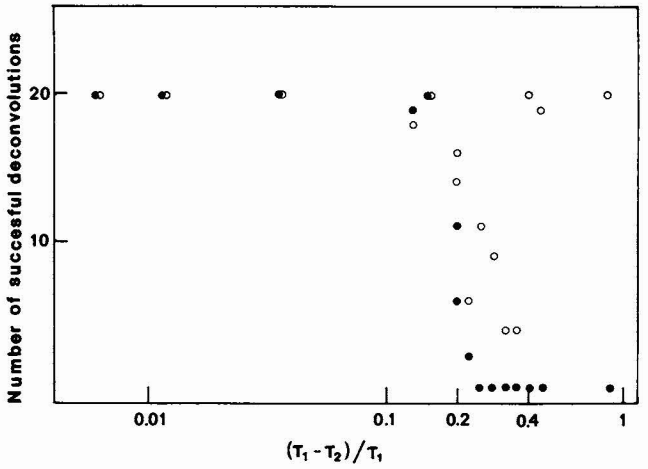

FIGURE 5 Deconvolution of a biexponential decay. Number of successful deconvolutions vs. $(\tau_1 - \tau_2)/\tau_1$ using: (●) a one-exponential function and (○) a two-exponential function.

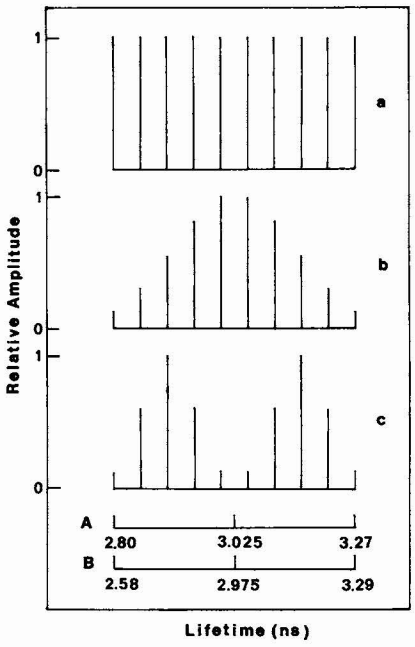

FIGURE 6 Functional form of the distributions. (*a*) uniform, (*b*) single gaussian, (*c*) double gaussian.

width/center ratios (R) of 0.155 and 0.238, respectively. While the A-type distributions can always be fitted with a single exponential function, for the B-type distributions this was never the case. Using multiexponential functions (up to 10 components) or increasing the number of decays used to obtain the distributions (up to 19) does not significantly modify the results.

We therefore conclude that the fluorescence decay analysis by iterative reconvolution would certainly fail in the case of real continuous distribution of lifetimes with $R \geq 0.2$, at least for distributions with center values near 3 ns. Of course, if such a failure occurs the use of better adapted methods such as the MEM (Livesey and Brochon, 1987)

TABLE 3 Number of successful deconvolutions of type A or B distributions using a multiexponential function

Functional form of the distribution	Type	One component	Two components	Three components
uniform	A	20/20	20/20	20/20
	B	0/20	0/20	0/20
single gaussian	A	20/20	20/20	16/20
	B	14/20	15/20	14/20
double gaussian	A	20/20	20/20	20/20
	B	17/20	18/20	17/20

or the exponential series method developed by Ware and coworkers (Siemiarczuk et al., 1990) is then fully recommended. More precisely, since the MEM and exponential series methods invariably work and supply decay parameters, they should be used only after failure of the iterative reconvolution procedure or if serious photophysical arguments for the existence of such lifetime distributions are at hand. In no case should their successful use be interpreted as a proof of the existence of a continuous distribution of lifetimes, as all too often happens. Conversely, if discrete exponential functions can be successfully fitted, this simply means that either they really exist or that the R values of the corresponding distributions do not exceed 0.2.

To summarize, when fitting a decay with a one-exponential function, a score of 20/20 indicates the presence of either a single component or a distribution of lifetimes with $R \leq 0.2$, a score between 0/20 and 20/20, the presence of either two close-lying discrete components with $0.15 \leq d \leq 0.4$, or a distribution of lifetimes with $R > 0.2$. If a score of 0/20 is obtained a two-exponential function should, of course, be tried before concluding.

CONCLUSIONS

The purpose of this paper was to present a new methodology for fluorescence decay analysis by iterative reconvolution based on a new insight in the statistics of SPTC data. In a recent publication (Lami and Piémont, 1992) we showed that the probability law of the CPC is a compound Poisson distribution. The χ^2_r weighting factors, i.e., the inverses of the variances, are no longer the inverses of the CPCs, as is the case for the Poisson law, but must be estimated from a sample of N decays. For a multiexponential decay a reasonable value is $N = 20$, although for a pure single-exponential decay, taking $N = 7$–8 already yields reliable results. For practical reasons N is always much less than the number of channels and therefore the efficiency of the estimation is very poor, i.e., in most cases the variances are underestimated. This means that they have to be multiplied by a corrective factor, the value of which depends slightly on *TNC*. Of course this factor must be carefully determined for each instrument and before each decay-time measurement with a monoexponential decaying fluorophore as a standard. In a very simple way it is then possible, applying Hotelling's statistic to the sample of N recorded decays, to improve the accuracy and at the same time better estimate the uncertainty of the recovered decay parameters and also detect with high sensitivity the presence of a distribution of lifetimes whose maximum width/center ratio exceeds 0.2. It is worth mentioning that among the various single-tryptophan residue proteins studied so far in our laboratory (mucus proteinase inhibitor (Faller et al., 1992), annexin V (C. Pigault and A. Wund, personal communication), Moloney murine leukemia virus nucleocapsid protein (Mely et al., 1993a), human immunodeficiency virus type I nucleocapsid protein NCp7 (Mely et al., 1993b)), not the slightest indication of fluorescence with a distribution of lifetimes with a maximum width/center ratio greater than 0.2 could be detected.

Making use of Ockham's Razor principle (Garrett, 1991), which states that the simplest theory to fit noisy data should be preferred, we therefore favor discrete components over distributions for protein fluorescence decay till proof of the opposite is given. Nevertheless, should such distributions be present for the abovementioned proteins, their width would not exceed 1.4 ns for the longest lifetime ($\tau \simeq 7$ ns) observed. According to a recent theoretical model describing the effect of harmonic conformational trajectories on protein fluorescence (Alcala, 1994), this narrow width of the distribution would then either mean that the harmonic conformational fluctuation period of the protein must be comparable to the fluorescence lifetime or that the variation of the tryptophan residue excited state lifetime, throughout the harmonic trajectory, is small.

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