

A comparative picosecond-resolved fluorescence study of tryptophan residues in iron-sulfur proteins

Victoria Dorovska-Taran^{a,b}, Arie van Hoek^a, Thomas A. Link^c, Antonie J.W.G. Visser^{a,*}, Wilfred R. Hagen^a

^aDepartment of Biochemistry, Agricultural University, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands

^bInstitute of Organic Chemistry Bulgarian Academy of Sciences, ul. Acad. Bonchev bl. 9 1113 Sofia, Bulgaria

^cUniversitätsklinikum Frankfurt, Zentrum für Biologische Chemie, Therapeutische Biochemie, Frankfurt/Main, Germany

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Abstract

The fluorescence intensity and anisotropy decays of the intrinsic tryptophan emission from six Fe/S proteins (ranging from the very simplest ones to enzyme complexes containing one, two or more Trp residues) were measured. All proteins were examined in the reduced and the oxidized state. In either redox state each protein exhibits ultrarapid tryptophan fluorescence decay on the picosecond timescale contributing up to 93% of the total decay. Correlation times in the range of 1 ns or less were found for all six iron-sulfur proteins reflecting internal Trp motion. In addition, some proteins exhibit longer correlation times reflecting segmental motion and overall protein tumbling. The ultrarapid fluorescence decay in iron-sulfur proteins indicates efficient radiationless energy transfer between distant tryptophan residues and iron-sulfur clusters. Such an energy transfer mechanism can be accounted for by referring to the three-dimensional structures of rubredoxin and ferredoxin in calculating the transfer efficiency of the single tryptophan–iron-sulfur couple.

Key words: Iron-sulfur protein; Tryptophan fluorescence quenching; Picosecond resolved fluorescence; Förster energy transfer

1. Introduction

Iron-sulfur proteins are ubiquitously present in all forms of life from archaebacteria to humans. They are essential parts of vital processes, e.g. respiration, photosynthesis, intermediary metabolism, and the activation of nitrogen, hydrogen, CO_x, SO_x, and methane. They function as electron transferring devices, as Lewis acid catalysts, as redox catalysts, and as regulatory systems in gene translation.

The intrinsic tryptophan fluorescence of an iron-sulfur protein containing one, two or more tryptophan residues represents an excellent chemical system for studying protein conformation and dynamics of amino acid residues directly implicated in the energy/electron transfer pathways. The significant overlap between the absorption spectrum of the iron-sulfur cluster and the emission spectrum of tryptophan makes radiationless energy transfer an extremely favoured process. However, because of the strong quenching of tryptophan emission in iron-sulfur proteins the detection and interpretation of emission signals with very low intensity pose problems, therefore a very small number of studies of these proteins by steady-state and time-resolved fluorescence methods has appeared [1,2].

In the present paper we report measurements of picosecond-resolved fluorescence decay of the tryptophan in six iron-sulfur proteins (ranging from the very simplest

ones containing only one tryptophan residue to an enzyme complex with five tryptophans) in different redox states. None of the observed ultrarapid picosecond fluorescence lifetimes have been reported previously.

2. Materials and methods

Megasphaera elsdenii rubredoxin was purified as described by [3]. The apoprotein (iron-depleted form) of rubredoxin was prepared by TCA precipitation followed by redissolving in buffer containing DTT. Spinach leaf ferredoxin was isolated as described in [4]. Purification of rubrerythrin from *Desulfovibrio vulgaris* has been described previously [5]. A water-soluble fragment of the Rieske iron-sulfur protein of the *bc₁* complex was prepared as described in [6] with some modifications [7]. *Desulfovibrio vulgaris* assimilatory SO₃ reductase was purified as in [8]. A 25 mM Tris-HCl, pH 7.5, solution was used as the buffer for rubredoxin, spinach ferredoxin, rubrerythrin, assimilatory sulfite reductase and Fe-hydrogenase. The buffer systems used for the Rieske soluble fragment were MES, pH 6.0, TAPS, pH 8.4, and CAPS, pH 10.0 all in a final concentration of 50 mM. The pH buffer values were adjusted with NaOH. Anaerobic reduction or re-oxidation of protein samples was carried out directly in 1 cm anaerobic quartz cuvettes by the addition of 5–15 µl of freshly prepared reductant or oxidant anaerobic solutions of Na₂S₂O₄ (threefold excess) or K₃Fe(CN)₆ (tenfold excess), respectively. The anaerobic reduction of *D. vulgaris* hydrogenase was performed by flushing with purified hydrogen; re-oxidation by protons was achieved by replacing the hydrogen with repeated vacuum/argon cycles for 30 min [9]. Absorption and steady-state fluorescence spectra were recorded on a Cary-14 spectrophotometer and a SLM-Aminco SPF-500C fluorometer. The optical density of each protein sample at 295 nm prepared for time-resolved fluorescence measurements did not exceed 0.1.

2.1. Time-resolved polarised fluorescence measurements

Time-resolved fluorescence and fluorescence anisotropy decays curves were measured by the time-correlated single-photon counting technique. The excitation source consisted of a frequency-doubled

* Corresponding author. Fax: (31) (8370) 84 801.

rhodamine 6G-dye laser giving 4 ps pulses at a repetition rate of 951.2 kHz and the excitation light at 295 nm was vertically polarized. Further technical details are given in [10]. Emission was measured through a combination of a WG335 cut-off filter (Schott) and a 348.8-nm interference filter of 5.4 nm bandpass (Schott). The instrumental response function, corresponding to the laser pulse convoluted with the detection response, was determined by measuring the fluorescence decay of *p*-terphenyl (BDH) in cyclohexane (spectroscopic grade, Merck) quenched by the addition of 30% (v/v) carbon tetrachloride resulting in a lifetime of tens of picoseconds [11]. The tryptophan fluorescence of each protein was sampled during 20 cycles of 10 s in each polarisation direction where the detection frequency of the parallel polarized component was set to 30 kHz to prevent pulse pile-up. The reference compound was sampled during 2 cycles of 10 s in each polarisation direction. Background (same solution but without protein) was usually sampled at one quarter of the sample acquisition time. Polarized fluorescence intensity components $I_{||}(t)$ and $I_{\perp}(t)$ were registered. From this the total fluorescence decay $S(t) = I_{||}(t) + 2I_{\perp}(t)$ and the fluorescence anisotropy decay $r(t) = (I_{||}(t) - I_{\perp}(t)) / (I_{||}(t) + 2I_{\perp}(t))$ was obtained. One complete measurement consisted of measuring the polarized (parallel and perpendicular components) fluorescence decays of the reference compound, the sample, the background and again the reference compound. The measuring system has been shown to accurately resolve fluorescence lifetimes of 30 ps [12]. All experiments were conducted at 20°C.

2.2. Data analysis

The maximum entropy data analysis method (MEM, Maximum Entropy Data Consultants Ltd., Cambridge, UK) was applied to recover lifetime and correlation time distributions from the total fluorescence decay, $S(t)$, and the anisotropy decay, $r(t)$. The principle of MEM has been described previously [13]. The advantage of this method is that a unique solution is found with no a priori knowledge of a decay model.

3. Results

3.1. Spectral overlap

The absorption spectra of the iron-sulfur proteins extensively overlap with the fluorescence emission of the tryptophan residues in these proteins (Fig. 1) creating the possibility of non-radiative energy transfer between excited tryptophans and iron-sulfur clusters.

3.2. Fluorescence decay

The fluorescence decay of oxidized and reduced states of rubredoxin, ferredoxin, rubrerythrin, sulfite reductase and hydrogenase at pH 7.5 was obtained. The Rieske soluble fragment was examined in its oxidized and reduced state at pH 6.0, 8.4 and 10. Time-resolved fluorescence decays of rubredoxin and of its apo-protein are shown together with the dynamic impulse response function in Fig. 2A. It can be observed already from the experimental curves that native rubredoxin has a much faster decay than the iron-free protein. An example of MEM analysis in fluorescence lifetime distributions is presented in Fig. 2B illustrating the presence of an ultrashort fluorescence lifetime component in native rubredoxin. Examples of time-resolved experimental fluorescence decays for oxidized hydrogenase and Rieske soluble fragment are shown in Fig. 3, providing evidence that rapid decay fluorescence components are also observed in other iron-sulfur proteins. An example of decay curves for oxidized and reduced ferredoxin is given in

Fig. 4A together with the results of MEM analysis in Fig. 4B. All fluorescence decays were found to be highly heterogeneous. The fluorescence lifetime distribution analysis recovered mostly four components. The values of the lifetimes (τ), and their fractional contributions (α) after MEM analysis of the fluorescence decays are collected in Table 1. In every case a very short lifetime component in the order of 20–60 ps has the most predominant contribution. The fractional contribution of the picosecond peak for the single tryptophan containing proteins, rubredoxin and ferredoxin, was between 80–95%, and for the proteins with two and more tryptophans 30–75%. This presence of a certain fraction of the short lifetime component indicates a multitude of microenvironments of the studied iron-sulfur proteins probably of similar origin as found for the flavin prosthetic group in glutathione reductase [14].

3.3. Fluorescence anisotropy decay

First it should be noted that the carrier signal for (sub)nanosecond anisotropy decay components arises from the slower, non-picosecond fluorescence decay components contributing to a minor extent to fluorescence intensity. It implies that the anisotropy decay can only be interpreted in a qualitative fashion. The correlation–time image of the fluorescence–anisotropy decay of rubredoxin was biphasic both in the oxidized and the reduced state. The fast component was in the order of 0.5 ns and the slow component 7 ns in both redox states. Results have been collected in Table 2. For the oxidized and reduced forms of spinach ferredoxin we could also identify complicated decays consisting of fast components and relatively slow rotational components arising from protein tumbling (see Table 2). The Rieske soluble fragment which contains two tryptophan residues exhib-

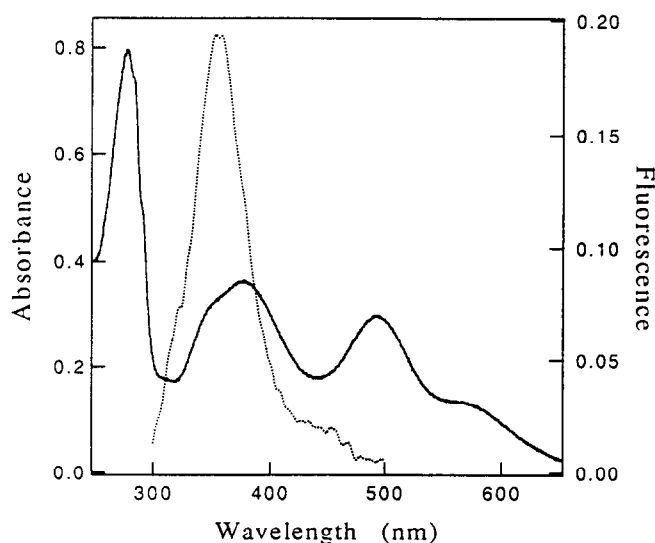


Fig. 1. Absorption (solid line) and fluorescence (dotted line) spectra of *M. elsdenii* rubredoxin in 25 mM Tris-HCl buffer, pH 7.5. Excitation wavelength 295 nm.

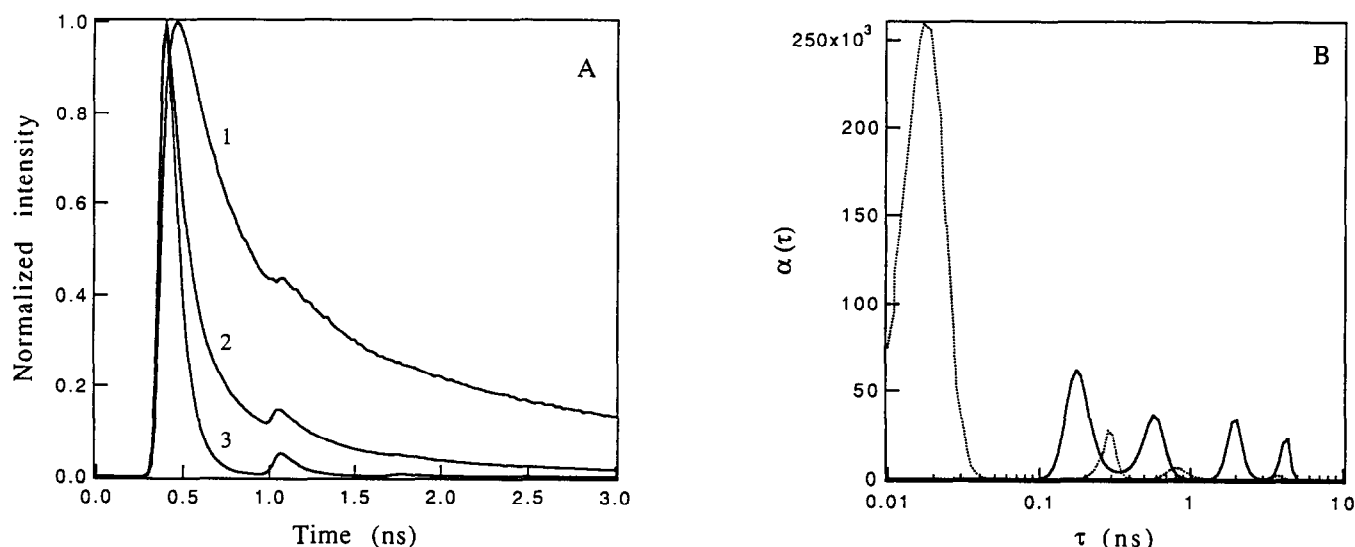


Fig. 2. (A) Experimental total fluorescence decay curves of *M. elsdenii* rubredoxin in 25 mM Tris-HCl buffer, pH 7.5. (1) Aporubredoxin (iron-depleted) (2) native rubredoxin; (3) reference. Excitation at 295, emission at 348.8 nm. (B) Fluorescence lifetime distribution of oxidized rubredoxin in 25 mM Tris-HCl buffer, pH 7.5: dotted line, native rubredoxin; solid line, aporubredoxin (iron-depleted).

its one main short correlation time which slightly increased from 1 to 2 ns as the pH increased from 6.5 to 10, with an additional slight increase upon reduction of the oxidized protein. Both rubrerythrin and sulfite reductase, which also contain two tryptophan residues, displayed in different redox states one fast and one long correlation time (see Table 2). The anisotropy decay of the five tryptophans in oxidized and reduced hydrogenase turned out to be even more complex (see Table 2).

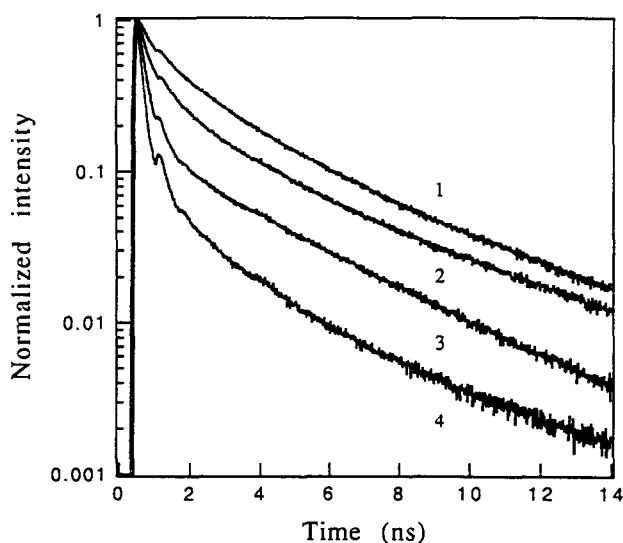


Fig. 3. Examples of experimental total fluorescence decay of iron-sulfur proteins in oxidized state: (1) *D. vulgaris* hydrogenase in 25 mM Tris-HCl buffer, pH 7.5; (2) *D. vulgaris* rubrerythrin in 25 mM Tris-HCl buffer, pH 7.5; (3) bovine heart Rieske soluble fragment in 50 mM MES, pH 6.0; (4) *D. vulgaris* assimilatory sulfite reductase in 25 mM Tris-HCl buffer, pH 7.5. Excitation at 295 nm, emission at 348.8 nm.

4. Discussion

The fluorescence decay of aqueous tryptophan solutions below pH 7 is biexponential with a minor component ($\tau_1 = 0.8$ ns) and a major component ($\tau_2 = 3.2$ ns) [15] and above pH 7 even a triple exponential with $\tau_1 = 0.5$ ns, $\tau_2 = 3$ ns, and $\tau_3 = 9.1$ ns [16]. Two shorter lifetimes were assigned to the zwitterionic form of tryptophan and the longest lifetime to anionic tryptophan, which shows that aqueous tryptophan is a poor model for tryptophan fluorescence in proteins. *N*-Acetyltryptophanamide (NATA) should be a better model system as its side chain mimics the peptide bond. Its fluorescence decay is monoexponential ($\tau = 2.9$ ns, pH 7.4) [17]. However, the fluorescence decay in proteins even with a single tryptophan residue is usually multiexponential. The common interpretation of such a result is the assumption of multiple protein conformers of tryptophan with one lifetime for each conformer [18]. It is likely that the lifetime in the ps timescale originated from efficient tryptophan quenching. Lifetimes in the ns region are expected to arise from non-quenched protein conformers [14]. Their amplitudes are very small due to rarely occurring orientations of the residue, which escape the quenching [19]. All iron-sulfur proteins studied exhibit unprecedented ultrarapid tryptophan fluorescence decay in the ps timescale. In all cases four exponentials are needed to obtain acceptable fits to the experimental data (Table 1).

The fractional contribution of the ps lifetime in rubredoxin and ferredoxin in both reduced and oxidized states is approximately 90%. This short lifetime could result from weak dipolar interactions between the excited indole ring or tryptophan and the iron-sulfur cluster. The

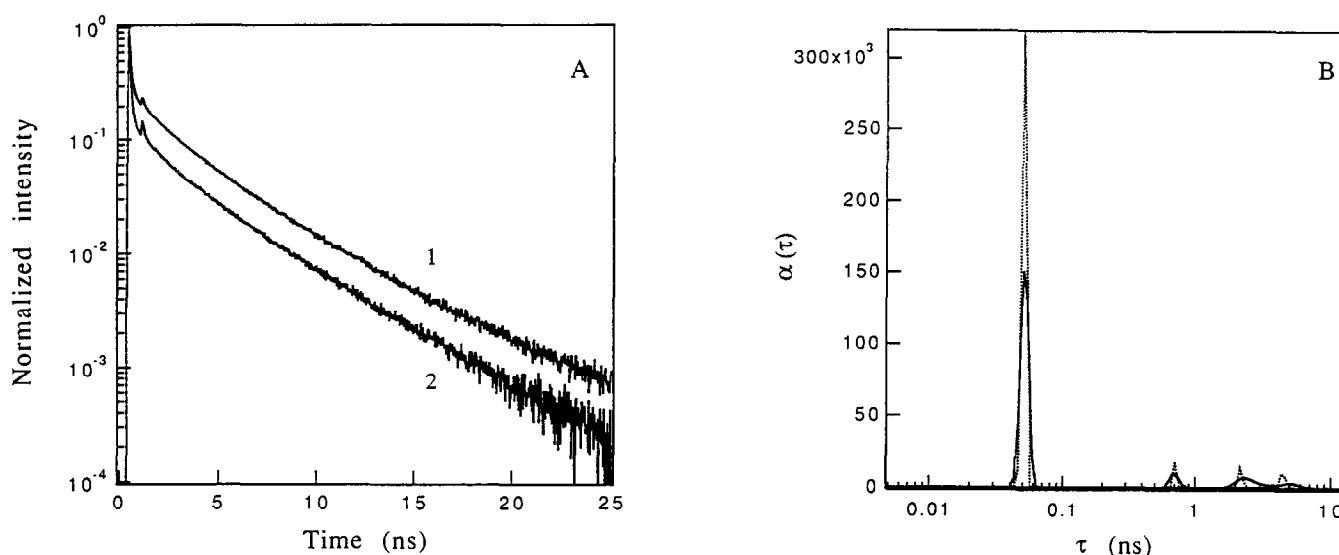


Fig. 4. (A) Experimental total fluorescence decay of spinach leaf ferredoxin in 25 mM Tris-HCl buffer, pH 7.5, in the reduced (1) and oxidized (2) state. Excitation at 295 nm, emission at 348.8 nm. (B) Fluorescence lifetime distribution of oxidized (solid line) and reduced (dotted line) spinach leaf ferredoxin in 25 mM Tris-HCl buffer, pH 7.5.

quenching of aromatic fluorescence by metal ions can involve a number of mechanisms, such as weak Förster dipole–dipole energy transfer, Dexter short range energy transfer (for a recent survey see [20]) or even through-space electron transfer. The most commonly occurring case is the Förster type of energy transfer. Energy transfer from Trp to the iron-sulfur cluster is dependent on the relative position and orientation of donor (trypto-

phan) and acceptor (the iron-sulfur cluster). The critical transfer distance R_0 (in nm) according to the Förster theory can be expressed by the following equation:

$$R_0 = 9.7 \times 10^2 (\kappa^2 \cdot n^{-4} \cdot Q_D \cdot J(\lambda))^{1/6}$$

Here κ^2 is the orientation factor, n is the refractive index of the solvent (1.4), Q_D is the quantum yield of the

Table 1
Fluorescence lifetimes of tryptophan residues in iron-sulfur proteins^a

Protein		M.W. (kDa)	Trp	Redox state	pH	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	α_4	τ_4 (ns)
1Fe(Cys) ₄	A	5.7	1	oxidized	7.5	0.95	0.02	0.03	0.35	0.01	0.83	0.02	3.71
<i>Megasphaera elsdenii</i> rubredoxin				reduced	7.5	0.93	0.03	0.05	0.33	0.01	0.80	0.01	2.99
apo-rubredoxin				oxidized	7.5	0.50	0.19	0.25	0.58	0.17	1.98	0.07	4.17
[2Fe-2S](Cys) ₄	B	≈11	1	oxidized	7.5	0.90	0.055	0.04	0.80	0.04	2.31	0.03	4.64
Spinach leaf ferredoxin				reduced	7.5	0.81	0.05	0.06	0.70	0.09	2.38	0.04	4.92
[2Fe-2S](Cys) ₂ (His) ₂	C	15	2	oxidized	6.0	0.42	0.04	0.50	0.20	0.03	0.84	0.05	3.60
bovine heart Rieske				reduced	6.0	0.57	0.03	0.36	0.25	0.03	1.10	0.04	3.50
soluble fragment				oxidized	8.4	0.74	0.02	0.16	0.25	0.08	0.70	0.02	3.70
				reduced	8.4	0.45	0.03	0.35	0.28	0.16	0.72	0.04	3.50
				oxidized	10.0	0.36	0.05	0.38	0.26	0.22	0.66	0.04	3.70
				reduced	10.0	0.25	0.09	0.43	0.34	0.27	0.75	0.05	4.10
1Fe and [Fe-μO-Fe](Glu,His)	D	21.5	2	oxidized	7.5	0.71	0.04	0.18	0.40	0.07	1.84	0.04	5.06
<i>Desulfovibrio vulgaris</i> rubrerythrin				reduced	7.5	0.73	0.03	0.09	0.18	0.13	0.55	0.05	1.67
[Fe/S] and siroheme	E	24	2	oxidized	7.5	0.55	0.06	0.41	0.16	0.03	0.94	0.01	3.00
<i>Desulfovibrio vulgaris</i> assimilatory sulfite reductase				reduced	7.5	0.62	0.03	0.19	0.18	0.11	0.80	0.07	3.00
[6Fe/S] and 2[4Fe-4S]	F	56	5	oxidized	7.5	0.42	0.02	0.18	0.22	0.22	0.90	0.18	3.48
<i>Desulfovibrio vulgaris</i> hydrogenase				reduced	7.5	0.53	0.02	0.17	0.22	0.17	0.95	0.13	3.28

^aObtained from the main contributions in the maximum entropy method of fluorescence decay analysis:

$$\sum_{i=1}^4 \alpha_i e^{-t/\tau_i}$$

in which τ_i represent the barycenters of the distribution and α_i the relative contributions. See Figs. 2B and 4B for graphical examples.

Table 2
Correlation times of tryptophan residues in iron-sulfur proteins^a

Protein ^b	Redox state	pH	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	β_3	ϕ_3 (ns)
A	oxidized	7.5	0.2	0.45 (0.02) ^c	0.06	7.0 (0.8) ^c		
	reduced	7.5	0.2	0.5 (0.02)	0.07	7.2 (0.9)		
apo-B	oxidized	7.5	0.2	0.9 (0.3)	0.05	5.8 (3.1)		
	reduced	7.5	0.05	0.9 (0.2)	0.06	3.4 (1.3)		
C	oxidized	7.5	0.06	0.8 (0.35)	0.02	2.4 (0.7)		
	reduced	6.0	0.2	0.85 (0.03)				
D	oxidized	6.0	0.15	0.9 (0.03)				
	reduced	8.4	0.15	1.4 (0.05)				
E	oxidized	8.4	0.15	1.6 (0.2)				
	reduced	10.0	0.15	1.6 (0.1)				
F	oxidized	10.0	0.15	2.2 (0.1)				
	reduced	7.5	0.03	1.7 (0.1)	0.1	67 (19)		
G	oxidized	7.5	0.03	1.3 (0.2)	0.1	57 (24)		
	reduced	7.5	0.1	1.05 (0.1)	0.09	34 (17)		
H	oxidized	7.5	0.03	0.9 (0.1)	0.15	24 (11)		
	reduced	7.5	0.05	0.7 (0.2)	0.01	2.8 (1.0)	0.09	48 (28) ^c
I	oxidized	7.5	0.1	0.25 (0.1)	0.03	2.5 (1.1)	0.07	66 (23)
	reduced	7.5	0.1	0.25 (0.1)	0.03	2.5 (1.1)	0.07	66 (23)

^a Obtained from the main contributions of the maximum entropy method of fluorescence anisotropy decay analysis. ϕ_1 is the correlation time (obtained from the barycenters of the distribution) and β_1 is the relative contribution. $\sum \beta_i$ represents the initial anisotropy.

^b Protein systems are detailed in Table 1.

^c Values in parentheses indicate the width of the distribution.

donor in the absence of the acceptor and $J(\lambda)$ is a measure of the spectral overlap integral between donor emission and acceptor absorption.

An assessment can be made for single tryptophan containing iron-sulfur proteins. The crystal structures of *M. elsdenii* rubredoxin and spinach ferredoxin are not known but from a solvent-surface image of the crystal structures of the closely related *Desulfovibrio gigas* rubredoxin [21] and *Spirulina platensis* ferredoxin (which contains Tyr in position 75 instead of tryptophan in spinach ferredoxin [22]) it is seen that the tryptophan (or tyrosine) residue and the iron-sulfur clusters are fully exposed to the solvent. The short component in the correlation time distribution of the studied proteins can be ascribed to rapid internal flexibility of the tryptophan residue. In addition, the absorption transition moment is not expected to have one unique direction in the iron-sulfur cluster since the light absorption arises from transfer of charge from the sulfur atoms to the iron. Because of these considerations (dynamic averaging of tryptophan and degeneracy of optical transitions of the iron-sulfur centre) we can therefore set in first approximation κ^2 equal to 0.67. The spectral overlap integrals (Fig. 1) for oxidized rubredoxin and ferredoxin were evaluated as $9.45 \times 10^{-15} \text{ cm}^3/\text{M}$ and $1.82 \times 10^{-14} \text{ cm}^3/\text{M}$, respectively. The (average) fluorescence quantum yield of aporubredoxin was determined by us as 0.14 which is in the same order of magnitude as found for apoferredoxin from *Halobacterium* of the Dead Sea [1]. The value for R_0 calculated for a $Q_D = 0.14$ is then in the order of 2.5 nm for both rubredoxin and ferredoxin. The transfer

efficiency, T , can be related to the donor-acceptor distance, r , and to the ratio of fluorescence lifetimes by:

$$T = 1 / \{ 1 + (r/R_0)^6 \} = 1 - (\tau_D/\tau_D^0)$$

in which τ_D is the average fluorescence lifetime of the donor (tryptophan) in the presence of acceptor and τ_D^0 is the lifetime in the absence of acceptor. In this way one can estimate the ratio of fluorescence lifetimes from the energy transfer efficiency and the expected lifetime component once the fluorescence lifetime in the apo-protein is known. The average distance between Trp³⁷ of different rubredoxins [21,23–25] and the iron is approximately 0.8 nm and between Tyr⁷⁵ and the cluster edge of ferredoxins [22,26,27] is approximately 1.2 nm. The calculated transfer efficiency is then between 99% and 100% for rubredoxin and ferredoxin. As the donor-acceptor distance is less than about 1.2 nm it is then not unreasonable to suggest that the quenching of the tryptophan fluorescence originates from energy transfer to the iron-sulfur cluster since, for example, a transfer efficiency of 99.8% implies that $\tau_D/\tau_D^0 \approx 0.002$ or τ_D is 20 ps when τ_D^0 is 10 ns.

From fluorescence lifetimes presented in Table 1 one can then immediately conclude that the picosecond lifetime components found in the majority of the iron-sulfur proteins are consistent with a Förster energy transfer mechanism. It is not known why these rapid components were not observed previously [2]. It should be pointed out that, depending on the donor-acceptor geometry and orientation in each iron-sulfur protein, other

quenching mechanisms, like Dexter short range energy transfer or electron transfer, may play a role.

From Tables 1 and 2 it can be seen that a change in redox state only leads to minor changes in the distribution pattern of the fluorescence lifetimes and not to changes in correlation times. The latter effect suggests that no major protein conformation change is involved in a change of redox state. The tendency observed with the fluorescence lifetimes can be explained by a change in the spectral overlap integral upon reduction, because the light absorption spectrum of the iron-sulfur cluster changes upon reduction. The effect of reduction on the spectral overlap modulates the critical transfer distance and will therefore affect the transfer rate and observed fluorescence lifetimes. However, because of the 1/6th power dependence of the critical transfer distance on the overlap integral, a twofold change in overlap integral, for instance, will have a minor influence on the final fluorescence lifetimes as is actually observed. In case of the Rieske protein a change in pH leads to a distinct change in the correlation time of internal motion. This observation suggests a pH-induced local protein conformational change. The correlation time distribution pattern is sometimes very complicated, e.g. hydrogenase with five tryptophan residues shows at least three distinct correlation times. In this case a multitude of decay modes is present arising from internal tryptophan motion, inter-tryptophan energy transfer and overall protein tumbling.

It should be emphasized that these polarized fluorescence decay results of a variety of iron-sulfur proteins provide a first global survey of the type of information present. A much more detailed, quantitative interpretation would require an investigation of each protein as a single biophysical object of study.

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