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Time-resolved fluorescence studies of flavodoxin

Demonstration of picosecond fluorescence lifetimes of FMN in *Desulfovibrio* flavodoxins

Antonie J.W.G. Visser, Arie van Hoek°, Tadeusz Kulinski* and Jean Le Gall*

Department of Biochemistry, Agricultural University, 6703 BC Wageningen, The Netherlands and + *Department of Biochemistry, The University of Georgia, Athens, GA 30602, USA*

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Picosecond-resolved fluorescence spectroscopy on FMN bound in flavodoxin from *Desulfovibrio vulgaris* and *Desulfovibrio gigas* revealed fluorescence lifetimes of about 30 ps. The lifetime shortening can be explained by charge transfer interaction in the flavin excited state with aromatic amino acid residues. In addition to the short lifetime component, nanosecond lifetimes were also observed in both flavodoxins. The latter fluorescence lifetime in *D. vulgaris* flavodoxin (5.6 ns) is even longer than that of free FMN (4.7 ns). A fixed lifetime analysis of the results from a diluted *D. vulgaris* flavodoxin solution yielded the dissociation constant of the FMN-apoflavodoxin complex: $K_d = 9.4 \pm 1.4$ nM at 20 °C and pH 7.0.

Flavodoxin; Flavin; FMN; Time-resolved fluorescence; Fluorescence lifetime; Charge transfer; *(Desulfovibrio)*

1. INTRODUCTION

Flavodoxins are well-characterized electron transfer proteins containing a single, noncovalently bound FMN as prosthetic group [1,2]. The molecular mass is, depending upon the source of organism, in the range of 16–25 kDa. High-resolution crystal structures of *Clostridium MP* and *Desulfovibrio vulgaris* flavodoxins have been reported [3,4]. Optical studies have been summarized in [2]. Raman spectra turned out to be characteristic for the flavodoxin species studied [5,6]. Recent NMR data provided strong support for tightly associated FMN in a compact flavodoxin conformation [7].

The fluorescence of FMN in flavodoxin is strongly quenched. It amounts to about 1–5% of the fluorescence of free FMN, the fluorescence quantum yield of which is comparable to that of riboflavin (Q_f = 0.26) [8]. The fluorescence is, however, not absent and can be accurately determined with sophisticated fluorescence equipment. In this letter a time-resolved fluorescence investigation of the FMN in two *Desulfovibrio* flavodoxins is reported. From the two selected flavodoxins the one from *D. vulgaris* has been the most extensively studied [9–12]. Owing to the high sensitivity, large dynamic range and good time-resolving power of the fluorometer, characteristic dynamic features of these flavodoxins are disclosed.

2. MATERIALS AND METHODS

Flavodoxins from *D. vulgaris* and *D. gigas* were

Correspondence address: A.J.W.G. Visser, Department of Biochemistry, Agricultural University, 6703 BC Wageningen, The Netherlands

° Permanent address: Department of Molecular Physics, Agricultural University, 6703 BC Wageningen, The Netherlands

* Permanent address: Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland

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isolated as described [9]. Just before measurement a sample from a stock solution was gel-permeated on Sephadex G-25 (Pharmacia) with 0.05 M sodium phosphate buffer, pH 7.0, in order to remove any dissociated FMN. The initial flavodoxin concentration used was 30 μM for *D. vulgaris* and 15 μM for *D. gigas*. The concentration was determined on a spectrophotometer using the extinction coefficients as reported in [10].

Time-resolved fluorescence measurements were performed with a mode-locked argon ion laser as the source of excitation at 458 nm and time-correlated single photon counting in detection. The detection wavelength was 531 nm. The system has been extensively described in [13,14]. Data analysis was performed with a deconvolution method as outlined recently [15].

3. RESULTS

3.1. *Simulations*

The temporal pulse width of the laser is about 100 ps, but it is broadened by the detector [13]. In order to investigate the feasibility of short lifetime determinations in the picosecond regime using a relatively broad impulse response function, a simulation was carried out. The results are given in fig.1. The experimental pulse is shown together with the convoluted fluorescence responses with lifetimes of 40 ps and 4.7 ns, respectively. The 4.7 ns lifetime can be associated with free FMN in water [16]. It can be clearly seen that the short component is created completely within the pulse. The fluorescence response has a slightly broader temporal width than the impulse response and is somewhat delayed. The leading edge of the short lifetime response is created distinctly earlier in time than the long lifetime response. Also shown in fig.1 are biexponential decay profiles consisting of the lifetime components of 40 ps and 4.7 ns in two different amplitude ratios (99:1 in favour of the short component and 1:1, respectively). These simulations demonstrate that, in order to recover picosecond lifetimes, analysis of experimental data has to be carried out over the whole pulse profile.

3.2. *Flavin fluorescence lifetimes in flavodoxin*

In flavodoxin the time-dependent FMN fluorescence is described by both ultrashort and relatively long lifetime components. In order to precisely determine the fluorescence lifetimes we decided to divide the decay curves into two parts, which are characteristic for the short- and long-time fluorescence behaviour, respectively.

In fig.2A an example of the short-time fluorescence decay analysis of *D. vulgaris* flavodoxin is presented. It is clearly evident in fig.2A that the fluorescence of flavodoxin-bound

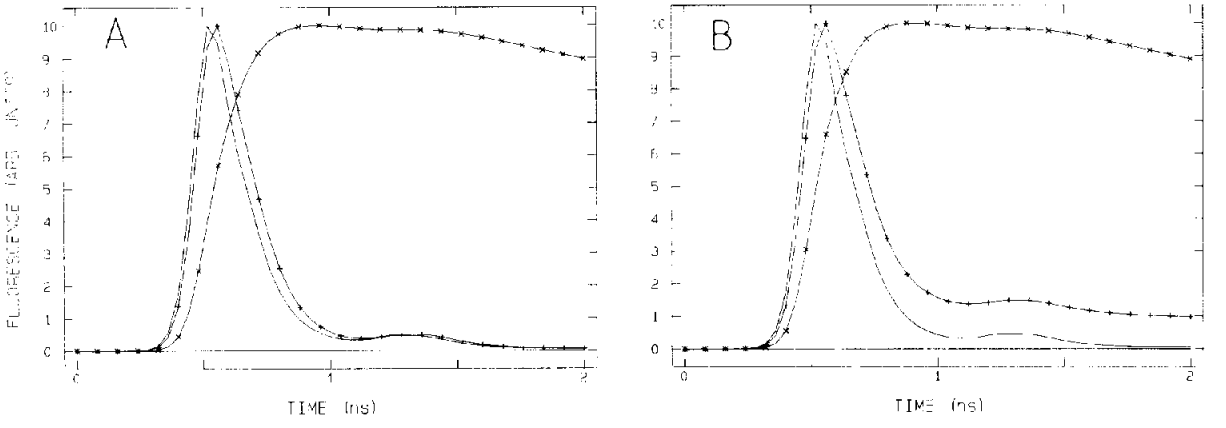

Fig.1. Simulation of fluorescence responses from a laser pulse of finite width. The laser pulse profile (——) was measured from the 992 cm^{-1} Raman frequency of benzene upon excitation at 458 nm and monitoring at 475 nm with an interference filter. The nominally 100 ps pulse is broadened to 200 ps by the detection system. (A) Time-resolved fluorescence profiles obtained by convolution of the pulse response with exponential decays: (+) τ = 40 ps, (×) τ = 4.7 ns. (B) Time-resolved fluorescence profiles resulting from biexponential decay models: (+) $0.99\exp(-t/40) + 0.01\exp(-t/4700)$, (×) $0.5\exp(-t/40) + 0.5\exp(-t/4700)$, with t expressed in ps.

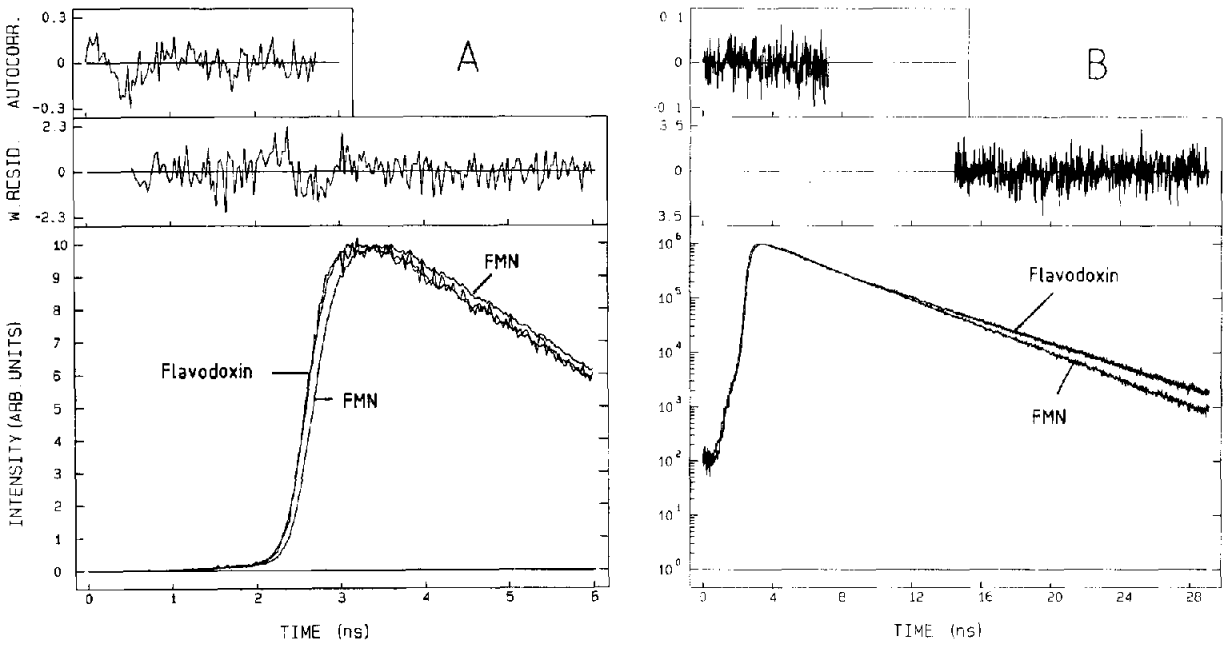

Fig.2. Analysis of fluorescence decay of 30 μM *Desulfovibrio vulgaris* flavodoxin at 20°C. In both panels three curves are shown, namely the experimental fluorescence responses of FMN and flavodoxin and the calculated fluorescence response of flavodoxin. On top of these curves are shown the weighted residuals between calculated and experimental fluorescence responses in each time channel and the autocorrelation of the residuals demonstrating the quality of the fit. FMN served as reference compound with τ = 4.7 ns. Peak channels contained approx. 35000 counts, the time equivalence per channel was 28 ps. (A) Analysis over the first 200 channels of flavodoxin fluorescence data, yielding a predominant component of 40 ps superimposed upon a slower decaying component. The longer lifetime cannot be accurately determined (see [15] for details). The scale is linear to show the apparent delay of FMN fluorescence with respect to flavodoxin fluorescence. (B) Analysis of the last 500 channels of flavodoxin fluorescence decay data yielding a single lifetime of 5.64 ns. The vertical scale is logarithmic to emphasize the different decay rates of free and bound FMN. The total fluorescence decay of flavodoxin is at least triple exponential yielding approximately 75% of the short component, 20% of the long component and 5% of an intermediate lifetime component.

FMN precedes that of free FMN. This is taken to indicate the presence of ultrafast fluorescence in flavodoxin. In fact, by using the fluorescence profile of free FMN as a reference with a lifetime of 4.7 ns, the value of the short component was found to be in the range of 30–40 ps (values are listed in table 1). In this analysis a biexponential decay served as a model function with the recovery of a predominantly present, short component and a less precisely determined longer component.

The analysis of the long fluorescence lifetime component of *D. vulgaris* flavodoxin is presented in fig.2B. Again as reference the fluorescence response of free FMN is shown. Interestingly, the fluorescence lifetime of the bound FMN (5.6 ns) is longer than that of free FMN. Lifetimes for several flavodoxin concentrations at two temperatures are listed in table 1. The fluorescence lifetime of *D. vulgaris* flavodoxin is slightly longer at 4°C. At 0.6 μM the long lifetime is somewhat shorter than the one at higher concentration. Because of the clear difference between the long lifetimes of free and bound FMN at both temperatures, a biexponential decay analysis with fixed lifetimes would yield the relative concentrations of free and bound FMN. Results of such an analysis are given in the legend of table 1. The dissociation constants, derived from this analysis, are 9.4 ± 1.4 nM (20°C) and 15.6 ± 2.3 nM (4°C), the errors being related to the standard errors in the preexponential factors. The dissociation constant is in good agreement with the one obtained previously (12.5 nM at

Table 1

Fluorescence decay parameters of FMN in flavodoxins from *Desulfovibrio vulgaris* and *Desulfovibrio gigas*

	Conc. (μM)	Temp. (°C)	Lifetime from leading edge fit (ps, ±3 ps)[a]	Lifetime from tail fit (ns, ±0.02 ns)[a]
D. vulgaris	30	20	40	5.64
	1.43	20	30	5.62
	0.59	20	30	5.57[b]
	30	4	32	5.85
	1.43	4	28	5.88
	0.59	4	30	5.75[c]
D. gigas	15	20	20	4.78
	1.5	20	22	4.79
	0.15	20	27	4.70
	15	4	20	4.86
	1.5	4	24	4.83
	0.15	4	26	4.80

[a] Standard errors are derived from fit

[b] Equal quality fit to $0.88\exp(-t/5.64) + 0.12\exp(-t/4.70)$ (t in ns)

[c] Equal quality fit to $0.85\exp(-t/5.85) + 0.15\exp(-t/4.85)$ (t in ns)

25°C in Tris buffer, pH 7.0 [11]). The endothermic association of FMN with apoflavodoxin [11] is also indicated by these time-resolved fluorescence data.

The results of the same analysis of the time-resolved fluorescence experiments, carried out with *D. gigas* flavodoxin, have been collected in table 1. The presence of the short lifetime component (20–30 ps) is also evident in *D. gigas* flavodoxin. The striking difference between the two flavodoxins is found in the long fluorescence component, which for *D. gigas* flavodoxin is the same as found for free FMN at both temperatures. Such a lack of distinction between the lifetimes of free and bound FMN impedes the determination of the dissociation constant as described for the *D. vulgaris* flavodoxin.

4. DISCUSSION

The strong quenching of the steady-state fluorescence of FMN bound in flavodoxin is due to interactions in both ground and excited state of the flavin. Ground-state interactions with aromatic amino acids are the main source of the so-called static quenching. Dynamic interactions occur in the first excited singlet state of the flavin leading to shortening of the fluorescence lifetime. In a recent paper [17] the application of picosecond-absorption spectroscopy on flavodoxin from *D. vulgaris* (strain Miyazaki) is reported. An exciplex absorption band was formed from an exciting laser pulse of 25 ps duration. The exciplex arose from electron transfer from tryptophan to excited flavin within 33 ps. Such behaviour is fully supported by our measured lifetimes of around 30 ps. The formation of the exciplex or charge-transfer state creates an extra deactivation channel of the excited singlet state with a concomitant shortening of the lifetime of the residual fluorescence. Dynamic processes can also be the result of rapid motion of both flavin and quencher molecules. The presence of picosecond fluorescence lifetimes in other flavoproteins has been demonstrated [18,19].

The interpretation of the long fluorescence lifetime is more ambiguous. The 5.6 ns component in *D. vulgaris* flavodoxin is longer than the 4.7 ns lifetime of free FMN. Two speculative explanations can be proposed. First, the charge-transfer state gives rise to longer-living fluorescence, although this state is assumed to be nonfluorescent. Second, the long lifetime component must arise from a small population of flavodoxins with a transiently more open and more fluorescent conformation leading to longer-lived fluorescence. The 5.6 ns lifetime is in agreement with the location of the flavin in a more apolar environment as provided by the tryptophan and tyrosine residues in the binding site [4]. As judged from the fluorescence lifetimes, the polarity of the FMN binding site must be different in *D. gigas* flavodoxin. The lower aromatic environment (absence of tryptophan) in the flavin binding region of *D. gigas* flavodoxin [20] is in agreement with such an interpretation.

Picosecond-resolved polarized optical spectroscopy and molecular dynamics calculations are planned in the near future to provide the experimental and theoretical framework of addressing these points.

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