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Application of a reference convolution method to tryptophan fluorescence in proteins

A refined description of rotational dynamics

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A reference method for the deconvolution of polarized fluorescence decay data is described. Fluorescence lifetime determinations for *p*-terphenyl, *p*-bis[2-(5-phenyloxazolyl)]benzene and *N*-acetyltryptophanamide ($AcTrpNH_2$) show that with this method more reliable fits of the decays can be made than with the scatterer method, which is most frequently used. Analysis of the $AcTrpNH_2$ decay with *p*-terphenyl as the reference compound yields an excellent fit with lifetimes of 2.985 ns for $AcTrpNH_2$ and 1.099 ns for *p*-terphenyl (20 °C), whereas the $AcTrpNH_2$ decay cannot be satisfactorily fitted when the scatterer method is used. The frequency of the detected photons is varied to determine the conditions where pulse pile-up starts to affect the measured decays. At detection frequencies of 5 kHz and 15 kHz, which corresponds to 1.7% and 5% respectively of the rate of the excitation photons no effects are found. Decays measured at 30 kHz (10%) are distorted, indicating that pile-up effects play a role at this frequency.

The fluorescence and fluorescence anisotropy decays of the tryptophan residues in the proteins human serum albumin, horse liver alcohol dehydrogenase and lysozyme have been reanalysed with the reference method. The single tryptophan residue of the albumin is shown to be characterized by a triple-exponential fluorescence decay. The anisotropy decay of albumin was found to be mono-exponential with a rotational correlation time of 26 ns (20 °C). The alcohol dehydrogenase has two different tryptophan residues to which single lifetimes are assigned. It is found that the rotational correlation time for the dehydrogenase changes with excitation wavelength (33 ns for λ_{ex} = 295 nm and 36 ns for λ_{ex} = 300 nm at 20 °C), indicating a nonspherical protein molecule. Lysozyme has six tryptophan residues, which give rise to a triple-exponential fluorescence decay. A single-exponential decay with a rotational correlation time of 3.8 ns is found for the anisotropy. This correlation time is significantly shorter than that arising from the overall rotation and probably originates from intramolecular, segmental motion.

Time-resolved fluorescence measurements of intrinsic probes in proteins can be a powerful tool to obtain information about the molecular structure and dynamic processes taking place on the fluorescence time scale, like rotation of the whole molecule or segments of it and interactions with other molecules, present in the solution [1, 2]. One class of intrinsic probes is formed by the tryptophan residues in proteins [3].

Interpretation of tryptophan fluorescence decay is a complex matter. In many cases, multiple exponential decays are found for proteins containing a single tryptophan [4]. This anomalous behaviour has been explained by the existence of three different $\alpha-\beta$ rotamers of tryptophan. According to the so-called 'modified conformer model' (MCM) [5–7] intramolecular charge transfer from the excited indole ring to electrophilic substituents on the 3-ethyl chain can occur. In each of the three $\alpha-\beta$ rotamers the distance and orientation of the indole ring relative to these substituents can be different, leading to different amounts of intramolecular charge transfer and thus to different lifetimes. Another complicating factor is that the light absorption of indole in the near-ultraviolet region involves two overlapping transitions, ^1L_a and ^1L_b [8, 9]. The relative contribution of these two transitions to the fluorescence is dependent on the excitation and emission wavelengths. Emission at 345 nm originates solely from the ^1L_a state, but excitation at 295 nm or 300 nm elicits both the ^1L_a and ^1L_b transitions [9]. Energy transfer between the two states can occur very efficiently [10, 11] and therefore the observed fluorescence is partially the result of this process. This explains why the anisotropy of the fluorescence at zero time never reaches the theoretical value of 0.4 even in case of immobilized fluorophores [12]. Changing the excitation wavelength from 295 nm to 300 nm gives a decrease of the contribution of the ^1L_b transition and thus an increase of the initial anisotropy.

At present, the most frequently used method for the time-resolved fluorescence measurements, is time-correlated single-photon counting [13]. The observed fluorescence, $S(t)$, is then a convolution of the delta pulse response, $s(t)$, of the sample, with the impulse response profile, $P(t)$. Once $P(t)$ is known, the decay parameters of the fluorescence can be obtained from $S(t)$ by a deconvolution procedure. A method to measure $P(t)$ is to use a scatterer, but this has the serious drawback that the measurements of $P(t)$ and $S(t)$ occur at different wavelengths and since most photomultipliers show some wavelength-dependent time dependence [14, 15] the $P(t)$

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Abbreviations. $AcTrpNH_2$, *N*-acetyl-tryptophanamide; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; HSA, human serum albumin; DW, Durbin-Watson parameter; LADH, horse-liver alcohol dehydrogenase; MCA, multichannel analyzer.

Enzymes. Horse liver alcohol dehydrogenase (EC 1.1.1.1); lysozyme (EC 3.2.1.17).

obtained is not the actual profile. To overcome this problem Zuker et al. [16] have recently developed a method in which the real $P(t)$ is obtained via the exponential fluorescence of a reference compound, which emits at the same wavelength as the sample under consideration. We have applied this method in a study of the tryptophan fluorescence and anisotropy decays of the proteins human serum albumin (HSA), horse liver alcohol dehydrogenase (LADH) and lysozyme, using *p*-terphenyl as reference compound and we have compared the results obtained with the 'reference method' to the ones obtained with the 'scatterer method'. Refinement of the procedure leads to a more profound interpretation of the experimental results and to new insights into the type of motions of these proteins.

MATERIALS AND METHODS

Chemicals and enzymes

(*p*-Terphenyl (scintillation grade) obtained from BDH and *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP, scintillation grade) obtained from Eastman Kodak, were dissolved in ethanol (Merck, fluorescent grade). AcTrpNH$_2$ (*N*-acetyl-L-tryptophanamide) purchased from Sigma was dissolved in 50 mM pH 7.0 potassium phosphate buffer. Oxygen quenching was prevented by bubbling the samples with oxygen-free argon. Defatted monomeric human serum albumin (HSA) was prepared as described for bovine serum albumin [17]. HSA (Sigma) was dissolved in water; charcoal was added and the pH of the solution was adjusted to 3.0 with 1 M HCl. After 2 h of stirring at room temperature, the charcoal was removed by centrifugation and the supernatant was adjusted to pH 8.0 with 1 M NaOH. Subsequently the supernatant was applied to a column (2.0 × 30 cm) of Sephadex G-150 (Pharmacia) equilibrated with 50 mM Tris, 0.2 M NaCl pH 8.0; 1-ml fractions were collected and only the last protein-containing fraction (monomer) was used for the experiments. LADH and lysozyme were obtained from Boehringer, LADH as a crystaline suspension in 20 mM potassium phosphate, 10% ethanol pH 7.0. 1 ml of the suspension was dialysed against 20 mM potassium phosphate pH 7.0 buffer for 24 h. Afterwards the undissolved material was removed by centrifugation. Lysozyme, which was supplied in a dry crystalline form, was dissolved in the same phosphate buffer and also dialysed for 24 h. All solutions were prepared with an $A_{295\,nm} = 0.1$, except the POPOP solution, which had an $A_{295\,nm}$ of 0.05. All experiments were done at 20 °C, unless otherwise indicated.

Experimental setup

The experimental setup for the polarized fluorescence decay measurements has been described previously [18, 19]. Some changes in the detection system were made to increase the sensitivity. A sheet of Polaroid (type HNP'B linear polarizer for ultraviolet radiation) was used to separate the parallel and the perpendicular components of the fluorescence. Every 10 s (controlled by the timer of the multichannel analyzer) this polarizer was rotated 90° from the original position in 0.3 s and the different components were stored in two different subgroups of the MCA (1024 channels). Typically this procedure was repeated 10 times. The detection wavelength was selected with interference filters (337, 345, 350 and 437 nm, 10-nm bandpass). The digital output of the MCA was sent to a Micro VAX II computer, where the data were evaluated.

DATA ANALYSIS

Fluorescence

The parallel, $I_{\parallel}(t)$, and the perpendicular, $I_{\perp}(t)$, polarized components of the fluorescence are measured. From these components the total fluorescence can be calculated:

$$S(t) = I_{\parallel}(t) + 2I_{\perp}(t) . \tag{1}$$

$S(t)$ is a convolution of the actual fluorescence, $s(t)$, with the impulse response profile, $P(t)$:

$$S(t) = P(t) * s(t) = \int_0^t P(t')\, s(t-t')\mathrm{d}t' . \tag{2}$$

This discussion will be restricted to (sum of) exponential decays, where:

$$s(t) = \sum_i \alpha_i \exp(-t/\tau_i) . \tag{3}$$

The τ_i's are the fluorescence lifetimes and the α_i's are a measure for the contribution of the different lifetimes to the total fluorescence decay. The fluorescence parameters α_i and τ_i must be determined from $S(t)$. The most popular among the different methods is that of least-squares fitting. In this procedure the weighted sum of squared residuals, WSSR, is minimized.

$$\mathrm{WSSR} = \sum_{k=1}^{n} w_k [S(t_k) - S_c(t_k)]^2 . \tag{4}$$

$S_c(t)$ is the calculated fit of $S(t)$, w_k is the weighting factor of data point $S(t_k)$ and n is the total number of data points. The weighting factors are given by the inverse of the variance of $S(t_k)$ [20–22].

$$w_k = 1.0/\mathrm{var}[S(t_k)] . \tag{5}$$

$$\mathrm{var}[S(t_k)] = \mathrm{var}[I_{\parallel}(t_k)] + 4\,\mathrm{var}[I_{\perp}(t_k)] . \tag{6}$$

For single-photon counting data, Poissonian noise is assumed so the variances of $I_{\parallel}(t_k)$ and $I_{\perp}(t_k)$ can be estimated as follows:

$$\mathrm{var}[I_{\parallel}(t_k)] = I_{\parallel}(t_k) . \tag{7a}$$

$$\mathrm{var}[I_{\perp}(t_k)] = I_{\perp}(t_k) . \tag{7b}$$

When background fluorescence must be subtracted $I_{\parallel}(t_k)$ and $I_{\perp}(t_k)$ are given by:

$$I_{\parallel}(t_k) = I_{s\parallel}(t_k) - I_{b\parallel}(t_k) \tag{8a}$$

$$I_{\perp}(t_k) = I_{s\perp}(t_k) - I_{b\perp}(t_k) \tag{8b}$$

where the subscripts s and b denote sample and background fluorescence respectively. In this case the Eqns (7) become:

$$\mathrm{var}[I_{\parallel}(t_k)] = I_{s\parallel}(t_k) + I_{b\parallel}(t_k) \tag{9a}$$

$$\mathrm{var}[I_{\perp}(t_k)] = I_{s\perp}(t_k) + I_{b\perp}(t_k) . \tag{9b}$$

Application of the proper weighting factors in the case of background fluorescence is very important in the fitting procedure.

As mentioned in the introduction, the use of a scatterer to measure $P(t)$ directly has serious disadvantages and therefore the use of reference compounds to obtain the impulse response profile has been proposed by several authors [16, 23–26]. Using the fluorescence, $S_r(t)$, of a reference compound with a single-exponential decay, with lifetime τ_r, the decay parameters can be obtained from:

$$S(t) = \sum_i \alpha_i S_r(t) + S_r(t) * \left\{ \sum_i \alpha_i \left(\frac{1}{\tau_r} - \frac{1}{\tau_i} \right) \exp(-t/\tau_i) \right\} . \tag{10}$$

In the weighting factors for the residues both the noise in $S(t)$ and $S_r(t)$ must be accounted for, therefore:

$$w_k = 1.0/\{\text{var}[S(t_k)] + \left(\sum_i \alpha_i\right)^2 \text{var}[S_r(t_k)]\}. \quad (11)$$

Minimization of the weighted-squared residuals can be performed with τ_r fixed, if an accurate value is known for it, or with τ_r as an extra iteration variable. A number of available reference compounds is well characterized [16, 27, 28], so in most cases τ_r will be known beforehand. A limitation of the method is that in the case that one of the τ_i values equals τ_r, this component will not be found in the analysis, because of elimination of the term $(1/\tau_r - 1/\tau_i)$ in Eqn (10).

Anisotropy

The anisotropy $r(t)$ of the fluorescence, is defined as follows:

$$r(t) = \frac{i_\parallel(t) - i_\perp(t)}{i_\parallel(t) + 2i_\perp(t)}. \quad (12)$$

Like $s(t)$, $r(t)$ can be analysed in terms of a series of exponentials:

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j) \quad (13)$$

where ϕ_j are the rotational correlation times. Experimentally one obtains $I_\parallel(t)$ and $I_\perp(t)$ and not $i_\parallel(t)$ and $i_\perp(t)$. Hence the only quantity that can be directly constructed is the so-called experimental anisotropy, $R(t)$:

$$R(t) = \frac{I_\parallel(t) - I_\perp(t)}{I_\parallel(t) + 2I_\perp(t)}. \quad (14)$$

The relationship between $R(t)$ and $r(t)$ is:

$$R(t) = \frac{P(t) * \{s(t)r(t)\}}{S(t)}. \quad (15)$$

When the α_i's and the τ_i's are known the anisotropy parameters can be obtained by fitting $R(t)$ to Eqn (15) (cf. [19]) but it is also possible to derive the expressions for $I_\parallel(t)$ and $I_\perp(t)$ and fit these quantities simultaneously with one set of parameters [29, 30].

Combination of Eqn (14) with Eqn (15) gives:

$$I_\parallel(t) = P(t) * \left\{\frac{2}{3} r(t)s(t) + \frac{1}{3} s(t)\right\} \quad (16a)$$

$$I_\perp(t) = P(t) * \left\{\frac{1}{3} s(t) - \frac{1}{3} r(t)s(t)\right\}. \quad (16b)$$

The weighting factors for the residuals, $w_{k\parallel}$ and $w_{k\perp}$, are in this case given by the inverse of the variances of $I_\parallel(t_k)$ and $I_\perp(t_k)$. The Eqns (15) and (16) can be used in the case that $P(t)$ is measured directly with a scatterer. When a reference is used the expressions for $I_\parallel(t)$ and $I_\perp(t)$ are (cf. [26]):

and the weighting of the residues:

$$w_{k\parallel} = 1/\left\{\text{var}[I_\parallel(t_k)] + \left(\frac{1}{3}\sum_i \alpha_i + \frac{2}{3}\sum_i\sum_j \alpha_i\beta_j\right)^2 \text{var}[S_r(t_k)]\right\} \quad (18a)$$

$$w_{k\perp} = 1/\left\{\text{var}[I_\perp(t_k)] + \left(\frac{1}{3}\sum_i \alpha_i - \frac{1}{3}\sum_i\sum_j \alpha_i\beta_j\right)^2 \text{var}[S_r(t_k)]\right\}. \quad (18b)$$

$S_r(t)$ can be measured with the polarizer set at 54.74° to the vertical or as $I_{r\parallel}(t) + 2I_{r\perp}(t)$.

In the derivation of the Eqns (17) and (18), it is assumed that when $s(t)$ and $r(t)$ are multiple exponential all the cross terms for the different lifetimes τ_i and correlation times ϕ_j must be accounted for. Physically this means that every lifetime component is coupled with every rotation. For instance, in the case of a mixture of two fluorescence compounds with each a single lifetime and correlation time, these lifetimes must only be associated with one of the correlation times. Hence, the model has to be adapted and some of the cross terms have to be omitted.

Computer programs

Two Fortran-77 computer programs were developed to analyse the polarized fluorescence decay data. In the first one $S(t)$ can be fitted with Eqn (2) or Eqn (10) yielding the fluorescence decay parameters α_i and τ_i. The fitting routine is according to the finite difference Levenberg-Marquardt algorithm, provided by the IMSL subroutine ZXSSQ [31]. The second program uses the α_i's and the τ_i's as input parameters and fits $I_\parallel(t)$ and $I_\perp(t)$ simultaneously to the Eqns (16) or (17) with the possibility to choose which cross term in the $s(t)r(t)$ product must be accounted for and which must be omitted. This program also uses subroutine ZXSSQ.

The quality of the fit is judged with visual inspection and with the help of various statistical functions and parameters, namely: the weighted residuals, the autocorrelation function of the weighted residuals, the number of sign changes in the autocorrelation function [32], the reduced χ^2 value and the Durbin-Watson parameter, DW [13]. The standard deviations of the fitted parameters can be estimated from the Hessian [20], which is one of the output parameters of subroutine ZXSSQ.

RESULTS AND DISCUSSION

Reference compounds

We have measured the fluorescence decays of *p*-terphenyl (λ_{em} = 345 nm) and POPOP (λ_{em} = 437 nm) upon 295-nm excitation. These two compounds are known to have a mono-

$$I_\parallel(t) = \frac{1}{3}\left(\sum_i \alpha_i + 2\sum_i\sum_j \alpha_i\beta_j\right) S_r(t) + \frac{1}{3} S_r(t) * \sum_i \left\{\alpha_i\left(\frac{1}{\tau_r} - \frac{1}{\tau_i}\right)\exp(-t/\tau_i)\right. \quad (17a)$$
$$\left. + \frac{2}{3} S_r(t) * \sum_i\sum_j \left\{\alpha_i\beta_j\left(\frac{1}{\tau_r} - \frac{1}{\tau_i} - \frac{1}{\phi_j}\right)\exp(-t/\tau_i - t/\phi_j)\right\}\right.$$

$$I_\perp(t) = \frac{1}{3}\left(\sum_i \alpha_i - \sum_i\sum_j \alpha_i\beta_j\right) S_r(t) + \frac{1}{3} S_r(t) * \sum_i \left\{\alpha_i\left(\frac{1}{\tau_r} - \frac{1}{\tau_i}\right)\exp(-t/\tau_i)\right. \quad (17b)$$
$$\left. - \frac{1}{3} S_r(t) * \sum_i\sum_j \left\{\alpha_i\beta_j\left(\frac{1}{\tau_r} - \frac{1}{\tau_i} - \frac{1}{\phi_j}\right)\exp(-t/\tau_i - t/\phi_j)\right\}\right.$$

Table 1. *Fluorescence decay parameters for* p-*terphenyl and POPOP at 20°C*
f = frequency of detected photons

Sample	f	τ	χ^2	DW
	kHz	ns		
p-Terphenyl	5	1.061 ± 0.001	1.95	1.17
	15	1.063 ± 0.001	1.91	1.14
	30	1.068 ± 0.001	2.40	0.91
POPOP	5	1.360 ± 0.002	1.31	1.51
	15	1.355 ± 0.002	1.44	1.29
	30	1.360 ± 0.003	2.05	0.92

Table 2. *Fluorescence decay parameters of* $AcTrpNH_2$ *at 20°C*
f is the frequency of detected photons. The impulse response profile was measured either with a scatterer (S) or was obtained via fluorescence of *p*-terphenyl (Ph_3), as indicated under Method

f	Method	τ	τ_r	χ^2	DW
kHz		ns			
5	S	2.953 ± 0.004	–	1.18	1.69
15	S	2.937 ± 0.003	–	1.22	1.73
30	S	2.902 ± 0.002	–	1.35	1.34
5	Ph_3	3.011 ± 0.006	1.108 ± 0.005	1.01	2.02
15	Ph_3	2.985 ± 0.006	1.099 ± 0.004	1.05	2.03
30	Ph_3	2.977 ± 0.008	1.119 ± 0.005	1.29	1.50

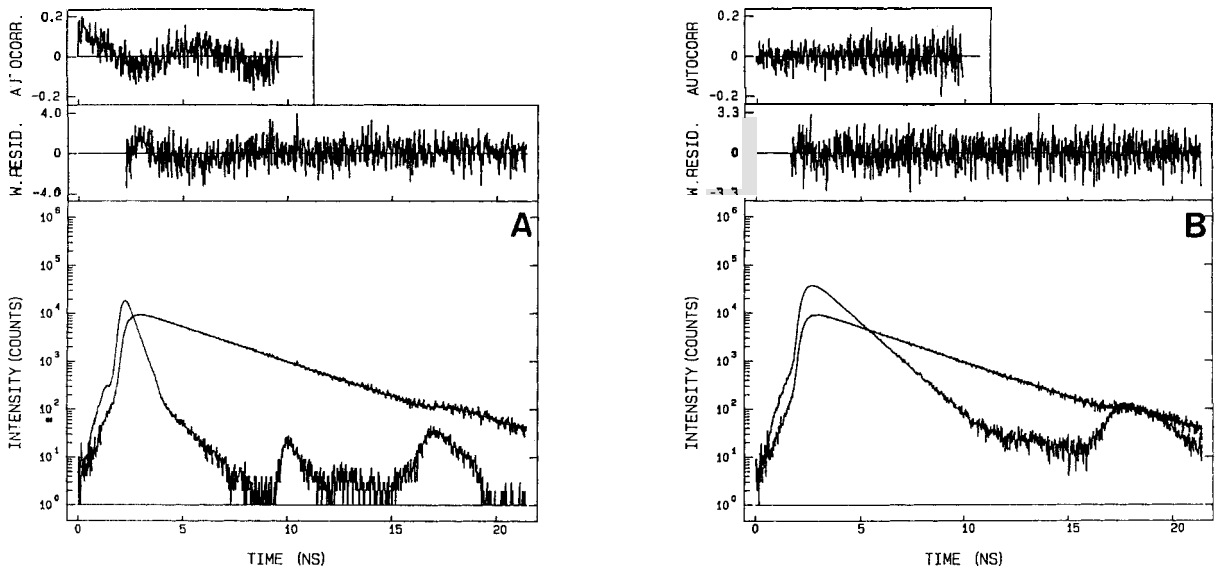

Fig. 1. *Fluorescence decay of* $AcTrpNH_2$ *at 20°C measured at 344 nm using 295-nm excitation.* (A) $AcTrpNH_2$ decay together with the impulse response profile which is obtained with a scatterer. (B) Decay curves of $AcTrpNH_2$ and of *p*-terphenyl, which are recorded under the same experimental conditions. The upper panels show the weighted residuals in each channel and the autocorrelation of these residuals. The decay parameters are listed in Table 2

exponential fluorescence decay [27]. Yet the fits with a single lifetime are very bad. Possibly these problems are due to pile-up effects. Usually we keep the frequency of the detected photons, f, at 15 kHz, which is 5% of the rate of the excitation photons. This time also f = 5 kHz and f = 30 kHz were tried. The results, presented in Table 1, show that the χ^2 and the Durbin-Watson parameter values (DW) for the 30-kHz fits are much worse than for the ones of the 5-kHz and 15 kHz decays. We may thus conclude that pile-up effects only occur at 30 kHz. The bad fitting of the results for 5 kHz and 15 kHz must have another origin, like a wavelength dependence of the detection system. When this dependence only results in a time shift, the quality of the fit should improve if we let this shift be an extra variable in the iteration [30]. No improvement was found, indicating that pulse-shape alterations with wavelength are dominant. In order to see whether these problems could be overcome with the use of the reference method, we have done some experiments with $AcTrpNH_2$ (λ_{ex} = 295 nm), measuring the impulse response profile directly with a scatterer or indirectly via the *p*-terphenyl fluorescence. These experiments were also done with the three start/stop pulse ratios. The results are listed in Table 2 and the 15-kHz decays are shown in Fig. 1. Excellent fits are obtained with the reference method at 5 kHz and 15 kHz. When the scatterer is used the quality of the fits is less, but not as bad as in the case of *p*-terphenyl and POPOP. The reason for this is that the $AcTrpNH_2$ lifetime is much longer and therefore the effect of the error in $P(t)$ using a scatterer is much less. We can conclude that, especially for short components in the fluorescence decay, the reference method yields more reliable results. Again 30 kHz was found to be too high and gave rise to pile-up effects. No difference is found between the results with at 5 kHz and 15 kHz. Consequently, it is better to use 15 kHz, because a better signal/noise ratio will be obtained in the same experimenting time.

During the analysis of the data with the reference method we let τ_r be an extra variable. Because the $AcTrpNH_2$ decay is also mono-exponential, this led to very good results for both the $AcTrpNH_2$ and the *p*-terphenyl lifetimes, which are in good agreement with values found by others [16, 25, 28].

The simultaneous analysis of the $AcTrpNH_2$ and *p*-terphenyl decays has also been performed on data obtained upon

300-nm excitation at two different emission wavelengths (337 and 350 nm) and three different temperatures (4, 20 and 30 °C). The resulting lifetimes are presented in Table 3. The fluorescence lifetime of $AcTrpNH_2$ is found to be rather independent of the excitation or emission wavelength but there is a clear dependence on temperature. With increasing temperature the lifetime decreases. The *p*-terphenyl lifetime seems to be quite insensitive to temperature changes. This was also observed by Zuker et al. [16].

PROTEINS

Human serum albumin

Human serum albumin (HSA) is a protein ($M_r = 69000$) which contains a single tryptophan residue. The fluorescence decay of this protein has been analysed before ($\lambda_{ex} = 295$ nm) [18, 33]. Two lifetimes were found, one of 3.3 ns and one of 7.8 ns. Using 300-nm excitation, we found in addition to the 3.3-ns and 7.8-ns lifetimes a third, short component in the fluorescence decay. The values of the α_i's and the τ_i's are listed in Table 4. There is a relatively large difference in the values found for τ_i when using the scatterer or the reference method. As explained before the wavelength dependence of the detection system will have the most pronounced effect on short components in the decay and therefore it is believed that the numbers obtained with the reference method are the most reliable ones. The average lifetime $\langle\tau\rangle$, which is defined as:

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \tag{19}$$

is in both cases found to be 6.0 ns, which is in very good agreement with the results published before.

During the analysis of this three-exponential decay a fixed value for τ_r had to be used. If τ_r was not fixed, the iteration process was terminated because of entrapment of χ^2 in local minima of the χ^2 hyperspace, leading to poor fits and wrong

Table 3. *Temperature and wavelength dependence of the fluorescence parameters of* $AcTrpNH_2$ and p-*terphenyl*
τ is the lifetime of $AcTrpNH_2$, τ_r is that of *p*-terphenyl

Temperature	λ_{em}	τ	τ_r
°C	nm	ns	
4	337	3.976 ± 0.010	1.060 ± 0.004
20		2.985 ± 0.006	1.064 ± 0.005
30		2.428 ± 0.004	1.057 ± 0.005
4	350	3.982 ± 0.010	1.071 ± 0.006
20		3.010 ± 0.007	1.069 ± 0.005
30		2.485 ± 0.004	1.073 ± 0.003

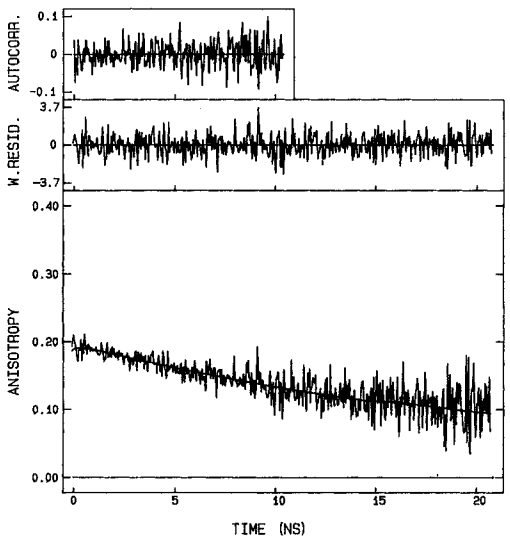

Fig. 2. *Anisotropy decay of HSA at 20 °C measured at 344 nm upon 300-nm excitation.* The experimental anisotropy (Eqn 13) is shown. The quantities which are actually fitted are $I_{\parallel}(t)$ and $I_{\perp}(t)$ but the fit of the experimental anisotropy is easily obtained from these values. The weighting factors for the residues are $W_k = 3\,S(t_k)/[2 + 3\,R(t_k) - 3\,R(t_k)^2 - 2\,R(T_k)^3]$ [22]. The total fluorescence is fitted with a three-component decay (Table 4). The anisotropy parameters are: $\beta = 0.19 \pm 0.01$ and $\phi = 26 \pm 0.6$ ns. These results are obtained using *p*-terphenyl as a reference compound and fixing a value of $\tau_r = 1.1$ ns

Table 4. *Fluorescence decay parameters for HSA, LADH and lysozyme at 20 °C*
The impulse response profiles was measured either with a scatterer (S) or obtained via *p*-terphenyl fluorescence (Ph_3) as indicated under Method

Protein	λ_{ex}	Method	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\langle\tau\rangle$	χ^2	DW
	nm			ns		ns		ns			
HSA	300	S	0.19 ± 0.01	0.55 ± 0.05	0.46 ± 0.01	3.7 ± 0.08	0.35 ± 0.01	7.7 ± 0.07	6.0	1.09	1.85
	300	Ph_3	0.41 ± 0.01	0.11 ± 0.02	0.29 ± 0.01	3.7 ± 0.01	0.30 ± 0.01	7.3 ± 0.07	6.0	1.05	1.89
LADH	295	S	0.61 ± 0.01	3.31 ± 0.04	0.39 ± 0.01	6.51 ± 0.04	–	–	5.09	1.22	1.71
	295	Ph_3	0.57 ± 0.01	3.38 ± 0.05	0.43 ± 0.02	6.23 ± 0.05	–	–	5.04	1.08	1.97
	300	S	0.67 ± 0.01	3.44 ± 0.04	0.33 ± 0.01	6.39 ± 0.06	–	–	4.83	1.16	1.79
	300	Ph_3	0.61 ± 0.01	3.44 ± 0.04	0.39 ± 0.02	5.95 ± 0.05	–	–	4.74	1.09	1.90
Lysozyme	295	S	0.62 ± 0.01	0.72 ± 0.01	0.38 ± 0.01	2.53 ± 0.01	–	–	1.95	5.50	0.34
	295	S	0.46 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	1.94 ± 0.02	0.04 ± 0.01	4.33 ± 0.06	2.00	1.19	1.94
	300	S	0.69 ± 0.01	0.98 ± 0.01	0.31 ± 0.01	2.89 ± 0.01	–	–	2.06	4.15	0.44
	300	Ph_3	0.75 ± 0.01	1.28 ± 0.01	0.25 ± 0.01	3.23 ± 0.02	–	–	2.17	3.03	0.61
	300	S	0.48 ± 0.01	0.55 ± 0.01	0.50 ± 0.01	2.08 ± 0.02	0.02 ± 0.01	5.94 ± 0.13	2.13	1.15	1.64
	300	Ph_3	0.42 ± 0.01	0.70 ± 0.02	0.56 ± 0.01	2.18 ± 0.02	0.02 ± 0.01	6.25 ± 0.16	2.22	1.03	1.83

τ_r values. Using 1.1 ns for τ_r as found with $AcTrpNH_2$ gave excellent results. The triple-exponential decay for the single tryptophan residue of HSA can be explained in terms of the modified conformer model as discussed in the introduction.

Analysis of the anisotropy decay of HSA at 20 °C gave identical results for the scatterer and the reference methods, because no short components are present in this decay. Fig. 2 shows the experimental and the fitted anisotropy decays. A single-exponential with $\beta = 0.19 \pm 0.01$ and $\phi = 26.0 \pm 0.6$ ns is found. It is possible to estimate the value of ϕ for a rigid hydrated sphere according to an empirical relationship

$$\phi_{calc} = M(\bar{v} + h)\eta/RT \qquad (20)$$

in which M is the molar mass, $\bar{v}$ is the partial specific volume (0.735 cm^3/g), h the degree of hydration (0.2 cm^3/g), η the viscosity (cP), R the gas constant and T the absolute temperature. For HSA at 20 °C Eqn (20) predicts a ϕ value of 26.5 ns, which is very close to the experimental value. This indicates that at 20 °C the Trp residue rotates with the protein as a whole and has no internal mobility. Our results agree with those of Munro et al. [34] who have used 300-nm synchrotron radiation to measure the anisotropy decay of HSA at 8 °C and at 41 °C. At 8 °C a mono-exponential decay was found with $\beta = 0.2$ and $\phi = 31.4$ ns. At 41 °C a second, short correlation time appears and the overall protein rotation is much faster than predicted with Eqn (20). At this temperature the protein has probably gained internal flexibility and cannot be considered as a rigid sphere.

In a previous publication [18] a double-exponential anisotropy decay at 21 °C was reported. These values were obtained by fitting the anisotropy without deconvolution. Upon reanalysis of these old data with our present method, we found that they also can be fitted with a single-exponential with the same correlation time as found with our new data. The after pulse, which is only taken into account when a deconvolution procedure is used, may have distorted the data.

Liver alcohol dehydrogenase

Liver alcohol dehydrogenase (LADH) is an ideal protein for time-resolved fluorescence because it is one of the best characterised proteins [35]. The active enzyme is a dimer made up of two identical ($M_r = 40000$) subunits. Each subunit contains two tryptophan residues. From X-ray data it is known that one of these residues (Trp-15) is located near the surface of the protein, while the other one (Trp-314) is buried inside the protein coil in a hydrophobic region, near the subunit interface. We have measured the tryptophan fluorescence lifetimes of LADH, using 295-nm or 300-nm excitation (Table 4). The differences between the results obtained with the scatterer and with the reference method are only small in this case, because the lifetimes are relatively long. From the χ^2 and DW values one may conclude that the fits with the reference method are slightly better than the ones with the scatterer method.

Similar experiments with LADH have been performed by other groups. Ross et al. [36] used 295-nm excitation and reported lifetimes which are comparable to ours. The small differences can be due to differences in the experimental conditions (i.e. buffer, temperature and emission wavelength). Based on KI quenching data, Ross et al. assign the short component to Trp-314 and the long one to Trp-15. Excitation at 300 nm is used by Barboy and Feitelson [37]. They report a single lifetime of 5 ns and interpret this by emission of Trp-314 only upon 300-nm excitation. Fitting of our data with a single exponential gave very bad results. A small decrease of the relative contribution of Trp-15 to the total fluorescence is found, but it does not totally disappear. This finding is supported by the steady-state quenching results of Ross et al. [36].

In the analysis of the anisotropy decay of LADH upon 295-nm and 300-nm excitation at 20 °C no differences between the results obtained with the scatterer and the reference method were found. The decay can be described by a single-exponential and because the rotational correlation times are relatively long a small error in $P(t)$ does not affect the results significantly. Fig. 3 shows the anisotropy decays of the protein upon 295-nm and 300-nm excitation. Excitation at 300 nm yields a higher initial anisotropy as was expected, but there is another effect in addition. The rotational correlation time at $\lambda_{ex} = 300$ nm is longer than at $\lambda_{ex} = 295$ nm (36.0 ± 0.9 ns and 33.0 ± 1.0 ns respectively). This phenomenon can only be explained by means of the non-spherical shape of the protein. Indeed it has been reported that the LADH molecule can be considered as a prolate ellipsoid, with long and short semiaxes of 11 nm and 6 nm respectively [35]. Tao [38] has derived a general expression for the anisotropy decay of ellipsoids of revolution:

$$r(t) = \frac{2}{5} P_2(\cos\lambda)[\beta_1(\theta)\exp(-t/\phi_1) + \beta_2(\theta)\exp(-t/\phi_2) + \beta_3(\theta)\exp(-t/\phi_3)] \qquad (21)$$

where $P_2(\cos\lambda)$ is the Legendre polynomial of order 2, λ the angle between the absorption and emission dipoles and θ the angle that the emission transition dipole subtends with the main symmetry axis of the ellipsoid. Three correlation times are needed to describe the anisotropy of an ellipsoid of revolution and their values depend on the axial ratio. For LADH this ratio is about 1.8. This means that the three ϕ values are nearly identical; thus a mono-exponential decay with a rotational correlation time which is the weighted average of ϕ_1, ϕ_2 and ϕ_3 is observed. For molecules with one chromophore the amplitudes (β_i) are given by:

$$\beta_1(\theta) = (3/2\cos^2\theta - 1/2)^2$$
$$\beta_2(\theta) = 3\cos^2\theta\sin^2\theta \qquad (22)$$
$$\beta_3(\theta) = 3/4\sin^4\theta.$$

LADH has four tryptophans and there are two different electronic transitions (^1L_b is almost perpendicular to ^1L_a). As a result the expressions which describe the LADH anisotropy decay are very complex: however it can be argued that changing the excitation wavelength from 295 nm to 300 nm causes changes in the parameters which determine the β values and thus in the observed average of the three rotational correlation times.

Lysozyme

Lysozyme is, like LADH, a protein for which much structural information is available [39]. The analysis of the tryptophan fluorescence decay of this enzyme is difficult, because there are six different Trp residues (28, 62, 63, 108, 111 and 123). Imoto et al. [40] have measured the steady-state fluorescence of the native protein and of modified derivatives in which Trp-62 and/or Trp-108 are specifically oxidized. Their results indicate that more than 80% of the fluorescence comes from these two residues. Moreover in the native protein, energy transfer seems to occur from Trp-108 to Trp-

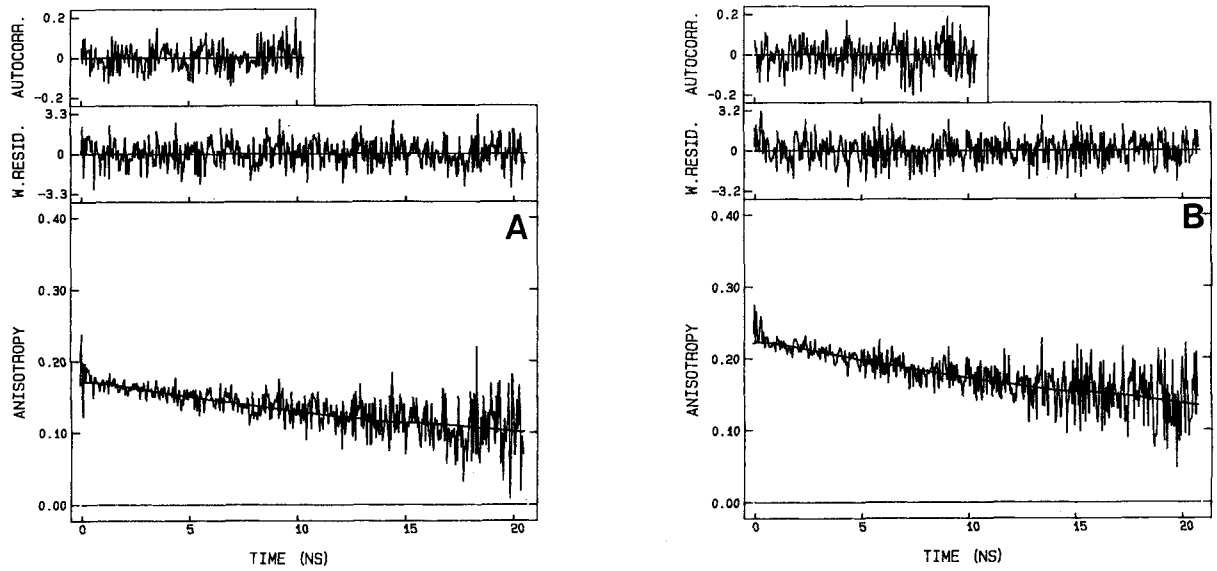

Fig. 3. *LADH anisotropy decay at 20°C.* Excitation wavelength is 295 nm (A) or 300 nm (B), emission wavelength is 344 nm. Fluorescence parameters are listed in Table 4. The anisotropies are fitted with a single exponential with $\beta = 0.17 \pm 0.01$ and $\phi = 33 \pm 1.0$ ns for $\lambda_{ex} = 295$ nm and $\beta = 0.22 \pm 0.01$ and $\phi = 36 \pm 0.9$ ns for $\lambda_{ex} = 300$ nm. *p*-Terphenyl was used as reference compound with $\tau_r = 1.1$ ns

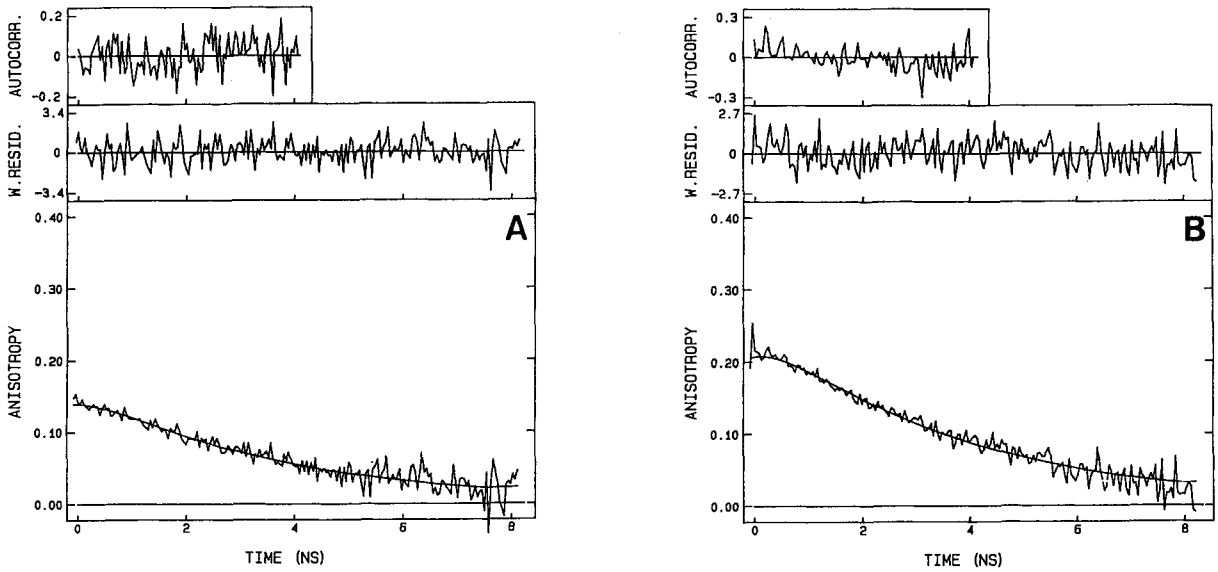

Fig. 4. *Anisotropy decays of lysozyme at 20°C, using 295-nm (A) and 300-nm (B) excitation.* Emission wavelength = 344 nm. The total fluorescence is fitted with three exponentials. Decay parameters are listed in Table 4. The anisotropy decays are described with a single exponential with $\beta = 0.14 \pm 0.01$, $\phi = 3.8 \pm 0.1$ ns and $\beta = 0.22 \pm 0.01$, $\phi = 3.8 \pm 0.1$ ns respectively

62. The lifetimes of the fluorescence have been measured by Formoso and Forster [41]. They find a double-exponential decay with lifetimes of about 0.5 ns and 2.26 ns at pH 8. Based on experiments with the modified derivatives, the 2.26-ns component was assigned to Trp-62 and the 0.5-ns component to Trp-108 and all other emitting Trp residues.

We have measured the native lysozyme decay upon 295-nm and 300-nm excitation. The lifetimes for the different fits are listed in Table 4 together with the χ^2 and DW values. The decay obtained with 295-nm excitation is analyzed using the impulse-response profile obtained with the scatterer. In this case the α_i and τ_i values obtained from a double-exponential fit are in very good agreement with the results of Formoso and Forster [41] but on the basis of the χ^2 and DW values and also of visual inspection of the plot of the weighted residuals these results have to be rejected. Fitting with three exponentials results in a considerable improvement. Analysis of the decay obtained with 300-nm excitation occurred both with the scatterer and the reference method. Again three exponentials are needed to obtain acceptable fits. The lifetimes are almost identical to the ones for $\lambda_{ex} = 295$ nm. The most important differences are found in the long component which becomes longer at $\lambda_{ex} = 300$ nm. It is not possible to assign the different lifetimes to specific residues on the basis of these experiments with the native enzyme only.

The anisotropy decays are shown in Fig. 4. Both decays are fitted with a single exponential, with a rotational correlation time of 3.8 ± 0.1 ns. With 300-nm excitation the initial

anisotropy is higher, as expected. A correlation time of 3.8 ns is low for a protein with a relative molecular mass, M_r = 13900. Chang et al. [42] have measured the fluorescence anisotropy decay of the eosin-lysozyme complex and they find a value of 5 ns for the overall rotational correlation time at 34°C. Assuming that ϕ is directly proportional to η/T (Eqn 20), this would correspond with 7.2 ns at 20°C. The same value is found from ^{13}C-NMR experiments [43]. A correlation time of 3.9 ns at 25°C was observed by Maliwal and Lakowicz [44] with their fluorescence depolarization technique. This value, which was also measured via tryptophan fluorescence, is very close to the one we find. Using an active-site inhibitor [*N*-acetyl-D-glucosamine or di(*N*-acetyl-D-glucosamine)], Maliwal and Lakowicz reported a significant increase in rotational correlation time. An explanation for these observations cannot easily be given. Internal motion of the tryptophan residues may influence the observed anisotropy decay. Molecular dynamics calculations [45] demonstrate the presence of picosecond internal movements of some tryptophan residues. However at present these oscillations cannot be observed with the single-photon counting technique. Nevertheless, they would result in a decrease of the initial anisotropy. Another possibility is that we observe the so-called hinge bending of lysozyme [46], that is the movement of two distinct domains of the protein relative to one another. This phenomenon could explain the increase in correlation time in the presence of active-site inhibitors [44], since it is known that binding of substrate reduces the hinge bending of lysozyme.

CONCLUDING REMARKS

Fluorescence lifetimes, obtained by the reference method, are in most cases very reliable. Only in the case that a lifetime component of the sample is too close to the reference lifetime will inconsistent results arise and in these cases other reference compounds or quenching of the reference fluorescence, as proposed by Löfroth [26], should be used.

The simultaneous analysis of the parallel and the perpendicular fluorescence components yields accurate rotational correlation times. Using this analysis technique, it is possible to distinguish between different correlation times of LADH upon 295-nm or 300-nm excitation. This observation indicates that the LADH molecule in solution does not behave as an isotropic rotor.

The relatively short correlation time found for lysozyme suggests that it is not the overall rotation of this protein that is observed but the hinge bending. This idea is supported by other experiments [44]. With our present equipment it is not possible to observe the picosecond internal motions of the tryptophan residues in proteins. However the fact, that the initial anisotropies at λ_{ex} = 300 nm never reach the value of 0.26, characteristic for totally immobilized tryptophans, suggests the presence of these motions. Implementation of a microchannel plate detector instead of a conventional photomultiplier tube improves the time resolution [47] and this is the first step to determine ultrafast motions experimentally in proteins.

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