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Time-Resolved Tryptophan Fluorescence in Flavodoxins

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The time-resolved fluorescence characteristics of tryptophan in flavodoxins isolated from the bacteria *Desulfovibrio gigas, Desulfovibrio vulgaris, Clostridium beijerinckii,* and *Megasphaera elsdenii* were examined. The fluorescence decays were recorded using pulsed synchrotron radiation as the excitation source and time-correlated single-photon counting in detection. The results were analyzed as lifetime distributions using the maximum entropy method. Comparison of the fluorescence decays of normal and flavin mononucleotide-depleted flavodoxins demonstrates that radiationless energy transfer from tryptophan to flavin occurs in all flavodoxins investigated. On comparing the lifetime distribution patterns of apo- and holoflavodoxins, it was noticed that a certain amount of apoprotein is present in all holoflavodoxin samples. The three-dimensional structure of two flavodoxins allowed us to compare experimental with theoretical transfer rates and the results were in fair agreement.

KEY WORDS: Flavodoxin; tryptophan fluorescence; resonance energy transfer; maximum entropy method; synchrotron radiation.

INTRODUCTION

Flavodoxins are relatively small proteins (14–23 kDa) which promote the transfer of electrons between two redox proteins as part of photosynthetic, nitrogen-reducing, sulfate-reducing, or hydrogen-evolving systems (see Ref. 1 for a review). The redox-active group is flavin mononucleotide (FMN) and the physiologically active redox states are the semiquinone and hydroquinone forms of bound FMN. Flavodoxins are found in several aerobic and anaerobic bacteria and in some algae. The three-dimensional structure, in different oxidation states, has been determined for several flavodoxins using X-ray crystallography and nuclear magnetic resonance [2–9]. From these structures rate constants of Förster-type energy transfer between tryptophan residues and flavin can be calculated and compared with the values experimentally determined from time-resolved tryptophan fluorescence. This approach is possible for the flavodoxins from *Desulfovibrio vulgaris* containing two tryptophan residues [10] and from *Clostridium beyerinckii* possessing three tryptophans [11]. In this paper time-resolved tryptophan fluorescence is examined for four flavodoxins. These flavodoxins all contain two to four tryptophans. A fluorescence decay analysis into discrete exponential components would be far too complicated to interpret the data. Because of this we analyzed the time-resolved tryptophan fluorescence data using the maximum-entropy method (MEM) [12,13]. In this way we could describe the results qualitatively and investigate common properties of different classes of flavodoxins.

MATERIALS AND METHODS

Preparation of Flavodoxins

Flavodoxins from *Desulfovibrio gigas, Desulfovibrio vulgaris, Clostridium beijerinckii,* and *Megas-*

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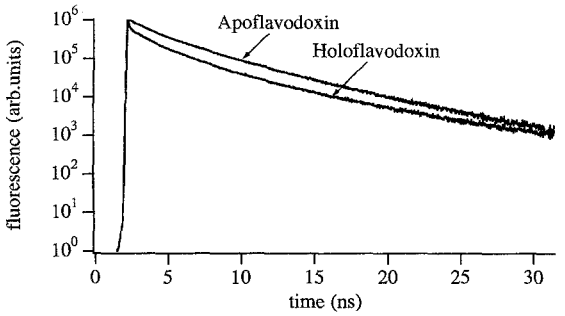

Fig. 1. Fluorescence decay of *Clostridium beijerinckii* apo- and holoflavodoxin (at 20°C).

phaera elsdenii were purified as described earlier [14–18]. The flavodoxins were dissolved in 0.1 *M* KP_i, pH 7.0, at protein concentrations of 10 μ*M*. Apoflavodoxins were prepared as described by Wassink and Mayhew [19]. Measurements were performed at temperatures between 4 and 30°C.

Time-Resolved Tryptophan Fluorescence Measurements

Fluorescence decay measurements were performed at beamline 32 of the MAX synchrotron in Lund, Sweden [20]. The instrumental response function amounted to 95 ps at full width of half-maximum. Apo- and holoflavodoxins were excited at 300 nm, and the fluorescence was detected using KV337 cutoff and OG330 bandpass filters (Schott, Mainz, Germany). The fluorescence decay was analyzed in terms of continuous lifetime distributions by means of the MEM [12,13,21]. This method has been shown to resolve nearby exponentials in mixtures of two compounds (either real mixtures or simulated cases) [22].

RESULTS AND DISCUSSION

As an example the fluorescence decays of *Cl. beijerinckii*, apo- and holoflavodoxin are presented in Fig. 1. This flavodoxin contains three tryptophan residues rather close to FMN. It is obvious that the fluorescence of holoflavodoxin initially decays much faster than that of apoflavodoxin (note that the intensity scale is logarithmic). For *D. vulgaris* flavodoxin this behavior has been ascribed to energy transfer from the excited tryptophan to the flavin acceptor, leading to drastic shortening of the decay [10]. It can also be seen in Fig. 1 that a much slower decay is present in the holoprotein. This slower decay has been ascribed to an excited-state reaction [10] which was more pronounced at longer emission wavelengths.

Because of the complexity of a multitryptophan protein system undergoing both energy transfer and excited-state reaction, the fluorescence decay kinetics of the apo- and holoflavodoxins were analyzed with the MEM. This representation of decay data can be considered as Laplace transform from the time domain to the lifetime domain. The patterns thus obtained allow for a qualitative description of the decay experiments taking into account only the information that is really contained in the data. The results of the MEM analyses are shown in Fig. 2. The MEM plots are shown both as amplitude (α) versus lifetime (τ) (Fig. 2A) and as $\alpha\tau$ versus τ (Fig. 2B), the latter format representing fluorescence intensity versus τ. The fluorescence decays of holoflavodoxin contain one major distribution at a relatively short lifetime and a few less intense components at longer lifetimes. The most pronounced feature in the decays of apoflavodoxin is the absence of the intense short-lifetime distributions compared to holoflavodoxin (the unresolved, small distribution at short lifetimes may be due to the presence of a small amount of holoflavodoxin escaping the conversion into apoprotein). The barycenter of this intense lifetime distribution ($\Sigma\alpha_i\tau_i/\Sigma\alpha_i$ calculated over this distribution) in *D. vulgaris* flavodoxin is positioned at about 750 ps, which perfectly matches the lifetime calculated in the case of energy transfer from the ^1L_a state of the remote tryptophan donor (Trp140) to the flavin acceptor [10] (see Fig. 3). In the *Desulfovibrio* holoflavodoxins there are also small contributions of shorter and longer lifetimes compared to the main distribution. The shorter distribution, between 50 and 100 ps, may arise from energy transfer from the proximal tryptophan to the flavin. We return to this point below. The two longer distributions observed in the holoprotein can be briefly explained as follows. The longest lifetime, about 6 ns, is present in all flavodoxins to a varying extent and it may be connected to an excited-state reaction alluded to previously [10]. The peak at a shorter time (2–3 ns) is at exactly the same position as for the apoprotein. It is therefore likely that this distribution is due to some apoflavodoxin present in the holoflavodoxin preparation. During isolation and purification of flavodoxin, some FMN is dissociated, leading to apoprotein which is copurified. Estimates of the relative amount of apoprotein can be obtained from an analysis of the distribution pattern. Results of these analyses indicated that 3.9% of the *Desulfovibrio* flavodoxin preparations originates from apoprotein. It is our conviction that this detection method for the presence of apoprotein is unique, sensitive, and quantitative.

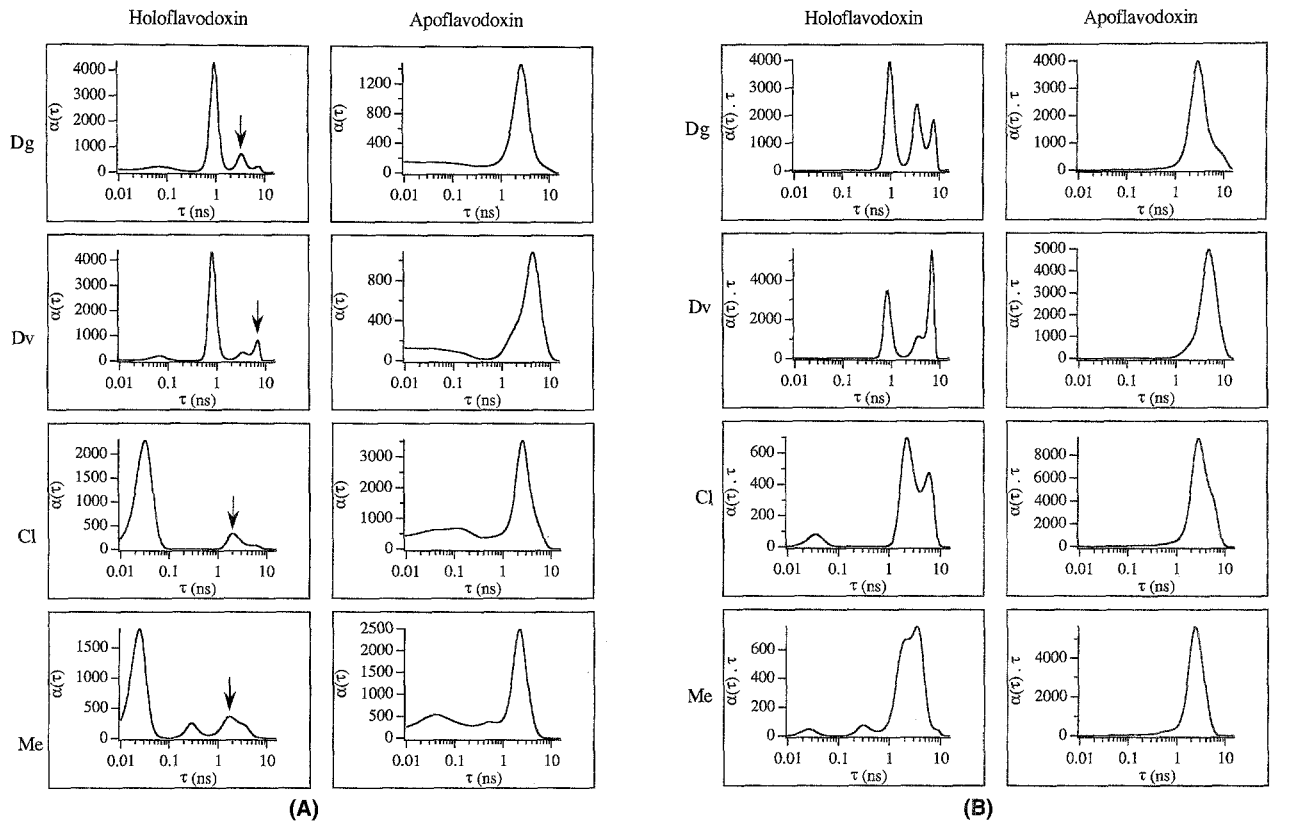

Fig. 2. Maximum entropy analyses of apo- and holoflavodoxins (at 20°C). (A) α versus τ. (B) ατ versus τ. Dg, *D. gigas* flavodoxin; Dv, *D. vulgaris* flavodoxin; Cl, *Cl. beijerinckii* flavodoxin; Me, *M. elsdenii* flavodoxin. The quality of the fits was excellent, $1.00 \leq \chi^2 \leq 1.07$. The arrows in the lifetime distribution of holoflavodoxin correspond to the main distribution in apoflavodoxin.

The intense distribution in *Cl. beijerinckii* holoflavodoxin is located at about 35 ps. Since this flavodoxin contains three tryptophan residues in close proximity to the flavin, more efficient energy transfer is expected. This results in a fluorescence lifetime which is shorter than in *D. vulgaris* flavodoxin. In the temperature range used (4–30°C), the barycenter of the lifetime distribution is hardly influenced, which is in agreement with energy transfer as a temperature-independent decay process. Furthermore, it was found that a large change in relative viscosity of the holoflavodoxin samples did not influence the position or lifetime distribution of the short lifetime (data not shown), again indicating that the short fluorescence lifetime originates from energy transfer. The lifetime distribution pattern of *M. elsdenii* flavodoxin shows a close similarity to that of *Cl. beijerinckii* flavodoxin. A comparison of the fluorescence decays of apo- and holoflavodoxins from the latter two sources indicates the presence of a certain amount of apoflavodoxin (4.2%) in the holoflavodoxin samples.

On the basis of the tryptophan fluorescence decay characteristics, the four flavodoxins can be divided into

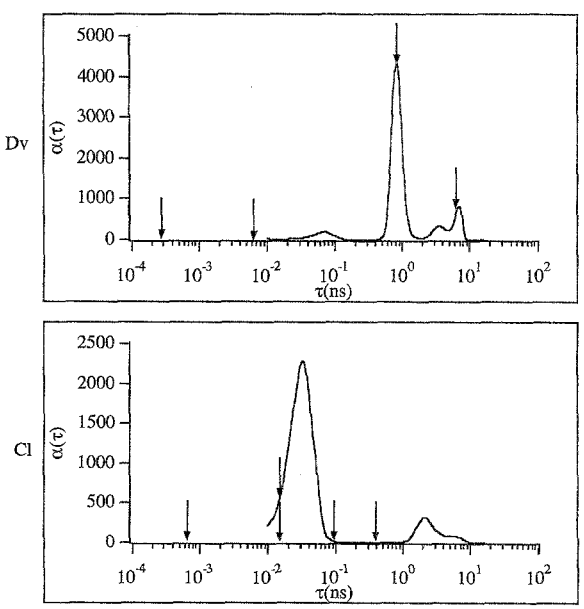

Fig. 3. Fluorescence lifetime distribution of *D. vulgaris* (Dv) and *Cl. beijerinckii* (Cl) flavodoxin (see Fig. 2) and energy transfer lifetimes, τ_{ET}, designated by arrows, with numerical values listed in Table I.

Table I. Fluorescence Lifetimes as a Result of Tryptophan-to-Flavin Energy Transfer Calculated for *D. vulgaris* and *Cl. beijerinckii* Flavodoxins

	Donor	→	Acceptor	τ_{ET} (ps)
D. vulgaris	Trp60 (^1L_a)		FMN	0.32
	Trp60 (^1L_b)		FMN	6.4
	Trp140 (^1L_a)		FMN	750
	Trp140 (^1L_b)		FMN	5300
Cl. beijerinckii	Trp6 (^1L_a)		FMN	15.6
	Trp6 (^1L_b)		FMN	400
	Trp90 (^1L_a)		FMN	0.70
	Trp90 (^1L_b)		FMN	0.02
	Trp95 (^1L_a)		FMN	15.4
	Trp95 (^1L_b)		FMN	101

two groups: one group (called the *rubrum* class) consists of the two *Desulfovibrio* flavodoxins possessing a remote tryptophan residue about 20 Å from the flavin (leading to a longer average lifetime), and the other (called the *pasteurianum* class) of the *Cl. beijerinckii* and *M. elsdenii* flavodoxins both having all tryptophan residues clustered together in the flavin active site (leading to a much shorter fluorescence lifetime because of efficient energy transfer). This division is rather arbitrary, but in accordance with division into main classes based on other properties of flavodoxins, such as spectroscopic characteristics, redox potentials, molecular weights, intermolecular electron transfer, and ability to bind riboflavin instead of FMN (for a recent survey see Ref. 23).

Finally, we wish to relate the Förster rate of transfer, which can be derived from the geometric parameters (orientation factor and distance) for each tryptophan–flavin couple in flavodoxins of known three-dimensional structure, such as those from *D. vulgaris* and *Cl. beijerinckii*, with the experimental rate as reflected by the short (reciprocal) lifetime distribution in the holoprotein. It is assumed that energy transfer can occur from both ^1L_a and ^1L_b states of tryptophan to the (second) absorption transition moment of the flavin; in other words, rapid interconversion between both electronic excited states of tryptophan is not taken into account [24]. The other assumption is that intertryptophan energy transfer also does not take place. Furthermore, only Förster transfer is considered, and not Dexter-type energy transfer or electron transfer. In Table I we summarize the expected fluorescence lifetime, τ_{ET}, as a result of tryptophan-to-flavin energy transfer, for each tryptophan residue in both flavodoxins (these results have been evaluated in Refs. 10 and 11). For clarity the energy transfer lifetimes, τ_{ET}, presented in Table I are plotted together with the lifetime distributions of both flavodoxins in Fig. 3. Given the crude approximations, the correlation with experiment is reasonable. Femtosecond lifetimes are, of course, beyond the scope of these experiments, but the remaining lifetimes correspond fairly well with the lifetime distributions measured. Therefore, we can infer that the observed lifetime barycenters do have a physical meaning.

CONCLUSIONS

As clearly demonstrated, the fluorescence of all holoflavodoxins decays more rapidly than that of apoflavodoxins. Maximum entropy analyses show that this difference is the result of a major short fluorescence lifetime distribution which is detected only in holoflavodoxins. Comparison with calculated results in flavodoxins of known structure indicates that this distribution originates from efficient energy transfer from tryptophan to flavin.

The fluorescence distribution found in the apoflavodoxins is always present to some degree in the holoflavodoxins. This can be explained by a small amount of copurified apoprotein in the holoflavodoxin samples.

Based on the barycenters of the main fluorescence lifetime distribution of the flavodoxins, the division of flavodoxins into two main classes is confirmed.

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REFERENCES

1. S. G. Mayhew and M. L. Ludwig (1975) in P. D. Boyer, (Ed.), *The Enzymes, Vol. XII* Academic Press, New York, pp. 57–118.
2. K. D. Watenpaugh, L. C. Sieker, and L. H. Jensen (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3857–3860.
3. R. M. Burnett, G. D. Darling, D. S. Kendall, M. E. LeQuesne, S. G. Mayhew, W. W. Smith, and M. L. Ludwig (1974) *J. Biol. Chem.* **249**, 4383–4392.
4. W. W. Smith, R. M. Burnett, G. D. Darling, and M. L. Ludwig (1977) *J. Mol. Biol.* **117**, 195–225.
5. M. L. Ludwig, K. A. Pattridge, W. W. Smith, L. H. Jensen, and K. D. Watenpaugh (1982) in V. Massey and C. H. Williams (Eds.), *Flavins and Flavoproteins*, Elsevier/North-Holland, Amsterdam, pp. 19–28.

6. W. W. Smith, K. A. Pattridge, M. L. Ludwig, G. A. Petsko, D. Tsernoglou, M. Tanaka, and K. T. Yasunobu (1983) *J. Mol. Biol.* **165**, 737–755.
7. C. P. M. van Mierlo, Ph. Lijnzaad, J. Vervoort, F. Müller, H. J. C. Berendsen, and J. de Vlieg (1990) *Eur. J. Biochem.* **194**, 185–198.
8. C. P. M. van Mierlo, B. P. J. van der Sanden, P. van Woensel, F. Müller, and J. Vervoort (1990) *Eur. J. Biochem.* **194**, 199–216.
9. W. Watt, A. Tulinsky, R. P. Swenson, and K. D. Watenpaugh (1991) *J. Mol. Biol.* **218**, 195–208.
10. R. Leenders, J. Vervoort, A. van Hoek, and A. J. W. G. Visser (1990) *Eur. Biophys. J.* **18**, 43–55.
11. R. Leenders, W. F. van Gunsteren, H. J. C. Berendsen, and A. J. W. G. Visser (1994) *Biophys. J.* **66**, 634–645.
12. A. K. Livesey, P. Licinio, and M. Delaye (1986) *J. Chem. Phys.* **84**, 5102–5107.
13. A. K. Livesey and J. C. Brochon (1987) *Biophys. J.* **52**, 693–706.
14. J. LeGall and E. C. Hatchikian (1969) *C.R. Acad. Sci. Paris* **264**, 2580–2583.
15. S. G. Mayhew and V. Massey (1969) *J. Biol. Chem.* **244**, 794–802.
16. S. G. Mayhew (1971) *Biochim. Biophys. Acta* **235**, 289–302.
17. K. Irie, K. Kobayashi, M. Kobayashi, and M. Ishimoto (1973) *J. Biochem. (Tokyo)* **73**, 353–366.
18. R. Leenders and A. J. W. G. Visser (1991) in B. Curti, S. Ronchi, and G. Zanetti (Eds.), *Flavins and Flavoproteins*, Walter de Gruyter, Berlin.
19. J. H. Wassink and S. G. Mayhew (1975) *Anal. Biochem.* **68**, 609–616.
20. R. Rigler, O. Kristenssen, J. Roslund, P. Thyberg, K. Oba, and M. Eriksson (1987) *Phys. Scripta* **T17**, 204–207.
21. R. Leenders, M. Kooijman, A. van Hoek, C. Veeger, and A. J. W. G. Visser (1993) *Eur. J. Biochem.* **211**, 37–45.
22. J. C. Brochon (1995) *Methods Enzymol.* **240**, 262–311.
23. S. G. Mayhew and G. Tollin (1993) in F. Müller (Ed.), *Chemistry and Biochemistry of Flavoenzymes, Vol. III*, CRC Press, Boca Raton, FL, pp. 389–426.
24. D. J. Ruggiero, D. C. Todd, and G. R. Fleming (1990) *J. Am. Chem. Soc.* **112**, 1003–1009.