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RAPID RELAXATION PROCESSES IN PIG HEART LIPOAMIDE DEHYDROGENASE REVEALED BY SUBNANOSECOND RESOLVED FLUOROMETRY

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The decay kinetics of the FAD-fluorescence in lipoamide dehydrogenase from pig heart have been reinvestigated using phase fluorometric methods and sophisticated laser pulse techniques. Both pulse and modulation methods lead to distinct heterogeneity in lifetimes. The two different techniques lead to good correspondence in the longer lifetime component of a biexponential decay model, whereas the more rapidly decaying component is distinctly shorter and has a larger amplitude using the phase technique with two available modulation frequencies (15 and 60 MHz). Lifetime measurements as a function of temperature and in the presence of D_2O instead of H_2O illustrate that the quenching of the FAD fluorescence in lipoamide dehydrogenase is mainly dynamic in nature and that solvent comes into contact with the fluorophor. Mobility of the flavin itself, free and bound to the enzyme, has been measured by both differential polarized phase fluorometry and experimental fluorescence anisotropy decay after ps laser pulse excitation. By employing flavin models it has been shown that both techniques have ps time resolution. Measurements with the latter more direct method indicate a rapid subnanosecond motion of the FAD bound within the enzyme, only visible at temperatures lower than about 15°C, where the protein rotational diffusion is slowed down. The significance of rapid transient conformational fluctuations for catalysis is discussed with reference to recently developed insights reported in the literature.

1. Introduction

Lipoamide dehydrogenase is a constituent of two multienzyme complexes, usually abbreviated as pyruvate and α-ketoglutarate dehydrogenase complexes [1]. The partial reaction, catalyzed by lipoamide dehydrogenase is the oxidation of dihydrolipoamide, covalently bound to the transacetylase component of the complex. The reaction is linked to reduction of NAD^+. Isolated from the complex the physiological activity seems to be connected with the dimeric form of the protein. The isolated enzyme exhibits one of the strongest flavin emissions encountered amongst flavoproteins. The noncovalently, tightly bound FAD can thus be considered as a natural probe for obtaining structural and dynamic information about the microenvironment of the active center of this enzyme. In a previous publication it was shown that the time dependence of the flavin fluorescence cannot be simply characterized by a single time constant [2]. A reasonable first approximation of the fluorescence decay process was obtained by a sum of two exponential terms with about equal amplitudes. This observation led to the assumption that the two FAD binding sites were not equivalent resulting in different FAD lifetimes.

The investigations were continued using different experimental approaches. Not only advanced laser pulse methods, but also phase and modulation techniques were employed to obtain the fluorescence lifetimes of the bound flavin. As far as we know, it will be the first time that both methods to obtain fluorescence lifetimes (phase and pulse fluorometry) were applied to the same system under identical conditions, thus facilitating a critical, independent comparison of the results. Another approach to be described is the determination of rapid types of motion in the (sub)nanosecond region that the free and bound FAD and molecular fragments near bound FAD undergo. This study shows that in the enzyme rather mobile structures are present and that channels are created allowing solvent and small molecul to enter the active site. Rapid fluctuations in structure concentrated in the flavin area may provide the short interacting distances and the internal energy for efficier catalysis to occur [3].

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2. Materials and methods

2.1. Materials

Lipoamide dehydrogenase was prepared from pig heart according to published procedures [4]. Solutions of 10 μM enzyme in 30 mM sodium phosphate buffer of pH 7.2 containing 0.3 mM EDTA were used in the experiments. All reagents were analytical grade and buffers were made from doubly distilled water. Deuterium oxide was obtained from Merck (Darmstadt). Replacement of H_2O by D_2O was obtained by a 24 hr dialysis against a hundredfold excess in volume of deuterated phosphate (pD = 7.6) in D_2O. The buffer was changed one time after 12 hr. 3-Methyllumiflavin was synthesized according to Hemmerich et al. [5]. FAD, purchased from Sigma Chemical Company, was additionally purified according to the procedure of Massey and Swoboda [6]. Pure FMN was obtained by dissociation from flavodoxin of *M.elsdenii* as previously described [7].

2.2. Methods

2.2.1. Absorption and emission spectra

Absorption spectra were measured on a Cary 14 spectrophotometer and emission spectra were recorded on an Aminco SPF-500. Quantum yields were determined as described previously [8].

2.2.2. Excited state lifetimes

Fluorescence lifetimes were determined either with the single photon counting technique or with a phase and modulation fluorometer. A description of the decay fluorometer system as well as of the method for analysis has been given elsewhere [9–11]. Fluorescence decay curves are usually distorted by the finite duration of the exciting pulse and by the response of the detection system. This is described by the so-called convolution integral:

$$F(t) = \int_0^t g(t')I(t - t')\mathrm{d}t', \qquad (1)$$

where $g(t)$ is the instrument response to an extremely narrow exciting pulse and $I(t)$ is the decay function, mostly represented by

$$I(t) = \sum_{i=1}^{n} \alpha_i \mathrm{e}^{-t/\tau_i}, \quad \text{with } n = 1 \text{ or } 2. \qquad (2)$$

Deconvolution was performed by the nonlinear least squares method [12]. The parameters (α_i, τ_i) for best fit of $I(t)$ were obtained after a search for the minimum value of χ^2 or Φ:

$$\chi^2 = (1/N) \sum_{i=1}^{N} [F(i) - F_c(i)]^2/F(i)$$

$$= (1/N) \sum_{i=1}^{N} W_i \Delta_i^2, \qquad (3a)$$

$$\Phi = (1/N_0) \sum_{i=1}^{N} W_i \Delta_i^2, \qquad (3b)$$

$F(i)$ denotes the experimental fluorescence curve in channel i, $F_c(i)$ the computed fluorescence response, W_i is the weighting factor for photon counting data, $W_i = 1/F(i)$, N is the total number of channels used and N_0 is a normalization factor, $N_0 = (1/N) \Sigma_{i=1}^{N} W_i$. For visual comparison plots of the weighted residuals were generated [12]. When an air-filled gated flash lamp as excitation source was used, the 358 nm line was selected using a Balzers 361 nm interference filter; the emission light was selected at 497, 520, 554 or 597 nm by a combination of Schott GG 495 or KV 520 cut off filters and of Balzers interference or band pass filters. Glan Thompson polarizers were inserted in the excitation and detection light paths. Usually the fluorescence lifetimes were obtained from the total decay:

$$S(t) = F_{\parallel}(t) + 2F_{\perp}(t), \qquad (4)$$

where $F_{\parallel}(t)$ is the time dependence of the emission polarized parallel to the polarization direction of the excitation and $F_{\perp}(t)$ is the component perpendicular to that direction.

An outline of the mode locked Ar-ion laser system as excitation source has been presented in a previous publication [13]. In this case the 476.7 nm line was chosen for excitation; detection light was isolated with Schott KV 520- and Balzers 554 nm-filters.

The phase fluorometer as well as applications to flavin model compounds has been extensively described elsewhere [8,9,14]. The modulation frequencies were 15 and 60 MHz. The 436 nm line of a 200 W mercury arc was selected with a 0.25 m monochromator of Applied Photophysics Inc.. The emission was filtered through a Schott GG 495. Since the excitation was performed

with linearly, vertically polarized light (Glan Thompson prism), a polarizer (Polacoat) was inserted into the emission light path oriented at 54.7° with respect to the vertical position [15]. The method for obtaining the parameters of a double exponential decay function from phase and modulation fluorometry is given in the Appendix.

In all experiments conducted with both pulse and modulation methods scattered light was negligible with respect to the fluorescence transmitted through the filters. The wavelength sensitive response of the photomultipliers used was shown to be of no importance [9,13 and 14]. Control experiments on fluorophors with a single fluorescence lifetime have proved the reliability of our measuring systems [9,13].

2.2.3. Rotational correlation times

The methodology for obtaining rotational correlation times from time resolved anisotropy measurements using laser excitation has already been detailed [13]. The experimental parameter giving an indication of the rate of rotation is the anisotropy defined as:

$$A(t) = \frac{F_{\parallel}(t) - F_{\perp}(t)}{F_{\parallel}(t) + 2F_{\perp}(t)}. \tag{5}$$

$A(t)$ adopts for a spherically shaped protein the following form:

$$A(t) = A_0 e^{-t/\phi}, \tag{6}$$

where $\phi = \eta V/KT = M_r(\bar{v} + h)\eta/KT$, V is the volume, η is the viscosity, T is the absolute temperature, K is the Boltzmann constant, $\bar{v}$ is the partial specific volume and h is the degree of hydration, M_r is the molecular weight. The excitation and emission wavelengths as described for the lifetime measurements were utilized. We used the nonlinear least squares method for analysis of the experimental emission anisotropy. Details of the analysis has been given in a previous publication [13].

The experimental set-up for and the principles of differential polarized phase fluorometry have been outlined by Weber [16–18]. From the tangent of the phase difference between the polarized emission components ($\tan \Delta = \tan(\delta_{\parallel} - \delta_{\perp})$, $\delta_{\parallel}$ and $\delta_{\perp}$ are the angular delays of the polarized components of the fluorescence with respect to the polarized excitation) a single rotational correlation time can be calculated assuming exponential decay from a homogeneous solution of fluorophors. The following formula was derived by Weber [17]:

$$\alpha^2 + \frac{2\alpha}{3 - p_0}\left(1 - \left|\frac{p_0}{\tan \Delta}\right| \omega\tau_0\right) + m^2(1 + \omega^2\tau_0^2) = 0, \tag{7}$$

τ_0 is the fluorescence lifetime, p_0 is the limiting polarisation, $\alpha = \tau_0/3\phi$, $m^2 = (1 - p_0^2)/(3 - p_0)^2$, ω is the angular modulation frequency of the exciting light. In our measurements was $\tan \Delta < 0$ and $p_0 > 0$, thus $|p_0/\tan \Delta| = -p_0/\tan \Delta$. Solving for ϕ gives the following expression:

$$\phi_{1,2} = \frac{\tau_0(p_0 - 3)}{3}\left\{\left(1 + \frac{p_0\omega\tau_0}{\tan \Delta}\right) \pm \left[\left(1 + \frac{p_0\omega\tau_0}{\tan \Delta}\right)^2 - (1 - p_0^2)(1 + \omega^2\tau_0^2)\right]^{1/2}\right\}^{-1}. \tag{8}$$

For τ_0 the phase lifetimes measured at 15 MHz were used, because these time constants were the best representation for the average lifetimes (see Appendix). A description of the experimental set up is presented elsewhere [9]. To remove polarization effects on the time resolution of the two photomultipliers, scramblers were placed between the analysers and the photomultipliers.

3. Results

3.1. Fluorescence lifetimes from phase fluorometry

Phase (τ_p) and modulation (τ_m) lifetimes of the fluorescence of FAD in lipoamide dehydrogenase as a function of temperature are presented in fig. 1. The shortest apparent lifetimes in the range 0–40° C are given by the time constants derived from the phase shift at 60 MHz. The longest time constants originated from modulation measurements at 60 MHz, whereas the phase lifetime determined at 15 MHz took values intermediate between the two lifetimes measured at 60 MHz. These data are fully consistent with nonhomogeneous decay as was outlined earlier using pulse fluorometry and using phase fluorometric results on lipoamide dehydrogenase isolated from *A.vinelandii* and *E.coli* [2,19]. As presented in the Appendix the averaged lifetime is repre-

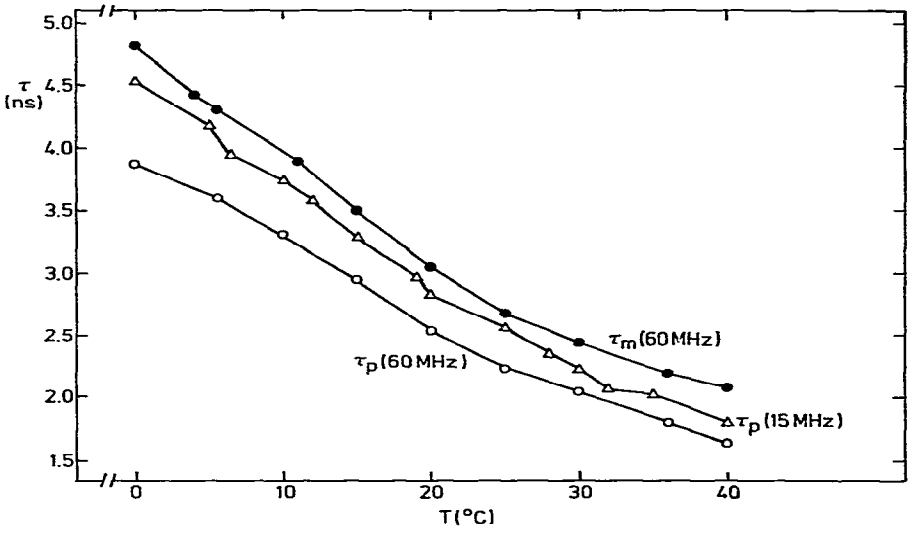

Fig. 1. Apparent lifetimes of FAD fluorescence in pig heart lipoamide dehydrogenase obtained from measurements of phase shift (τ_p) and demodulation (τ_m) as a function of temperature. ○ τ_p (60 MHz); ● τ_m (60 MHz); △ τ_p (15 MHz).

sented at best by τ_p at 15 MHz, especially when the individual lifetimes are short (< 2.5 ns). From fig. 1 it is clear that τ_p(15 MHz) is decreased from 4.5 ns at 0°C to 1.8 ns at 40°C. In fig. 2A the ratio of quantum yields of lipoamide dehydrogenase and FMN (Q/Q_0) is plotted as a function of temperature. For comparison the ratio of the lifetimes (τ/τ_0) is shown in the same figure. From fig. 2A it is clear, that $\tau/\tau_0 > Q/Q_0$. This

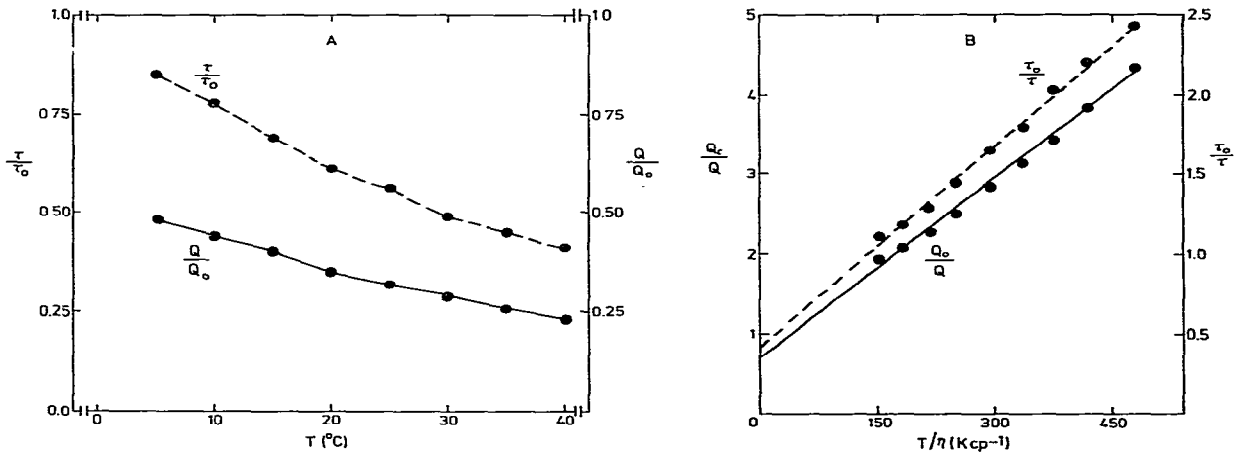

Fig. 2. A. Temperature dependence of ratio of quantum yields (Q/Q_0) and of lifetimes (τ/τ_0). B. Q_0/Q and τ_0/τ as a function of T/η. Values with subscript 0 denote FMN fluorescence, values without subscript refer to fluorescence properties of FAD in pig heart lipoamide dehydrogenase.

implies, that next to dynamic quenching a static contribution is present [20]. The fluorescence yield and lifetime of FMN are only slightly dependent on temperature, while the lifetime is completely single exponential at all temperatures [21]. The ratio of quantum yields of lipoamide dehydrogenase and FMN follows almost the same dependence on temperature as the ratio of lifetimes. From a comparison of the averaged lifetimes of FAD in lipoamide dehydrogenase to those of FMN at various temperatures the effective activation energy for the quenching process can directly be determined from an Arrhenius plot (not shown). The value obtained is 22.7 kJ/mole, typical for a diffusion limited rate of quenching. To quantitate the thermal quenching as described by Bushueva et al. [22] the reciprocal ratio of quantum yields and lifetimes from above was plotted against T/η (T = temperature (K) and η = viscosity of water (cp)). The results are shown in fig. 2B. The slope and intercept of the quantum yield data have values of 0.0074 cp K^{-1} and 0.70 respectively and from the lifetime data these values are 0.0042 cp K^{-1} and 0.42. Referring to the results of Bushueva et al. [22] such values of the slopes are indicative for efficient dynamic quenching. At infinite viscosity no dynamic quenching is present and therefore a good comparison of yields and lifetimes as such is possible. Both intercepts are smaller than one which shows that the flavin bound to the protein has a higher quantum yield and a longer lifetime in the limit of infinite viscosity. This is probably due to a more apolar environment of the bound flavin compared with FMN in aqueous solution. Studies with model flavin compounds show that both yields and lifetimes increase upon diminishing the polarity of the solvent [8].

Following the procedure derived in the Appendix we also determined the parameters of a double exponential decay from the measured apparent lifetimes shown in fig. 1. The results at several temperatures are collected in table 1. Striking is, that the short lifetime component is much shorter and has a larger amplitude relative to the results, obtained with pulse fluorometry. Also incorporated are the second order averaged lifetimes ($\langle\tau\rangle = \Sigma_{i=1}^{2}\alpha_i\tau_i^2/\Sigma_{i=1}^{2}\alpha_i\tau_i$) and the apparent lifetimes from the phase shift at 15 MHz. Noteworthy is the excellent agreement between the averaged lifetimes and the lifetimes measured at a modulation frequency of 15 MHz.

3.2. Pulse fluorometry of lipoamide dehydrogenase

Some of the previously reported pulse fluorometry experiments were repeated under different experimental circumstances [2]. Firstly, the availability of ps laser pulses with wavelengths in the first absorption band of the flavin provides a direct comparison of the results with the previous results, in which a relatively long flash lamp excitation (> 2 ns) into the second absorption band was employed. Secondly, a method was devel-

Table 1
Lifetimes ($\tau_{1,2}$) and amplitudes ($\alpha_{1,2}$) of bi-exponential fluorescence decay of FAD in pig heart lipoamide dehydrogenase calculated from phase fluorometric data

T (°C)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ [a] (ns)	τ_p (15 MHz) (ns)
0	0.74	0.10	0.26	4.82	4.56	4.51 [b]
5	0.73	0.10	0.27	4.45	4.20	4.16
10	0.62	0.18	0.38	4.04	3.78	3.75 [c]
	0.74	0.10	0.26	4.04	3.78	
15	0.68	0.13	0.32	3.55	3.30	3.28
20	0.76	0.11	0.24	3.13	2.85	2.83
25	0.81	0.10	0.19	2.96	2.60	2.57
30	0.76	0.10	0.24	2.51	2.24	2.23
35	0.78	0.10	0.22	2.33	2.04	2.02
40	0.80	0.10	0.20	2.14	1.82	1.80 [b]

[a] The average lifetime $\langle\tau\rangle$ is defined as $\Sigma_{i=1}^{2}\alpha_i\tau_i^2/\Sigma_{i=1}^{2}\alpha_i\tau_i$. [b] Approximate solutions. [c] Two solutions for the same set of data.

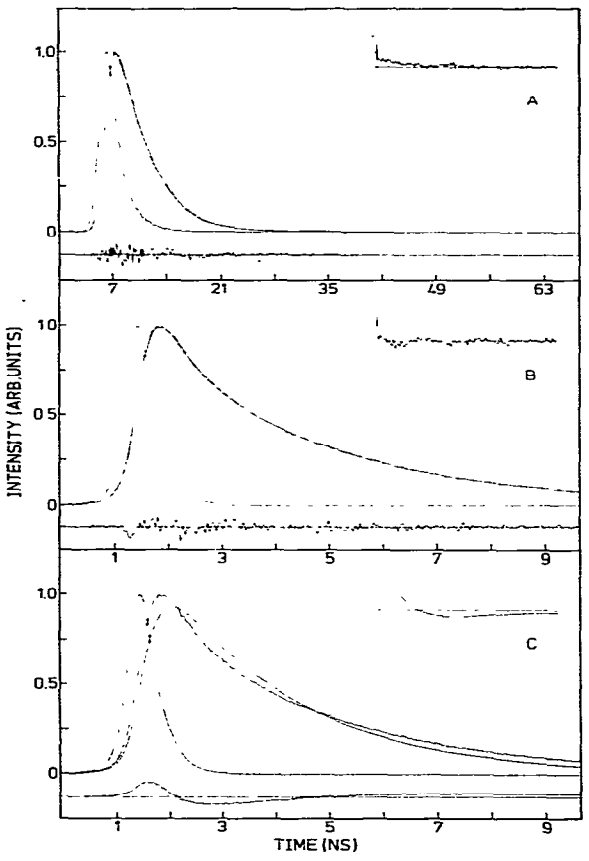

Fig. 3.A. Excitation profile from flash lamp, experimental fluorescence decay and decay calculated assuming a bi-exponential decay law. The difference between computed and experimental decay in each channel is plotted at the bottom, the autocorrelation function of these residuals is given in the upper right part. B. As in A, but with excitation pulse from a mode locked Ar ion laser. C. As in B, but under the assumption of a single exponential. Temperature = 20–21°C. Emission viewed through Schott KV 520 and Balzers 554 nm. Parameter values are given in tables 2 and 3.

oped to obtain also the lifetime at the red edge of the fluorescence spectrum (595 nm) [9]. As an illustration results obtained by two modes of excitation (flash lamp and laser) are given in fig. 3A and B. The experimental results of both were fitted with a double exponential decay. It was impossible to fit the data with a mono-exponential decay law as judged from the presented functions of weighted residuals and autocorrelation of residuals (fig. 3C). The short lifetime component as obtained by laser excitation is somewhat shorter than the one obtained by flash lamp excitation, whereas the longer lifetimes and the average lifetimes are in better agreement. The results of a single and double exponentia analysis with laser excited flavin fluorescence are incorporated in table 2. The agreement between the values of the longer lifetime components obtained by this method and from phase fluorometry (cf. table 1) is quite acceptable.

The most important outcome of the "red edge" experiments using flash lamp excitation is that the lifetimes were constant across the emission band as is illustrated in table 3. Only the amplitudes showed a slight change going to longer detection wavelengths. The values obtained at the other wavelengths are in good agreement with the previously determined parameters [2].

Fig. 4 shows the experimental and computed fluorescence response of the flavin in lipoamide dehydrogenase when water was displaced by deuterium oxide. Also in this case the fluorescence decay is heterogeneous but the average lifetime is 1.2 times longer than the one in aqueous solution. Although an isotope effect might be important it is striking that the viscosity of D_2O is 1.2 times larger than that of H_2O ($\eta_{D_2O}/\eta_{H_2O} = 1.2$), which would illustrate that the quenching by protein residues is diffusion controlled and that D_2O is accessible to FAD. The displacement of H_2O by D_2O did also shift the fluorescence spectrum slightly, but did not change the shape of the band (fig. 5).

3.3. Rotational motion as studied with differential polarized phase fluorometry

Both the lifetimes (τ_0) and the differential tangent (tan Δ) (cf. section 2) were measured between 4 and 40°C. According to eq. (8) two solutions of the rotational correlation times (ϕ) are obtained, one of which is physically meaningful and results in appropriate values for ϕ. For each temperature both roots are evaluated and from the temperature dependence of the correlation times the physically meaningful solution is selected. In principle a bell shaped curve for tan Δ is to be expected if the experiments are carried out over a wide range of temperatures and/or viscosities [18]. For lipoamide dehydrogenase the following values at 7.0°C

Table 2
Lifetimes of FAD fluorescence in pig heart lipoamide dehydrogenase upon laser excitation

	Single exponential fit [a]	Double exponential fit [a]					
T (°C)	τ (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Φ_2/Φ_1
4	3.3	0.36	0.6	0.64	4.1	3.8	0.51
10	2,9	0.39	0.4	0.61	3.4	3.2	0.47
20	2.4	0.54	0.3	0.46	3.1	2.8	0.29
20 [b]	3.2	0.53	0.3	0.47	4.0	3.7	0.10
30	1.8	0.58	0.3	0.42	2.5	2.2	0.28
40	1.4	0.51	0.5	0.49	2.0	1.7	0.75

[a] Symbols refer to convolution of the laser pulse profile with either a single or a double exponential function $\Sigma_{i=1}^{n}\alpha_i e^{-t/\tau i}$, with $n = 1$ or $n = 2$ and $\Sigma_{i=1}^{2}\alpha_i = 1$. Φ_2/Φ_1 is the ratio of fitting criterions of double and single exponential analysis (see section 2).
[b] D_2O instead of H_2O.

were obtained: τ (15 MHz) = 3.87 ns, |tan Δ| (60 MHz) = 0.041. At 39.5°C these values were: τ (15 MHz) = 1.80 ns, |tan Δ| (60 MHz) = 0.024. Assuming a limiting polarization (p_0) of 0.45 the correlation times are calculated with eq. (8) for temperatures between 4 and 40°C and are plotted against η/T in fig. 6. The reason for taking $p_0 = 0.45$ may become clear by referring to the experimental data shown in the inset of fig. 6, in which the degree of polarization is plotted aginst temperature on an expanded scale. Between 0° and 2°C there is a distinct drop in p from 0.45 to 0.43, the latter value being preserved between 2° and 40°C notwithstanding the large decrease in average lifetime. The distinct reversible change in polarization from 0° to 2°C is fully reproducible and must be due to a structural change in the direct neighborhood of FAD.

To get an idea about the relevance of the data obtained with this method several experiments with 3-

Table 3
Lifetimes of FAD fluorescence in pig heart lipoamide dehydrogenase at several emission wavelengths [a]

		Single exponential analysis	Double exponential analysis					
T (°C)	λ_{em} (nm)	τ (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	Φ_2/Φ_1
1	497	4.4	0.44	0.8	0.56	5.0	4.5	0.08
	520	4.4	0.33	1.5	0.67	5.1	4.6	0.19
	554	4.5	0.26	1.5	0.74	5.0	4.6	0.62
	597	4.6	0.28	1.1	0.72	5.1	4.7	0.19
21	497	2.8	0.48	0.8	0.52	3.4	2.9	0.10
	520	2.6	0.51	0.9	0.49	3.3	2.8	0.15
	554	2.9	0.46	0.9	0.54	3.5	3.0	0.07
	597	2.9	0.36	0.8	0.64	3.3	3.0	0.17

[a] Excitation with flash lamp (λ_{exc} = 358 nm). See table 2 for details.

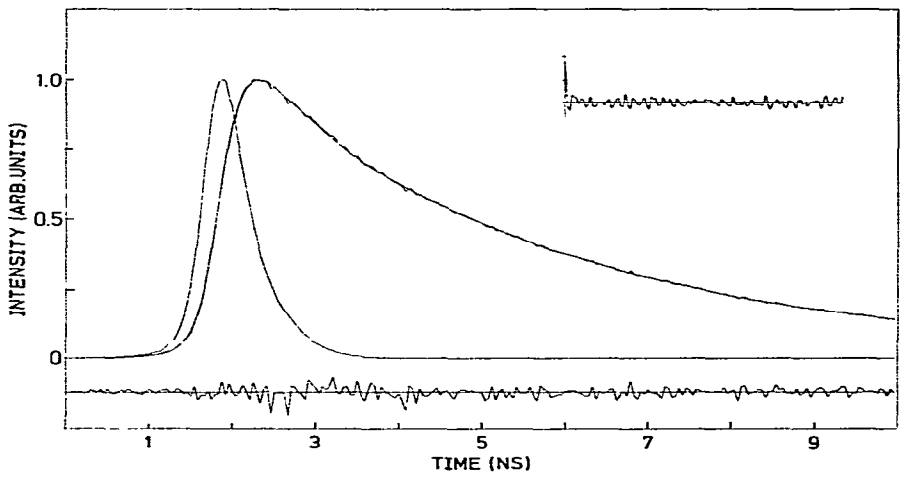

Fig. 4. Laser pulse profile, experimental and calculated bi-exponential fluorescence response of FAD in lipoamide dehydrogenase in D_2O (pD = 7.6), temperature = 20°C. Details are given in fig. 3. Parameter values are mentioned in table 2.

methyllumiflavin in water and in glycerol were carried out. For the free flavin in aqueous solution the phase difference between the two polarized emission components (Δ) could only be resolved using the higher modulation frequency (60 MHz). The resulting correlation times assuming $p_0 = 0.47$ [8] are presented in fig. 7A and illustrate that the time resolution is in the ps region. Using highly viscous glycerol as solvent values for ϕ in the ns region are obtained as shown in fig. 7B.

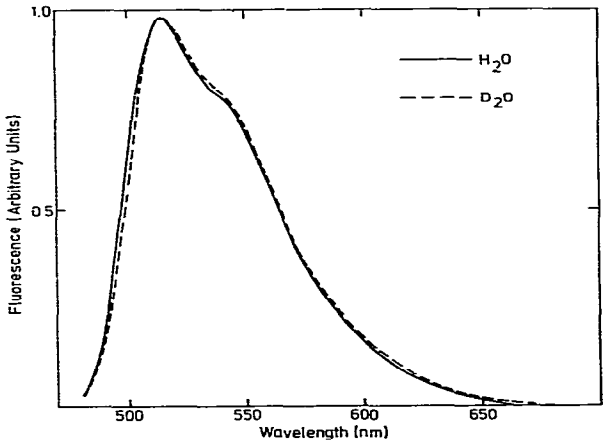

Fig. 5. Normalized fluorescence spectra of FAD in pig heart lipoamide dehydrogenase in H_2O (solid line) and in D_2O (dashed line). Excitation wavelength = 476 nm, temperature = 20°C.

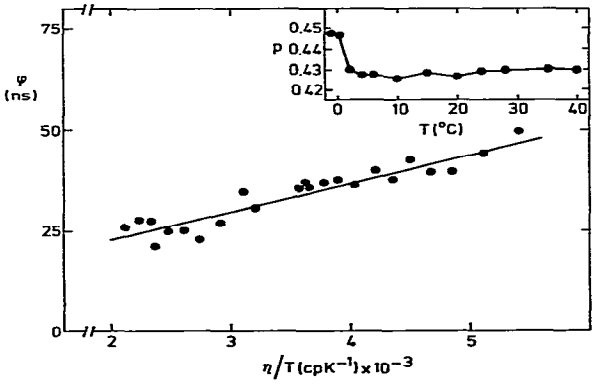

Fig. 6. Temperature dependence of the rotational correlation time of FAD in pig heart lipoamide dehydrogenase determined from differential polarized phase fluorometry. The straight line is the result of a linear regression analysis to the equation $\phi = 7.11 \times 10^3\ \eta/T + 8.7$. Inset: the course of the steady state degree of polarization (p) as a function of temperature.

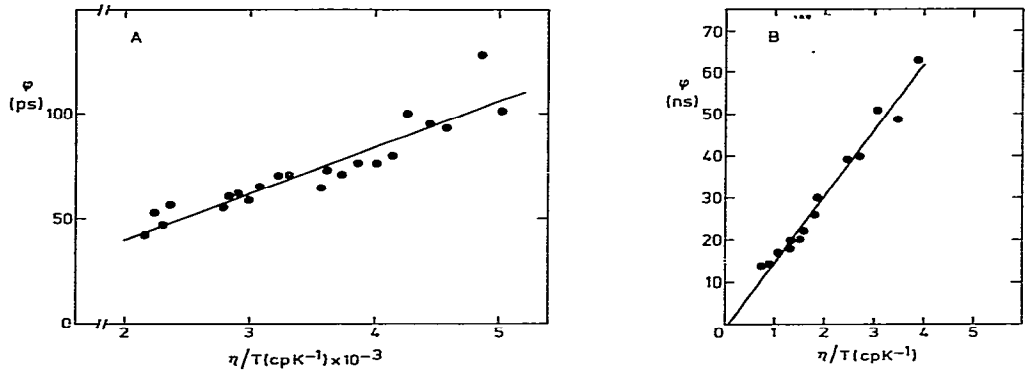

Fig. 7. Rotational correlation times of 3-methyllumiflavin obtained from differential polarized phase fluorometry. A. 10 μM in water, the straight line is a linear regression fit to the equation $\phi = 22.04\ \eta/T - 0.0039$; B. 10 μM in glycerol, the straight line is a fit to the equation $\phi = 15.68\ \eta/T - 1.07$. Details are mentioned in the text.

3.4. Rotational correlation times from experimental time-dependent anisotropy

The use of ps laser pulses for excitation of the flavin brings the determination of rapid rotational motion within the limits of experimental approach. Excitation with a pulse of linearly, vertically polarized light selects an ensemble of molecules with their absorption transition moments aligned along this direction. This photoselection disappears because of rotational Brownian motion of the molecules. The rate of randomization depends among others on the size and shape of the molecules and on the internal rotation occurring in the macromolecules. In order to estimate the lower limit of correlation times that can be measured with this technique anisotropy experiments were carried out with the cofactor FAD free in solution at 10°C. Fig. 8A shows the exciting laser pulse, the parallel and perpendicular polarized fluorescence components as a function of time. Directly after excitation there is a distinct difference in intensity between the two components, but the difference disappears in time as randomization oc-

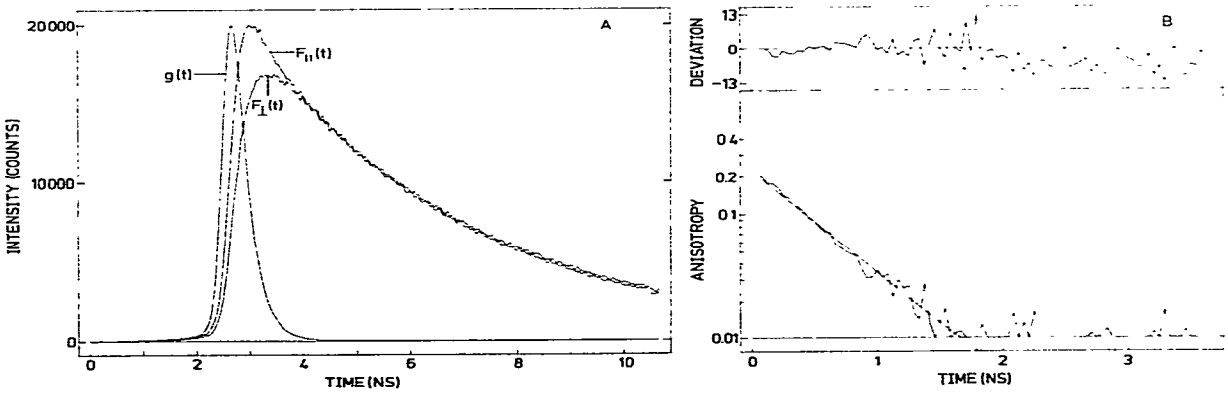

Fig. 8. Rotational correlation time of free FAD in solution (10 μM) at 10°C from laser induced polarized fluorescence decay. A. Laser pulse, parallel and perpendicular polarized emission components. B. Experimental anisotropy and fit with a single ϕ of 460 ps. The deviation function (upper curve) is defined as $(A_c(i) - A(i))/\sqrt{A(i)}$, where $A(i)$ is the content of the ith channel and $A_c(i)$ is the calculated value of this content.

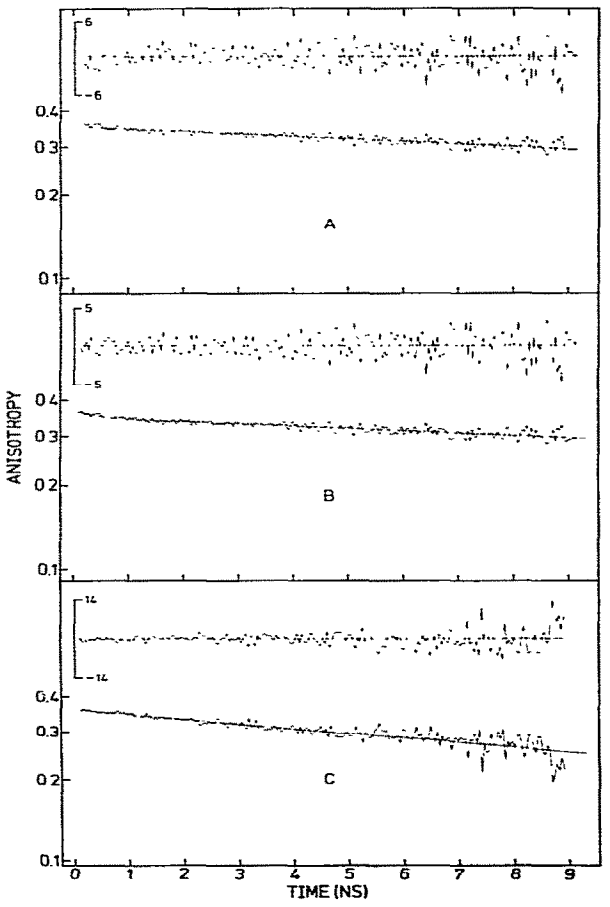

Fig. 9. Rotational correlation times of FAD in pig heart lipoamide dehydrogenase from experimental decay of anisotropy. A. Experimental anisotropy and fit with a single exponential, $T = 4°C$. B. same experimental data as in A, fit with a double exponential. C. a single exponential fit and experimental data obtained at 40°C. Deviation functions as in fig. 8. The values of the parameters are given in table 4.

curs by rapid Brownian rotation. The emission anisotropy can be constituted from the two components and the data obtained can be analyzed with the nonlinear least squares method to obtain the correlation times directly. The experimental anisotropy and the computed one assuming a single correlation time of 0.46 ns is given in fig. 8B. The analysis was started at a time coincident with the peak of the exciting pulse. Analysis of the experimental data was also performed starting at times later than the pulse, but this hardly affected the value of the correlation time. On top of the anisotropy data the deviation function has been displayed. This function fluctuates around the baseline showing that the motion is reasonably represented by a single correlation time.

The same experiments were carried out for FAD bound to lipoamide dehydrogenase. In this case a distinct difference between the two emission components also exists, however the difference decreases only slightly during the observation time of 12 ns. The emission anisotropy derived from the two components is given in fig. 9A at a temperature of 4°C. At temperatures up to about 10°C there is evidence for a rapid type of movement superimposed on the much slower rotational diffusion of the protein itself. The analysis of the curve shown in fig. 9A was performed assuming a single time constant for motion and one can notice that at times shortly after the pulse the fitting is not perfect. The same data set is again presented in fig. 9B and the smooth drawn curve is now obtained by a fit with two different correlation times. At increasing temperature the slopes corresponding to slow motion become less horizontal, resulting in shorter rotational correlation times. Since the amplitude of the rapid rotation is very small compared to the slower one and a relatively small number of channels is involved, the experimental anisotropy at higher temperatures cannot be resolved anymore into two contributions. Fitting with two components at temperatures $\geqslant 20°C$, always produced unequivocal results. To illustrate this the experimental curve obtained at 40°C and the single exponential fit to this curve are given in fig. 9C. Clearly a perfect fit is obtained The sharp drop in the steady state emission anisotropy (or polarization) between 0° and 2°C is already indicative for a rapid internal oscillation of the flavin molecule. Due to the improved time resolution of our system this internal movement can be made visible. The correlation times at several temperatures are collected in table 4. The single correlation times are slightly longer than the ones obtained by differential phase fluorometry, although they are of the same order of magnitude.

4. Discussion

The heterogeneity in lifetimes of flavin fluorescence

Table 4
Rotational correlation times from experimental anisotropy decay of pig heart lipoamide dehydrogenase

T (°C)	A_1	ϕ_1 (ns)	A_2	ϕ_2 (ns)	χ^2
4	0.34	53 ± 1			2.5
	0.018 ± 0.004	0.5 ± 0.2	0.332	57 ± 2	2.3
10	0.34	45 ± 1			3.8
	0.018 ± 0.004	0.5 ± 0.2	0.332	49 ± 2	3.7
20	0.35	42 ± 1			2.5
30	0.34	36 ± 1			3.8
40	0.34	26 ± 0.5			6.0

in lipoamide dehydrogenase as found earlier is also confirmed in this study using two different techniques for obtaining fluorescence lifetimes, namely the pulse method and the phase- and modulation method. The values of the long lifetime component and of the average lifetime at different temperatures obtained with these techniques are in good agreement. The short lifetime obtained from the phase method is however much shorter (in the order of 100 ps) and has a higher amplitude (cf. table 1). The reason that the results in this respect do not correspond is not known, but one important point should be emphasized. Only two modulation frequencies were employed and lifetimes were determined from measurements of the two phase shifts and of the relative decrease in modulation at 60 MHz. The accuracy of the results depends strongly on the real lifetime values, e.g. subnanosecond lifetimes can be measured far more precisely from the phase shift at the higher modulation frequency (60 MHz) [9]. Therefore no absolutely true values can be expected from these results. However, the results are internally consistent and are suitable for comparative purposes. It is to be preferred in order to obtain absolute values to investigate heterogeneous decay using phase shifts at a whole range of modulation frequencies in order to avoid possible artefacts like inappropriate weighting of values.

Comparing the lifetimes obtained from the fluorescence decay there is a slight discrepancy between the results from flash lamp excited fluorescence and the ones from laser induced fluorescence. The long lifetimes are in good agreement, whereas the short component is distinctly longer in those experiments, where for excitation into the second absorption band of the flavin a flash lamp was used. Internal conversion to the lowest excited singlet state is extremely rapid excluding this as a possible cause. However, the pulse from the flash lamp (FWHM = 2.5 ns) is much longer than the laser pulse seen by the system (FWHM = 0.5 ns) and also much longer than the shorter lifetime. The probable systematic lengthening of the short lifetime might therefore be related to the more rigorous deconvolution required to extract the decay parameters from flash lamp data. From the results presented in table 3 it is also clear that the relative amplitudes of a double exponential decay function show rather scattering values in the case of lamp excitation.

There is no doubt that the thermal quenching of the flavin fluorescence in lipoamide dehydrogenase is dynamic in nature, since the average lifetime is shortened at higher temperatures. The quenching is induced by collisional contact with neighbouring molecules during the lifetime of the flavin fluorescence. The rapid collisions that are required are caused by local structural fluctuations of the protein. The rapid mobility of protein structures have already been demonstrated with fluorescence techniques. Lakowicz and Weber employed oxygen quenching to find that even tryptophan residues buried in the interior of proteins are accessible to oxygen molecules [23]. Grinvald and Steinberg concluded to rapid relaxation reactions in chicken pepsinogen [24]. Bushueva et al., from thermal quenching of protein fluorescence [22] and Barboy and Feitelson from induce quenching in alcohol dehydrogenase from horse liver [25], also demonstrated the presence of structural fluctuations. All these studies were performed using the intrinsic probe tryptophan.

The flavin in lipoamide dehydrogenase can act as a natural fluorescence probe as well, although the fact that the dimeric enzyme contains two FAD molecules might complicate the interpretation somewhat. The average lifetime measured at room temperature in D_2O is about 1.2 times longer than the lifetime measured in aqueous solutions. This is to be expected from the slightly larger viscosity of D_2O, which diminishes the dynamic quenchi by other residues resulting in a slightly longer lifetime of flavin fluorescence. However an isotope effect on the excited state lifetime cannot be fully excluded. For instance, due to the long time of dialysis a proton of an amino acid residue located in a hydrophobic pocket could be exchanged for deuterium. It seems contradictory that the shape of the emission spectrum (fig. 5)

is characteristic for a flavin in a more or less apolar environment, while there is full exchange of H_2O and D_2O within the active site.

The heterogeneity in lifetimes was initially explained by the assumption that the dimeric protein contains two catalytic FAD sites in different environments, an explanation justified by the equal contributions to the fluorescence decay. Since in all results (cf. tables 1, 2 and 3) the amplitude ratio in a biexponential decay is not strictly unity one should consider alternative possibilities. Also the observations that lipoamide dehydrogenase bound in a multienzyme complex (pyruvate dehydrogenase complex) does not show in all cases equal weights to the two lifetime components makes this interpretation questionable [26]. The quenching residues in the protein move towards the flavin somewhat hindered because of the constraints imposed by the protein framework. The quenching itself might lead to departure from single exponential decay. A distribution of lifetimes instead of simply two components can then be expected. Another explanation for the nonexponential decay is the assumption of a reversible excited state reaction. Flavin in the excited state is electrophilic and rapid reversible electron transfer may occur between flavin and protein residues, e.g. a sulfhydryl, tryptophanyl, histidyl or tyrosyl moiety. In fact, these photoreactions have been demonstrated recently in laser induced photo-CIDNP experiments [27]. Explanations of this type for nonexponential decay have been proposed by Gafni and Brand [28] and Robinson et al. [29] for compounds other than flavins.

The mobility of the flavin itself can be determined from the time dependence of the emission anisotropy after laser pulse excitation. The resolution is in the ps time range as illustrated by the experiment with FAD in solution leading to a value of the rotational correlation time of about 450 ps. Differential polarized phase fluorometry also has ps time resolution, but the disadvantage is that the method is less direct, since one should determine in addition the degree of polarization and the average lifetime of the fluorescence as well. In a strict sense eqs. (7) and (8) are only applicable to a homogeneous population of fluorophores undergoing free isotropic rotations. We did not investigate the effect on these expressions of heterogeneity in lifetimes or the presence of hindered depolarizing rotations [18]. Both techniques give evidence that the FAD is mainly rigidly bound to the apoenzyme and rotates together with the whole protein. As an example the rotational correlation time measured at 20° is 42 ns (cf. table 4). From $\phi = M_r(\bar{v} + h)\eta/KT$ one gets a value of 38 ns for a 100 000 dalton hydrated spherical protein at 20°C, assuming $\bar{v} = 0.73$ cm^3/g and $h = 0.2$ cm^3/g. Experimental and calculated values are thus in very good agreement. Another important conclusion is that the rotation can be assigned to the entire dimeric protein and that there does not exist independent flexibility of the subunits in the nanosecond time range. In addition there is evidence that bound FAD exhibits some subnanosecond motion. The first indication of the latter effect comes from measurements of the (steady state) degree of polarization (p) as a function of temperature, where a sharp transition is visible between 0 and 2°C, while p remains further constant between 2 and 40°C (fig. 6). The sharp change might reflect a change in viscosity near FAD due to a different structure of the bound water molecules. Further evidence is provided by the time dependent emission anisotropy $A(t)$ at temperatures below 10°C, where a distinct rapid component can be discerned, although the amplitude is small relative to that corresponding with the overall rotation of the protein. The fast rotation can only be observed at the lower temperatures where the protein rotation is slowed down. One might argue that this fast component is due to some dissociated FAD, but this seems improbable since the dissociation constant of the FAD-apoenzyme complex is extremely low and suggests that under the conditions used all FAD is bound to the protein. Another factor to be considered is the occurrence of energy transfer between the two coenzyme molecules bound to the dimeric enzyme leading to a rapid initial depolarization. Although this possibility cannot be fully excluded, it seems improbable based on two grounds. The overlap between absorption and emission spectra necessary for efficient transfer is small resulting in a short R_0-value. When FAD is dissociated from lipoamide dehydrogenase in the pyruvate dehydrogenase complex from *E.coli*, recombination with a minimum amount of coenzyme (less than half of the sites occupied) resulted in the same rapid component in the decay of the emission anisotropy (A.J.W.G. Visser, unpublished observations). Similar subnanosecond motions have been observed recently by Munro et al. [30] from the anisotropy decay of proteins containing a single tryptophan residue. The short subnanosecond correlation time in these cases was interpreted to arise from a rapid rotation of a tryp-

tophan residue within a cone of a certain semiangle [30]. Using the same expression from reference [30] for rotation diffusion within a cone of semiangle α we find from the data in table 4 $\alpha = 8-9°$. This corresponds with a small but significant oscillation.

The importance of these rapid dynamic processes for enzyme catalysis is not quite ascertained. However, examples are accumulating in the literature, which emphasize the importance of these phenomena for the present knowledge of catalysis. McCammon et al. used molecular dynamics to simulate ps motion of tyrosin side chains in proteins [31]. The small amplitude motions are the results of either collisions or coupling of a large number of vibrations of protein residues. The fluctuations in structure produce a fluid and flexible system allowing easy accessibility of the solvent to interior groups. Cooper came to the important conclusion that discrete protein molecules (*not* ensembles of protein molecules) can undergo sizeable fluctuations in thermodynamic properties [3]. Transient conformational fluctuations are thus thermodynamically inevitable. Gavish has used the concept of structural fluctuations in a so-called transient strain model to explain enzymatic reactions [32,33]. In his view the dynamic state of an enzyme substrate complex can be described by structural fluctuations, whose randomness gives the complex a finite probability to reach a transient strain to overcome the energy barrier in the reaction. Two competing processes of the solvent and other molecules are important, namely random collisions, which increase the kinetic energy of various motional modes and frictional forces, which dissipate the energy again by viscous damping and reduce the amplitude of the fluctuations [32]. With respect to the active site of lipoamide dehydrogenase it can be stated that there are regions of considerable mobility present, which verifies the idea of Weber that also this protein is "a kicking and screaming stochastic molecule" [34].

Appendix

Phase and modulation fluorometry in the case of heterogeneous emission

It is the purpose of this section to give a short summary of the mathematical protocol necessary to obtain the parameters of a double exponential decay from phase and modulation fluorometry. The definitions and procedures as outlined by Jablonski are followed [35].

A decay ("probability") function can be defined as:

$$P(t) = I(t) \Big/ \int_0^\infty I(t)\,dt, \tag{A.1}$$

where $I(t)$ is the intensity of the emitted light as a function of time created by an infinitely short light pulse. $P(t)$ is normalized:

$$\int_0^\infty P(t)\,dt = 1. \tag{A.2}$$

An average time or lifetime can be defined as:

$$\langle\tau\rangle = \int_0^\infty P(t)\, t\, dt. \tag{A.3}$$

For example, if $I(t)$ follows a single exponential function ($I(t) = e^{-t/\tau}$) one obtains:

$$P(t) = (1/\tau)e^{-t/\tau} \rightarrow \langle\tau\rangle = \tau. \tag{A.4}$$

Or in general, if $I(t)$ is a sum of exponential terms ($I(t) = \Sigma_{i=1}^{n} \alpha_i e^{-t/\tau_i}$):

$$P(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \Big/ \sum_{i=1}^{n} \alpha_i \tau_i$$

$$\rightarrow \langle\tau\rangle = \sum_{i=1}^{n} \alpha_i \tau_i^2 \Big/ \sum_{i=1}^{n} \alpha_i \tau_i. \tag{A.5}$$

Jablonski [35] has shown that upon excitation with modulated light (ω = angular frequency of modulation) the time dependence of the fluorescence intensity is given by:

$$I(t) = \int_0^\infty f(t - t')\, P(t')\, dt', \tag{A.6}$$

where $f(t - t')$ is the pumping function:

$$f(t - t') = A + B \cos\{(\omega t - t')\}, \tag{A.7}$$

with $A \geqslant B \geqslant 0$ and the degree of modulation of the exciting light $M' = B/A$.

After integration and rearrangement the following time dependence of the fluorescence intensity is ob-

tained:

$$I(t) = A + b\cos(\omega t - \theta), \tag{A.8}$$

where

$$\theta = \arctan(\langle\sin\omega t\rangle/\langle\cos\omega t\rangle), \tag{A.9}$$

and

$$b = B[\langle\cos\omega t\rangle^2 + \langle\sin\omega t\rangle^2]^{1/2}. \tag{A.10}$$

$\langle\sin\omega t\rangle$ and $\langle\cos\omega t\rangle$ are mean values, averaged over the decay function, e.g.:

$$\langle\sin\omega t\rangle = \int_0^\infty P(t)\sin\omega t\,dt. \tag{A.11}$$

It can be shown from eqs. (A.4), (A.9), (A.10) and (A.11) that for a single exponential decay with lifetime τ the phase shift (θ) and the degree of modulation of the fluorescence ($M = b/A$) are related by:

$$\theta = \arctan\omega\tau \rightarrow \tau_p = (\tan\theta)/\omega, \tag{A.12}$$

and

$$D = M/M' = (1+\omega^2\tau^2)^{-1/2} \rightarrow \tau_m = (1/D^2 - 1)^{1/2}/\omega, \tag{A.13}$$

in which τ_p is the apparent lifetime as measured from the phase shift and τ_m is the apparent lifetime as obtained from a decrease in the degree of modulation (demodulation D). For a single decay process $\tau_p = \tau_m$.

In the case that the time course of the fluorescence follows a double exponential decay law (eq. (A.5) with $n = 2$) the expressions for τ_p and τ_m become (cf. eqs. (A.5), (A.9), (A.10) and (A.11):

$$\tau_p = \frac{\tan\theta}{\omega} = \frac{R\tau_1^2/(1+\omega^2\tau_1^2) + \tau_2^2/(1+\omega^2\tau_2^2)}{R\tau_1/(1+\omega^2\tau_1^2) + \tau_2/(1+\omega^2\tau_2^2)}. \tag{A.14}$$

R is the amplitude ratio α_1/α_2, τ_m is given by eq. (A.13), but D follows now from:

$$D = \frac{1}{R\tau_1 + \tau_2}\{[R\tau_1/(1+\omega^2\tau_1^2) + \tau_2/(1+\omega^2\tau_2^2)]^2 + \omega^2[R\tau_1^2/(1+\omega^2\tau_1^2) + \tau_2^2/(1+\omega^2\tau_2^2)]^2\}^{1/2}. \tag{A.15}$$

It is obvious that the double exponential function consists of three undetermined parameters, namely R, τ_1 and τ_2. In principle three different lifetime determinations are necessary to solve these parameters from three nonlinear equations. In our phase fluorometer only two modulation frequencies were employed, namely 15 and 60 MHz. Since the lifetimes to be determined are within the region of 0.1–6.0 ns, it was decided to measure τ_p and τ_m at 60 MHz and τ_p at 15MHz. The inaccuracy for τ_m at 15 MHz is too large for lifetimes < 2 ns and cannot be used [9]. With the values of τ_p (60 MHz), τ_p (15 MHz) and τ_m (60 MHz) the parameters R, τ_1 and τ_2 were obtained from a solution of three non-linear equations as given by eqs. (A.14) and (A.15). A computer program was written to obtain the real solutions. In the range of 0.5–5 ns the values of the dispersion terms $\omega^2\tau^2$ are collected in table A.1. Inspection of table A1 learns that at 15 MHz $\omega^2\tau^2 < 1$ for both lifetimes 0.5 and 5.0 ns. If $\omega^2\tau^2$ is neglected with respect to unity eq. (A.14) simplifies to eq. (A.5) for the second order averaged lifetime.

Table A1
τ versus $\omega^2\tau^2$ for two modulation frequencies

τ (ns)	$\omega^2\tau^2$	
	ν = 15 MHz	ν = 60 MHz
0.5	2.22×10^{-3}	3.55×10^{-2}
2.0	3.55×10^{-2}	5.68×10^{-1}
5.0	2.22×10^{-1}	3.55

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