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.

Keywords: time-resolved fluorescence, fluorescence anisotropy decay, global analysis, associative fitting model.

1. Introduction

Time-resolved polarized fluorescence spectroscopy has been of great value to study 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 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. 1992 a,b,c; Leenders et al. 1993 a,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 a 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 fluorescence lifetime in the picosecond range of the flavin. In addition, the mere presence of Tyr159 also results in a nanosecond fluorescence depolarization process of the flavin which was attributed to excited-state charge transfer yielding an out-of-plane motion of the flavin transition moment. At this stage one can only speculate about the role of this particular tyrosine residue (which is also present in several other flavoproteins with related function). One role 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 loose 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, which presence has been previously described in proteins, membranes and proteolipid

systems (Ludescher et al. 1987; Lee et al. 1990; van Paridon et al. 1988; Peng et al. 1990 a,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.

2. Experimental

The preparation and characterization of the Y159A Npx mutant from *Enterococcus faecalis*, as well as the experimental setup for time-resolved polarized fluorescence, has been detailed previously (Visser et al. 1998). The enzyme was dissolved in 50 mM potassium phosphate pH 7.0 plus 0.6 mM EDTA (concentration 10 μM). FAD, obtained from Sigma, has been dissolved in 50 mM potassium phosphate pH 7.5 (concentration 10 μM). 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 12 nm band width.

3. Data Analysis

3.1. Models

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

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

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

where A_i and t_i are, respectively, the amplitude and lifetime of the *i*-th component of fluorescence, b_i and f_i are, respectively, the initial anisotropy and rotational correlation time of the *i*-

th component of anisotropy, 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 $I_{\wedge}(t)$ 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 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 time-resolved anisotropy: each decay component of fluorescence is linked to a particular decay component of anisotropy. In the latter case, parallel $I_{||}(t)$ and perpendicular $I_{\wedge}(t)$ components of fluorescence intensity can be written as (Lakowicz 1983):

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

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

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

3.2. 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 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 non-associative 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 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 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.

4. Results

The analysis in the low temperature region (277-303K) was performed in terms of four fluorescence components *(N=4)* and one anisotropy component *(M=1)*. The results of analysis are presented in Table 1 (277-303K). Typical examples of the fits are shown in figure 1 for the total fluorescence decay and in figure 2 for the anisotropy decay (for clarity the time range of all graphs was reduced from 30 ns to 20 ns). Despite the fact that the rotational correlation time f_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 f_1 of 129 ns at 277 K and of 85 ns at 303 K can be expected (Visser et al. 1998). Since the value of f_1 is about two to four times longer than the total detection range (30 ns) it is therefore hardly possible to confidently recover such dependence. A tiny contribution of a rapid component is observed in the anisotropy decay at 303 K (see figure 2), which is absent in the decays at lower temperatures. This rapid component must be due to a tiny fraction of dissociated FAD (*vide infra*).

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 fluorescence and two exponentials for anisotropy *(M=2)* (see figure 3 for total fluorescence and figure 4 for anisotropy; again for clarity 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 the analysis of 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 non-overlapping confidence interval is for the shortest lifetimes of Npx and free FAD (0.017 ns and 0.014 ns, respectively). However, these lifetimes can not 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. Only one lifetime component is clearly distinct, namely 2 ns for free FAD and 3 ns for Npx. The fluorescence anisotropy decay of free FAD can be well approximated by a single exponential with 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 both “free FAD” parameters (2 ns lifetime and 0.1 ns correlation time) was clearly observed in the fluorescence data of Npx at 313 K (see Table 2).

Due to the fact that the fluorescence lifetimes of free and bound FAD are highly coincident, it is impossible to directly determine the molar fractions of free (f_1) and bound (f_2) FAD. A simplification can be made by assuming that free and bound FAD are characterized by single "average" fluorescence lifetimes (Peng et al. 1990a), each one being linked to a specific correlation time. The contributions of the corresponding fluorescence components will then yield the approximate fractions of free and bound FAD. Analysis showed that the average lifetime of free FAD at 313 K is ca. 2.0 ns and the one of Npx at 303 K (where FAD is still bound) is ca. 2.4 ns. The ratio of fractions of free and bound FAD is then f_1/f_2 = 1.0/2.3 yielding a molar fraction of free FAD f_1 = 0.3. Another approach has been proposed by Lee et al. (1992) and utilizes the steady-state anisotropies, which can be obtained by integration of the experimental anisotropies. The equation 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 fluorescence quantum yields, which can be approximated by the ratio of average lifetimes. Substituting the observed values $\langle r \rangle$ = 0.25, $\langle r_1 \rangle$ = 0.012, $\langle r_2 \rangle$ = 0.30 and R = 0.83 into eq. 7 gives f_1 = 0.2 which is in fair agreement with the value that is more roughly estimated from the average lifetimes.

5. Discussion

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, the 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 figure 2). The apparent rise in the anisotropy (see figure 4) is due to the fact that two distinct species are present: 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.

Although it is not demonstrated in this investigation, the denaturation process can be followed in detail by measurement of the time-resolved polarized fluorescence at a series of temperatures below and above the denaturation temperature. It would then be possible to investigate whether (or not) the dissociation of FAD from the tetrameric protein occurs in a highly cooperative manner. In addition, the free energy of denaturation can be established by implementing a particular thermodynamic scheme of denaturation. By gradually cooling down the protein solution and monitoring the time-resolved anisotropy, one can also establish whether (reversible) renaturation takes place at the same temperature. These experimental and analytical protocols are, of course, not confined to Npx, but can be carried out to each flavoprotein. The approach will be particularly useful to compare the denaturation temperatures of wild-type and mutant flavoproteins.

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.

6. Acknowledgments

We thank Nina Visser for providing the experimental data of the mutant NADH peroxidase and Petra van den Berg for providing the experimental data of FAD at 313 K prior to publication. Fellowships from Wageningen Agricultural University (to A.V.D. and V.V.S.) are gratefully acknowledged. This research was supported by The Netherlands Foundation for Chemical Research, with financial aid from The Netherlands Organization for Scientific Research (A.J.W.G.V.), by National Institutes of Health Grant GM-35394 (A.C.) and by National Science Foundation Grant INT-9400123 (A.C.).

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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.

	Fluorescence				**Anisotropy**
	Global lifetimes				
	t_1	t_2	t_3	t_4	f_1
	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				
Temperature	**A_1**	**A_2**	**A_3**	**A_4**	b_1
277 K	0.035 0.016;0.062	0.34 0.32;0.36	0.075 0.066;0.083	1.29 0.48;1.67	0.37 0.36;0.37
283 K	0.031 0.014;0.055	0.34 0.32;0.35	0.074 0.066;0.082	1.31 0.54;1.67	0.36 0.36;0.37
293 K	0.027 0.011;0.049	0.33 0.31;0.34	0.077 0.069;0.085	1.43 0.43; 1.80	0.36 0.35;0.36
303 K	0.020 0.009;0.038	0.31 0.30;0.31	0.097 0.087;0.105	1.64 0.72;2.13	0.34 0.34;0.35

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

Y159A Npx						
Component 1					**Component 2**	
Fluorescence				**Anisotropy**	**Fluorescence**	**Anisotropy**
t_1	t_2	t_3	t_4	f_1	t_1	f_1
5.04 4.29;5.58	3.11 2.80;3.6	0.71 0.51;0.93	0.020 0.017;0.036	89 37; -	1.62 1.46;1.85	0.11 0.07;0.20
A_1	A_2	A_3	A_4	b_1	A_1	b_1
0.023 0.001; -	0.17 0.10; 0.19	0.062 0.039;0.080	1.23 0.32;1.79	0.32 0.3; -	0.16 0.13;0.20	0.36 0.13; -

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

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

free FAD					
Fluorescence				**Anisotropy**	
t_1	t_2	t_3	t_4	f_1	f_2
4.89 4.34;7.15	2.06 1.74;2.73	1.09 0.69;1.33	0.014 0.012;0.015	0.10 0.08;0.13	18.9 2.3; -
$\mathbf{A_1}$	$\mathbf{A_2}$	$\mathbf{A_3}$	$\mathbf{A_4}$	b_1	b_2
0.031 0.008;0.049	0.22 0.14;0.30	0.15 0.06;0.25	1.66 1.38;2.61	0.34 0.29; -	0.009 0.005;0.012

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

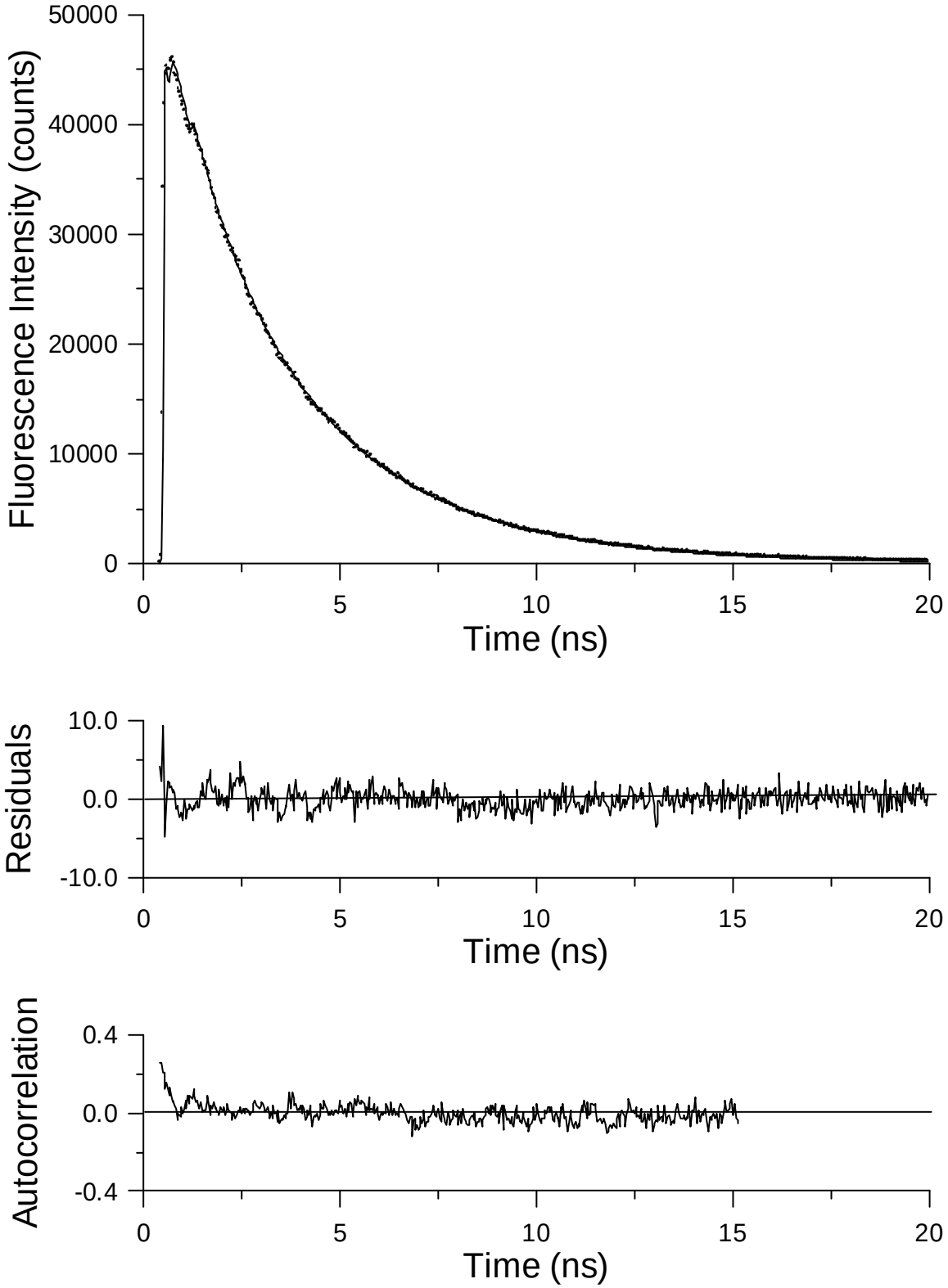
Legends to Figures

Fig. 1. Experimental (points) and fitted (solid line) curves of total fluorescence decay of Y159A Npx measured at 303 K.

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

Fig. 3. Experimental (points) and fitted (solid line) curves of total fluorescence decay of Y159A Npx measured at 313 K.

Fig. 4. Experimental (points) and fitted (solid line) curves of time-dependence of fluorescence anisotropy of Y159A Npx measured at 313 K.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20000
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10
15
20
Time (ns)
Residuals
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0.0
-10.0
0
5
10
15
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Time (ns)
Autocorrelation
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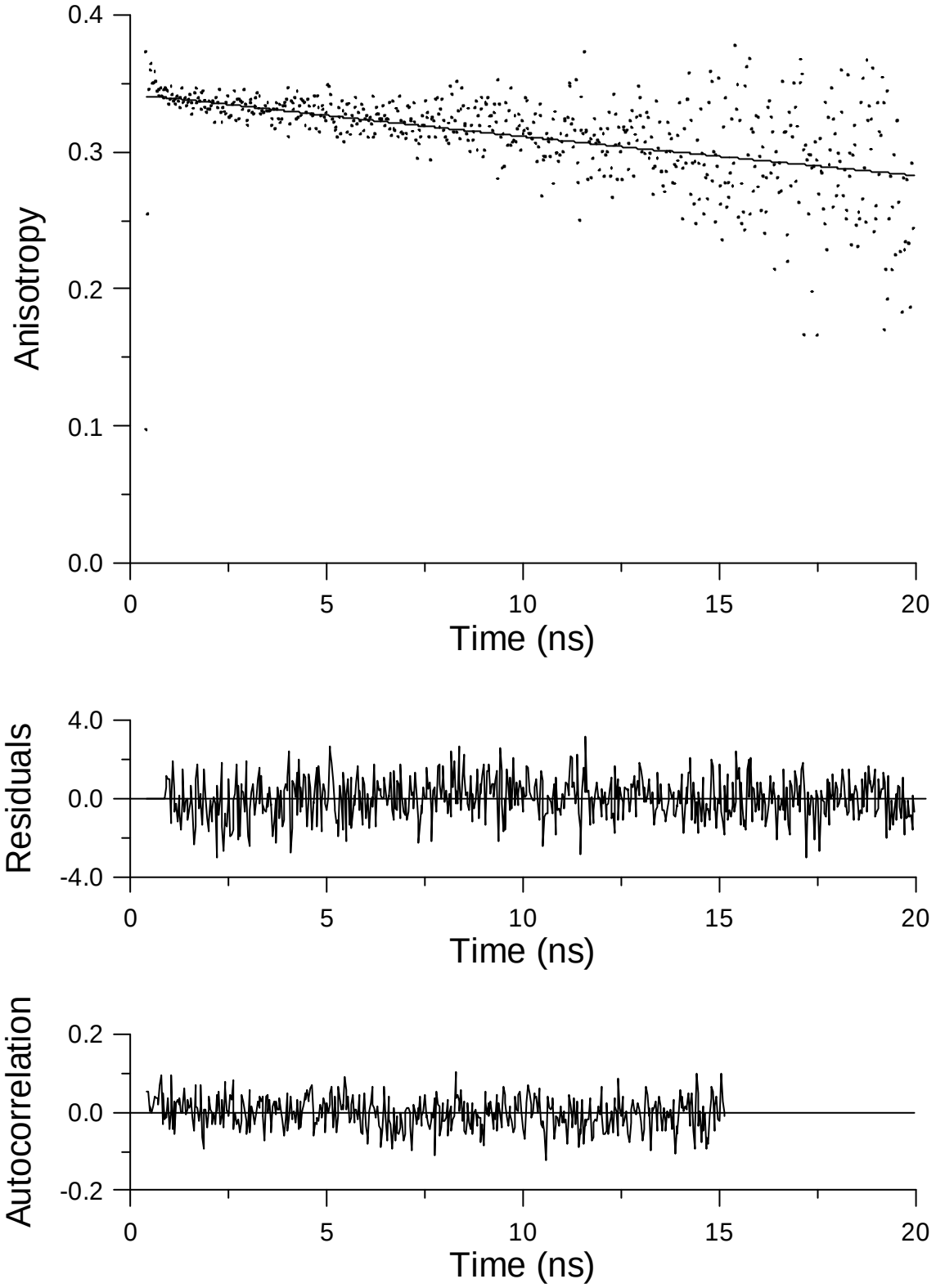
0
5
10
15
20
Time (ns)

Fig. 1.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Time (ns)
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0
5
10
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Time (ns)
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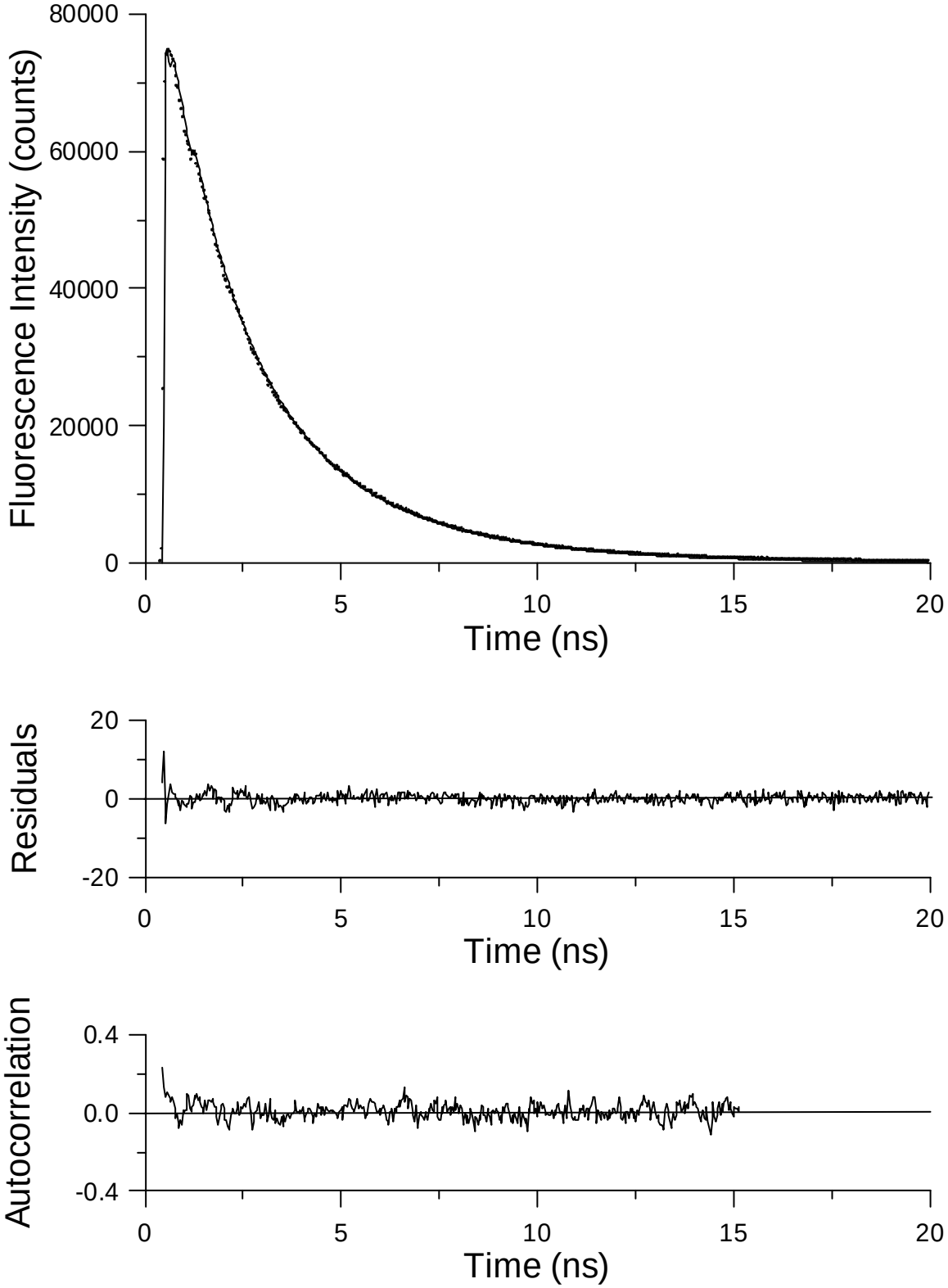
Time (ns)

Fig. 2.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Time (ns)
Residuals
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-20
0
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Time (ns)
Autocorrelation
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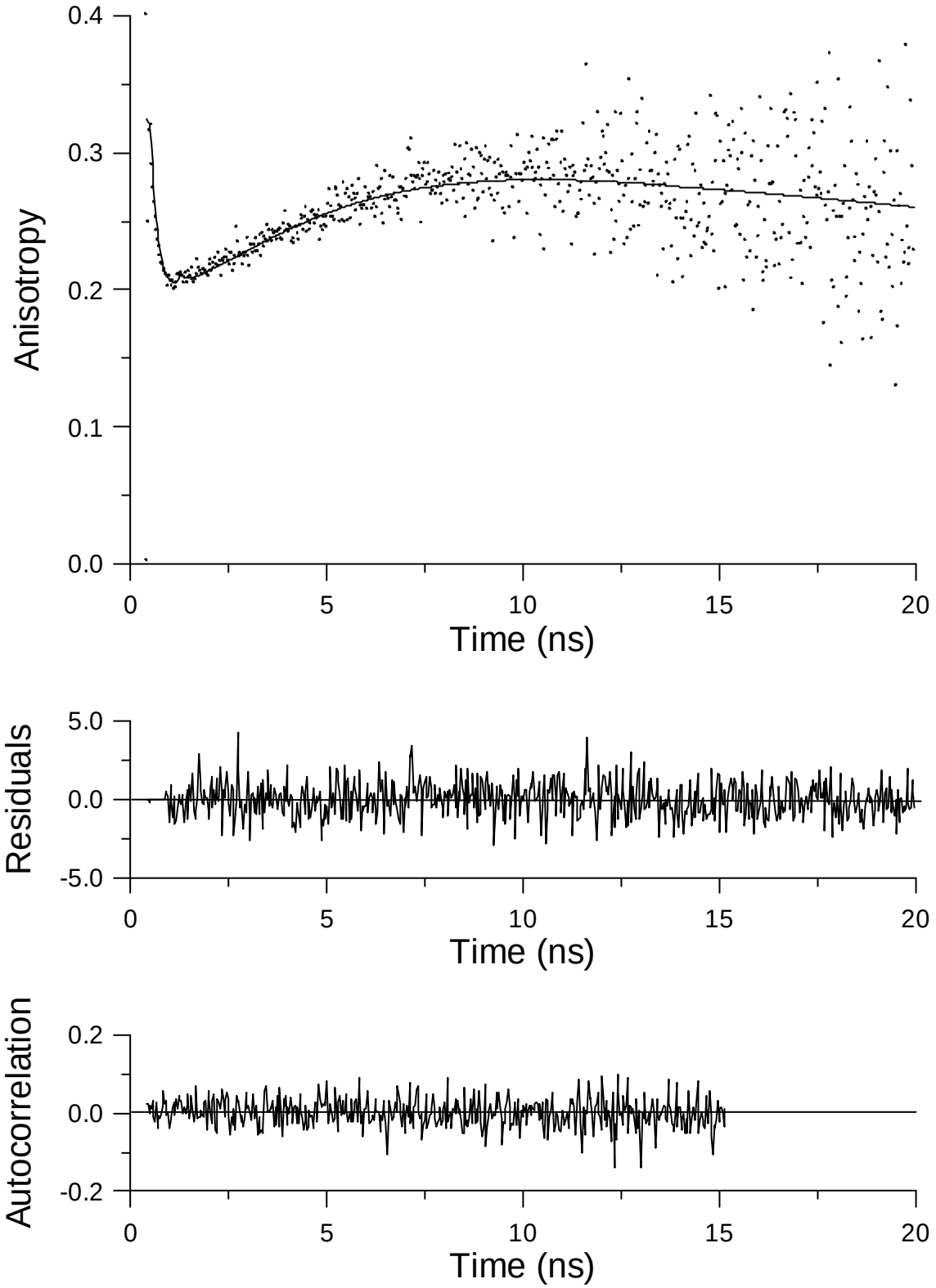
Time (ns)

Fig. 3.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Time (ns)
Residuals
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-5.0
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5
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15
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Time (ns)
Autocorrelation
0.2
0.0
-0.2
0
5
10
15
20
Time (ns)

Fig. 4.