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Thermal stability of a flavoprotein assessed from associative analysis of polarized time-resolved fluorescence spectroscopy

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Abstract Upon gradually heating a particular mutant of the flavoprotein NADH peroxidase, it was found from the peculiar time-resolved fluorescence anisotropy pattern of the flavin prosthetic group (FAD) that, at elevated temperature, FAD is released from the tetrameric enzyme. Since in this case a mixture of free and enzyme-bound FAD contributes to the time-dependent fluorescence anisotropy, its analysis can only be accomplished by an associative fitting model, in which specific fluorescence lifetimes of both species are linked to specific correlation times. In this letter the general approach to the associative polarized fluorescence decay analysis is described. The procedure can be used for other flavoproteins to determine the temperature at which the onset of thermal denaturation will start, leading to release of the flavin prosthetic group.

Key words Time-resolved fluorescence · Fluorescence anisotropy decay · Global analysis · Associative fitting model

Introduction

Time-resolved polarized fluorescence spectroscopy has been of great value to study the dynamical properties of tryptophan-containing proteins (Beechem and Brand 1985; Demchenko 1992; Millar 1996). Other fluorescent proteins are flavoproteins containing the naturally fluorescent flavin prosthetic group such as FMN or FAD as the reporting molecule. By using time-resolved fluorescence spectroscopy we have previously investigated a number of flavoproteins, from which very specific molecular properties of the flavin active site could be derived (Bastiaens et al. 1992a, b, c; Leenders et al. 1993a, b; Visser et al. 1997; van den Berg et al. 1998). Very recently, we have investigated the time-resolved fluorescence properties of FAD in tetrameric NADH peroxidase (Npx) from *Enterococcus faecalis* and three mutant enzymes (Visser et al. 1998). By using an Y159A mutant enzyme it turned out that Tyr159, which is in van der Waals contact with the flavin, has a significant influence on the photophysical properties of the flavin. Tyr159 is responsible for very efficient quenching, leading to a very prominent flavin fluorescence lifetime of picosecond duration. The role of Tyr159 may be the stabilization of the active site of the free enzyme. Support for this idea came from two experiments (Visser et al. 1998). Firstly, the Y159A Npx was observed to lose activity on prolonged storage at 253 K. The second experiment is connected to the temperature dependence of the time-resolved fluorescence anisotropy of Y159A Npx. The time-dependent fluorescence anisotropy showed at 313 K a very rapid decay arising from dissociated FAD and an apparent rise, which is due to FAD still bound to the tetrameric enzyme.

In this letter we present an approach how to analyze this peculiar time-dependent anisotropy pattern, whose presence has been previously described in proteins, membranes and proteolipid systems (Ludescher et al. 1987; van Paridon et al. 1988; Lee et al. 1990; Peng et al. 1990a, b). We used the Y159A mutant of Npx as an illustrative example, since the time dependence of both total fluorescence and anisotropy is much simpler than for the wild-type enzyme.

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Experimental

The preparation and characterization of the *E. faecalis* Y159A Npx mutant, as well as the experimental setup for time-resolved polarized fluorescence, has been detailed previously (van den Berg et al. 1998; Visser et al. 1998). The enzyme was dissolved (concentration 10 μM) in 50 mM potassium phosphate (pH 7.0) plus 0.6 mM EDTA. FAD, obtained from Sigma, was dissolved (concentration 10 μM) in 50 mM potassium phosphate (pH 7.5). The excitation wavelength was 460 nm and the emission wavelength was selected by a cutoff filter (550 nm) and a line filter (558 nm) with a 12 nm band width.

Data analysis

Models

Fluorescence $I(t)$ and anisotropy $r(t)$ decay curves were analyzed by a sum of exponential terms:

$$I(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i) \quad (1)$$

$$r(t) = \sum_{i=1}^{M} \beta_i \exp(-t/\phi_i) \quad (2)$$

where A_i and τ_i are, respectively, the amplitude and lifetime of the ith component of fluorescence, β_i and ϕ_i are, respectively, the initial anisotropy and rotational correlation time of the ith component of anisotropy, and N and M are, respectively, the number of fluorescent and anisotropy components.

As long as all components of the fluorescence contribute to the anisotropy, the analysis can be performed in a non-associative fashion, in which parallel $I_{||}(t)$ and perpendicular $I_{\perp}(t)$ intensity components take the following form (Lakowicz 1983):

$$I_{||}(t) = I(t)[1 + 2r(t)] \quad (3)$$

$$I_{\perp}(t) = I(t)[1 - r(t)] \quad (4)$$

In the case of Y159A Npx at one particular temperature (313 K), one has a mixture of anisotropies arising from free and bound FAD, which can be analyzed using an associative model for the time-resolved anisotropy: each decay component of fluorescence is linked to a particular decay component of the anisotropy. In the latter case, parallel $I_{||}(t)$ and perpendicular $I_{\perp}(t)$ components of the fluorescence intensity can be written as (Lakowicz 1983):

$$I_{||}(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i)\left\{1 + 2\sum_{j=1}^{M} t_{ij}\beta_j \exp(-t/\phi_j)\right\} \quad (5)$$

$$I_{\perp}(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i)\left\{1 - \sum_{j=1}^{M} t_{ij}\beta_j \exp(-t/\phi_j)\right\} \quad (6)$$

where $t_{ij} = 1$, if fluorescence and anisotropy components are associated, and $t_{ij} = 0$, if not.

Methods

Data analysis was performed with the Fluorescence Data Processor (FDP) software developed in the Systems Analysis Department, Belarusian State University (Minsk, Belarus). The temperature dependence of the total fluorescence and anisotropy decays can be visualized by three-dimensional graphics of the data provided by the FDP software.

The parameters of the fluorescence and anisotropy decays, measured in the low temperature region (277–303 K), were obtained by a global approach (Beechem 1992) provided by the FDP software. According to this approach, the fluorescence decays of the Y159A Npx sample measured at different temperatures were simultaneously fitted. The nonassociative model [described by Eqs. (3) and (4)] with the linked fluorescence and anisotropy decay times was applied. For the experiment at the highest temperature (313 K), analysis has been performed separately using the associative model [described by Eqs. (5) and (6)] for the anisotropy.

The fitting procedure consists of two steps. First, the total fluorescence decays were analyzed and the fluorescence decay parameters were obtained. In a second step the anisotropy decay parameters were estimated subsequently by simultaneously fitting of parallel and perpendicular polarized components of the fluorescence (van Hoek et al. 1987) with fluorescence parameters fixed to the values obtained after the first step. For the associative model, this procedure should be concluded by a third step with all parameters (arising from both total fluorescence and anisotropy) being adjustable.

The error estimation of the recovered parameters has been performed by the exhaustive search method (Beechem 1992). The goodness of fit was judged by the χ^2 statistical criterion and by visual inspection of the time dependence of weighted residuals and their autocorrelation functions.

Results

The analysis in the low temperature region (277–303 K) was performed in terms of four fluorescence components ($N = 4$) and one anisotropy component ($M = 1$). The results of the analysis are presented in Table 1. Typical examples of the fits are shown in Fig. 1 for the total fluorescence decay and in Fig. 2 for the anisotropy decay (for reasons of clarity the time range of all graphs was reduced from 30 ns to 20 ns). Despite the fact that

Table 1 Estimated fluorescence and anisotropy decay parameters of Y159A Npx and their confidence intervals at the 67% level as a function of temperature in the range 277–303 K

Temperature	Fluorescence				Anisotropy
	Global lifetimes				
	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	ϕ_1 (ns)
	5.77 5.24; 6.82	3.35 3.25; 3.48	0.93 0.83; 1.06	0.017 0.016; 0.019	104 85; 129
	Temperature dependence of amplitudes				
	A_1	A_2	A_3	A_4	β_1
277 K	0.020 0.009; 0.035	0.20 0.18; 0.21	0.040 0.038; 0.048	0.74 0.28; 0.96	0.37 0.36; 0.37
283 K	0.018 0.008; 0.031	0.19 0.18; 0.20	0.042 0.038; 0.047	0.75 0.31; 0.95	0.36 0.36; 0.37
293 K	0.014 0.006; 0.026	0.175 0.17; 0.19	0.041 0.037; 0.046	0.77 0.23; 0.97	0.36 0.35; 0.36
303 K	0.010 0.004; 0.018	0.15 0.14; 0.16	0.047 0.042; 0.051	0.79 0.35; 1.03	0.34 0.34; 0.35

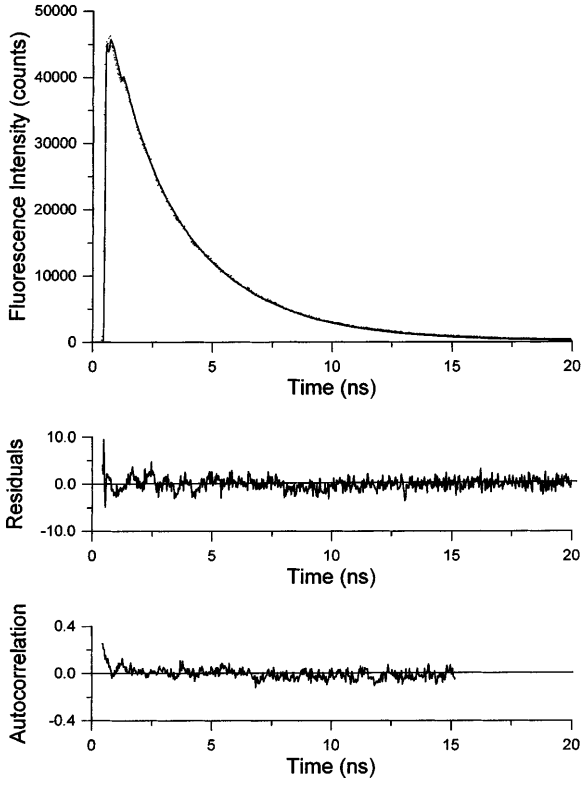

Fig. 1 Experimental (*points*) and fitted (*solid line*) curves of total fluorescence decay of Y159A Npx measured at 303 K. The small dips close to the maximum intensity arise from reflections in the emission light path as explained by Kroes et al. (1998)

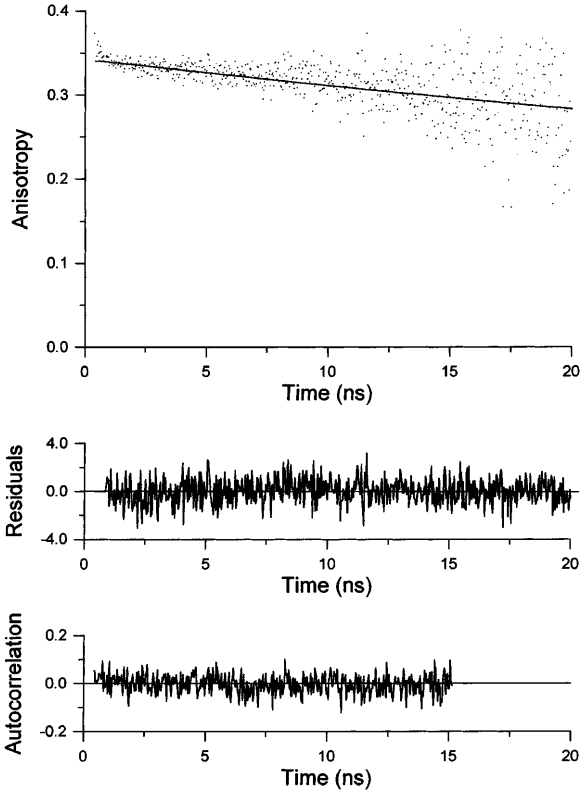

Fig. 2 Experimental (*points*) and fitted (*solid line*) curves of fluorescence anisotropy decay of Y159A Npx measured at 303 K

the rotational correlation time ϕ_1 was linked and kept constant over all temperatures, it should slightly decrease because of the decrease of water viscosity and the increase of temperature. Assuming a globular shape of the Npx tetramer, a correlation time ϕ_1 of 132 ns at 277 K and of 80 ns at 303 K can be expected (Visser et al. 1998). Since the value of ϕ_1 is about 2–4 times longer than the total detection range (30 ns), it is therefore hardly possible to confidently recover such dependence. Indeed, the confidence limits of rotational correlation times span the extreme values of ϕ_1 between 277 and 303 K. A tiny contribution of a rapid component is observed in the anisotropy decay at 303 K (see Fig. 2), which is absent in the decays at lower temperatures. This rapid component must be due to a tiny fraction of dissociated FAD (see below).

For the highest temperature (313 K), the fit results are presented in Table 2. A good fit quality is achieved only by using five exponentials ($N=5$) for the fluorescence and two exponentials for the anisotropy ($M=2$) (see Fig. 3 for the total fluorescence and Fig. 4 for the anisotropy; again for clarity reasons the graphs are shown until 20 ns). The extra components in the total fluorescence and anisotropy decays can be explained by the presence of free FAD. This conclusion is supported by analysis of the experimental data of free FAD measured at 313 K (Table 3). The total fluorescence decay of FAD contains four exponential components. Two of them (with lifetimes of about 4.89 ns and 1.09 ns) fall in the confidence interval of the exponential components of Npx (lifetimes of about 5.77 ns and 0.93 ns). The only nonoverlapping confidence interval is for the shortest lifetimes of Npx and free FAD (0.017 ns and 0.014 ns, respectively). However, these lifetimes cannot be precisely retrieved, since they are twice less than the used time channel width of 30 ps. Furthermore, they hardly contribute to the slower anisotropy decay. In the near future we will investigate this analysis of picosecond fluorescence decay times using simulated decay data with an experimental instrumental response function. Only one lifetime component is clearly distinct, namely 2 ns for free FAD and 3 ns for Npx. The fluorescence

Table 2 Estimated fluorescence and anisotropy decay parameters of Y159A Npx and their confidence intervals at the 67% level at 313 K[a]

Component 1					Component 2	
Fluorescence				Anisotropy	Fluorescence	Anisotropy
τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	ϕ_1 (ns)	τ_1 (ns)	ϕ_1 (ns)
5.04	3.11	0.71	0.020	89	1.62	0.11
4.29; 5.58	2.80; 3.6	0.51; 0.93	0.017; 0.036	37; –	1.46; 1.85	0.07; 0.20
A_1	A_2	A_3	A_4	β_1	A_1	β_1
0.012	0.10	0.038	0.75	0.32	0.10	0.36
0.001; –	0.06; 0.12	0.024; 0.049	0.19; 1.09	0.3; –	0.08; 0.12	0.13; –

[a] The upper bound (marked with –) of anisotropy parameters is not defined in some cases

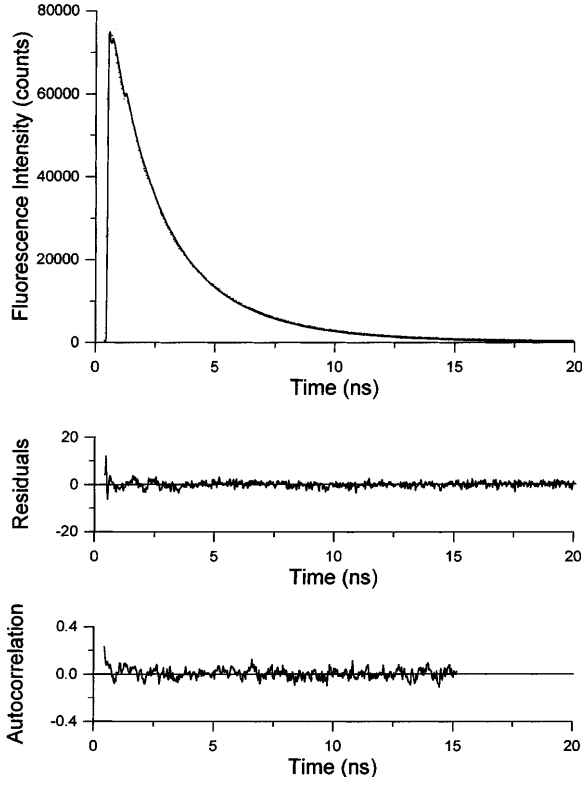

Fig. 3 Experimental (*points*) and fitted (*solid line*) curves of total fluorescence decay of Y159A Npx measured at 313 K. Further details are given in the legend of Fig. 1

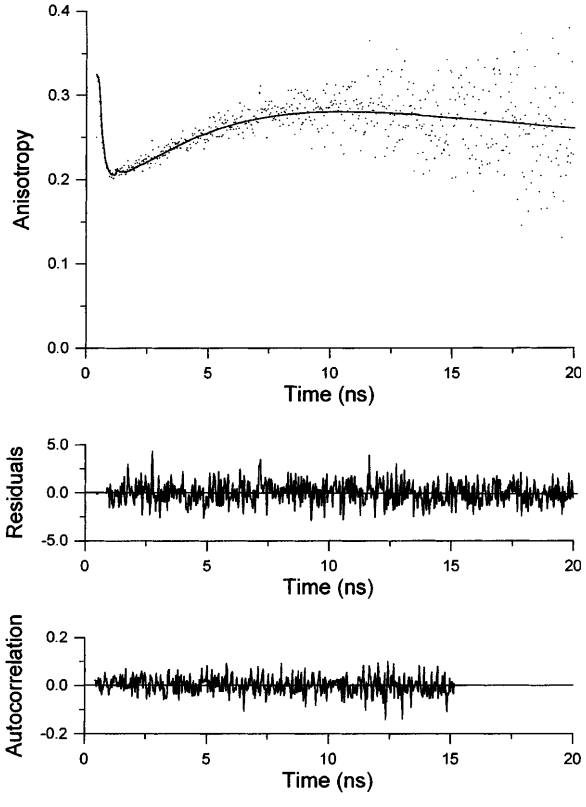

Fig. 4 Experimental (*points*) and fitted (*solid line*) curves of time dependence of fluorescence anisotropy of Y159A Npx measured at 313 K

Table 3 Estimated fluorescence and anisotropy decay parameters of free FAD and their confidence intervals at the 67% level at 313 K[a]

Fluorescence				Anisotropy	
τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	ϕ_1 (ns)	ϕ_2 (ns)
4.89	2.06	1.09	0.014	0.10	18.9
4.34; 7.15	1.74; 2.73	0.69; 1.33	0.012; 0.015	0.08; 0.13	2.3; –
A_1	A_2	A_3	A_4	β_1	β_2
0.015	0.11	0.065	0.81	0.34	0.009
0.004; 0.024	0.07; 0.15	0.03; 0.12	0.67; 1.27	0.29; –	0.005; 0.012

[a] The upper bound (marked with –) of anisotropy parameters is not defined in some cases

anisotropy decay of free FAD can be well approximated by a single exponential with a rotational correlation time of 0.1 ns. This correlation time is much shorter than that of the bound one of ca. 90 ns. The presence of "free FAD" parameters (2.3 ns average lifetime and 0.1 ns correlation time) was clearly observed in the fluorescence data of Npx at 313 K (see Table 2).

In a previous study (Visser et al. 1997) the different fluorescence lifetimes could be clearly associated to the different rotational correlation times of free and protein-bound flavins. Owing to the fact that the fluorescence lifetimes of free and bound FAD in Y159A Npx are highly coincident, it is impossible to directly determine the molar fractions of free (f_1) and bound (f_2) FAD. Therefore an approximation proposed by Lee et al. (1992) is made, based on the knowledge of average anisotropies and lifetimes. The average anisotropies can be obtained by integration of the experimental time-dependent anisotropies. Furthermore, free and bound FAD can be characterized by second-order average fluorescence lifetimes (Peng et al. 1990a). The equation derived by Lee et al. (1992) is:

$$f_1 = (\langle r\rangle - \langle r_2\rangle)/[R(\langle r_1\rangle - \langle r\rangle) + \langle r\rangle - \langle r_2\rangle] \quad (7)$$

where R is the ratio of the fluorescence quantum yields of free and bound FAD, approximated by the ratio of their average lifetimes. Analysis showed that the average fluorescence lifetime of free FAD at 313 K is 2.34 ns and that of Npx at 303 K (where FAD is still bound) is 3.32 ns. Substituting the observed values $\langle r\rangle = 0.25$, $\langle r_1\rangle = 0.012$, $\langle r_2\rangle = 0.30$ and $R = 0.70$ into Eq. (7) gives $f_1 = 0.23$ and therefore $f_2 = 0.77$.

Conclusions

Time-resolved fluorescence anisotropy is, in contrast to its steady-state counterpart, a powerful method to determine the thermal denaturation temperature of flavoproteins. The flavin prosthetic group is bound to its protein by a superposition of forces arising from hydrogen bonding, hydrophobic and electrostatic interactions (regarding the latter interaction, note that the two phosphate groups of FAD are negatively charged). When the protein is denatured, these interactions will be broken and the flavin prosthetic group is dissociated. Release of the flavin prosthetic group from the protein leads to a rapid decay component in the anisotropy-time profile. In contrast, the onset of the rapid component will not directly show up in steady-state fluorescence anisotropy (see Fig. 2). The apparent rise in the anisotropy (see Fig. 4) is due to the fact that two distinct species are present under equilibrium conditions: free FAD and FAD bound to the Npx mutant enzyme. Each species has its own fluorescence decay times and rotational correlation time, although in this particular case only one fluorescence lifetime component is clearly different, as well as are both correlation times. The relative amount of free and enzyme-bound FAD can only be recovered by an associative analysis of the fluorescence anisotropy decay, in which the distinct fluorescence lifetime is associated to its characteristic correlation time.

Finally, a physiological argument should be addressed. The bacteria producing the Y159A Npx protein were grown at 310 K. It is doubtful that much FAD dissociation is going on in vivo. All the purification steps were carried out at 277 K. Therefore the FAD dissociation observed at 313 K is related to the in vitro situation.

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