Redox Properties of Electron-Transfer Flavoprotein Ubiquinone Oxidoreductase As Determined by EPR–Spectroelectrochemistry†

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ABSTRACT: We have determined the formal potential values for each electron transfer to electron transfer flavoprotein–ubiquinone oxidoreductase (ETF-QO), in order to further characterize the thermodynamics of electron transport from various acyl-CoA thioesters to the mitochondrial ubiquinone pool. ETF-QO contains one $[4Fe\text{-}4S]^{2+,1+}$ cluster and one FAD prosthetic group. A preliminary visible-spectroelectrochemical titration showed that the two redox centers were reduced almost simultaneously. Since the visible spectra of the chromophores overlap, it was not possible to resolve the formal potential value for each electron transfer to the protein using this method. Accordingly, an EPR–spectroelectrochemical cell was designed so that each formal potential value could be resolved by EPR quantitation of the flavin semiquinone and the reduced iron–sulfur cluster during the titration. The formal potential values for electron transfer to ETF-ubiquinone oxidoreductase at pH 7.5 and 4 °C were $E_1°' = +0.028$ V and $E_2°' = -0.006$ V for the first and second electron transfers, respectively, to the FAD and $E°' = +0.047$ V for the iron–sulfur cluster. The thermodynamics of electron transport from the acyl-CoA substrates of β-oxidation to the mitochondrial electron transport chain have been fully resolved with completion of this work. The results are discussed in terms of their significance to the overall electron transport process from β-oxidation.

Electron-transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO)[1] is an iron–sulfur flavoprotein in the inner mitochondrial membrane. ETF-QO accepts electrons from electron-transfer flavoprotein (ETF), which oxidizes at least eight primary flavoprotein dehydrogenases in the mitochondrial matrix. These primary flavoprotein dehydrogenases include the short-, medium-, and long-chain acyl-CoA dehydrogenases in the fatty acid β-oxidation pathway (Beinert, 1963), 2-methylbutyryl-CoA, isovaleryl-CoA, and glutaryl-CoA dehydrogenases from amino acid oxidative pathways (Ikeda et al., 1983; Gholson et al., 1962), and the *N*-methyl dehydrogenases, dimethylglycine and sarcosine dehydrogenases (Wittwer & Wagner, 1981). The *N*-methyl dehydrogenases also serve to supply the intramitochondrial folate pool. ETF-QO transfers electrons to the ubiquinone pool which is also accessed by the NADH and succinate ubiquinone oxidoreductases, complexes I and II (Frerman, 1987). Thus, ETF-QO serves as the membrane-bound electron transport link for the electrons derived from a variety of oxidative processes to the main mitochondrial respiratory chain.

Inherited deficiency of ETF-QO or ETF has been shown to cause the metabolic disorder glutaric acidemia type II (GAII) (Frerman & Goodman, 1985; Loehr et al., 1990). GAII patients usually excrete sarcosine and the free acids, or their metabolites, derived from acyl-CoA substrates of the primary flavoprotein dehydrogenases.

ETF-QO was initially isolated from beef heart mitochondria by Ruzicka and Beinert (1977) and characterized as a single polypeptide containing a 4Fe-4S cluster and one FAD prosthetic group. ETF-QO isolated from pig liver submitochondrial particles is used in the present work. This protein has been purified and extensively characterized by Beckmann and Frerman (1985). The molecular weight, visible spectrum, and EPR characteristics of pig liver ETF-QO are similar to those of the protein isolated from beef heart. The molecular weight of the protein from pig liver was initially estimated to be 69 000 by SDS–polyacrylamide gel electrophoresis (Beckmann & Frerman, 1985). More recently, a molecular weight of 64 800 was calculated from the sequence of the cloned cDNA (Goodman et al., 1992). The assembly of the iron and sulfide atoms into a $[4Fe\text{-}4S]^{2+,1+}$ cluster was confirmed initially by resonance Raman spectroscopy (Schmidt et al., 1983) and more recently by magnetic circular dichroism and EPR spectroscopy (Johnson et al., 1987).

ETF-QO can accept a maximum of three electrons: two electrons for full reduction of the FAD prosthetic group and one electron for reduction of the iron–sulfur cluster. ETF-QO can be fully reduced by chemical reductants, such as sodium dithionite, but is only reduced by two electrons using catalytic amounts of ETF (Beckmann & Frerman, 1985). EPR investigation has shown that the FAD-anionic semiquinone and the reduced iron–sulfur cluster are present in the two-electron reduced form of ETF-QO (Johnson et al., 1987).

The thermodynamics of electron transport from the acyl-CoA substrates of the β-oxidation process to the mitochondrial electron transport chain have been under intensive study in this and other laboratories (Lenn et al., 1990; Stankovich & Soltysik, 1987; Frerman, 1987; Ramsay et al., 1987; Gustafson

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[1] Abbreviations: $E°'$, formal potential value; $E_1°'$, formal potential value for first electron transfer; $E_2°'$, formal potential value for second electron transfer; E_m, midpoint potential value for overall two-electron transfer; ETF, electron-transfer flavoprotein; ETF-QO, electron-transfer flavoprotein-ubiquinone oxidoreductase; GAII, glutaric acidemia type II; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MCAD, medium-chain acyl-CoA dehydrogenase; SHE, standard hydrogen electrode.

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et al., 1986; Husain et al., 1984). An important aspect of the current study is the determination of the formal potential values of the FAD and iron–sulfur cluster in ETF-QO. Through a detailed understanding of this electron transport system, it may ultimately be possible to determine whether there is a kinetic or a thermodynamic basis for the observed low activity of the mutant forms of ETF-QO found in GAII patients. In a study involving potentiometric measurements on pigeon heart submitochondrial particles, Ohnishi et al. (1972) measured the formal potential value for an iron–sulfur cluster that was later associated with ETF-QO. Subsequently, Ruzicka and Beinert (1977) estimated the midpoint potential value for the FAD prosthetic group relative to this formal potential value for the iron–sulfur cluster by comparing the intensities of the EPR signals from the reduced iron–sulfur cluster and the FAD semiquinone during a reductive titration of purified ETF-QO from beef heart mitochondria.

We have now rigorously determined the three formal potential values for purified ETF-QO from pig liver mitochondria through the coordinated use of EPR and spectroelectrochemical techniques. A novel EPR–spectroelectrochemical cell was designed which allowed all three formal potential values to be independently determined for the first time from a single sample. We also discuss the significance of these results in relation to the overall electron transport process from β-oxidation.

MATERIALS AND METHODS

Materials

ETF-ubiquinone oxidoreductase was prepared from pig liver and isolated from submitochondrial particles which had been stored at −70 °C, according to the procedures of Watmough et al. (1991). Water was glass-distilled. The chemicals used and their sources are as follows: methyl viologen and gallocyanine (British Drug House), phenazine methosulfate and 2,6-dichlorophenolindophenol (Sigma Chemical Co.), indigo disulfonate (indigo carmine) (Manufacturing Chemists), HEPES (*N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) (Fisher Scientific Co.), and lauryl D-maltoside (Fluka Chemical Corp.). Lumiflavin-3-acetate was the generous gift of Dr. Sandro Ghisla, University of Konstanz, Konstanz, West Germany. Pyocyanine was photochemically prepared from phenazine methosulfate according to the method of McIlwain (1937).

Methods

Molar Absorptivity Determination. Amino acid quantitation was performed at the Microchemical Facility, at the University of Minnesota, on oxidized ETF-QO samples containing approximately 25 μg of protein in 400 μL of 8 mM HEPES. The absorption spectrum of this sample was recorded, and then the sample was hydrolyzed in 6 N HCl for 24 h at 110 °C. The amino acid contents were compared to external standards and reported as nanomoles per sample. Nanomoles of amino acid per sample was converted to nanomoles of ETF-QO per sample by dividing by the number of amino acid residues of that type in the cDNA sequence of ETF-QO. The molar absorptivity at 280 nm was calculated from the measured absorbance of the 400-μL sample and the concentration of ETF-QO present in the sample, as determined by the average nanomoles of ETF-QO per sample from Thr, Ser, Pro, Ala, Ile, Leu, Tyr, Phe, His, Lys, and Arg. These amino acids were chosen for the molar absorptivity calculation because the number of each amino acid present in the sequence as calculated from the amino acid analysis matched the number of each amino acid present as calculated from the actual cDNA sequence. The molar absorptivity values for ETF-QO at 280 and 424 nm were determined to be 179 ± 13 and 23.6 ± 1.7 mM^{-1} cm^{-1}, respectively.

Potentiometric Titrations. All experiments were performed in 0.020 M enzyme grade HEPES buffer at pH 7.5. The protein was stored in 20% (v/v) glycerol to maintain activity through freeze/thaw storage cycles; however, glycerol was removed prior to the electrochemical experiments, in order to speed the attainment of equilibrium during titration. The detergent, lauryl maltoside, was added to the experimental solution, in the amount of 0.1 mg/mL, to prevent the protein from aggregating in solution.

Potential measurements were made using either a BAS-100 electrochemical analyzer or an Orion Research Model 601A digital ionalyzer. The potential of the Ag/AgCl reference electrode was determined using a standard solution of potassium ferricyanide and potassium ferrocyanide each at 0.050 M, in 0.10 M potassium phosphate buffer at pH 7.0 and 25 °C. Under these conditions, the formal potential value for this couple is +0.425 V versus the SHE (O'Reilly, 1973). The potential measurements made during potentiometric titration of ETF-QO were at 4 °C. The temperature dependence of the Ag/AgCl reference electrode formal potential value was determined to be +0.004 V on going from 25 to 4 °C. The method for measuring this potential difference has been previously described (Stankovich & Fox, 1984). All potential values are reported versus the standard hydrogen electrode (SHE). Spectrophotometric measurements were made using a Varian Cary 210 or Cary 219 spectrophotometer with thermostatted cell compartments.

Potentiometric titrations were performed using dithionite as a reductant and methyl viologen to mediate the transfer of electrons to the protein's redox centers. Initially, the protein was titrated with three redox indicators present in the experimental solution, in order to determine the redox potential range in which the protein was reduced. Each redox indicator was titrated alone, using the visible–spectroelectrochemical method, in order to later subtract the indicator spectra from the potentiometric titration spectra of ETF-QO and to determine their redox potential values at pH 7.5 and 4 °C. The indicators used in the initial potentiometric titration were indigo disulfonate (E_m = −0.089 V), pyocyanine (E_m = 0.000 V), and gallocyanine (E_m = +0.049 V). The maximal redox buffering capacity for this mixture of dyes includes the potential range from −0.119 to +0.079 V versus the SHE. From the first titration, it appeared that the protein was fully reduced before the redox potential range of indigo disulfonate was reached. Therefore, in subsequent experiments only pyocyanine and gallocyanine were used to indicate the potential of the protein solution. They have a maximal redox buffering capacity of from −0.030 to +0.079 V versus the SHE.

In the visible–spectroelectrochemical titrations, 1.0 μM pyocyanine and gallocyanine, 20 μM methyl viologen, and from 4 to 10 μM ETF-QO were used. In the EPR–spectroelectrochemical titrations, 4.0 μM pyocyanine and gallocyanine, 50 μM methyl viologen, and from 36 to 54 μM ETF-QO were used. Dithionite solutions were prepared to a concentration of 5.0 mM and were then standardized by titration of lumiflavin-3-acetate.

EPR–Spectroelectrochemical Titrations

EPR–Spectroelectrochemical Cell. The visible–spectroelectrochemical technique could not be used to resolve the

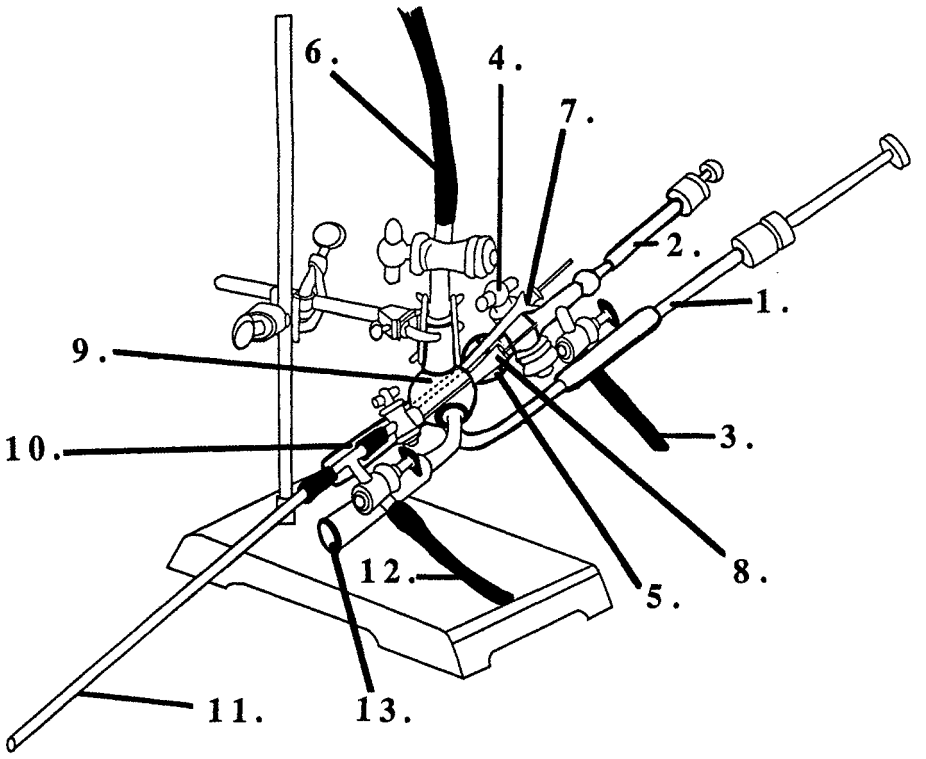

FIGURE 1: EPR–spectroelectrochemical cell: 1, syringe containing sodium dithionite; 2, syringe to transfer aliquot from experimental cuvette to EPR tube; 3, gas line to top section; 4, stopcock to top section; 5, ball and socket joint; 6, gas line to main cell; 7, reference electrode; 8, working electrode; 9, main body of cell; 10, visible cuvette; 11, EPR tube; 12, gas line to bottom section; 13, holder for Cary spectrophotometer.

formal potential values of the redox centers present in ETF-QO, since both redox centers appeared to be reducing almost simultaneously and the visible spectra of the FAD prosthetic group and iron–sulfur cluster overlap. ETF-QO contains two paramagnetic redox species during reductive titration, the FAD semiquinone and the reduced iron–sulfur cluster. Therefore, the problem of spectral overlap was resolved by designing a cell (Figure 1) in which both the visible spectrum and the EPR spectrum could be monitored during potentiometric titration.

The main body of the cell consists of a visible cuvette with a path length of 0.58 cm and the capacity to hold 2.9 mL of experimental solution. A silver/silver chloride reference electrode and a gold working electrode are used for potential measurement. A gas-tight Hamilton syringe containing a chemical reductant (or oxidant) is located in a side arm of the cell. The main body of the cell is maintained under positive argon pressure throughout the titration to minimize oxygen leakage. When equilibrium is reached (when the measured cell potential and absorption spectrum remain constant for at least 10 min), a 250-μL aliquot of experimental solution is withdrawn from the cuvette and is transferred to an anaerobic EPR tube.

The top section of the cell can be made anaerobic independently and contains a port through which a Hamilton syringe, containing a 42-cm needle, can be inserted via a septum. The syringe is inserted into the spectroelectrochemical cuvette and a 250-μL aliquot of equilibrium solution is withdrawn. When the bottom section of the cell has been made anaerobic, the transfer needle is rotated and inserted down into the EPR tube, the solution is deposited, and the needle is withdrawn. The stopcock between the main cell and bottom section is shut and the EPR tube, while still connected, is frozen in liquid nitrogen and removed. In this way, the transfer of the sample to the EPR tube is accomplished without ever removing the syringe from the anaerobic cell. Further, the experimental solution is not repeatedly frozen and thawed during titration; instead, an aliquot is transferred quantitatively to the EPR tube while the rest of the experimental solution remains in the cell.

The cell exhibited virtually no oxygen leakage when kept under positive argon pressure throughout the titration. The potential of the protein solution was measured after each solution transfer to an EPR tube, in order to determine the amount of hysteresis introduced by oxygen leakage during the transfer process. The potential of the experimental solution was no more than 0.005 V more positive after the transfer had taken place. It was determined that virtually no reoxidation occurred from the time the EPR samples were frozen to the time when they were measured (18 h).

EPR Spectroscopy. X-band EPR measurements were made on a Varian E-109 spectrometer which was digitally interfaced to a laboratory microcomputer (Lipscomb & Salo, 1983). The spectrometer was equipped with an Oxford Instruments ESR-910 liquid helium cryostat, a Hewlett Packard 436A power meter, and a Hewlett Packard 5350B microwave frequency counter. EPR spectral manipulations and integrations were performed using a program written by Dr. David R. Jollie. The standard used for quantitation was a 1.0 mM $Cu(ClO_4)_2$ solution. Spin quantitations were performed using spectra measured at nonsaturating microwave power levels according to previously described procedures (Aasa & Vänngård, 1975; Fee, 1978). The EPR spectra used for quantitation of the semiquinone were recorded at 4 μW and 25 K, while the EPR spectra used for quantitation of the iron–sulfur cluster were recorded at 10 μW and 10 K.

Calculations. The formal potential of the $[4Fe\text{-}4S]^{2+,1+}$ cluster was calculated by a computerized nonlinear regression fit (Duggleby, 1981) to a plot of E versus [ox]/[red] using the Nernst equation:

$$E = E^{\circ\prime} + (0.055/n) \log ([ox]/[red]) \quad (1)$$

where E is the measured equilibrium potential at each point in the titration, $E^{\circ\prime}$ is the formal potential value for the reduction of the iron–sulfur cluster at 4 °C, and n equals 1 for the iron–sulfur cluster reduction. At each point in the titration, the concentration of reduced iron–sulfur cluster was determined from double integration of its EPR spectrum. The concentration of oxidized iron–sulfur cluster was determined by subtracting the amount of reduced species from the initial concentration of protein present before titration and by correcting for volume changes during titration. The initial protein concentration was calculated from the visible absorption spectrum of fully oxidized protein.

The formal potential values for the first ($E_1^{\circ\prime}$) and second ($E_2^{\circ\prime}$) electron transfers to the FAD prosthetic group were determined from the amount of semiquinone formed as a function of reducing equivalents (x) added. The maximum fraction of semiquinone formed during titration (M) is related to the difference between $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ by the equation (Clark, 1960)

$$E_1^{\circ\prime} - E_2^{\circ\prime} = 0.110 \log [2M/(1 - M)] \quad (2)$$

From the value of $E_1^{\circ\prime} - E_2^{\circ\prime}$, theoretical curves for the relative amounts of oxidized, semiquinone, and fully reduced FAD can be calculated for any value of x using the following equations:

$$[\text{ox}] = Suv/(uv + u + 1) \quad (3)$$

$$[\text{semi}] = Su/(uv + u + 1) \quad (4)$$

$$[\text{red}] = S/(uv + u + 1) \quad (5)$$

where S = [ox] + [semi] + [red], u = [semi]/[red], and v = [ox]/[semi]. A nonlinear regression fit to a plot of the measured equilibrium potential at each point in the titration versus [ox]/[semi] or [semi]/[red] yielded the formal potential values for each electron transfer to FAD.

RESULTS

The visible absorption spectra obtained for the reductive titration of ETF-QO by dithionite indicated that a plot of the absorbance, at 404 nm, versus reducing equivalents added was linear, breaking sharply at three reducing equivalents per protein molecule. This was in accordance with previous measurements by Beckmann and Frerman (1985). A linear plot is observed only at 404 nm indicating that the magnitude of the change in absorbance due to the iron–sulfur cluster and FAD upon reduction are similar at this wavelength. Since this is a linear relationship, the number of equivalents added for each point in all subsequent redox titrations was calculated from the measured absorbance at 404 nm after the subtraction of redox indicator dye spectra. The molar absorptivity values calculated for oxidized and fully reduced ETF-QO at 404 nm were 23.2 and 9.8 mM^{-1} cm^{-1}, respectively.

Accurately determined molar absorptivity values were essential in the determination of formal potential values, since all concentrations of redox species were calculated relative to the initial concentration of oxidized ETF-QO as calculated from its visible absorption spectrum. Thus, the molar absorptivity values at various wavelengths, for oxidized ETF-QO, were determined on the basis of protein determination by amino acid analysis. The molar absorptivity values were 179 ± 13 and 23.6 ± 1.7 mM^{-1} cm^{-1}, at 280 and 424 nm, respectively, in good agreement with the previously determined molar absorptivity value at 424 nm (24.0 mM^{-1} cm^{-1}) (Beckman & Frerman, 1985). The good correlation between the equivalents added and the calculated concentration of FAD and iron–sulfur clusters present suggests that the molar absorptivity value determined here, on the basis of quantitative amino acid analysis, provides an accurate means for the determination of protein concentration.

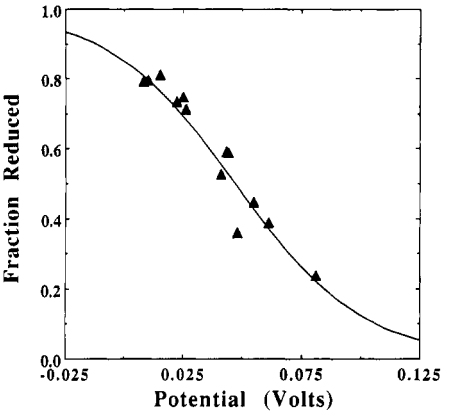

FIGURE 2: Potentiometric titration curve for the iron–sulfur cluster in ETF-QO: plotted as the fraction reduced versus measured potential. Data were collected from four individual titrations. The experimental solution contained 4.0 μM pyocyanine and gallocyanine and 50 μM methyl viologen. Data were collected at 4 °C and at pH 7.5.

Previous studies have shown that the oxidation product from dithionite reduction, the sulfite ion, forms an adduct with the flavin prosthetic group in many flavoproteins, particularly those which form the red anionic radical upon one-electron reduction (Massey et al., 1969). The formation of this adduct is characterized by the bleaching of the visible absorption spectrum of the oxidized flavoprotein during titration with sulfite ion. For this work, a 20-fold excess of sodium sulfite was added to 14 μM ETF-QO and incubated for 4 h at 15 °C. No change in the visible absorption spectrum was observed which indicated that this adduct was not formed with the flavin in ETF-QO.

Johnson et al. (1987) initially characterized ETF-QO from pig liver using EPR spectroscopy. The EPR spectrum consisted of an isotropic signal due to the FAD-anionic semiquinone with a g-value of 2.003 and a rhombic signal due to the $[4Fe\text{-}4S]^{1+}$ cluster with g-values at g_x = 1.885, g_y = 1.939, and g_z = 2.085. No other EPR signals were observed from 0 to 400 mT for temperatures between 4 and 100 K and up to a microwave power of 200 mW. Similar results were observed in this work. At a power of 10 μW, the EPR signal due to the reduced iron–sulfur cluster disappears at 35–40 K. Microwave power saturation profiles were obtained for this work in order to determine the best conditions for quantitation of each species. Half-saturation values ($P_{1/2}$) [see, for example, Rutter et al. (1984)] of 0.47 and 0.43 mW were obtained for the FAD semiquinone at 25 K and the iron–sulfur cluster at 10 K, respectively.

A plot of the fraction reduced versus measured potential for the $[4Fe\text{-}4S]^{2+,1+}$ cluster of ETF-QO is shown in Figure 2. The formal potential value for the iron–sulfur cluster was determined by regression analysis to be $E^{\circ\prime}$ = +0.047 V, at pH 7.5, and 4 °C. The regression analysis yielded an n value of 0.88, indicating that one electron is transferred as expected. It was of interest to prepare EPR samples of fully reduced ETF-QO, in order to compare the percent recovery of the fully reduced iron–sulfur cluster determined by EPR quantitation with the initial concentration of oxidized ETF-QO. The EPR data indicated an average of 81 ± 4% recovery of fully reduced iron–sulfur cluster, when compared to the

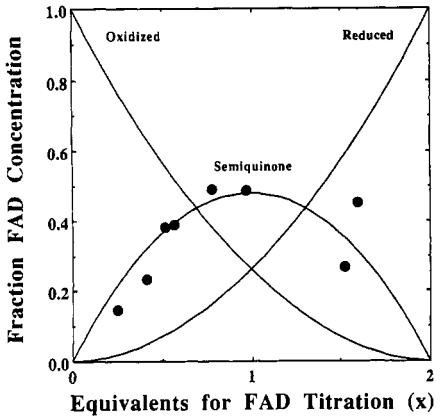

FIGURE 3: Plot of fraction of FAD semiquinone formed versus reducing equivalents added for FAD titration.

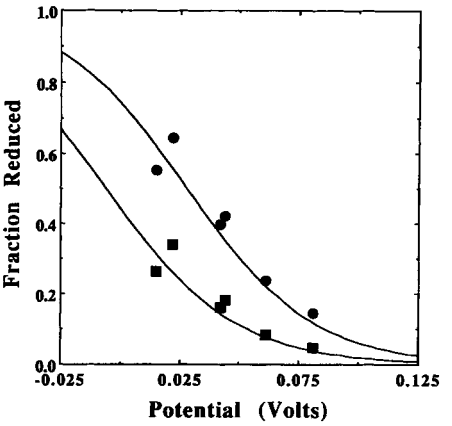

FIGURE 4: Potentiometric titration curve for the first (●) and second (■) electron transfers to the FAD prosthetic group in ETF-QO: plotted as fraction reduced versus measured potential for each electron transfer. Experimental conditions were identical to those in Figure 2.

theoretical concentration at the beginning of titration, as calculated by a molar absorptivity value of 23 600 M^{-1} cm^{-1} (424 nm). It is unlikely that this slightly low quantitation is due to the presence of protein missing the iron–sulfur cluster, due to the good correlation between protein, iron, and sulfide observed. Johnson et al. (1987) observed an 84 ± 10% recovery of fully reduced iron–sulfur cluster, during dithionite titration, by using a molar absorptivity value of 24 000 M^{-1} cm^{-1} (424 nm) to calculate the initial protein concentration. They also reduced ETF-QO with an excess of its natural reductant, ETF, and obtained a 72 ± 10% recovery of reduced iron–sulfur cluster showing that similar low recoveries are obtained independent of the reduction technique.

Low EPR-based quantitations of 4Fe-4S clusters found in other proteins have been attributed in several cases to the conversion of a portion of the reduced cluster population to an $S = 3/2$ state. The EPR spectra of ETF-QO at various stages of reduction were inspected for signals that could arise from an $S = 3/2$ state, but none were observed. In order to determine whether the missing spins could be attributed to conversion of the cluster to another form, such as the $[3Fe\text{-}4S]^{1+,0}$ cluster, EPR samples in the present work were reoxidized and remeasured. No signal near $g = 2.01$ indicating the presence of a $[3Fe\text{-}4S]^{1+,0}$ cluster was detected. It is possible that a spin–spin interaction exists between the reduced iron–sulfur cluster and FAD semiquinone, but we were unable to detect any EPR line-shape distortion which would indicate this interaction. A 5–10% error inherent in any quantitative EPR measurement because of instrumental limitations (Warren & Fitzgerald, 1977) may also account, at least in part, for the discrepancy. Due to the uncertainty introduced by the substoichiometric cluster quantitation, we estimate that an error of ±0.010 V exists in the formal potential value determination of the iron–sulfur cluster in ETF-QO. Therefore, this value can be most accurately represented by $E^{\circ\prime} = +0.047 \pm 0.010$ V.

Figure 3 represents a plot of the fraction of the total FAD present during a redox titration as oxidized, semiquinone, and reduced species versus the number of added reducing equivalents for FAD titration (x). A best fit to the semiquinone data was established at a potential difference between the first and second formal potential values ($E_1^{\circ\prime} - E_2^{\circ\prime}$) of +0.029 V and at a maximum fraction of semiquinone formation of 0.48. Figure 4 is a plot of the fraction reduced versus measured potential, for the first and second electron transfers to FAD, as calculated from the data shown in Figure 3 (see Methods). The formal potential values for the first and second electron transfers were determined to be $E_1^{\circ\prime} = +0.028$ V and $E_2^{\circ\prime} = -0.006$ V, respectively, at pH 7.5 and 4 °C. The regression analyses yielded n values of 0.91 and 0.89 for the first and second electron transfers, respectively. We estimate that an error of ±0.015 V exists in the formal potential value determination for each electron transfer to the FAD in ETF-QO, so these values can be accurately represented by $E_1^{\circ\prime} = +0.028$ and $E_2^{\circ\prime} = -0.006 \pm 0.015$ V. The reversibility of the ETF-QO system was verified in two titrations using 2,6-dichlorophenolindophenol and potassium ferricyanide as oxidants. Similar potentials and visible spectra were obtained in both reductive and oxidative directions.

DISCUSSION

EPR–Spectroelectrochemical Cell. The EPR–spectroelectrochemical cell in Figure 1 was designed in order to determine the formal potential values of the FAD prosthetic group and the $[4Fe\text{-}4S]^{2+,1+}$ cluster in ETF-QO. The novel design of this cell allows for coordinated visible, EPR, and potentiometric measurements on a protein sample during a single titration. This cell design also minimizes denaturation of the protein sample by avoiding the transport of experimental solution back and forth between the spectroelectrochemical cuvette and an EPR tube. Moreover, repeated freeze/thaw cycles of the sample are avoided since a new aliquot of the experimental solution is withdrawn and frozen to define each titration point. This is important since many proteins can be quite unstable due to repeated freeze/thaw cycles that occur in other EPR potentiometric titration cells. The cell also allows for anaerobic sample transfer from the cuvette to the EPR tube.

Although a maximum of only five EPR tubes could be acquired during a single titration, the tubes contain protein which is of high integrity, since protein manipulation and the total period of titration is minimized. This procedure carries the liability that more than one titration is necessary to gain an adequate amount of data for formal potential value determinations, but the samples that result are more likely to represent the true state of the protein.

A problem encountered in potentiometric titration methods, in which the amounts of oxidized and reduced species are measured at a much lower temperature than the potential values, is that the electrons may redistribute among multiple redox centers in a protein during freezing. A room temperature EPR titration would indicate whether a flavin's formal potential values have shifted from those determined in low-temperature work. This is beyond the scope of the present work since a special apparatus is required (Porras & Palmer, 1982).

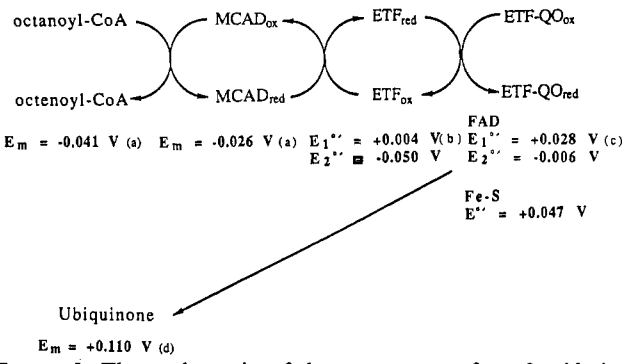

FIGURE 5: Thermodynamics of electron transport from β-oxidation to the mitochondrial electron transport chain: (a) pH 7.6, midpoint potential value for MCAD is in the presence of substrate and product (Lenn et al., 1990); (b) pH 7.5 (Husain et al., 1984); (c) pH 7.5, present work; (d) pH 7.0 (Morrison et al., 1982).

Thermodynamics of Electron Transfer from β-Oxidation. In vivo, ETF-QO is reduced by ETF in the inner mitochondrial matrix and is subsequently reoxidized by ubiquinone inside the inner mitochondrial membrane. Thus, ETF-QO is the membrane-bound link between electrons derived from specific reactions, which occur in the matrix, and a common membrane-bound electron transport chain. It has been suggested that electrons are transferred from ETF to ETF-QO, via the FAD prosthetic group, and then subsequently leave ETF-QO, via the iron–sulfur cluster, to reduce ubiquinone (Frerman, 1988).

The thermodynamics specific to the electron-transfer reactions from the β-oxidation process to the electron transport chain are represented in Figure 5. It has been suggested that the only electron transfer that ETF utilizes in vivo is the first electron transfer and that the FAD prosthetic group cycles between the oxidized and semiquinone forms during steady-state turnover (Ramsay et al., 1987; Frerman, 1987). However, full reduction of the ETF flavin can be achieved coulometrically or by chemical reduction. The formal potential values of ETF from pig liver have been determined and are $E_1^{\circ\prime}$ = +0.004 V and $E_2^{\circ\prime}$ = –0.050 V (Husain et al., 1984). As shown in Figure 5, medium-chain acyl-CoA dehydrogenase (MCAD) bound to product (E_m = –0.026 V) is thermodynamically capable of catalyzing single electron transfer steps to two ETF molecules to form two ETF semiquinone molecules ($E_1^{\circ\prime}$ = +0.004 V). Acceptance of a second electron by an ETF molecule from MCAD is not as thermodynamically favorable ($E_2^{\circ\prime}$ = –0.050 V) as well as being kinetically slow. This is in accord with the kinetic data of Gorelick et al. (1985) which showed that electrons leave product-bound MCAD by a radical mechanism.

It had been previously shown that ETF-QO reduction by ETF resulted in only the FAD semiquinone and reduced iron–sulfur cluster being formed (Beckmann & Frerman, 1985). The current studies indicate that the transfer of electrons from the ETF semiquinone ($E_1^{\circ\prime}$ = +0.004 V) to the FAD prosthetic group in ETF-QO ($E_1^{\circ\prime}$ = +0.028 V and $E_2^{\circ\prime}$ = –0.006 V) will be thermodynamically favorable for the first electron transfer and that the second electron transfer should be approximately 50% complete. However, the transfer of the third electron to the ETF-QO red-anionic flavin semiquinone may be kinetically slow, when ETF semiquinone is the reductant, since it is also a red-anionic semiquinone. The reduction of red-anionic semiquinones, by other negatively charged reducing agents, tends to be kinetically slow even when thermodynamically feasible (Stankovich & Fox, 1983; Van den Berghe-Snorek & Stankovich, 1985).

Ohnishi et al. (1972) first identified the iron–sulfur cluster of ETF-QO in pigeon heart submitochondrial particles; however, at that time they could not identify the protein to which this cluster belonged. They made EPR and potentiometric measurements during reductive titration of the submitochondrial particles and determined that the formal potential value for this particular iron–sulfur cluster was +0.040 V at pH 7.2. The formal potential value of the [4Fe-4S]$^{2+,1+}$ cluster in ETF-QO determined in the present work is $E^{\circ\prime}$ = +0.047 ± 0.010 V. This value is in good agreement with the value obtained by Ohnishi et al. and indicates that the membrane environment does not change the formal potential value of the iron–sulfur cluster to a large extent.

Ruzicka and Beinert (1977) performed a reductive titration on ETF-QO from beef heart and measured the amounts of flavin semiquinone and reduced iron–sulfur cluster present; however, potential measurements were not made during the titration. By assuming the reported value for the formal potential of the iron–sulfur cluster, they estimated that the midpoint potential value (two-electron) for the FAD was about 0.055 V more negative than the formal potential value of the iron–sulfur cluster, or E_m = –0.015 V. It appeared that a maximum of approximately 50% semiquinone was formed during this titration. The formal potential values of the FAD prosthetic group determined in the present work are $E_1^{\circ\prime}$ = +0.028 and $E_2^{\circ\prime}$ = –0.006 ± 0.015 V, indicating that a substantial amount of the semiquinone is thermodynamically stabilized. The midpoint potential value for the overall two-electron transfer can be calculated and is E_m = +0.011 V. This is 0.026 V more positive than the E_m value which Ruzicka and Beinert estimated from the relative EPR signal intensities of the iron–sulfur cluster and semiquinone. The latter titrations were performed in 10 mM Tris-HCl buffer at pH 7.4 which exhibits a much larger temperature-dependent change in pH than the HEPES buffer used in the present study (Williams-Smith et al., 1977). Consequently, it appears that the midpoint potential estimate by Ruzicka and Beinert for the FAD prosthetic group was quite accurate, when corrected for buffer-dependent pH changes which were not recognized at the time that they did their work.

Iron–Sulfur Cluster in ETF-QO. The [4Fe-4S]$^{2+,1+}$ cluster in ETF-QO is of particular interest, because it utilizes the same redox states as the "ferredoxin"-type clusters, yet it exhibits a formal potential value approximately 0.300–0.650 V more positive than the formal potential values observed for most other ferredoxin-type clusters. In fact, the formal potential value of this cluster is midway between the ferredoxins ($E^{\circ\prime} \approx$ –0.420 V) and the high potential iron–sulfur proteins (HiPIPs) ($E^{\circ\prime} \approx$ +0.350 V) which function between the 3+ and 2+ redox states. Although the specific factors that control the formal potential values of the ferredoxins and HiPIPs are not understood, several differences between the two protein types have been observed. Since no crystal structure is yet available for this protein, we do not know what factors are contributing to the stabilization of the reduced iron–sulfur cluster in ETF-QO making its reduction more favorable than the clusters found in ferredoxins. For example, the 1+ redox state in ferredoxins appears to be stabilized by increased solvent accessibility and a larger number of hydrogen bonds from the polypeptide to the cluster (Carter, 1977; Stout, 1982). These factors may also be contributing to the stabilization of the 1+ redox state in ETF-QO.

The clusters of two other [4Fe-4S]$^{2+,1+}$ containing flavoproteins, trimethylamine dehydrogenase and adenylyl sulfate reductase, exhibit formal potential values similar to those of the ETF-QO cluster (Stankovich & Steenkamp, 1987; Lampreia et al., 1990). The primary sequence of adenylyl

sulfate reductase is not known; however, it can be seen from the primary sequence (Barber et al., 1992) and crystal structure of trimethylamine dehydrogenase that the distribution of cysteine residues that ligand the cluster in an α/β loop (Lim et al., 1986) is similar to the distribution of the likely liganding cysteines in ETF-QO (Goodman et al., 1991). Further, although the cluster domain of ETF-QO contains hydrophobic residues, 9 of the 32 residues are amides and carboxylates, rendering the microenvironment relatively polar, which may help to stabilize the fully reduced form of the cluster and thus cause its formal potential value to become more positive.

A Model for Electron Transfer. It has been suggested that in vivo the semiquinone form of ETF is the reductant of ETF-QO and that this is accomplished through two separate electron transfers (Frerman, 1987). The ETF semiquinone reduces ETF-QO to the two-electron reduced form which consists of the FAD semiquinone ($E_1^{\circ\prime} = +0.028$ V) and the fully reduced iron–sulfur cluster ($E^{\circ\prime} = +0.047$ V). The formal potential values obtained for these electron transfers suggest that both of these electrons should be transferred simultaneously from ETF-QO to fully reduce a ubiquinone molecule ($E_m = +0.110$ V). Since the iron–sulfur cluster is an obligatory one-electron donor, the ubisemiquinone molecule must be formed during the electron-transfer process and ETF-QO is apparently capable of stabilizing the ubisemiquinone without any additional protein factors being present (Frerman, 1987). It was proposed from this model that ETF-QO cycles between the two-electron and one-electron reduced forms in the steady state (Frerman, 1987). The actual redox states that ETF-QO utilizes during steady-state turnover are dependent upon the actual lifetime of the ubisemiquinone versus the rate at which the electrons are transferred into ETF-QO from the ETF semiquinone. Further kinetic studies are needed to define the ETF-QO redox states which are actually utilized during steady-state turnover.

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