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Flavin binding site differences between lipoamide dehydrogenase and glutathione reductase as revealed by static and time-resolved flavin fluorescence

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Subnanosecond-resolved fluorescence measurements of the FAD bound in glutathione reductase and lipoamide dehydrogenase revealed characteristic differences in dynamic properties of both enzymes, which are considered to have common structural features. The flavin fluorescence in glutathione reductase is quenched mainly via a dynamic mechanism, in agreement with enhanced flexibility of the flavin as inferred from rapid depolarization of the fluorescence.

Glutathione reductase; Lipoamide dehydrogenase; Fluorescence anisotropy; Rotational correlation time; Flavin-binding site; Flexibility

1. INTRODUCTION

Glutathione reductase and lipoamide dehydrogenase are FAD containing flavoproteins, which have been extensively studied [1–3]. While both enzymes catalyze different reactions, the structure of the active site shows common features in the two proteins. A disulfide bridge between two cysteines is in close contact with FAD. Electrons supplied by NADH (lipoamide dehydrogenase) or by NADPH (glutathione reductase) are transferred from reduced FAD to the vicinal cystine residue and then to the other substrate, lipoic acid and glutathione, respectively.

The 3-dimensional structure of glutathione reductase has been solved at high resolution [2]. The functional form is a dimeric protein of 100 kDa. Both subunits are involved in binding glutathione. Both FAD and NADPH are bound in an extended conformation to the enzyme. Lipoamide dehydrogenase is a constituent of the pyruvate dehydrogenase multienzyme complex. In the reaction catalyzed by the complex the electron flow is from dihydrolipoamide, covalently bound to the complex, to NAD^+. The enzyme, isolated from the complex, is dimeric, as well, with a molecular mass of 102.5 kDa. The lipoamide dehydrogenase from *Azotobacter vinelandii* has been crystallized [4].

In this letter we describe a study on static and time-resolved fluorescence properties of glutathione reductase from human erythrocytes and lipoamide dehydrogenase from *Escherichia coli*. Because of the contact between the cystine sulfur atoms and the flavin, the FAD fluorescence is expected to be strongly quenched [5]. This is indeed the case for glutathione reductase. In lipoamide dehydrogenase, however, the fluorescence quantum yield is relatively high. Also the average fluorescence lifetime is quite long [6]. Our time-resolved fluorescence and fluorescence anisotropy results provide clear evidence, that the flavin microenvironments in both enzymes are different.

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2. MATERIALS AND METHODS

Glutathione reductase from human erythrocytes was a gift from Dr B. Mannervik, University of Stockholm. Lipoamide dehydrogenase was resolved from the pyruvate dehydrogenase complex according to standard procedures [7]. Both enzymes were used as 0.1–1 μM solutions in 50 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA.

Fluorescence spectra were measured on an Aminco SPF-500 spectrofluorimeter. In time-resolved fluorescence experiments the line at 458 nm of a mode-locked argon ion laser was used to excite the samples. The fluorescence was monitored via a 531 nm interference filter. Details of the experimental set-up and the single photon detection method have been presented elsewhere [8]. Data analysis was performed as described [9].

3. RESULTS AND DISCUSSION

3.1. *Steady-state fluorescence*

The FAD fluorescence spectra taken from both enzymes revealed no difference in emission maxima (located at about 520 nm) or change in spectral shape (characteristic shoulder at 545 nm). The fluorescence quantum efficiencies, however, are widely different. The fluorescence quantum efficiency of glutathione reductase is only 7.7% of that of lipoamide dehydrogenase (taken as 100%, see table 1).

3.2. *Fluorescence decay*

In fig.1 the time-resolved fluorescence patterns and corresponding fits of both enzymes are presented. The fluorescence decays are in both cases complex.

For glutathione reductase a decay model of three exponential terms was not sufficient to obtain an optimum fit. A sum of four exponential functions with lifetimes and relative amplitudes as listed in table 1 had to be used. It is difficult to provide a good physical description of this multiexponential decay. The fluorescence decay is dominated by a short lifetime component. The shortening of the fluorescence lifetime from e.g. 4.7 ns (FMN in water [10]) to 50 ps must be ascribed to dynamic quenching by, for instance, sulfur atoms of cystine or cysteine residues. In general, dynamic quenching is dictated by a diffusion-controlled collisional mechanism leading to shortening of the fluorescence lifetime. In a protein the quenching of the FAD fluorescence is caused by the amino acid residues in the vicinity of the flavin and unlimited diffusion is excluded because of the spatial constraints imposed by the polypeptide chain. Hindered diffusion may be in part responsible for the nonexponential decay pattern. The four-component fit must be considered as a purely mathematical description and it indicates a multitude of microenvironments of the flavin. A

Table 1

Fluorescence characteristics of glutathione reductase (GR) and lipoamide dehydrogenase (LD) at 20°C

	Steady-state fluorescence	
	Wavelength emission maximum (nm)	Relative quantum efficiency (%)
GR	520	7.7
LD	520	100

	Fluorescence lifetimes		
	α	τ (ns)	Average τ (ns)
GR	0.949 ± 0.09	0.048 ± 0.003	0.11
	0.037 ± 0.003	0.66 ± 0.03	
	0.011 ± 0.001	2.3 ± 0.3	
	0.003 ± 0.001	5.5 ± 0.9	
LD	0.35 ± 0.03	0.15 ± 0.02	1.82
	0.24 ± 0.01	1.54 ± 0.08	
	0.41 ± 0.01	3.39 ± 0.03	

	Correlation times			
	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)
GR	0.32 ± 0.01	3.57 ± 0.04	–	–
LD	0.32 ± 0.01	24.4 ± 0.6	–	–
	0.11 ± 0.03	12 ± 3	0.21 ± 0.03	39

Standard deviations are derived from fit

Average $\tau = \Sigma_i \alpha_i \tau_i$

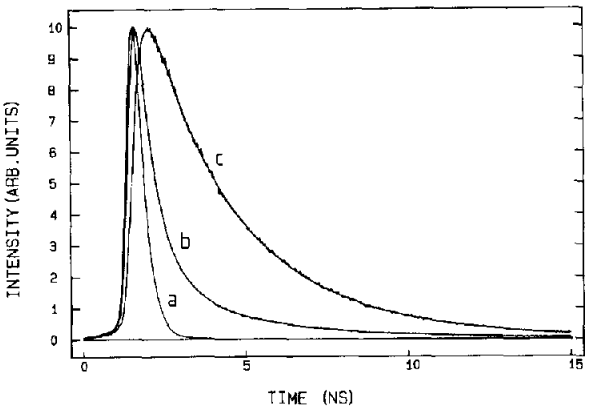

Fig.1. Fluorescence decay curves and their fits of glutathione reductase from human erythrocytes (curve b) and lipoamide dehydrogenase from *E. coli* (curve c). Also the laser excitation response curve is shown (curve a). The fit to the fluorescence of glutathione reductase is a sum of four exponential terms; the fluorescence of lipoamide dehydrogenase was fitted to a triple exponential function. Time constants (τ) and relative amplitudes (α) are collected in table 1.

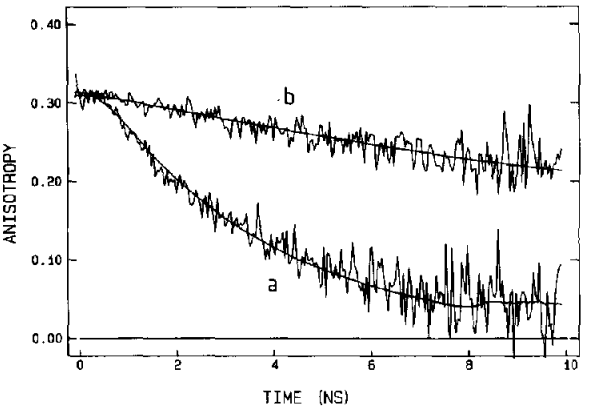

Fig.2. Fluorescence anisotropy decay curves (noisy curves) and their fits (smooth lines) of glutathione reductase (a) and lipoamide dehydrogenase (b). In both enzymes the anisotropy decay is exponential with correlation times (ϕ) collected in table 1.

lifetime distribution may be a better representation, although the distribution is expected to be broad. In earlier studies we have shown that the fluorescence decay of FAD in lipoamide dehydrogenase is heterogeneous as well [11]. For an optimum fit a triple exponential function had to be used (parameters in table 1). The contribution of the short lifetime component is smaller than for glutathione reductase, indicating that the dynamic quenching mechanism is less efficient in lipoamide dehydrogenase. The same arguments as for glutathione reductase hold for this flavoprotein. A distribution of lifetimes ranging from 0.2 to 5.0 ns may be a better description than three lifetime components as presented in table 1.

The ratio of average lifetimes of both proteins appropriately reflects the ratio of quantum efficiencies of the flavin fluorescence in both enzymes (see table 1).

3.3. *Fluorescence anisotropy decay*

Assuming homogeneous rotation, i.e. all fluorescence lifetime components are associated with the same rotation, the anisotropy decays of both proteins gave rise to surprisingly simple patterns as illustrated in fig.2 for both enzymes.

The fluorescence anisotropy decay of glutathione reductase can be fitted to a single exponential function with a correlation time of 3.6 ns (also listed in table 1). A time constant of 3.6 ns is too short to account for rotation of the whole protein. The correlation time of the whole protein can be calculated to be in the order of 38 ns at 20°C on the basis of an empirical formula relating correlation time ϕ with the M_r of a hydrated, spherical protein: ϕ (in ns) = $3.84 \times 10^{-4} \cdot M_r$ [12]. The about 10-fold shorter correlation time suggests that a segmental motion of the protein is the predominant type of rotational dynamics of the enzyme-bound FAD. Depolarization owing to energy transfer from one FAD to the other within the dimeric protein can be excluded, since the anisotropy decays to zero. When energy transfer among rigidly held chromophores takes place, the anisotropy decays to a constant anisotropy [13].

As in glutathione reductase the initial anisotropy decay of lipoamide dehydrogenase can be interpreted more unambiguously than the fluorescence decay pattern, since a single exponential decay is observed. The recovered correlation time of 24 ns, however, is too short to account for a spherical rotor of 102 kDa (39 ns at 20°C, see also table 1). Exactly the same result was obtained earlier for the *Azotobacter vinelandii* lipoamide dehydrogenase [14,15]. The shorter correlation time than expected

was ascribed to a hinged subunit movement. We have quantified this motion by fitting the experimental data according to a biexponential decay model. The anisotropy decay could be fitted equally well with a fixed correlation time of 39 ns and a shorter correlation time of 12 ns (see table 1). The 12 ns correlation time can tentatively be interpreted by a restricted subunit motion. The angular amplitude of this motion follows from the preexponential factor of the longer correlation time according to a relation as derived earlier [16]. From the data in table 1 the amplitude amounts to 30°.

4. CONCLUSION

The flavin binding sites of glutathione reductase and lipoamide dehydrogenase have different dynamic properties as revealed with time-resolved FAD fluorescence. The fluorescence of FAD in glutathione reductase is quenched via a predominantly dynamic mechanism as deduced from the shorter average fluorescence lifetime. In agreement with this dynamic quenching is the fact that the FAD is more flexibly bound in glutathione reductase than in lipoamide dehydrogenase. It would be interesting to investigate whether such a distinction could be inferred from the X-ray diffraction results of crystalline *A. vinelandii* lipoamide dehydrogenase.

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