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Tryptophan Fluorescence in Electron-Transfer Flavoprotein:Ubiquinone Oxidoreductase: Fluorescence Quenching by a Brominated Pseudosubstrate†

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ABSTRACT: We have studied the intrinsic fluorescence of the 12 tryptophan residues of electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). The fluorescence emission spectrum (λ_{ex} 295 nm) showed that the fluorescence is due to the tryptophan residues and that the contribution of the 22 tyrosine residues is minor. The emission maximum (λ_m 334 nm) and the bandwidth ($\Delta\lambda_{1/2}$ 56 nm) suggest that the tryptophans lie in hydrophobic environments in the oxidized protein. Further, these tryptophans are inaccessible to a range of ionic and nonionic collisional quenching agents, indicating that they are buried in the protein. Enzymatic or chemical reduction of ETF:QO results in a 5% increase in fluorescence with no change of λ_m or $\Delta\lambda_{1/2}$. This change is reversible upon reoxidation and is likely to reflect a conformational change in the protein. The ubiquinone analogue $Q_0(CH_2)_{10}Br$, a pseudosubstrate of ETF:QO (K_m = 2.6 μM; k_{cat} = 210 s^{-1}), specifically quenches the fluorescence of one tryptophan residue (K_d = 1.6–3.2 μM) in equilibrium fluorescence titrations. The ubiquinone homologue UQ-2 (K_m = 2 μM; k_{cat} = 162 s^{-1}) and the analogue $Q_0(CH_2)_{10}OH$ (K_m = 2 μM; k_{cat} = 132 s^{-1}) do not quench tryptophan fluorescence; thus the brominated analogue acts as a static heavy atom quencher. We also describe a rapid purification for ETF:QO based on extraction of liver submitochondrial particles with Triton X-100 and three chromatographic steps, which results in yields 3 times higher than previously published methods.

Electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) is an iron–sulfur flavoprotein located on the inner face of the inner mitochondrial membrane. This integral membrane protein catalyzes the oxidation of electron-transfer flavoprotein (ETF)[1] and the reduction of ubiquinone. It thus functions with ETF as an intermediate electron carrier between eight primary flavoprotein dehydrogenases and the ubiquinone pool (Frerman 1987). These primary dehydrogenases are the three acyl-CoA dehydrogenases of β-oxidation, isovaleryl-CoA dehydrogenase, 2-methylbutyryl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, sarcosine dehydrogenase, and dimethylglycine dehydrogenase (Frerman, 1988).

ETF:QO contains a single noncovalently bound molecule of FAD together with a $[4Fe\text{-}4S]^{1+,2+}$ cluster (Ruzicka & Beinert, 1977; Beckmann & Frerman, 1985a) and can exist in four oxidation states. Enzymatic reduction yields a two-

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[1] Abbreviations: deazaflavin, 10-*N*-methyl-3-*N*-(sulfopropyl)-5-deazaflavin; ETF_{ox}, electron-transfer flavoprotein (oxidized); ETF_{1e^-}, electron-transfer flavoprotein (one electron reduced); ETF_{2e^-}, electron-transfer flavoprotein (two electron reduced); Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MCAD, medium-chain acyl-CoA dehydrogenase; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; $Q_0(CH_2)_{10}Br$, 6-(10-bromodecyl)ubiquinone; $Q_0(CH_2)_{10}OH$, 6-(10-hydroxydecyl)ubiquinone; $Q_0(CH_2)_7H$, 6-heptylubiquinone; $Q_0(CH_2)_9H$, 6-nonylubiquinone; UQ-1, ubiquinone 1; UQ-2, ubiquinone 2.

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electron-reduced enzyme that contains the one-electron-reduced cluster and the anionic flavin semiquinone (Johnson et al., 1987). The three-electron-reduced protein containing the flavin hydroquinone and a reduced cluster can only be attained by chemical reduction with low-potential reductants (Beckmann & Frerman, 1985a). By analogy with NADH:ubiquinone oxidoreductase (complex I) and succinate:ubiquinone oxidoreductase (complex II), the iron–sulfur cluster is the likely reductant of ubiquinone (Ragan, 1987; Ohnishi, 1987). However, unlike ETF:QO, these complex enzymes require other protein components to exhibit ubiquinone reductase activity (Frerman, 1987). Thus far, only the kinetics of UQ-1 reduction by ETF:QO have been investigated (Ramsay et al., 1987), but nothing is known about the ubiquinone binding site in this monomeric ubiquinone oxidoreductase.

The intrinsic fluorescence of proteins is primarily due to tryptophan residues (Lakowicz, 1983). Tryptophan fluorescence is extremely sensitive to environment (Burstein et al., 1973), and this property has been exploited to study changes in the structure of proteins and map tryptophan residues relative to other chromophores and fluorophores (Stryer, 1978). Those changes of structure related to function and redox state in mixed-valance cytochrome *c* oxidoreductase have been studied in this way (Copeland et al., 1987, 1988; Ferreira-Rajabi & Hill, 1989). This approach has also been employed to investigate the binding of ubiquinone homologues and the dependence of ubiquinone binding on the redox state of ubiquinol:cytochrome *c* oxidoreductase (Samworth et al., 1988). However, such studies are difficult to interpret since both systems are multipolypeptide complexes.

Aromatic residues have been implicated in the ubiquinone binding sites of the photosynthetic reaction center and the redox sites of a number of bacterial, plant, and animal oxidation–reduction proteins. Thus our rationale was that the fluorescence properties of tryptophan residues could be a useful tool to investigate structure–function relationships in electron-transfer reactions of the protein, including the reaction at the intrinsic quinone binding site. Further, this approach might elucidate some aspects of the electron-transfer reaction between the redox centers, which have been estimated to be separated by about 1 nm on the basis of the extremely weak coupling observed between the centers in the two-electron-reduced protein (Johnson et al., 1987).

Materials and Methods

Reagents. MCAD was prepared essentially as described (McKean et al., 1979) and had an A_{270nm}:A_{448nm} ratio of 5.3–5.6; its concentration was determined by using E_{448nm} = 15.2 mM^{-1} cm^{-1} (Gorelick et al., 1985). ETF was purified as described by Husain and Steenkamp (1983) and had an A_{270nm}:A_{436nm} of 5.8–6.0; its concentration was determined by using E_{436nm} = 13.4 mM^{-1} cm^{-1} (McKean et al., 1983). Glucose oxidase (type V from *Aspergillus niger*), catalase, and octanoyl-CoA were purchased from Sigma. Urea (Microselect) and Q_0 were purchased from Fluka. The urea was prepared as a concentrated stock that was deionized with a mixed-bed resin (Bio-Rex RG 501-X8) prior to use. Acrylamide (four times recrystallized) was purchased from Serva, and dodecyl maltoside was purchased from Boehringer-Mannheim.

The short-chain ubiquinone homologues UQ-1 and UQ-2 were gifts from Eisai Corp., and the ubiquinone analogues $Q_0(CH_2)_7H$ and $Q_0(CH_2)_9H$ were gifts of Dr. B. L. Trumpower (Dartmouth Medical School). The ubiquinone analogues $Q_0(CH_2)_{10}Br$ and $Q_0(CH_2)_{10}OH$ were synthesized from Q_0 as described by Yu and Yu (1982). Deazaflavin was a gift from Dr. Betty Sue Masters (Medical College of Wisconsin).

Ultrapure water (Milli Q, Millipore Corp.) was used in all experiments, and all other reagents were of the highest available grade.

Assay of ETF:QO. ETF:QO was assayed spectrophotometrically in a 0.4-mL volume containing 20 mM HEPES, 1 μM MCAD, 1 μM ETF, 100 μM octanoyl-CoA, and 70 μM UQ-1, pH 7.4. Reactions were initiated by the addition of ETF:QO. Activity was calculated from the decrease in absorbance at 275 nm due to the reduction of UQ-1 with a ΔE_{275} of 7.3 mM^{-1} cm^{-1}, which takes into account the formation of *trans*-oct-2-enoyl-CoA (Ramsay et al., 1987). This extinction coefficient was determined in this laboratory and is in good agreement with the value of 7.4 mM^{-1} cm^{-1} reported by Ramsay et al. (1987) in the assay buffer without detergent. For the kinetic studies, difference extinction coefficients of the other quinones were similarly determined in 20 mM Hepes, pH 7.5, containing 0.1 mg mL^{-1} dodecyl maltoside at the red-shifted absorption maxima and corrected for the formation of *trans*-oct-2-enoyl-CoA at those absorption maxima. The experimentally determined difference extinction coefficients used in the kinetic experiments were the following: UQ-1, ΔE_{277nm} = 7.1 mM^{-1} cm^{-1}; UQ-2, ΔE_{277nm} = 7.6 mM^{-1} cm^{-1}; $Q_0(CH_2)_{10}OH$, ΔE_{280nm} = 7.6 mM^{-1} cm^{-1}; $Q_0(CH_2)_{10}Br$, ΔE_{280nm} = 8.3 mM^{-1} cm^{-1}; $Q_0(CH_2)_7H$, ΔE_{279nm} = 7.5 mM^{-1} cm^{-1}; $Q_0(CH_2)_9H$, ΔE_{280nm} = 5.0 mM^{-1} cm^{-1}.

In the kinetic assays and equilibrium fluorescence titrations, the concentrations of the longer side-chain ubiquinone analogues and homologues were always less than 20 μM, since the absorbance at the maximum becomes nonlinear with respect to concentration. This is presumably due to aggregation in the buffer–detergent system employed.

Assay of ETF:QO by comproportionation of ETF_{ox} and ETF_{2e^-} was carried out as previously described (Beckmann & Frerman, 1985b). All assays were done at 25 °C.

Other Methods. Protein was assayed as described by Miller (1959). Flavin and non-heme iron content was determined as previously described (Beckmann & Frerman, 1985a).

Purification of ETF:QO. ETF:QO was purified from pig liver submitochondrial particles (Beckmann & Frerman 1985a) that were stored at −70 °C in isolation medium (10 mM Tris-HCl, 10 mM succinate, 250 mM sucrose, 0.1 mM DTT, pH 7.4). The particles were thawed and washed with isolation medium by centrifugation (100000*g* × 90 min), then resuspended in isolation medium containing 2% Triton X-100, and extracted for a minimum of 4 h at 4 °C. Insoluble material was removed by centrifugation (100000*g* × 90 min) and the concentration of Tris in the supernatant adjusted to 0.1 M with 1 M Tris-HCl, pH 7.4.

The supernatant was mixed batchwise with DEAE-Bio-Gel previously equilibrated with 0.1 M Tris-HCl, 0.1% Triton X-100, and 0.1 mM DTT, pH 7.4. Mixing was carried out for a minimum of 2 h with gentle agitation. The gel was then washed by centrifugation four times with the same buffer to remove any unbound protein. The gel was then loaded into a column (60 × 5 cm) to give a bed height of 35 cm. Protein was eluted with a 2-L linear gradient formed with the starting buffer and 0.35 M Tris-HCl, 0.1% (w/v) Triton X-100, and 0.1 mM DTT, pH 7.4. The fractions that contained the majority (>90%) of octanoyl-CoA:UQ-1 reductase activity were pooled and concentrated to about 150 mL.

The concentrated pool was dialyzed for 12 h against 0.1 M Tris-HCl, 0.1 M KCl, 0.1% (w/v) Triton X-100, and 0.1 mM DTT, pH 7.4 (four changes of 1 L). The enzyme was loaded onto a column (32 × 5 cm) of DEAE-Sepharose equilibrated

Table I: Purification of ETF:QO

	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
crude particles	113000	nd	nd	
Triton X-100 extract	60000	nd	nd	
DEAE-Bio-Gel	860	2690	3.13	100
DEAE-Sepharose	78	2131	27.3	79
hydroxylapatite (pool 1)	26.4	775	29.4	29
hydroxylapatite (pool 2)	33.1	929	28.6	35

with the same buffer, and the column was washed with that buffer until the A_{280nm} of the eluent returned to 0.04. Protein was eluted with a 2-L linear gradient of KCl (0.1–0.4 M) in 0.1 M Tris-HCl, 0.1% (w/v) Triton X-100, and 0.1 mM DTT, pH 7.4. Those fractions that contained the highest specific activities of octanoyl-CoA:UQ-1 reductase activity (which elute just after a heme protein) were pooled and concentrated to 100 mL.

This concentrated pool was dialyzed against 10 mM potassium phosphate, 0.1% (w/v) Triton X-100, and 0.1 mM DTT, pH 7.4 (three changes of 1 L). The enzyme was then loaded on a column (5 × 13 cm) of hydroxylapatite equilibrated with the same buffer. The column was washed with 1.5 L of starting buffer and then eluted with a 1.5-L linear gradient formed from starting buffer and 120 mM potassium phosphate, 0.1% Triton X-100, and 0.1 mM DTT, pH 7.4. Fractions with a constant ratio of octanoyl-CoA:UQ-1 reductase activity to A_{430nm} were pooled and concentrated to 1–2 mg mL^{-1} protein.

In most preparations ETF:QO activity eluted from the hydroxylapatite column as a major peak with a prominent shoulder. These were always pooled separately (Table I), but there was little or no difference in the specific activity of the two pools. Further, each gave a single band (67 kDa) on SDS–polyacrylamide gel electrophoresis, and the two pools had identical absorption and fluorescence spectra.

The preparation was passed over a column (1 × 5 cm) of Bio-Beads SM-2 equilibrated with 20 mM potassium phosphate, pH 7.4, to remove Triton X-100, and dialyzed against 20 mM potassium phosphate, pH 7.4. Glycerol was added to 20% and the enzyme was frozen at −70 °C in aliquots. A typical purification is summarized in Table I. The treatment with Bio-Beads appears to remove Triton X-100 effectively, which is critical for fluorescence measurements. Further treatment of the enzyme with Extractigel D (Pierce) had no effect on the fluorescence or ultraviolet absorption properties of the protein.

The preparation gave a single band on SDS–polyacrylamide gels when 20 μg was subjected to electrophoresis and stained with Coomassie Blue G250. The flavin:Fe ratio of a typical preparation was 1:4.2, and the specific activity in the octanoyl-CoA:UQ-1 reductase assay was usually 30–33 μmol min^{-1} mg^{-1}. The latter is in good agreement with the specific activity reported by Ramsay et al., using the same concentrations of ETF and UQ-1 (Ramsay et al., 1987). The turnover number assayed by comproportionation of ETF_{ox} and ETF_{2e^-} was 90 s^{-1} (assayed at pH 7.8 with 1 μM ETF_{ox} and 1.5 μM ETF_{2e^-}), which agrees well with the value obtained previously with a different purification procedure (Beckmann & Frerman, 1985b).

Fluorometric and Spectrophotometric Measurements. Steady-state fluorescence measurements were made with a Perkin-Elmer LS-5 fluorometer and the spectra displayed on a Kipp & Zonen BD40 strip chart recorder under conditions described in the text. Absorption spectra were determined with a Shimadzu UV-3000 spectrophotometer operating in the double-beam mode under conditions described in the text.

Treatment of Fluorescence Data. In quenching experiments inner filter effects due to protein, quinones, or acrylamide were corrected by the formula

$$F_c = F \text{ antilog } [[(A_{ex} + A_{em})]/2] \quad (1)$$

where F_c is the corrected fluorescence intensity, F is the observed intensity, A_{ex} is the absorbance of the solution at the wavelength of excitation, and A_{em} is the absorbance of the solution at the wavelength of emission (Lakowicz, 1983).

Stern–Volmer quenching constants (K_{SV}) were determined for systems with a static quenching component by using a modified Stern–Volmer equation (Eftink & Ghiron, 1976):

$$(F_0/F_q) \exp(V[Q]) = 1 + K_{SV}[Q] \quad (2)$$

where F_0 is the intensity of fluorescence at a given wavelength in the absence of any quenching agent, F_q is the intensity of fluorescence at the same wavelength in the presence of a known concentration [Q] of quenching agent, V is the static quenching constant, and K_{SV} is the Stern–Volmer constant.

To determine if the small amount of quenching of tryptophan fluorescence we expected due to interaction of ubiquinone homologues and analogues was due to binding, the data were analyzed with a modified Stern–Volmer plot (Leher, 1971; Samworth et al., 1988). This predicts that if a fraction of the total tryptophan residues in a protein (f_a) is accessible to a specific quenching agent, the Stern–Volmer equation can be rewritten:

$$F_0/\Delta F = 1/f_a K_a[Q] + 1/f_a \quad (3)$$

where $\Delta F = F_0 - F_q$, K_a is the association constant of the quenching agent, and the other symbols are as previously defined.

Estimates for K_d (K_a^{-1}) and f_a were calculated for each experiment by using a nonlinear regression analysis (Marquardt, 1963) done on a personal computer with software supplied by SAS Institute Inc., Box 8000, Cary, NC 27511.

Reduction of ETF:QO. The enzymatic or photochemical reduction of ETF:QO was monitored spectrophotometrically by following the decrease in absorbance at 404 nm (Beckmann & Frerman, 1985a); an extinction coefficient of 4.2 mM^{-1} cm^{-1} per electron was used.

The photochemical reduction of ETF:QO was done at 4 °C under strict anaerobic conditions in 20 mM HEPES, 0.1 mg mL^{-1} dodecyl maltoside, and 10 mM EDTA, pH 7.5, containing 0.8 μM ETF:QO and 40 nM deazaflavin. At this concentration, deazaflavin had no detectable contribution to the absorption or fluorescence spectra. Reduction was achieved as previously described (Beckmann & Frerman, 1985a).

Substrate reduction of ETF:QO was achieved by the titration under anaerobic conditions of ETF:QO with octanoyl-CoA and catalytic concentrations of MCAD and ETF. Titrations were done at 10 °C in 20 mM HEPES, 0.1 mg mL^{-1} dodecyl maltoside, 12 nM MCAD, 12 nM ETF, and 0.2% β-D-glucose, pH 7.5. Glucose oxidase (70 milliunits) and catalase (250 milliunits) were added to scavenge any remaining oxygen. At the concentrations used, the flavoproteins MCAD and ETF and glucose oxidase do not contribute to the absorption or fluorescence spectra. Reduction was achieved by the addition of octanoyl-CoA from an anaerobic concentrated stock.

Results

Excitation and Emission Spectra of ETF:QO. Excitation of ETF:QO at 280 or 295 nm results in an emission spectrum

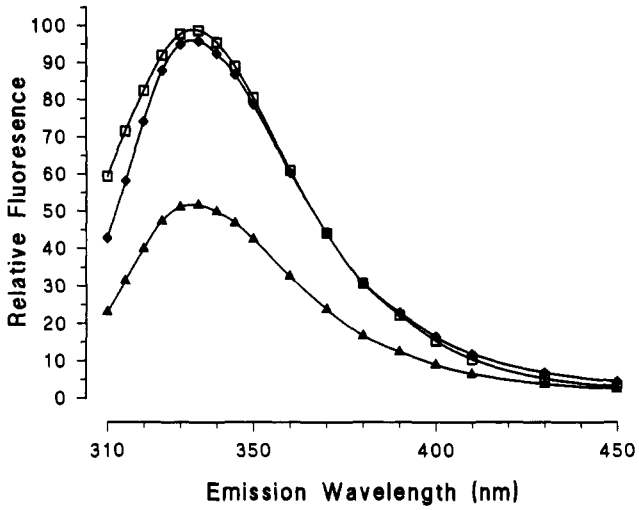

FIGURE 1: Fluorescence emission spectra of oxidized ETF:QO. Emission spectra were determined by using excitation wavelengths of 280 nm (□) and 295 nm (▲). The calculated emission spectrum (◆) is the normalized spectrum generated by multiplying by a constant that is the ratio of the emission intensities at 360 nm produced by excitation at 295 and 280 nm. Spectra were determined in 20 mM Hepes and 0.1 mg mL^{-1} dodecyl maltoside, pH 7.5 at 25 °C.

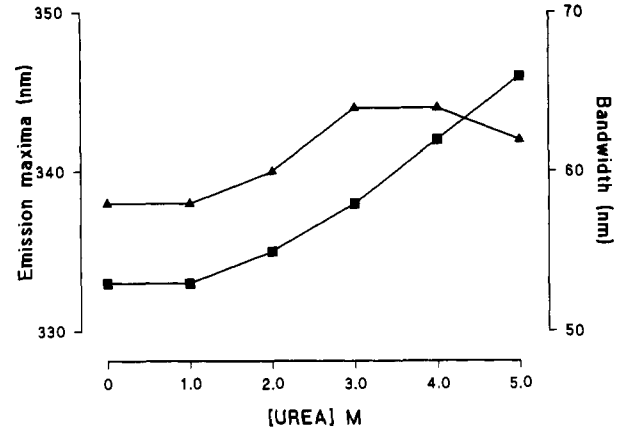

FIGURE 2: Effect of urea concentration on the fluorescence of tryptophan residues in oxidized ETF:QO. Experimental conditions were as given in the legend to Figure 1. Urea was added from a fresh 10 M stock that was treated with mixed-bed resin. The time of exposure to urea was 10 min. There was no change in the fluorescence intensity over the concentration range given; the changes in emission maximum (■) and bandwidth (▲) are shown.

with a maximum at 334 nm (Figure 1). The excitation spectrum (λ_{em} 334 nm) shows a maximum at 282 nm (not shown), which is consistant with tryptophan fluorescence. ETF:QO contains 22 tyrosine residues that could contribute to the intrinsic fluorescence (Beckmann & Frerman, 1985a). The tyrosine contribution was determined by normalizing the emission spectra relative to the emission at 360 nm, where fluorescence originates only from tryptophan. The normalized spectrum (Figure 1) indicates that the fluorescence is almost entirely due to tryptophan with only a small fraction of the emission between 310 and 335 nm due to tyrosine. The emission maximum at 334 nm compares with the emission maximum at 345 nm of tryptophan in aqueous solution. This blue shift of 11 nm together with the bandwidth of the emission spectrum at half the emission maximum (58 nm) would indicate that all 12 tryptophan residues exist in overall hydrophobic environments.

Effect of Collisional Quenchers on the Emission Spectrum of ETF:QO. To further investigate the environment of the tryptophan residues in ETF:QO, we examined the effect of three collisional quenching agents: CsCl, NaI, and acrylamide. Neither Cs^+ ions nor I^- ions had any effect on the fluorescence of ETF:QO. However, both quenched the fluorescence of 2.5 μM indole under identical conditions. The observed Stern–Volmer constants obtained with this model compound were 5.5 M^{-1} with Cs^+ ions and 35 M^{-1} with I^- ions. These are close to the values for K_{SV} reported by Hill et al. (1986) for the quenching of indole by these agents in aqueous solution. This would indicate that the fluorophore is still accessible to these quenchers in 0.1 mg mL^{-1} dodecyl maltoside, which is the critical micellar concentration of this detergent in distilled water (Ljungdahl et al., 1987). Higher concentrations of dodecyl maltoside cause partitioning of the indole into a mixed micelle, which makes it inaccessible to quenchers (Hill et al., 1986).

Acrylamide slightly quenches the tryptophan fluorescence of ETF:QO at high concentrations (K_{SV} = 1.6 M^{-1}); however, this is insignificant compared with the quenching at very low concentrations of the model compound (K_{SV} = 32 M^{-1}). Acrylamide is a small nonpolar molecule that is a highly efficient collisional quencher which might be expected to partition into the protein at higher concentrations and quench the buried tryptophans. The quenching by acrylamide is linear with concentration, with no negative deviation of the curve that might indicate that it had preferential access to a population of tryptophan residues.

Effect of Urea Concentration and pH on the Emission Spectrum of ETF:QO. Figure 2 shows the effect of increasing concentrations of urea on the emission spectrum of ETF:QO. The concentration-dependent red shift of the emission maxima and increase in bandwidth of the spectrum are consistent with the protein unfolding and the tryptophan residues becoming more accessible to solvent. Thus, at 5 M urea, the emission spectrum is close to that of tryptophan in aqueous solution. It is of interest that the tryptophan environments are apparently quite stable in low concentrations of urea. Also, the transient change in the bandwidth of the spectrum between 3 and 4 M urea suggests that the tryptophan residues at that stage in the unfolding exist in more than one environment. Throughout the range of urea concentrations, there was no increase in the tryptophan fluorescence, indicating the absence of quenching of tryptophan fluorescence by other tryptophan residues or by the flavin in the protein. Further, the tryptophan fluorescence intensity, λ_m, and $\Delta\lambda_{1/2}$ were constant over the pH range 6.5–8.5.

Effect of Redox State on the Fluorescence of ETF:QO. Fluorescence was investigated as a function of oxidation–reduction state of the protein. In the initial experiments, ETF:QO was reduced enzymatically by titration with octanoyl-CoA under anaerobic conditions in the presence of catalytic amounts of ETF and MCAD. The extent of reduction was followed spectrophotometrically (Figure 3A); an extinction coefficient at 404 nm of 4.22 mM^{-1} cm^{-1} per electron equivalent was used (Beckmann & Frerman, 1985a).

The emission spectrum of the protein containing one electron or two electrons showed increased fluorescence at 334 nm (Figure 3B). The fluorescence increases 5%–7% after correction for dilution by the added substrate. Titrating the reduced protein with hexacyanoferrate(III) reoxidizes it and reverses the increase in fluorescence at 334 nm to the level of the oxidized protein. This reversal of the increase in fluorescence was also observed when the protein was allowed to reoxidize in air.

An identical increase in fluorescence was obtained when the protein was reduced photochemically, and the increased level

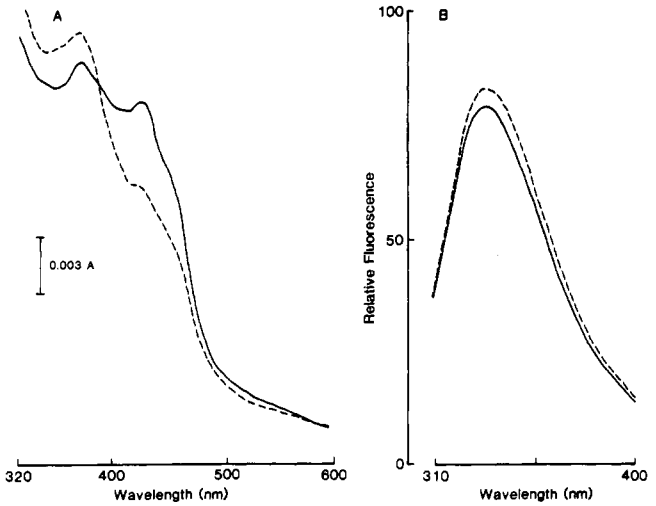

FIGURE 3: Effect of redox state on the fluorescence of ETF:QO. ETF:QO was titrated with octanoyl-CoA under anaerobic conditions in the presence of catalytic concentrations of MCAD and ETF at 10 °C, as described under Materials and Methods. The visible absorption spectra of the oxidized and one-electron-reduced states of ETF:QO (solid and dashed lines, respectively) are shown in panel A, and the fluorescence emission spectra of the oxidized and one-electron-reduced forms of ETF:QO are shown in panel B. There was no further increase of fluorescence upon the addition of a second electron equivalent.

Table II: Kinetic Constants for Quinone Reduction by ETF:QO

substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
6-nonylubiquinone	1.6	246	147
6-(10-hydroxydecyl)ubiquinone	1.8	132	73.3
6-heptylubiquinone	2.0	182	89.7
ubiquinone 2	2.3	162	70.4
6-(10-bromodecyl)ubiquinone	2.6	210	80.8
ubiquinone 1	4.5	84	18.7

[a] The quinone was the varied substrate; all other conditions were as described under Materials and Methods.

of fluorescence was maintained when the protein became three electron reduced. Reoxidation of photochemically reduced protein also resulted in the decrease of fluorescence to the level characteristic of the oxidized protein.

Kinetics of Ubiquinone Reduction. The kinetic constants of a range of ubiquinone homologues and analogues that were potential quenchers of any exposed tryptophan residues in the ubiquinone binding site were measured (Table II). These studies were done in the presence of 0.1 mg mL^{-1} dodecyl maltoside for two reasons: first, we wished to ensure that the protein was monomeric and that aggregation did not affect quinone binding, and second, previous studies indicated that in the presence of this concentration of dodecyl maltoside the turnover number of ETF:QO with UQ-1 and UQ-2 as substrates was enhanced 2.3- and 6.4-fold, respectively (Frerman, 1990).

The experimentally determined kinetic constants are shown in Table II. These indicate that while the value of K_m is broadly related to the overall polarity of the side chain, k_{cat} is influenced by both chain length (hydrophobicity) and the nature of the substituent on the end of the side chain (bromine being more electronegative than hydroxyl). The best measure of catalytic efficiency k_{cat}/K_m (Fersht, 1982) indicates that an analogue with a long aliphatic side chain, i.e., $Q_0(CH_2)_9H$, is the optimum substrate that we tested and that substrates with a similar number of carbon atoms, but with a polar substituent, are not as good substrates. These data infer that addition of the polar substituents, hydroxyl or bromine, or the addition of two double bonds, as in the case of UQ-1, is

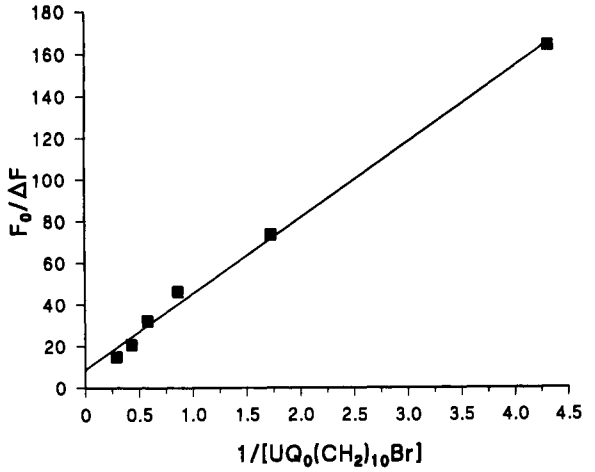

FIGURE 4: Modified Stern–Volmer plot for quenching tryptophan fluorescence in ETF:QO by the ubiquinone analogue $Q_0(CH_2)_{10}Br$. ETF:QO was titrated with $Q_0(CH_2)_{10}Br$ under the conditions given in the legend to Figure 1, and the data were plotted according to a modified Stern–Volmer equation (3) that permits calculation of a binding constant and the fraction of fluorophores available to the quenching agent. Appropriate corrections were made for dilutions during the titration and for inner filter effects.

equivalent to the loss of two to three methylene groups.

Quenching of Fluorescence by $Q_0(CH_2)_{10}Br$. Of the ubiquinone homologues and analogues tested, only $Q_0(CH_2)_{10}Br$ quenched tryptophan fluorescence, reducing the total fluorescence by about 10% after appropriate corrections for dilution and inner filter effects were applied. Since we had already determined that the tryptophan residues of ETF:QO are inaccessible to small ionic quenching agents and that $Q_0(CH_2)_{10}Br$ is a substrate for the enzyme (K_m = 2.6 μM, k_{cat} = 210 s^{-1}), it appeared that this substrate is acting as a static quencher by heavy atom quenching in a hydrophobic ubiquinone binding site. To gain some insight into the fraction of tryptophan residues accessible to quenching (f_a) in this manner and an estimate of the dissociation constant (K_d) for this interaction, we treated the data in a modified Stern–Volmer plot (eq 3) (Leher, 1971; Samworth et al., 1988). In three experiments, values for K_d and f_a, respectively, were as follows: experiment I, 1.62 ± 0.55 μM and 0.09 ± 0.02; experiment II, 2.02 ± 0.89 μM and 0.11 ± 0.03; experiment III, 3.19 ± 0.65 μM and 0.08 ± 0.01. On the basis of amino acid analysis (Beckmann & Frerman, 1985a) and the derived amino acid sequence from a cDNA (S. I. Goodman, personal communication), it is known that the protein contains 12 tryptophan residues. The values of f_a from these analyses indicate quenching of approximately one tryptophan residue, assuming that the quantum yields of all tryptophan residues are identical. Data from experiment III are shown in Figure 4. The observed dissociation constants for $Q_0(CH_2)_{10}Br$ (calculated) are somewhat lower than the values obtained by a similar experimental method for the interaction of a range of ubiquinone homologues and analogues with the bc_1 complex from beef heart (Samworth et al., 1988).

Discussion

Structure–function relationships in ETF:QO are of particular interest for several reasons. First, unlike NADH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase, it is a monomeric protein that contains an intrinsic ubiquinone binding site that does not require auxilary proteins to reduce ubiquinone or stabilize ubisemiquinone intermediates. Second, after a step down to one-electron transfer at the level of the primary ETF-linked dehydrogenases, ETF:QO catalyzes

a step up to two-electron transfer. Third, the mechanism of electron transfer between the flavin and the cluster is poorly understood. Finally, mutations in this protein result in the metabolic disease glutaric aciduria type II, an often fatal inborn error of metabolism (Loehr et al., 1990).

The aromatic amino acids tyrosine and tryptophan participate directly in electron-transfer reactions in several proteins including *Escherichia coli* ribonucleotide reductase (Stubbe, 1990), *Saccharomyces cerevisiae* cytochrome *c* peroxidase (Sivaraja et al., 1989; Prince & George, 1990), and photosystem II (Debus et al., 1988a,b) by the formation of radicals. No evidence for such radicals was found in the EPR spectrum of ETF:QO (Beckmann & Frerman, 1985a; Johnson et al., 1987). However, the 12 tryptophan, 22 tyrosine, and 24 phenylalanine residues may contribute to favorable environments for electron transfer in the ETF or ubiquinone binding sites and/or electron transfer between the flavin and the iron–sulfur cluster.

We have measured the steady-state fluorescence of ETF:QO tryptophan residues as a probe of protein structure in oxidized ETF:QO and the one- and two-electron-reduced states that are achieved enzymatically and the three-electron-reduced state that is achieved by photochemical reduction (Beckmann & Frerman, 1985a). The fluorescence emission maximum (λ_m) of the oxidized protein is 334 nm and the bandwidth at half peak height ($\Delta\lambda_{1/2}$) is 58 nm. Enpirical modeling of protein tryptophan fluorescence by Burstein et al. (1973) would suggest that these results can be interpreted by a model in which 80% of the residues lie in hydrophobic environments and 20% are exposed to solvent. However, analysis of the accessibility of tryptophan residues using the ionic collisional quenchers, I^- and Cs^+, indicates that the tryptophan residues of ETF:QO are inaccessible. Acrylamide is polar but nonionic and has access to all but the most buried tryptophan residues (Eftink & Ghiron, 1976). The tryptophan residues of ETF:QO are somewhat accessible to acrylamide (K_{SV} = 1.69 M^{-1}). This value compares with a K_{SV} of 0.3 M^{-1} obtained with aldolase, in which the tryptophan residues are completely buried (Hill et al., 1986), and cytochrome oxidase (K_{SV} = 2 M^{-1}), a multisubunit integral membrane protein complex (Hill et al., 1986; Capaldi et al., 1988). Thus, the data presented here are consistent with a protein molecule in which all 12 tryptophan residues are buried in moderately hydrophobic environments.

Recently, five overlapping fragments of cDNA encoding ETF:QO have been cloned and sequenced (S. I. Goodman, personal communication). All 12 tryptophans are encoded within the cDNA sequence, and hydropathy analysis of the derived amino acid sequence indicates that 7 of the 12 tryptophan residues lie in hydrophilic microenvironments. Taken with our data, these results suggest that the protein is folded to sequester these residues from solvent.

The effect of increasing concentrations of urea on the intrinsic tryptophan fluorescence of ETF:QO is also consistent with the idea that the tryptophans are buried in moderately nonpolar environments. The emission maximum shifts to 350 nm and broadens to 60 nm, a value that is consistent with tryptophan in an aqueous environment, as the concentration of urea reaches 5 M. These data imply that the protein gradually denatures to finally expose a core that contains the majority of the tryptophan residues. This result cannot be due to dissociation of aggregates, since the protein is monomeric under the experimental conditions, although it does aggregate to a hexamer in the absence of detergent. It is of interest that tryptophan fluorescence does not increase at high urea concentrations, suggesting there is no mutual quenching of tryptophan residues or quenching of tryptophan by the flavin prosthetic group.

Enzymatic or chemical reduction of ETF:QO results in about a 5% increase in tryptophan fluorescence with no detectable change of either the emission maximum or $\Delta\lambda_{1/2}$. Transfer of one electron equivalent to the protein is sufficient to cause the increased tryptophan fluorescence. This electron rapidly equilibrates between the flavin and iron–sulfur cluster (Ruzicka & Beinert, 1977) and is not localized on a single center. Transfer of a second or third electron equivalent causes no further increase in tryptophan fluorescence. The increased fluorescence is related to the change in the oxidation–reduction state of the protein, since the increase is reversed by reoxidation of the protein in air or in the presence of ferricyanide. Reversibility is observed from the one-, two-, or three-electron-reduced states. We have not yet determined whether this increased fluorescence is kinetically equivalent to the rate of reduction of the protein.

There is a tryptophan residue in the quinone binding site. None of a series of ubiquinone homologues and analogues containing isoprenyl, aliphatic, or hydroxyl-substituted aliphatic side chains quench tryptophan fluorescence in equilibrium titrations. However, the brominated analogue, $Q_0(CH_2)_{10}Br$, quenches tryptophan fluorescence to a maximum of about 10%. Brominated molecules are efficient quenching agents resulting from heavy atom quenching, which requires intimate contact between the fluorophore and quencher (East & Lee, 1982; Berlman, 1973; Parker, 1968). This explains why the brominated analogue quenches when the other analogues and homologues do not. If all the tryptophans in the protein are assumed to have identical fluorescence properties, the data indicate that the fluorescence of one tryptophan residue is quenched when the analogue is bound. The calculated dissociation constant for equilibrium binding of the analogue is 1.6–3.2 μM, which compares with the kinetically determined K_m, 2.6 μM, at the same concentration of lauryl maltoside in 20 mM HEPES buffer, pH 7.5. The quenching is due to a static quenching process, rather than any large difference in the binding properties of the quinones, since no great difference exists between the kinetic constants of the reaction with the brominated analogue and those ubiquinone homologues and analogues that do not quench tryptophan fluorescence (Table II). No identity has been found between the primary amino acid sequence of ETF:QO and either those conserved sequences proposed to be involved in quinone binding and semiquinone stabilization (Hearst & Sauer, 1984) or the 9.5-kDa quinone binding protein of ubiquinol–cytochrome *c* oxidoreductase (Usui et al., 1990).

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NMR Structural Refinement of a Tandem G·A Mismatched Decamer d(CCAA*GA*TTGG)$_2$ via the Hybrid Matrix Procedure†

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ABSTRACT: A complete relaxation matrix approach employing a matrix eigenvalue/eigenvector solution to the Bloch equations is used to evaluate the NMR solution structure of a tandemly positioned G·A double mismatch decamer oligodeoxyribonucleotide duplex, d(CCAA*GA*TTGG)$_2$. An iterative refinement method using a hybrid relaxation matrix combined with restrained molecular dynamics calculations is shown to provide structures having good agreement with the experimentally derived structures. Distances incorporated into the MD simulations have been calculated from the relaxation rate matrix evaluated from a hybrid NOESY volume matrix whose elements are obtained from the merging of experimental and calculated NOESY intensities. Starting from both A- and B-DNA and mismatch syn and anti models, it is possible to calculate structures that are in good atomic RMS agreement with each other (<1.6 Å RMS) but differ from the reported crystal structure (>3.6 Å). Importantly, the hybrid matrix derived structures are in excellent agreement with the experimental solution conformation as determined by comparison of the 200-ms simulated and experimental NOESY spectra, while the crystallographic data provide spectra that are grossly different.

Mismatched bases in DNA are naturally occurring, and while they may lead to mutations during replication and transcription, they are also known to be repaired. The efficiency of repair of mismatched bases appears to be dependent upon both the nature of the mismatch and the sequences flanking the mismatch. The G·A-type mismatch has been shown to reflect these observations (Kramer et al., 1984; Fazakerley, 1986) and thus provides an excellent model to decipher the fine structural characteristics that impart its unique recognizable properties.

The G·A mismatches have been shown to adopt three grossly

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