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Flavin dynamics in oxidized *Clostridium beijerinckii* flavodoxin as assessed by time-resolved polarized fluorescence

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The time-resolved fluorescence characteristics of flavin in oxidized flavodoxin isolated from the anaerobic bacterium *Clostridium beijerinckii* have been examined. The fluorescence intensity decays were analyzed using the maximum-entropy method. It is demonstrated that there exist large differences in fluorescence behaviour between free and protein-bound FMN. Three fluorescence lifetime components are found in oxidized flavodoxin, two of which are not present in the fluorescence-intensity decay of free FMN. The main component is distributed at 30 ps, with relative contribution of 90%. Another minor component (4% contribution) is distributed at 0.5 ns. The third component is distributed at 4.8 ns (6%), coinciding with the main distribution present in the fluorescence decay of free FMN. The results allowed us to determine the dissociation constant, $K_d = 2.61 \times 10^{-10}$ M (at 20°C).

Collisional fluorescence-quenching experiments revealed that the flavin moiety responsible for the longest fluorescence lifetime is, at least partially, exposed to the solvent. The shortest lifetime is not affected significantly, indicating that it possibly originates from an active-site conformation in which the flavin is more or less buried in the protein and not accessible to iodide.

The fluorescence anisotropy behaviour of free and protein-bound FMN was examined and analyzed with the maximum-entropy method. It was found that an excess of apoflavodoxin is required to detect differences between free and protein-bound FMN. In free FMN one single distribution of rotational correlation times is detected, whereas in flavodoxin the anisotropy decay is composed of more than one distribution.

Associative analysis of fluorescence anisotropy decays shows that part of the 4.8 ns fluorescence lifetime present in the flavodoxin fluorescence decay, is coupled to a rotational correlation time similar to that of free FMN in solution, while another part of this lifetime is coupled to a longer correlation time of about 1 ns. This finding is in accordance with earlier studies [Barman, B. G. & Tollin, G. (1972) *Biochemistry 11*, 4746–4754] in which it was proposed that the first binding step of the flavin to the protein involves the phosphate group rather than another part of the FMN.

The two shortest fluorescence lifetimes, which do not carry information on the long-term rotational behaviour of the protein, seem nonetheless to be associated with a longer rotational correlation time which is comparable to overall protein tumbling. These lifetime components probably originate from a complex in which the flavin-ring system is more or less immobilized within the protein matrix.

Flavodoxins are low-molecular-mass electron-transport proteins (14–25 kDa) which can substitute for ferredoxin in a number of reactions (Mayhew, 1971; Mayhew and Ludwig, 1975; Mayhew and Tollin, 1992). Flavodoxins can be isolated from some algae and several anaerobic and aerobic bacteria grown under iron-containing or iron-deficient conditions (Knight and Hardy, 1966; Benemann et al., 1969; Mayhew, 1971; Vetter and Knappe, 1971; Hatchikian et al., 1972; van Lin and Bothe, 1972; Irie et al., 1973). The redox-active centre of flavodoxins is the non-covalently bound flavin mononucleotide (FMN). Flavodoxins can exist in different oxidation states, i.e. oxidized, one-electron reduced (semiquinone), and two-electron-reduced (hydroquinone). The physiologically relevant redox states are the semiquinone and hydroquinone ones.

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In the seventies the three-dimensional structure of oxidized *Clostridium beijerinckii* flavodoxin was resolved using X-ray crystallography (Burnett et al., 1974). The structures of the semiquinone and reduced clostridial flavodoxin show a large overall similarity with the oxidized protein (Smith et al., 1977; Ludwig et al., 1982). The major difference between these two oxidation states is the direction of the carbonyl group of Gly57. Upon reduction the carbonyl rotates towards the flavin facilitating hydrogen bonding between the carbonyl oxygen and the hydrogen attached to flavin atom N5. Recently the structure of *Megasphaera elsdenii* flavodoxin in different oxidation states was determined using two-dimensional NMR and restrained molecular-dynamics simulations (van Mierlo et al., 1990a,b). The overall structures of

C. beijerinckii and *M. elsdenii* flavodoxin were shown to be highly similar. These three-dimensional structures mainly yield information about the static properties of the flavodoxins. However, knowledge of the dynamic behaviour of proteins is essential in understanding the way in which they function (Karplus and McGammon, 1983; Kraut, 1988; Farnum et al., 1991).

Spectroscopic techniques in general, and fluorescence measurements in particular, have been widely used to characterize microenvironments of intrinsically and extrinsically bound chromophores (Rigler and Ehrenberg, 1973, 1976; Cundall and Dale, 1983; Lakowicz, 1983; Beechem and Brand, 1985; Leenders et al., 1990, 1993; Bastiaens et al., 1992a,b,c). In this respect tryptophan residues are the most intensively studied chromophores in proteins. These fluorescence techniques can also be applied to study other chromophores, for example flavins in various flavoproteins. In this study we used time-resolved fluorescence and fluorescence-anisotropy techniques to obtain information about the dynamic characteristics of flavin bound in oxidized *C. beijerinckii* flavodoxin. The time-resolved fluorescence studies of reduced flavodoxins have been reported (Leenders et al., 1993). Although clostridial flavodoxin has a low flavin-fluorescence quantum yield ($Q < 0.01$), it has the advantage that it contains several intrinsic chromophoric groups which are located in or near the active site of the protein (the flavin and a number of tryptophan residues). These chromophores are ideally suited for fluorescence studies in which information about the flexibility of the active site can be obtained.

In this contribution, attention is focused on the fluorescence properties of the oxidized prosthetic group FMN bound in flavodoxin. As a result of the equilibrium between protein-bound and dissociated FMN, differences in fluorescence behaviour between free and protein-bound FMN can be used to study the association process and binding properties of the FMN, and the obtained insight in motional behaviour may contribute to the understanding of the process of electron transfer of flavodoxins.

MATERIALS AND METHODS

Preparation of the flavodoxins

The preparation of *C. beijerinckii* flavodoxin was as described previously (Leenders and Visser, 1991). The flavodoxins were dissolved in 0.1 M potassium phosphate, pH 7.0, at protein concentrations of about 10 μM (determined spectrophotometrically). At these concentrations reabsorption effects are eliminated. All chemicals used were of the highest purity available and only Millipore-filtered water was used. Apoflavodoxins (30 μM in 0.1 M potassium phosphate, pH 7.0) were prepared as described by Wassink and Mayhew (1975). Experiments were conducted between 4 °C and 30 °C.

Fluorescence-quenching experiments

Collisional fluorescence-quenching experiments using KI were performed as described by Lehrer (1971). Samples containing *C. beijerinckii* flavodoxin were quenched as well as samples containing free FMN in solution (quenched for comparison). The effects of KI quenching were studied by means of steady-state and time-resolved fluorescence experiments.

Time-resolved fluorescence and fluorescence anisotropy measurements

Fluorescence-decay measurements using a mode-locked argon-ion laser/synchronously pumped dye laser system as the source of excitation, have been described extensively (van Hoek et al., 1983, 1987; van Hoek and Visser, 1985; Visser et al., 1985; Vos et al., 1987). The most recent description of the system has been given by van Hoek and Visser (1992). The protein-bound FMN as well as the FMN in aqueous buffer solution were excited (457.9 nm) in their first absorption band. Erythrosin-B was used as a reference compound to describe the instrument response (Erythrosin-B fluorescence decays single-exponentially with a typical lifetime of about 80 ps at 4 °C). After excitation the polarized fluorescence was monitored using a Schott line filter (Schott 557.6, with half-band width of 12.6 nm) in combination with a Schott cut-off filter (KV550).

The fluorescence and fluorescence-anisotropy decays were analyzed in terms of continuous lifetime distributions by means of the maximum-entropy method. In this method (Livesey et al., 1986; Livesey and Brochon, 1987), a distribution of lifetimes, $\alpha(\tau)$, is calculated which results in a maximum value of the entropy, S (Jaynes, 1983),

$$S = -\int_0^\infty \alpha(\tau) \log \frac{\alpha(\tau)}{m(\tau)} \, d\tau \qquad (1)$$

where $m(\tau)$ is the initial guess for the distribution of fluorescence lifetimes. In all cases the entropy, S, is maximized under the condition that χ^2 is minimized to unity,

$$\chi^2 = \frac{1}{N} \sum_{k=1}^{N} \frac{\{I_c(k) - I_o(k)\}^2}{I_o(k)} \qquad (2)$$

where N is the number of channels, $I_c(k)$ and $I_o(k)$ are the calculated and observed number of photons in channel k, respectively. Furthermore, $I_o(k)$ is an estimate for the variance in channel k. The obtained distribution will yield the most uncorrelated solution. When no *a priori* knowledge about the shape of the distribution was available, $m(\tau)$ was set to a flat distribution in $\log(\tau)$ space (Livesey and Brochon, 1987; Mérola et al., 1989; Bastiaens et al., 1992c). Since fluorescence intensities and anisotropy contributions are only represented correctly in $\log(\tau)$ and $\log(\phi)$ space, respectively, we divided the logarithmic time axis into 150 equally spaced intervals. $\mathrm{Log}(\tau)$ space was in the range 0.01 − 15 ns, whereas $\log(\phi)$ space was in the range range 0.05 − 30 ns. Integration of the amplitudes $\beta(\phi)$ yields the initial anisotropy, $r(0)$.

The time-resolved fluorescence-anisotropy behaviour was also analyzed using the maximum-entropy method in an associative manner (for details see Bastiaens et al., 1992d). In this analysis of the fluorescence and fluorescence-anisotropy decay, the entropy of the cross-product $\alpha(\tau) \cdot \beta(\phi)$ is maximized (again under the constraint of minimum χ^2),

$$S = -\int_0^\infty \alpha(\tau)\,\beta(\phi) \log \frac{\alpha(\tau)\,\beta(\phi)}{m(\tau)} \, d\tau \qquad (3)$$

where $m(\tau)$ is the initial guess of the $\alpha(\tau) \cdot \beta(\phi)$ distribution. The logarithmic time axes were divided into 40 equally spaced intervals. Since this type of analysis yields information about specific correlations between fluorescence lifetimes and rotational correlation times it can substantially contribute to the understanding of multi-exponential fluorescence and fluorescence anisotropy decays. All analyses were performed on a Silicon Graphics 4D/35 computer.

RESULTS AND DISCUSSION

Quenching of flavin fluorescence in flavodoxin

It is known from earlier studies that flavin fluorescence can be quenched significantly upon binding with the apoflavoprotein (see review Mayhew and Ludwig, 1975). Steady-state fluorescence experiments showed that the reason for this quenching must be the interaction of the isoalloxazine ring system with specific parts of the protein. In particular, disulfide bridges and aromatic amino acid side chains are potential quenchers of flavin fluorescence in flavoproteins. In case of tryptophan side chains, using flavinyl-tryptophan model compounds, it was shown by steady-state fluorescence measurements (MacKenzie et al., 1969) and proton magnetic resonance experiments (Föry et al., 1970; Johnson and McCormick, 1973) that the isoalloxazine and indole rings are coplanar. This ground-state complex clearly influences fluorescence characteristics of the flavin ring system. This is the basis for the so-called static quenching of flavin fluorescence (Lakowicz, 1983).

Time-resolved fluorescence

The time-resolved fluorescence decays of *C. beijerinckii* flavodoxin and free FMN in solution were measured at different temperatures. The fluorescence decays recorded at 4 °C are presented in Fig. 1A. The protein-bound FMN fluorescence initially decays much faster than the fluorescence of free FMN, indicating the influence of the protein environment upon FMN fluorescence. The decay curves were analyzed using the maximum-entropy method (MEM), to obtain information about the distribution of lifetimes in the fluorescence decays. The results of these analyses are shown in Fig. 1B and C. The quality of the fits was excellent, $1.02 \leq \chi^2 \leq 1.06$. It is clear that two short-lifetime components (about 30 ps and 0.5 ns) are distributed in the protein-bound FMN fluorescence which are not present in the fluorescence decay of free FMN. This difference in lifetime distributions directly results from binding of the FMN to the protein, leading to quenching by the protein environment. The very short lifetime (30 ps) has already been noticed for *Desulfovibrio* flavodoxins (Visser et al., 1987) and glutathione reductase (Bastiaens et al., 1992c). It has been ascribed to exciplex formation and electron transfer from a donor to the flavin, which brings the flavin excited-state lifetime into the picosecond range. A third fluorescence-lifetime distribution is found in flavodoxin which coincides with the main lifetime distribution found in free FMN in solution (4.8 ns).

It is known that an equilibrium exists between free and protein-bound FMN. Therefore, the 4.8-ns fluorescence lifetime in flavodoxin may well originate from dissociated FMN. Barman and Tollin (1972) examined the relaxation kinetics of flavin analogs binding to *Azotobacter vinelandii* apoflavodoxin and it was demonstrated that binding of phosphorylated flavin analogs has to be described by a two-step mechanism,

$$P + F \rightleftharpoons X \rightleftharpoons FP \qquad (4)$$

where P is the apoflavodoxin, F is the flavin analog, FP is the flavodoxin in which the flavin is firmly bound, and X is an intermediate structure in which the flavin is bound to the apoflavodoxin in some way. Due to the relatively small dissociation constant K_d, only a very small fraction of dissociated FMN is present in a flavodoxin solution. However, because of its much higher fluorescence quantum yield, as compared to protein-bound FMN, it will contribute significantly to the decay of flavin fluorescence in flavodoxin samples. To demonstrate this, the effect of temperature on the fluorescence behaviour of flavin and flavodoxin was examined. At higher temperatures (≥ 20 °C) the barycenter of the lifetime distribution of the flavin slightly decreases (from 4.8 ns to 4.7 ns). The temperature dependence of the fluorescence decay of clostridial flavodoxin, given in terms of lifetime distributions, is illustrated in Fig. 2. It is obvious that

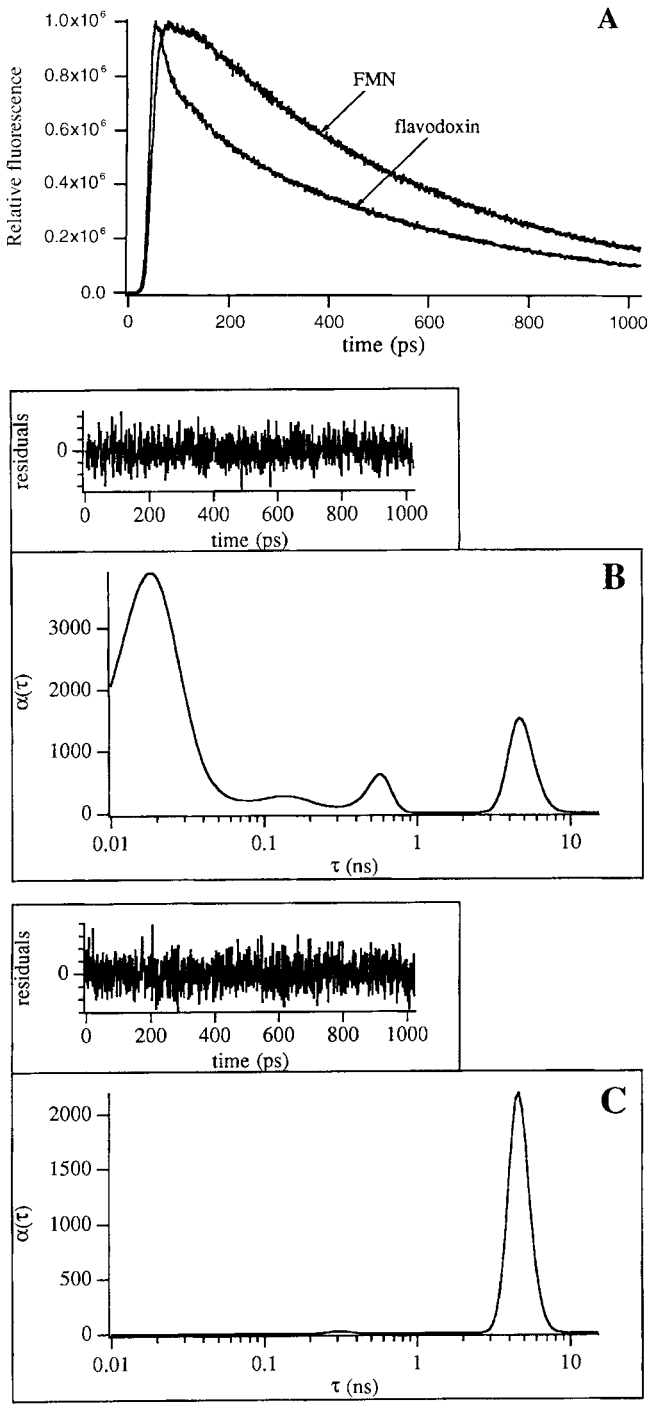

Fig. 1. Fluorescence decays (at 4 °C) of clostridial flavodoxin and free FMN in solution. (A) The results of the maximum-entropy analysis are also presented. (B), Clostridial flavodoxin; (C), FMN in solution. The fit quality was excellent; χ^2 is 1.02 and 1.06, for flavodoxin and FMN, respectively.

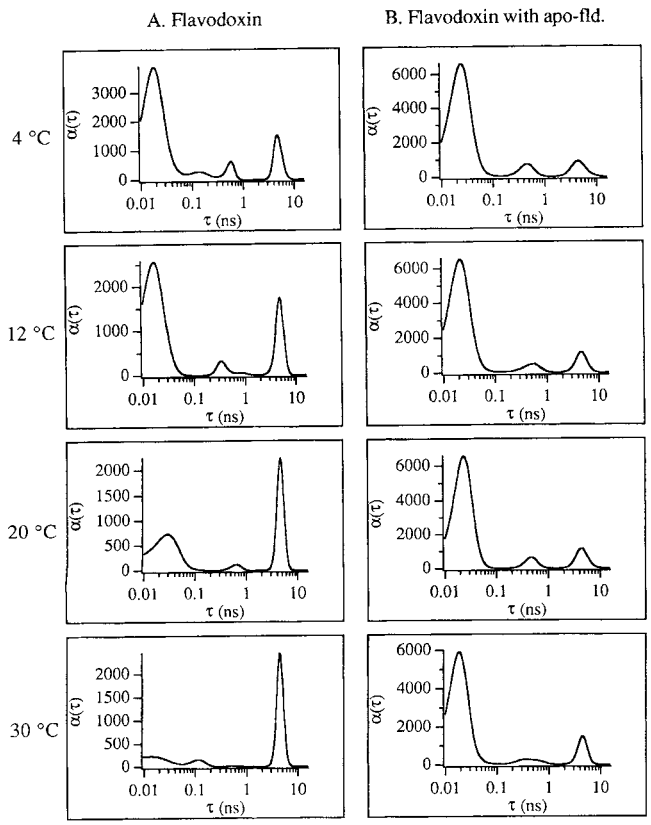

Fig. 2. Temperature dependence of the flavodoxin fluorescence decay. (A) The fluorescence lifetime distributions are calculated with the maximum entropy method. Addition of apoflavodoxin to the flavodoxin sample clearly influences the lifetime distribution at different temperatures (B). The fit quality was very good; χ^2 between 1.01 and 1.08. fld, flavodoxin.

the contribution of the short-lifetime component decreases at higher temperatures, in favour of the contribution of the 4.8-ns-fluorescence-lifetime component. The most reasonable explanation is that at higher temperatures part of the population of the non-covalently bound FMN chromophores dissociates from the protein (Eqn 4), resulting in a larger contribution of the 4.8-ns-fluorescence-lifetime component. To examine if this distribution at 4.8 ns originates from dissociated FMN, apoflavodoxin was added to the clostridial flavodoxin sample (the apoprotein does not absorb light at the excitation wavelength). Addition of 80 µl apoflavodoxin resulted in a significant decrease of the contribution of the 4.8-ns-fluorescence-lifetime component (see Fig. 2B), which is the result of a shift in the equilibrium. The dissociated flavin binds to the added apoflavodoxin to form holoflavodoxin. Addition of an excess of apoflavodoxin, however, did not result in complete loss of the 4.8-ns-fluorescence-lifetime component. In most flavodoxin samples used in the experiments a slight excess of apoflavodoxin was present.

Analysis of the fluorescence decays of flavodoxin samples containing apoflavodoxin demonstrated that the lifetime distribution is no longer temperature dependent. Only at 30°C does the contribution of the 4.8-ns component increase somewhat, indicating a small shift of the equilibrium. The short lifetime component is always predominantly present (about 80–90%), whereas the intermediate lifetime (distributed between 0.2 ns and 1.0 ns) contributes for only about 4%. The rest of the flavodoxin-fluorescence decay is composed of the 4.8-ns component.

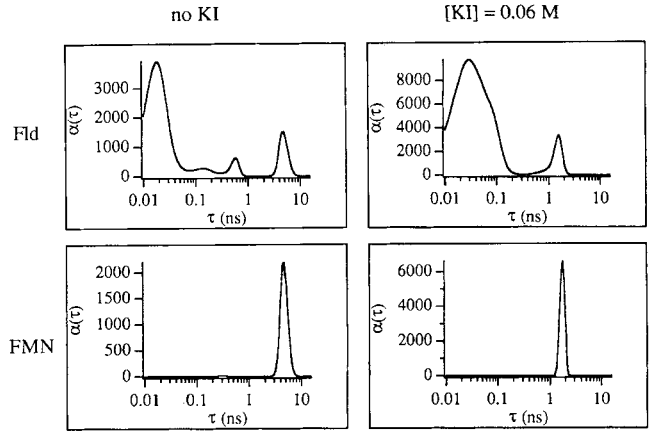

Fig. 3. Quenching experiments with clostridial flavodoxin. ([KI] is 0.06 M; no apoflavodoxin was added). The results of KI quenching of FMN in solution are given for comparison. It is clear that both samples contain a similar flavin moiety. The intermediate lifetimes in the flavodoxin (Fld) also seem to be quenched by the iodide.

Another piece of evidence for the equilibrium between protein-bound and dissociated flavin is given by KI quenching experiments with flavodoxin samples to which no apoflavodoxin was added (Fig. 3). The 4.8-ns fluorescence lifetime is quenched significantly in both flavodoxin and FMN in solution, indicating that both samples contain a similar flavin moiety.

Determination of the dissociation constant

For the ease of calculation it is assumed that the 4.8-ns-fluorescence-lifetime distribution in flavodoxin completely originates from dissociated flavin (Fig. 2; flavodoxin with no added apoprotein) and the other predominant lifetime is related to flavin bound in the flavodoxin. Relative fluorescence quantum yields can then be obtained from the ratio of lifetimes

$$\frac{Q_F}{Q_f} = \frac{\tau_F}{\tau_f} \tag{5}$$

where Q_F and Q_f are the quantum yields of FMN and flavodoxin, respectively, and the corresponding lifetimes are calculated from the barycenters of the lifetime distributions. From the ratio of these barycenters (4.8 ns and 30 ps for FMN and flavodoxin, respectively) the relative quantum yield can be calculated, Q_F/Q_f is 160. Integration of the lifetime distribution at 20°C yields that 55% of the total fluorescence originates from flavodoxin and 45% from dissociated flavin. When these values are corrected for the quantum-yield ratio, 99.49% of the flavin is bound in flavodoxin and 0.51% is dissociated. When the reaction in Eqn (4) is considered to occur via a one-step mechanism (no intermediate state X) and apoflavodoxin and dissociated flavin are present in equimolar concentrations (the total flavin concentration is 10 µM), the dissociation constant K_d at equilibrium is then given by

$$K_d = \frac{[P] \cdot [F]}{[PF]} \tag{6}$$

The dissociation constant at 20°C can then be calculated, $K_d = 2.61 \times 10^{-10}$ M. This value is in good agreement with the one determined for *M. elsdenii* flavodoxin, K_d is 4.3×10^{-10} M (Mayhew, 1971). The standard enthalpy change of

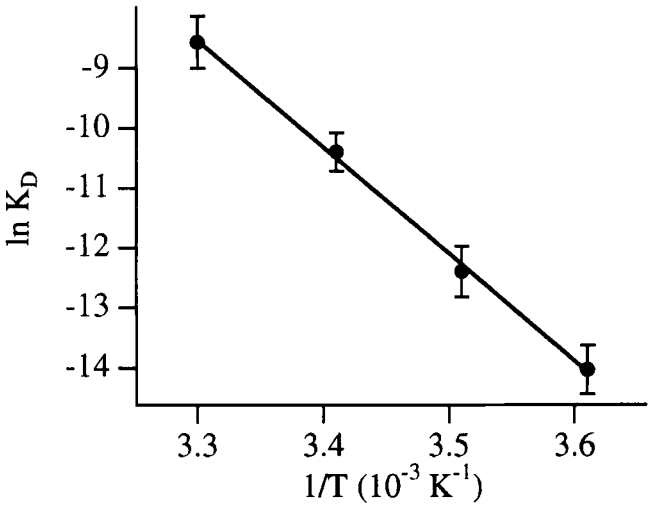

Fig. 4. van't Hoff plot of the equilibrium constant (in molar fractions) of flavin dissociating from clostridial flavodoxin. The solid line is a linear fit to the experimental data. Standard deviations are estimated from multiple experiments.

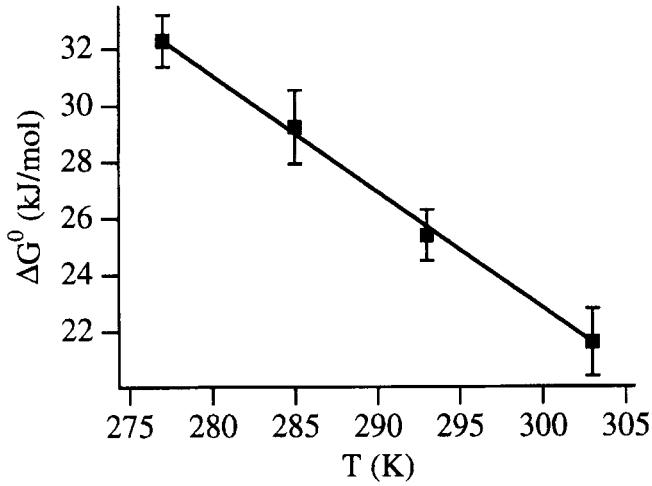

Fig. 5. Free energy of flavin dissociation as a function of temperature. The solid line is a linear fit to the experimental data. Standard deviations are estimated from multiple experiments.

the dissociation reaction, ΔH^0, is determined by the fractional dissociation constant ($k_\alpha = \gamma^2/(1-\gamma)$, where γ is the molar fraction dissociated flavin) at various temperatures and using the van't Hoff equation

$$\frac{\mathrm{d}\ln K_d}{\mathrm{d}(1/T)} = -\frac{\Delta H^0}{R} \qquad (7)$$

where R is the gas constant (8.31 J · K^{-1} · mol^{-1}) and T is the temperature in K. From the slope of the van't Hoff plot (Fig. 4) the standard enthalpy change of the dissociation reaction, ΔH^0,was calculated to be 144.8 ± 12.4 kJ/mol. The standard entropy difference, ΔS^0, is obtained from the temperature dependence of the free energy ΔG^0 (Table 1),

$$\frac{\mathrm{d}\Delta G^0}{\mathrm{d}T} = -\Delta S^0 \qquad (8)$$

From the slope of the plot of ΔG^0 (Fig. 5) the standard entropy difference, ΔS^0, was determined as 406 ± 35 J · mol^{-1} · K^{-1}. By comparing values at 20°C (ΔH^0 = 144.8 kJ mol and $T\Delta S^0$ = 119.1 kJ/mol) it is suggested that the enthalpy contribution prevails in the dissociation reaction.

Table 1. The thermodynamic parameters describing the equilibrium of flavin, free and bound in clostridial flavodoxin, as a function of temperature. fld, flavodoxin.

t	[FMN]	[fld]	K_d	ΔG^0
°C	μM		M	kJ/mol
4	0.009	9.991	1.01×10^{-11}	32.2
12	0.022	9.978	3.25×10^{-11}	29.0
20	0.051	9.949	2.61×10^{-10}	25.7
30	0.137	9.863	1.90×10^{-9}	21.6

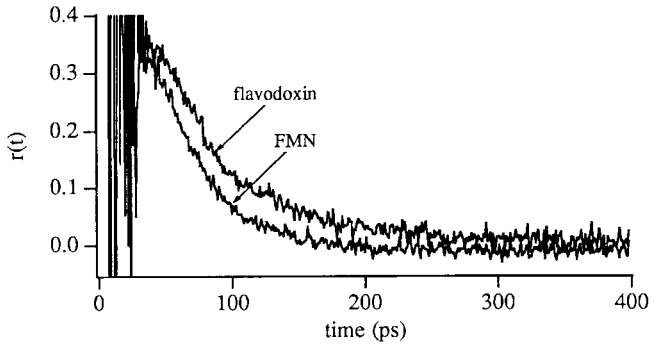

Fig. 6. The anisotropy decays of free FMN and *C. beijerinckii* flavodoxin at 20°C.

Time-resolved fluorescence is one of the very few techniques with which dissociation constants, K_d, can be determined directly in very dilute solutions (thermodynamically ideal).

Time-resolved fluorescence anisotropy

The time-resolved fluorescence anisotropy decays of oxidized *C. beijerinckii* flavodoxin did not differ significantly from the anisotropy decay of free FMN unless an amount of apoflavodoxin was added to the flavodoxin samples. This difference in anisotropy decay is illustrated in Fig. 6. The fluorescence anisotropy decay of flavodoxin is delayed as compared to the anisotropy decay of free FMN, indicating that in flavodoxin the rotation is more hindered than in free flavin (both decays are nonetheless very rapid). The fluorescence anisotropy decays of free FMN in solution, analyzed using the maximum-entropy method, is presented in Fig. 7. It is obvious that the rotational-correlation time, ϕ, of free FMN in solution behaves according to the Stokes-Einstein relationship,

$$\phi = \frac{\eta V}{k T} \qquad (9)$$

where η is the viscosity, V is the molecular volume, k is Boltzmann's constant, and T is the temperature.

Analysis of the clostridial flavodoxin anisotropy decay using the maximum entropy method, shows that the decay has to be described by more than one distribution (Fig. 7). At 4°C a distribution is found at 290 ps, which is ascribed to partially dissociated FMN. A second distribution is found at about 1.2 ns. According to the Stokes-Einstein relation in

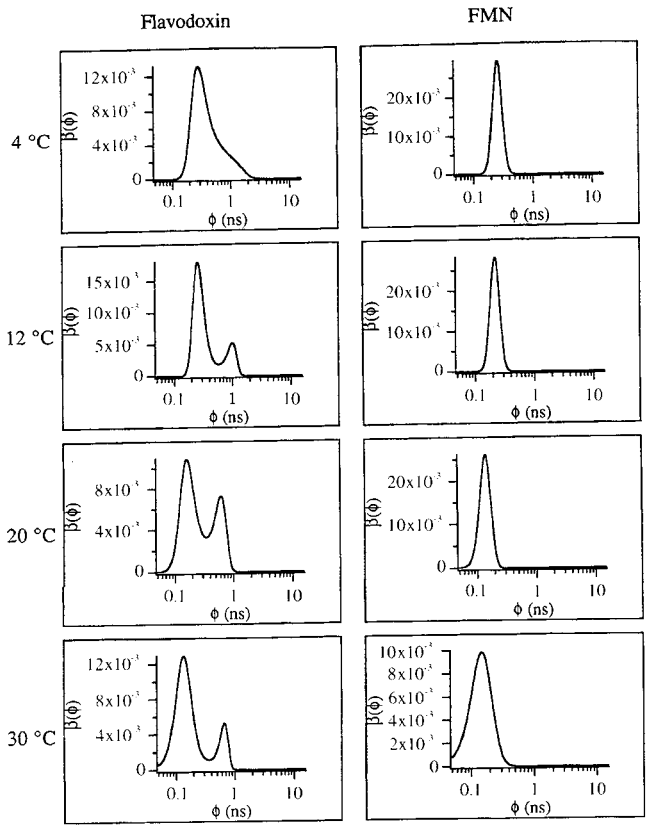

Fig. 7. Maximum entropy analyses of the anisotropy decay of clostridial flavodoxin and free FMN in solution. Measurements are performed between 4 °C and 30 °C. The position of the shortest distribution in the clostridial flavodoxin anisotropy decay is clearly identical to that in free FMN. A longer rotational correlation time is only present in flavodoxin.

combination with the empirically determined relation of Visser et al. (1983),

$$\phi(T) = \eta(T)\, 3.8\times10^{-4} M_r \qquad (10)$$

a protein of M_r of 15000 has a rotational correlation time of about 8.5 ns (at 4 °C). No significant distribution is found corresponding to protein tumbling, indicating that FMN which is bound to the apoflavodoxin is not immobilized within the protein matrix, but has a certain degree of motional flexibility. This is expressed by the rotational correlation time of about 1.2 ns. Integration of the amplitudes, $\beta(\phi)$, yields initial anisotropies of 0.35 for both the FMN and flavodoxin. For other flavoproteins identical initial anisotropies have been demonstrated (Bastiaens et al., 1992b).

One should keep in mind that the very short fluorescence lifetime, which may be coupled to an immobilized flavin moiety, decays so fast that information about the relatively long rotational correlation time is completely lost, irrespective of temperature.

Associative analyses of polarized fluorescence decays

To interpret the polarized fluorescence decays in an unambiguous way we have carried out associative analyses of both flavodoxin and FMN in solution. In this way more information is obtained about the components present in the anisotropy decay of clostridial flavodoxin at different temperatures. The decays were analyzed in terms of rotational correlation times which are coupled to specific fluorescence lifetimes (Eqn 3). The initial anisotropy was fixed at 0.35 in the analysis of both flavodoxin and free FMN. The results of the analyses are given in Fig. 8.

Some very interesting details can be extracted from Fig. 8. First of all we notice that more than one distribution can be found in the flavodoxin anisotropy decay. One of the most pronounced features is the fact that the very short fluorescence lifetime (30 ps) can hardly be coupled specifically with certain rotational-correlation times. As expected, accurate information about longer rotational-correlation times cannot be retrieved from picosecond fluorescence lifetimes. Only at 4 °C is it valid to remark that this ultrashort lifetime seems correlated with a larger rotational-correlation time (more than 5 ns) possibly corresponding with the one expected from overall protein tumbling (8.5 ns). This implies that this associative behaviour originates from a flavin moiety which is immobilized within the protein matrix. Another very pronounced feature (at 4 °C) is the presence of a correlation between the fluorescence lifetime distributed at 4.8 ns and the rotational correlation time distributed at 280 ps. Since this correlation exactly corresponds with the one found in the associative analysis of free FMN and since the size of its rotational correlation time perfectly matches the one expected from extrapolation of the already published correlation time of 155 ps at 20 °C, it is ascribed to dissociated FMN. So, even at lower temperatures (between 4 °C and 20 °C), where no significant dissociation is expected there always seems to be some dissociated FMN present. Furthermore, another correlation is detected between the 4.8-ns-fluorescence-lifetime distribution and a rotational correlation time which is longer than the one found for free FMN. This is best illustrated with measurements at 20 °C (Fig. 8).

The origin of this correlation can be ascribed to a flavin moiety which is flexibly bound to the protein in such a way that the electronic structure of the flavin chromophore is not influenced and therefore no effect on the fluorescence characteristics of the chromophore is detected. The rotational correlation time is larger than for free FMN because the motion of the isoalloxazine ring system is somewhat hindered by the protein environment. A possible explanation is that binding of this moiety occurs via the phosphate group of the FMN. Since earlier studies (Edmondson and Tollin, 1971) demonstrated that the presence of the phosphate group is indispensable for FMN binding to the protein matrix, it might therefore be that the correlation found between the 4.8-ns fluorescence lifetime and the 1-ns rotational correlation time originates from an intermediate step in the binding process. The correlations found in flavodoxin associated with lifetimes between 0.1 ns and 1.5 ns may originate from iso-κ solutions ($1/\kappa = 1/\tau_1 + 1/\phi_2 = 1/\tau_2 + 1/\phi_1$).

Conclusions

In the fluorescence decay of clostridial flavodoxin a trimodal lifetime distribution is demonstrated. The shortest component of about 30 ps originates from the interaction between flavin and the closely located tryptophan residue (Trp90). The fluorescence-lifetime component of about 4.8 ns, present in the flavodoxin decay, coincides with the only distribution present in the fluorescence decay of free FMN. A third lifetime component is distributed at about 0.5 ns. The presence of fluorescence lifetimes of tens of picoseconds in flavodoxins with heavily quenched flavin fluores-

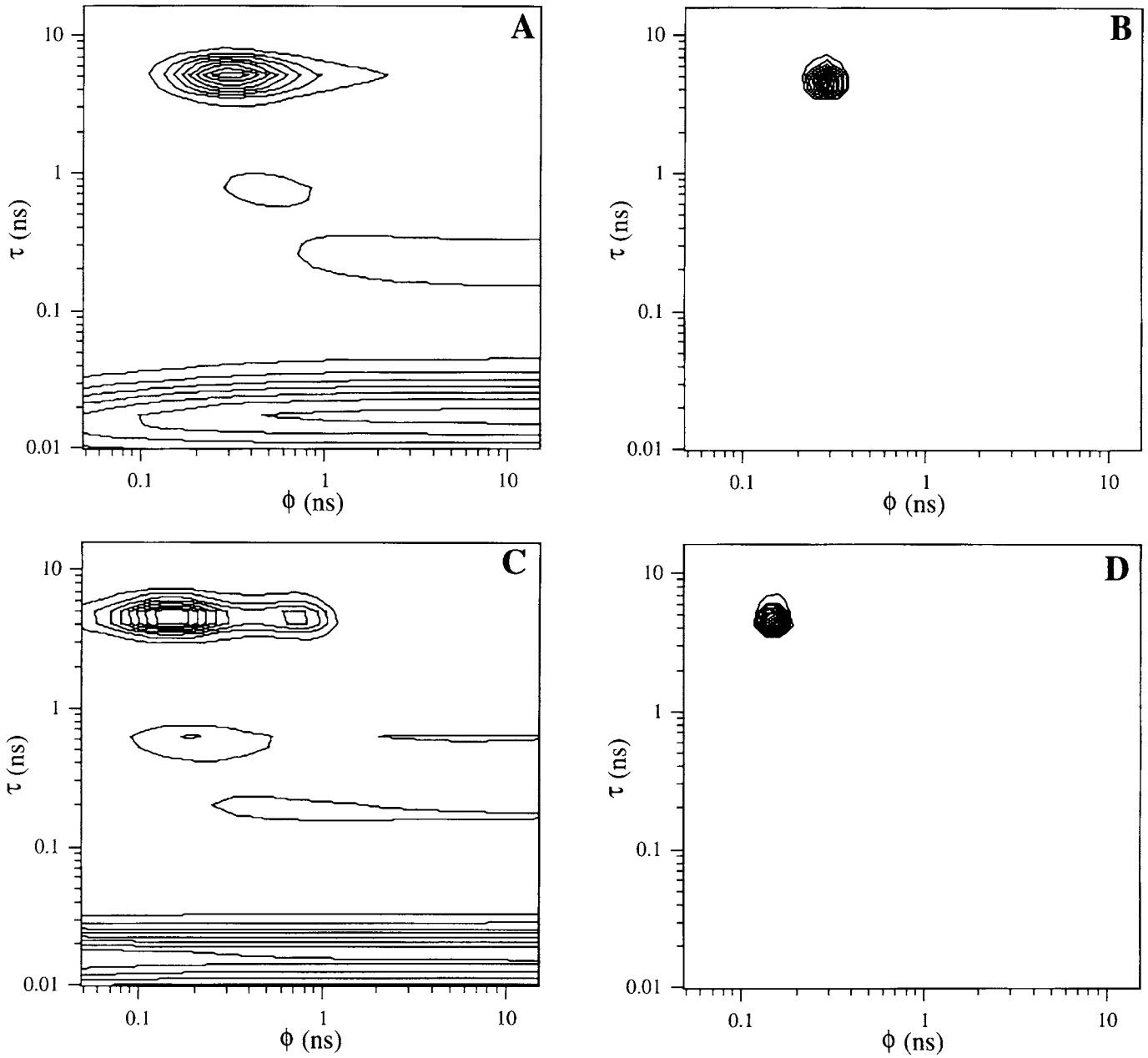

Fig. 8. Contour plots of the associative analysis of the fluorescence and fluorescence anisotropy decays of clostridial flavodoxin at different temperatures, 4°C and 20°C (see text for details). The initial anisotropy was fixed at 0.35 during the analyses. The results for FMN in solution are also given. (A), flavodoxin at 4°C; (B), FMN at 4°C; (C), flavodoxin at 20°C; (D), FMN at 20°C.

cence (non-fluorescent flavoproteins) seems to be a general property.

The relative contribution of the fluorescence lifetimes is clearly temperature dependent, in agreement with the existing equilibrium between protein-bound and dissociated flavin. From these experiments the dissociation constant could be determined, $K_d = 2.61 \times 10^{-10}$ M (at 20°C). A titration study, in which clostridial apoflavodoxin was added to the protein sample, resulted in a significant change in the distribution of fluorescence lifetimes, reflecting the shifted equilibrium between protein-bound and dissociated FMN. Addition of an excess of apoflavodoxin did not result in complete loss of the 4.8-ns fluorescence component.

Collisional fluorescence-quenching experiments with flavodoxin samples that did not contain added apoflavodoxin, showed that the 4.8-ns fluorescence component is affected significantly by iodide. This indicates that a population of flavin molecules, responsible for the 4.8-ns fluorescence lifetime, is accessible to iodide.

The fluorescence-anisotropy decays were also analyzed in an associative way, and it was found that in free FMN one single distribution of rotational-correlation times is present, whereas in flavodoxin the anisotropy decay is composed of more than one distribution. In flavodoxin it was demonstrated that part of the 4.8-ns fluorescence lifetime is specifically coupled to the rotational correlation time of free (dissociated) FMN, but another part of this lifetime is coupled to a longer correlation time (about 1.2 ns). This component is assigned to the intermediate state in which the flavin is flexibly bound. This is in agreement with earlier temperature-

jump studies (Barman and Tollin, 1972) which showed that binding of free FMN to the apoflavodoxin occurs via a two-step mechanism. In the first binding step, the phosphate group is involved which then triggers a conformational change of the protein. Although later studies (reviewed by Mayhew and Tollin, 1992) suggested that flavin binding occurs in a single step, it should be pointed out that current time-correlated single photon fluorescence measurements of high dynamic range allow the observation of low concentrations of intermediate protein conformations (see for instance Bastiaens et al., 1992c) which is not possible with conventional relaxation techniques. At 4 °C the shortest fluorescence lifetime seems to be coupled with a rotational correlation time corresponding to the whole protein meaning that the flavin moiety responsible for the short lifetime is immobilized within the protein matrix.

The different conformational substates probably play a role in the electron-transfer process. However, time-resolved polarized-fluorescence studies in which the flavodoxin is bound to natural donor and acceptor redox partner proteins have to be performed to demonstrate the exact role of the fluctuations as found in this study.

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