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Electron paramagnetic resonance and magnetic circular dichroism studies of electron-transfer flavoprotein-ubiquinone oxidoreductase from pig liver

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Pig liver electron-transfer flavoprotein-ubiquinone oxidoreductase has been investigated by room temperature UV-visible, low-temperature electron paramagnetic resonance and low-temperature magnetic circular dichroism spectroscopies. The results provide unambiguous evidence for the presence of a single [4Fe-4S] cluster that is diamagnetic in the isolated enzyme and becomes paramagnetic with an $S=1/2$ ground state on reduction with dithionite or enzymatically with the physiological electron donor. The EPR data for samples at pH 7.8 indicate that FAD is reduced by one electron to the anionic semiquinone form in the enzymatically reduced enzyme, and by two electrons to the hydroquinone form by excess dithionite. The possibility of weak spin-spin interaction between the FAD semiquinone and the $[4Fe\text{-}4S]^{1+}$ center is discussed in the light of the observation of a small increase in the linewidth of the Fe-S EPR in enzymatically reduced samples.

EPR; MCD; Electron-transfer flavoprotein-ubiquinone oxidoreductase; Iron-sulfur protein; Flavoprotein

1. INTRODUCTION

Electron-transfer flavoprotein-ubiquinone oxidoreductase is a membrane-bound iron-sulfur flavoprotein that provides the entry port for electrons derived from β-oxidation of fatty acids into the mitochondrial electron transport chain [1,2]. Based on a comparison of the available analytical and spectroscopic data, the samples of ETF-QO that have been purified to homogeneity from beef heart [2] and pig liver [3] mitochondria are very similar. Both have a single subunit with an $M_r \cong 70000$, one FAD per molecule, approx. 4 non-heme iron atoms and 4 acid labile sulfides per FAD, as well as indistinguishable UV-visible and EPR characteristics. The EPR spectra of dithionite reduced samples [1–3], and resonance Raman data for the isolated pig liver enzyme [4], are consistent with the non-heme iron being present in the form of a $[4Fe\text{-}4S]^{1+,2+}$ cluster.

Here, we report UV-visible absorption, low-temperature MCD and quantitative EPR studies of pig liver ETF-QO as isolated, reduced with excess dithionite, and enzymatically reduced with the physiological electron donor, ETF. Previous equilibrium spectrophotometric titrations showed that ETF-QO accepts a maximum of two electrons

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Abbreviations: ETF, electron-transfer flavoprotein; ETF-QO, electron-transfer flavoprotein-ubiquinone oxidoreductase; G-AD, general acyl-CoA dehydrogenase; Q, ubiquinone; MCD, magnetic circular dichroism; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

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from ETF [3], whereas it can accept three electron equivalents from dithionite or by photochemical reduction [2,3]. The EPR results reported here establish the redox state of the constituent prosthetic groups in dithionite and enzymatically reduced samples and raise the possibility of weak spin-spin interaction between the FAD semiquinone and the paramagnetic form of the iron-sulfur cluster in the enzymatically reduced enzyme. The MCD data provide unambiguous confirmation that the iron sulfur center is a ferredoxin-type $[4Fe\text{-}4S]^{1+,2+}$ cluster.

2. MATERIALS AND METHODS

ETF-QO, ETF and G-AD were isolated from pig liver mitochondria using the published procedures [3,5,6]. *Clostridium pasteurianum* ferredoxin was isolated and reconstituted from apo-protein as described by Mortenson et al. [7] and Rabinowitz [8]. Chaps and octanoyl-CoA were purchased from Sigma and P-L Biochemicals, respectively. All other reagents were of the highest purity available.

The samples of ETF-QO used for spectroscopic investigations had properties analogous to those used in previous studies [3], i.e. Fe/S/FAD 3.5:3.4:1, with approximately one FAD per enzyme molecule. Concentrations were based on ϵ_{424} = 24 $mM^{-1} \cdot cm^{-1}$ for the enzyme prepared [3]. Samples were in 100 mM Tris-HCl buffer, pH 7.8, containing 8 mM Chaps, with 40% (v/v) glycerol. Glycerol was present to enable the formation of glasses on freezing samples for MCD spectroscopy. Dithionite-reduced samples were prepared by anaerobic addition of a 10-fold stoichiometric excess of sodium dithionite. Enzymatically reduced samples were prepared by making anaerobic solutions 1 μM in G-AD and ETF, followed by addition of a 5-fold stoichiometric excess of octanoyl-CoA. Samples were taken for spectroscopic analysis after no further change in the absorption spectrum was apparent (approx. 20 min).

Reconstituted samples of *C. pasteurianum* ferredoxin (A_{400}/A_{280} = 0.79) in 0.15 M Tris-HCl buffer, pH 7.4, with 50% (v/v) ethylene glycol, were reduced anaerobically with a 20-fold stoichiometric excess of sodium dithionite. The concentration was based on ϵ_{390} = 30.6 $mM^{-1} \cdot cm^{-1}$ for the oxidized protein [8].

UV-visible absorption measurements were made using a Cary 219 spectrophotometer. MCD spectra were recorded using an Oxford Instruments SM3, split-coil, superconducting magnet mated to a Jasco J500C spectropolarimeter. Spectra were recorded digitally using a Jasco DP-500N data processor interfaced to an IBM PC-XT microcomputer. Sample temperatures were measured using calibrated carbon glass resistors (Lake Shore Cryogenics) placed both directly above and below the sample and controlled by a Rh/Fe resistor and heater connected to an Oxford Instruments DTC2 temperature controller. Magnetic field calibration was carried out with a transverse Hall probe (Lake Shore Cryogenics). MCD spectra are corrected for natural CD and expressed as the difference in the molar extinction coefficients for left and right circularly polarized light, $\Delta\epsilon = \epsilon_L - \epsilon_R$. Magnetic fields, pathlengths and sample concentrations are given in the figure legends. EPR spectra were recorded on an X-band IBM ER200D spectrometer interfaced to an IBM 9000 computer for data handling and manipulations. Low temperatures were achieved using an Oxford Instruments ESR-9 flow cryostat. Spectra were quantified by double integration under non-saturating conditions using 1 mM CuEDTA as the standard.

3. RESULTS

UV-visible spectra for the samples of pig liver ETF-QO used in the low-temperature EPR and MCD studies are shown in fig.1. The broad shoulders at 315 nm and 420 nm in the dithionite-reduced spectrum arise from excess dithionite and a trace impurity of ferrocytochrome, respectively. Allowing for this, the spectra of the enzymatically reduced and dithionite-reduced enzyme correspond closely to those reported previously for two- and three-electron reduced ETF-QO, respectively [3].

EPR spectra for samples of the enzymatically reduced and dithionite-reduced ETF-QO at pH 7.8 are shown in fig.2. (Isolated enzyme samples exhibited a very weak isotropic EPR signal centered around g = 2.003.) Both spectra are a superposition of two signals: an isotropic radical signal centered at g = 2.003 from FAD semiquinone that

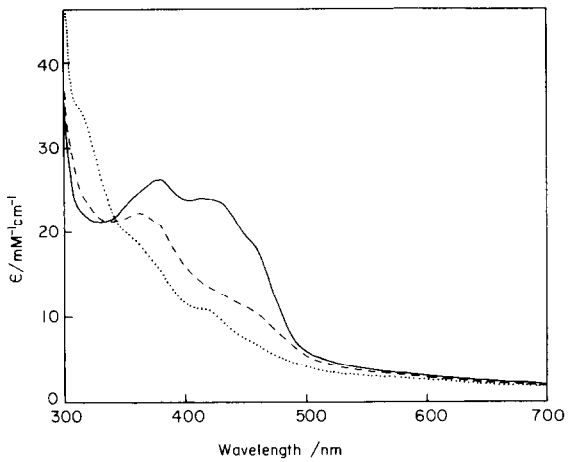

Fig.1. Room temperature absorption spectra for the isolated enzyme, enzymatically reduced and dithionite-reduced ETF-QO. Solid line, isolated enzyme; broken line, enzymatically reduced; dotted line, dithionite-reduced form.

is readily observed at room temperature and a rhombic signal, g = 2.085, 1.939, 1.885, from an iron-sulfur cluster that is only observable below 30 K. No other EPRs were observed over the field range 0 to 400 mT for temperatures between 4 and 100 K with microwave powers up to 200 mW. Spin quantitations for the two types of EPR are tabulated in table 1. Within experimental error, the rhombic signal accounts for 1 spin/4Fe atoms in both the dithionite and enzymatically reduced enzyme, whereas the FAD semiquinone radical is only present in significant concentrations in enzymatically reduced ETF-QO. The linewidth of the g = 2.003 resonance, 1.46 ± 0.02 mT under non-saturating conditions, is consistent with the anionic form of the FAD semiquinone [9,10].

While the EPR spectra for the enzymatically reduced enzyme do not show any clearly defined evidence of spin-spin interaction between the two paramagnetic prosthetic groups, it is noteworthy that the signal from the $[4Fe\text{-}4S]^{1+}$ center is slightly broadened compared to the dithionite-reduced sample. Based on spectra for three independent samples, the halfwidth of the low-field resonance at g = 2.085 was found to increase by 0.4 ± 0.2 mT for the enzymatically reduced samples. However, the spin relaxation characteristics of the EPR from the $[4Fe\text{-}4S]^{1+}$ center are essentially unchanged in the enzymatically and dithionite-reduced samples,

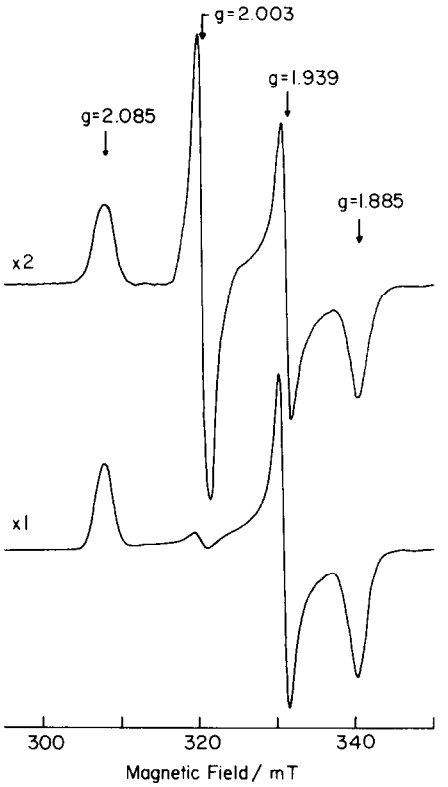

Fig.2. EPR spectra for enzymatically reduced and dithionite-reduced ETF-QO. (Upper spectrum) Enzymatically reduced ETF-QO (39 μM). (Lower spectrum) Dithionite-reduced ETF-QO (69 μM). Conditions: temperature, 13 K; microwave power, 1 mW; modulation amplitude, 0.63 mT; frequency 8.99 GHz. The EPR from the FAD semiquinone radical at g = 2.003 is strongly saturated under these conditions. The multiplication factors indicate the relative gains of the two spectra.

as judged by the half-relaxation parameter ($P_{1/2}$ [11] at 11 K = 6 ± 1 mW and 4 ± 1 mW for the dithionite and enzymatically reduced samples, respectively).

Low-temperature MCD spectra for dithionite-reduced ETF-QO are shown in fig.3. The spectrum displays temperature-dependent MCD bands throughout the region investigated, 300–800 nm, with the exception of the sharp temperature-independent derivative centered at around 550 nm that arises from the diamagnetic low-spin ferrocytochrome impurity. Previous studies have shown that the form and temperature dependence of the low-temperature MCD spectrum from

Table 1

Spin concentrations of EPR signals

	Spins/molecule[a]	
	$g = 2.003$[b]	$g = 2.085$, 1.939, 1.885[c]
Isolated enzyme	<0.01	–
Enzymatically reduced	0.60	0.72
Dithionite reduced	0.05	0.84

[a] Average values of three independent samples. Values are accurate to ± 10%. Sample concentrations are based on $\epsilon_{424} = 24\ mM^{-1} \cdot cm^{-1}$ for the isolated enzyme

[b] Spin quantitation performed at 100 K with 10 μW microwave power

[c] Spin concentration performed at 11 K and 100 μW, after subtraction of $g = 2.003$ signal

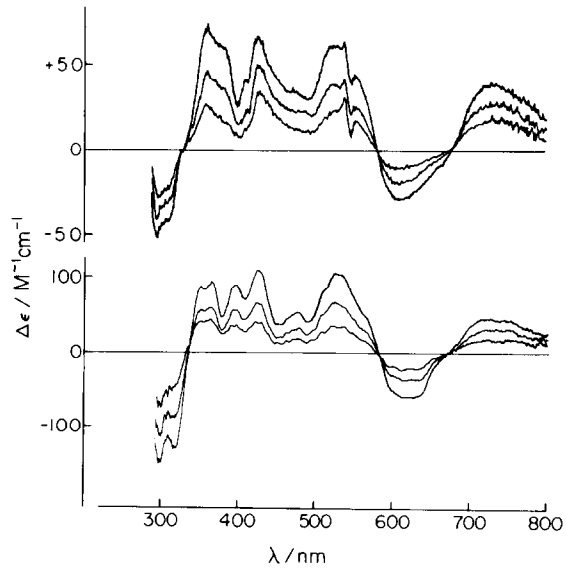

Fig.3. Low-temperature MCD spectra for dithionite-reduced ETF-QO and *C. pasteurianum* ferredoxin. (Upper panel) MCD spectra of dithionite-reduced ETF-QO (69 μM) at 1.67 K, 4.22 K and 8.5 K; magnetic field 4.5 T; pathlength 0.16 cm. (Lower panel) MCD spectra of dithionite-reduced reconstituted *C. pasteurianum* ferredoxin (120 μM) at 1.55 K, 4.22 K, and 8.8 K; magnetic field 4.5 T; pathlength 0.16 cm. The intensity of all MCD bands in both sets of spectra increase with decreasing temperature.

paramagnetic iron-sulfur clusters are indicative of cluster type [12]. For dithionite-reduced ETF-QO both the form and intensity of the MCD spectrum are entirely consistent with one $[4Fe\text{-}4S]^{1+}$ center. This is illustrated in fig.3 which compares the MCD spectrum of dithionite-reduced ETF-QO with that of dithionite-reduced reconstituted *C. pasteurianum* ferredoxin under analogous conditions. Anaerobically handled, reconstituted *C. pasteurianum* ferredoxin was used since it contains exclusively two $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ in the dithionite-reduced form, and shows no evidence of oxidative cluster degradation which is often apparent in the aerobically isolated protein [13]. MCD magnetization data for each of the prominent positive features in the spectra of dithionite-reduced ETF-QO indicate an isolated doublet ground state and are fitted well by theoretical curves based on the EPR g values (not shown). The temperature-dependent MCD bands, therefore, arise exclusively from a chromophore with an $S = 1/2$ ground state.

MCD spectra for the isolated enzyme and enzymatically reduced form were dominated by contributions from the low-spin ferricytochrome impurity in the Soret region (not shown). As noted previously, low-spin ferricytochromes exhibit very intense MCD spectra that are at least 100-times more intense than those of paramagnetic iron-sulfur centers [14]. However, enzymatically reduced ETF-QO did exhibit temperature-dependent features in the 500–800 nm region analogous to those of the $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ center in the dithionite-reduced enzyme.

4. DISCUSSION

The EPR and MCD results presented here provide unambiguous evidence for one ferredoxin-type $[4Fe\text{-}4S]^{1+,2+}$ cluster in pig liver ETF-QO. The [4Fe-4S] cluster is diamagnetic in the isolated, oxidized enzyme and becomes paramagnetic with an $S = 1/2$ ground state in the enzymatically and dithionite-reduced enzyme. The spectroscopic data support the conclusions made from equilibrium spectrophotometric titrations which indicated that ETF-QO accepts a maximum of two electrons from ETF, whereas it can be reduced by three electrons by dithionite or photochemically with deazaflavin. Moreover they indicate that the [4Fe-4S] cluster and the FAD have both been

reduced by one electron in the enzymatically reduced form, and that the additional electron in dithionite or photochemical reduction is utilized primarily to reduce the FAD anionic semiquinone radical to the hydroquinone form. While the midpoint for the latter reduction is yet to be determined, the results are consistent with current estimates of the midpoint potentials for the $[4Fe\text{-}4S]^{1+,2+}$ and $FAD/FAD^{\cdot -}$ couples; ≈60 mV and ≈0 mV at pH 7.8, respectively [2,3,15].

Finally we turn our attention to the possibility of spin-spin interaction between the FAD anionic semiquinone and $[4Fe\text{-}4S]^{1+}$ prosthetic groups in the enzymatically reduced enzyme. Among iron-sulfur flavoproteins such an interaction is most marked in bacterial trimethylamine dehydrogenase which contains a [4Fe-4S] cluster and a novel covalently-bound FMN [16], separated by approx. 4 Å [17]. In this enzyme the interaction is manifested by a complex multiline resonance in the $g = 2$ region and an intense half field resonance around $g = 4$. Neither feature is apparent in enzymatically reduced ETF-QO. In addition, no significant change in the relaxation properties of the $[4Fe\text{-}4S]^{1+}$ was found to accompany reduction of FAD from the paramagnetic semiquinone to the diamagnetic hydroquinone form. However, the slight narrowing of the EPR signal from the $[4Fe\text{-}4S]^{1+}$ cluster that accompanies reduction of the paramagnetic semiquinone to the diamagnetic hydroquinone form is most readily interpreted in terms of loss of a weak spin-spin interaction. Alternative explanations would need to invoke a protein conformational change that perturbs the environment of the Fe-S center. Potentiometric titrations monitored by EPR spectroscopy as well as investigations of the spin relaxation behavior of the FAD semiquinone in samples depleted in the Fe-S cluster, are planned to try and differentiate between these possibilities.

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