Thompson, R. C. (1973) *Biochemistry 12*, 47.
Thompson, R. C. (1977) *Methods Enzymol. 46*, 220.
Westerik, J. O., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195.
Wilk, S., & Orlowski, M. (1982) *J. Chromatogr. 249*, 121.
Wilk, S., & Orlowski, M. (1983) *J. Neurochem. 41*, 69.
Yoshimoto, T., & Walter, R. (1977) *Biochim. Biophys. Acta 485*, 391.

Electron-Transfer Flavoprotein-Ubiquinone Oxidoreductase from Pig Liver: Purification and Molecular, Redox, and Catalytic Properties†

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ABSTRACT: Electron-transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) was purified to homogeneity from pig liver submitochondrial particles. It is comparable in molecular weight and general properties to ETF-QO from beef heart [Ruzicka, F. J., & Beinert, H. (1977) *J. Biol. Chem. 252*, 8440–8445], and the electron spin resonance signals of the reduced iron–sulfur cluster are essentially identical. ETF-QO catalyzes the transfer of electrons from electron-transfer flavoprotein (ETF) to nitro blue tetrazolium, with a sluggish reaction turnover number of about 10–30 min^{-1}. In contrast, the enzyme rapidly disproportionates ETF semiquinone, with a turnover number of 200 s^{-1}. The reverse reaction, comproportionation of oxidized and hydroquinone ETF, provides an enzymatic assay for ETF-QO with picomolar sensitivity. Equilibrium spectrophotometric titrations show that ETF-QO accepts a maximum of two electrons from ETF and accepts three electron equivalents from dithionite or by photochemical reduction. All electrons from the enzymatically or chemically reduced protein can be transferred to 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone (PB), and this reaction is readily reversible. Reduction of ETF-QO by 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzohydroquinone is pH dependent and indicates the enzyme to have a redox potential that decreases by 47 mV per pH unit. Therefore, ETF-QO binds one to two protons upon reduction. The E_0' at pH 7.3 is 38 mV. The ability of ETF-QO to catalyze the equilibration of ETF redox states has been used to evaluate the equilibrium $2ETF_{sq} + nH^+ \rightleftarrows ETF_{ox} + ETF_{hq}$. The pH dependence of the equilibrium indicates that $n = 1$ and is consistent with the assignment of ETF semiquinone (ETF_{sq}) and hydroquinone flavin species as true anions. The one-electron reduction potential of oxidized ETF is predicted to be independent of pH.

As many as eight primary flavoprotein dehydrogenases of the mitochondrial matrix (Crane et al., 1956; Hauge et al., 1956; Noda et al., 1980; Ikeda et al., 1983; Ikeda & Tanaka, 1983; Frisell & MacKenzie, 1962) are oxidized by a common flavoprotein, electron-transfer flavoprotein (ETF).[1] Anaerobic steady-state reduction of ETF by catalytic concentrations of G-AD results in the formation of the one-electron-reduced ETF anionic flavin semiquinone, which is a potent product inhibitor of the primary dehydrogenase (Beckmann et al., 1981). The fully reduced ETF hydroquinone is formed enzymatically but at a 10-fold slower rate subsequent to the one-electron reduction (Reinsch et al., 1980; Hall & Lambeth, 1980). ETF semiquinone is also readily obtained by chemical reduction with dithionite (Gorelick et al., 1982). The stabilization of the radical has been suggested to be kinetic rather than thermodynamic (Massey & Hemmerich, 1980; Hussin et al., 1984), since the semiquinone disproportionates to a mixture of all three redox states over the period of days (Gorelick et al., 1982). Since the equilibrium constant for disproportionation is near 1, it is difficult to evaluate the importance of the thermodynamic properties of ETF redox states in determining how this flavoprotein functions.

After reduction by a primary dehydrogenase, ETF is apparently reoxidized by a 4Fe-4S flavoprotein, ETF-QO (Ruzicka & Beinert, 1975, 1977; Schmidt et al., 1983). The specific function of the iron–sulfur flavoprotein is suggested by three lines of evidence. First, freeze–quench studies with the solubilized ETF-QO demonstrated the kinetic competence of the electron transfer from ETF to ETF-QO (Ruzicka & Beinert, 1977). Second, there is a specific increase of ETF-QO in brown adipose tissue during cold acclimation of guinea pigs. Brown adipose tissue oxidizes fatty acids to support thermogenesis (Flatmark et al., 1982). Third, immunoreactive enzyme is undetectable in liver submitochondrial particles derived from a patient with the heritable metabolic disorder glutaric acidemia type II (Goodman & Frerman, 1984). Patients with

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[1] Abbreviations: ETF, electron-transfer flavoprotein; G-AD, general (or medium-chain) acyl-CoA dehydrogenase; ETF-QO, electron transfer flavoprotein-ubiquinone oxidoreductase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Q, ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; PB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; PB-H_2, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzohydroquinone; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; NBT, nitro blue tetrazolium; TN, turnover number; AMPD, 2-amino-2-methyl-1,3-propanediol; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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this disease excrete large amounts of metabolites derived from substrates of the acyl-CoA dehydrogenases.

The physiological redox states of the ETF and ETF-QO from previous studies cannot be explicitly defined. For example, although it is known that full reduction of the iron–sulfur cluster of ETF-QO is achieved by enzymatic reduction (Ruzicka & Beinert, 1977), there is no information regarding the total number of electrons that ETF-QO accepts in this process. Also, it is not known whether electron transfer from ETF to ETF-QO occurs from the ETF semiquinone or the hydroquinone. Although the reductive half-reaction of ETF has been extensively studied, no detailed investigations of the oxidative half-reaction have been conducted. The purposes of the present investigation were to characterize the ETF-QO protein, to establish a sensitive catalytic assay for the enzyme, and to elucidate some of the redox properties of both ETF and ETF-QO.

Experimental Procedures

Materials. Methanesulfonic acid (4 N), containing 0.2% tryptamine, and methyl acetimidate were obtained from Pierce Chemical Co. Ampholine solution, with a pH range of 4–6.5, was from Pharmacia. Spheroidal hydroxylapatite was from BDH Biochemicals, and CHAPS was from Sigma. Butyryl-CoA and octanoyl-CoA were from P-L Biochemicals. Q_0 was purchased from Fluka Chemical Corp. and was used to synthesize the Q_1 analogue, PB, as described by Wan & Folkers (1978). The purified product had the same NMR δ values and R_f value on silica gel G thin-layer chromatography developed with hexane–chloroform–ether (10:10:1) as reported. The 5-deazaflavin was a gift from Dr. Helmut Beinert, University of Wisconsin—Madison. All other reagents were obtained from commercial sources and were the highest purity available.

Enzymes. G-AD was prepared from pig liver mitochondria essentially as described by McKean et al. (1979). The purified enzyme had a 270-nm:446-nm absorbance ratio of 5.6. ETF was also prepared by the procedure of Husain & Steenkamp (1983). ETF preparations used in this investigation had 270-nm:436-nm absorbance ratios of 5.8–6.0.

ETF-QO was prepared from submitochondrial particles, which had been stored at −70 °C, with the following modifications of the procedure described by Ruzicka & Beinert (1977). The particles were resuspended with a Potter-Elvehjem homogenizer and a tightly fitting Teflon pestle in 10 mM Tris-HCl, 5 mM potassium succinate, 250 mM sucrose, and 1 mM dithiothreitol, pH 7.4, to a protein concentration of 50 mg/mL. After the initial cholate extraction (0.18 mg of cholate/mg of protein) and centrifugation, the supernatant was fractionated with solid ammonium sulfate rather than being discarded. The protein precipitating between 50% and 60% saturation was dissolved in a minimum volume of 10 mM Tris-HCl, pH 7.4, and applied to a Sephadex G-75 column (5 cm × 50 cm) equilibrated with the same buffer. Fractions eluting in the void volume were pooled and applied to a DEAE-Bio-Gel column (4 cm × 10 cm). After being washed with 0.1 M Tris-HCl, pH 7.4, the column was washed with about 500 mL of 0.1 M Tris-HCl containing 0.1% (w/v) Triton X-100. During this wash, a majority of the heme proteins was eluted, leaving the fainter yellow-green ETF-QO bound near the top of the bed. ETF-QO was then eluted with a linear gradient (1000-mL total volume) from 0.1 to 0.3 M Tris-HCl, both of which contained 0.1% Triton. The centrifugal pellets from the first cholate extract were also resuspended and treated as previously described. Most of the ETF-QO is released from the membranes during the first extraction. Following DEAE-Bio-Gel chromatography, fractions containing NBT reductase activity (see below) were pooled and concentrated to about 5 mg/mL with an Amicon PM-30 membrane. The sample was dialyzed against 10 mM Tris-HCl, pH 7.4, followed by the addition of 100 mM CHAPS to a final concentration of 8 mM. Samples (10 mg of protein) were then loaded onto a column (1.5 cm × 10 cm) containing spheroidal hydroxylapatite equilibrated with 10 mM Tris-HCl–8 mM CHAPS, pH 7.4. After a wash with 10–20 mL of the same buffer, the ETF-QO was eluted with a linear gradient (150-mL total volume) from 10 mM Tris-HCl to 10 mM Tris-HCl containing 100 mM potassium phosphate, pH 7.4; both buffers contained 8 mM CHAPS. ETF-QO elutes at approximately 10 mM phosphate (Figure 1). Figure 2

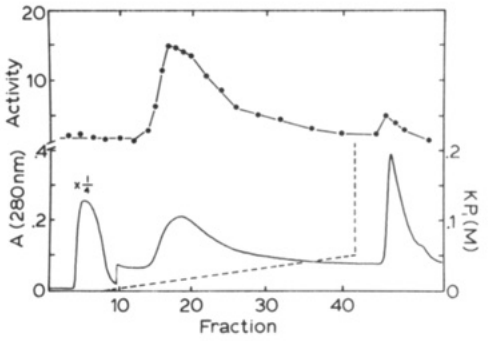

FIGURE 1: Elution of ETF-QO from hydroxylapatite. The column (1.5 × 10 cm) was equilibrated with 10 mM Tris-HCl–8 mM CHAPS, pH 7.4, loaded with 10 mg of ETF-QO (60% pure), washed with about 20 mL of the same buffer, and then eluted with a 150-mL potassium phosphate gradient as shown. At the end of the gradient, the column was washed with 250 mM KP_i. All buffers contained 10 mM Tris-HCl and 8 mM CHAPS, at pH 7.4. ETF-QO activity was monitored by the NBT reaction described in the text and is expressed as an increase in absorbance of 0.0025 per 4 min per 50-μL aliquot.

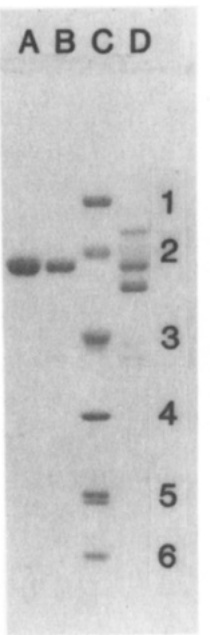

FIGURE 2: Polyacrylamide gel electropherogram in the presence of SDS of ETF-QO before (lane D) and after (lanes A and B) hydroxylapatite chromatography. The gel contained a linear gradient of total acrylamide from 8% to 15% at a constant ratio of acrylamide to bis(acrylamide) of 30:0.8. Lane C contains the following molecular weight marker proteins (4 μg each): 1, phosphorylase *b* (94 000); 2, bovine serum albumin (67 000); 3, ovalbumin (43 000); 4, carbonic anhydrase (30 000); 5, soybean trypsin inhibitor (20 100); 6, α-lactalbumin (14 400). Lanes A, B, and D contained 7, 4, and 7 μg of protein, respectively. The gel was stained with 0.006% Coomassie blue G-250 in 3% perchloric acid and destained in water.

Table I: Purification of ETF-QO from Pig Liver Submitochondrial Particles

sample	protein (mg)	units/mg ($\times 10^3$)[a]	units[a]	n-fold	yield (%)
submitochondrial particles	178 000	ND[b]	ND		
cholate extract	50 600	ND	ND		
50–60% ammonium sulfate/G-75	8 660	1.72	14.9	1	100
DEAE-Bio-Gel	192	41.2	7.91	23.8	53
hydroxylapatite	48	93.0	4.47	54.0	30

[a] Units are expressed as micromoles of monoformazan produced per minute in the standard NBT-linked assay. [b] ND, not detectable.

shows an SDS–polyacrylamide gel of a sample before and after hydroxylapatite chromatography. Samples were then concentrated to 1–2 mg/mL, made to contain 20% glycerol, and frozen in 1-mL aliquots at −70 °C. The enzyme has been stored for several months at −70 °C without any changes in either spectral or catalytic properties. Glycerol is required to prevent an approximate 50% inactivation by a single freeze–thaw cycle. Table I summarizes a typical purification of ETF-QO. No NBT reductase activity (see below) could be detected with crude submitochondrial particles or with the cholate extract. However, competing endogenous electron acceptors of the respiratory chain are expected to interfere with this assay.

Methods. Protein concentrations during enzyme preparations were determined by the Biuret reaction (Schleif & Wensink, 1981). For purified samples, the more sensitive method of Lowry et al. (1951), as modified by Miller (1959), was used. Bovine serum albumin was the standard and was assayed spectrophotometrically with $E^{1\%}_{280nm} = 6.6$. The color yield of purified ETF-QO in the Lowry assay is 96% that of the bovine serum albumin standard.

Carboxamidomethylated enzyme was prepared as follows. A 12-nmol sample of ETF-QO was dissolved in 0.7 mL of argon-saturated buffer containing 0.5 M Tris, 10 mM EDTA, 6 M guanidine hydrochloride, and 210 nmol of dithiothreitol, at pH 8.5. The anaerobic mixture was incubated in the dark at 23 °C for 1 h, with intermittent mixing, followed by the addition of 1.04 μmol of iodoacetamide. After 20 min of reaction, 14 μmol of β-mercaptoethanol was added to quench the alkylation. The carboxamidomethylated protein was then dialyzed against three changes of distilled water and recovered by lyophilization.

Flavin was determined by the method of Siegel (1978). The procedure was modified in order to avoid boiling the samples, which can hydrolyze FAD. Each 2.0-mL sample in the cuvette initially contained 0.10 M sodium phosphate, 0.2% SDS, and 20–50 nM flavin, at a pH of 7.7. After 10 min, the fluorescence was recorded, 0.20 mL of 1 N HCl was added, and the fluorescence at pH 2.6 was then determined. Internal FAD and FMN standards were used for determination of flavin in unknown samples. FAD and FMN standards were purified by chromatography on Whatman DE-52 DEAE-cellulose in 0.1 M potassium phosphate, pH 6.7, in the dark at 5 °C (Massey & Swoboda, 1963).

Iron was determined following the modified procedure of Beinert (1978). Acid-labile sulfide was determined by the methylene blue chromogen method of Fogo & Popowsky (1949) as modified by Siegel et al. (1973). Identical values of ETF-QO sulfur content were obtained with either internal or external standards.

Phospholipid associated with the purified enzyme was determined after extraction of the protein by the method of Bligh & Dyer (1959). Lipid phosphorus in the $CHCl_3$ was determined by the method of Chen et al. (1959) as modified by Ames & Dubin (1960).

Amino acid analyses were carried out on ETF-QO after hydrolysis at 115 °C for 24 and 48 h in methanesulfonic acid as described by Simpson et al. (1976). Total cysteine was determined after oxidation with performic acid as described by Moore (1963), except that the addition of HBr was omitted. After oxidation, the sample was frozen, lyophilized, and hydrolyzed with methanesulfonic acid. This procedure was found to prevent the total destruction of tyrosine and histidine residues as found in the original method (Moore, 1963). The analyses were carried out for us by Dr. Daniel Omilianowski, University of Wisconsin—Madison. Tryptophan was determined spectrophotometrically in samples of carboxamidomethylated protein from which the redox centers had been removed (Edelhoch, 1967).

Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the procedures of Weber et al. (1972) and Laemmli (1979). Gels were stained either with 0.25% Coomassie blue R-250 in 50% methanol containing 10% acetic acid or with 0.006% Coomassie blue G-250 in 3% perchloric acid. Gels stained with Coomassie G-250 were destained in water.

Isoelectric focusing was carried out in 6% polyacrylamide gels (0.5 cm × 11.5 cm) containing 5% glycerol, 0.05% Triton X-100, and 1.55% ampholyte solution. The upper cathodic reservoir contained 0.4% ethanolamine, pH 10, containing 5% glycerol, and the lower anodic reservoir contained 0.2% phosphoric acid (Wrigley, 1971). The pH gradients were determined in triplicate by eluting 1-cm gel slices with 1 mL of glass-distilled water; the pH of the eluates was measured with a calibrated glass electrode.

Spectrophotometric Titrations. Optical absorption spectra were measured with a Perkin-Elmer 559 spectrophotometer. Samples were maintained at 15 °C unless indicated otherwise. In experiments performed under anaerobic conditions, the semi-microcuvette was sealed with a rubber stopper and the sample subjected to at least 10 cycles of evacuation and purging with purified argon. A total of 7 units of glucose oxidase containing 45 units of catalase was then added. Samples contained 20 mM glucose prior to evacuation. The most oxygen-sensitive reduced flavoenzyme used in this study is ETF semiquinone, which was stable for at least 2 h under these conditions, except for a slow nonenzymatic disproportionation (Gorelick et al., 1982). Photoreduction was achieved by irradiating samples in a cuvette, cooled in an ice–water bath, from a distance of 10 cm from the lense of a slide projector equipped with a 300-W lamp. The anaerobic samples contained about 0.5 μM 5-deazaflavin and 1 mM EDTA. ETF-QO is stable in the presence of this versene. Titrations with degassed titrant were performed with a 25-μL Hamilton gas-tight syringe pierced through the rubber septum. ETF semiquinone was generated quantitatively by titration with sodium dithionite dissolved in 0.10 M sodium pyrophosphate, pH 9.

Solutions of PB and PB-H_2 used in titrations were prepared as follows. Due to the limited solubility of PB in water, a 100× stock solution was first made with methanol as solvent. This was then diluted with ethylene glycol, to give a final concentration of 1 mM. PB-H_2 was made by reduction with sodium borohydride after degassing the ethylene glycol solution (Trumpower et al., 1972). The reduced PB solution was made anaerobic by evacuation and purging with argon. PB was quantitated by using $\epsilon_{277nm} = 14.3\ mM^{-1}\ cm^{-1}$ (Schatz, 1967) in methanol.

Fluorescence spectra and intensity measurements were obtained with a Perkin-Elmer LS-5 ratio spectrofluorometer, with samples maintained at 25 °C. EPR spectra were recorded for us by Dr. Roger Sealy at the National Biomedical ESR Center, Milwaukee, WI. The standard Varian E-109 spectrometer was equipped with an Air Products LTD-3-110 helium-transfer system and was regulated with a Model APD-E temperature controller.

Enzyme Assays. Steady-state kinetic rates of NBT-coupled assays were measured spectrophotometrically at 23 °C. The K_m of G-AD for octanoyl-CoA and ETF and the reaction turnover number were determined by the dichlorophenolindophenol-coupled assay as previously described (McKean et al., 1979). The presence of 0.1% Triton X-100 had no effect on these parameters. ETF-QO activity was monitored in a coupled assay, in which the reduction of NBT to the monoformazan is observed by absorbance at 530 nm with an experimentally determined extinction coefficient of 19.8 mM^{-1} cm^{-1}. This value agrees with the value determined by Bielski et al. (1980) and is pH independent between 6.0 and 10.0. The concentration of oxidized NBT was determined spectrophotometrically, ϵ_{257nm} = 61 mM^{-1} cm^{-1} (Bielski et al., 1980). The standard NBT-coupled assay contained 20 μM octanoyl-CoA, 0.5 μM G-AD, 0.5 μM ETF, 70 μM NBT, and 0.1% Triton X-100 in 0.7 mL of 20 mM Tris-HCl, pH 8. This assay is useful for monitoring ETF-QO activity as the enzyme elutes from the DEAE-Bio-Gel or hydroxylapatite columns or for determining the effect of various treatments on enzyme stability. The standard assay is linear up to about 30 nM ETF-QO. Despite the utility of this standard assay, it may not be strictly valid for determination of kinetic constants. Although saturation behavior is observed with ETF, the steady-state kinetic parameters are dependent on reagent concentrations in an indeterminate fashion. This observation is likely due to the highly coupled nature of this assay.

The ability of ETF-QO to catalyze the equilibrium of ETF redox states provides a sensitive assay of ETF-QO activity. Although the reaction may be observed in either direction, the most sensitive and economical assay utilizes the comproportionation reaction. In the disproportionation assay, 0.6-mL reaction mixtures were prepared containing 20 mM Tris-HCl, pH 7.4, containing 20 mM glucose and ETF (varied from 2 to 15 μM in individual experiments). The cuvettes were sealed with a 1 cm thick rubber stopper, and the reaction mixtures were made anaerobic by 10 cycles of evacuation and purging with argon; 3 units of glucose oxidase containing 20 units of catalase was then injected. The absorption spectrum of oxidized ETF was recorded, and then ETF was reduced quantitatively to the semiquinone with dithionite (Husain & Steenkamp, 1983). Reactions were initiated by the addition of ETF-QO, and the decrease in absorbance at 370 nm was followed. Initial velocities were determined by analyses of single progress curves (Orsi & Tipton, 1979). In the comproportion assay, the decrease in oxidized ETF flavin fluorescense is observed continuously with time and the initial velocity subsequently determined by replotting in an integrated fashion (Orsi & Tipton, 1979). The ETF hydroquinone cosubstrate is generated by anaerobic titration of 50–80 μM ETF with dithionite (50 mM in 0.1 M sodium pyrophosphate, pH 9). In the assay, reaction mixtures are prepared containing Tris-HCl, pH 7.4, and glucose such that final concentrations of both are 20 mM. The assay mixtures are then sealed with a 1 cm thick rubber stopper and made anaerobic by evacuation and purging with argon as described above; glucose oxidase containing catalase is injected as above, and the reaction mixtures are equilibrated at 25 °C for 10 min. ETF hydroquinone is then transferred from the stock solution to the assay cuvette with a 25-μL Hamilton gas-tight syringe. This mixture serves as the fluorescence blank; degassed oxidized ETF is added, and the reaction is initiated with ETF-QO. Reaction mixtures typically contained 20 mM Tris-HCl, pH 7.4, 0.1–2.5 μM ETF hydroquinone, 0.1–1.0 μM oxidized ETF, and 20–80 pM ETF-QO per 0.80-mL assay. Fluorescence measurements were made with a Perkin-Elmer LS-5 spectrofluorometer, with excitation and emission wavelengths of 340 and 495 nm, respectively (Beckmann & Frerman, 1983a).

Table II: Properties of Mammalian ETF-Ubiquinone Oxidoreductases

property	Ruzicka & Beinert (1977)	this work
source	beef heart	pig liver
absorbance max (nm)	424	424
ϵ_{max} (mM^{-1} cm^{-1})	~27	24
M_r (flavin)	~74 000	73 000
M_r (subunit)	~66 000	69 000[a]
isoelectric point	ND[b]	5.2
Fe:S:FAD	3.7:3.6:1	3.5:3.4:1
ESR spectrum[c]		
g_x	1.886	1.883
g_y	1.939	1.939
g_z	2.086	2.084

[a] Determined by SDS–polyacrylamide gel electrophoresis in varying gel porosities. [b] ND, not determined. [c] Spectrum of the dithionite-reduced protein determined at 11–13 K.

Table III: Amino Acid Composition of Pig Liver ETF-QO[a]

	hydrolysis time (h)[b]		
amino acid	24	48	24[c]
Asx	57.2	57.3	59.0
Thr	32.7	31.6	32.9
Ser	27.9	25.4	28.1
Glx	63.6	64.7	68.0
Pro	42.4	35.0	43.4
Gly	64.7	65.1	65.7
Ala	40.5	40.9	41.2
Val	31.5	39.3	28.6
$^1/_2$-Cys			8.8
Met	9.5	8.2	12.9
Ile	30.7	32.6	31.4
Leu	60.2	60.8	59.8
Tyr	22.3	21.2	17.2
Phe	24.3	25.1	24.0
Lys[d]	41.8	42.4	42.6
His	22.6	22.7	23.8
Arg	27.8	28.2	27.9
Trp[e]	11.9		

[a] Reported as numbers of residues per 69 000 g of protein. [b] Hydrolysis was carried out in vacuo at 115 °C in 4 N methanesulfonic acid. [c] Sample oxidized for 4 h with performic acid prior to hydrolysis. [d] A total of 42.5 amino groups per 69 000 g of protein (Lowry) was determined by trinitrophenylation of carboxamidomethylated enzyme. [e] Determined spectrophotometrically in 6 M guanidine hydrochloride–20 mM sodium phosphate, pH 6.5.

Results

Properties of ETF-QO. The general properties of pig liver ETF-QO are comparable to the beef heart enzyme (Ruzicka & Beinert, 1977) and are summarized in Table II. Resonance Raman spectra have confirmed the assignment of the Fe-S cluster to a single 4Fe-4S type (Schmidt et al., 1983). Table III summarizes the amino acid content of ETF-QO determined after hydrolysis in 4 N methanesulfonic acid. The cysteine content is sufficiently high to provide the likely thiol ligands for a 4Fe-4S cluster. The enzyme is also quite rich in hydrophobic residues, including phenylalanine, tyrosine, and

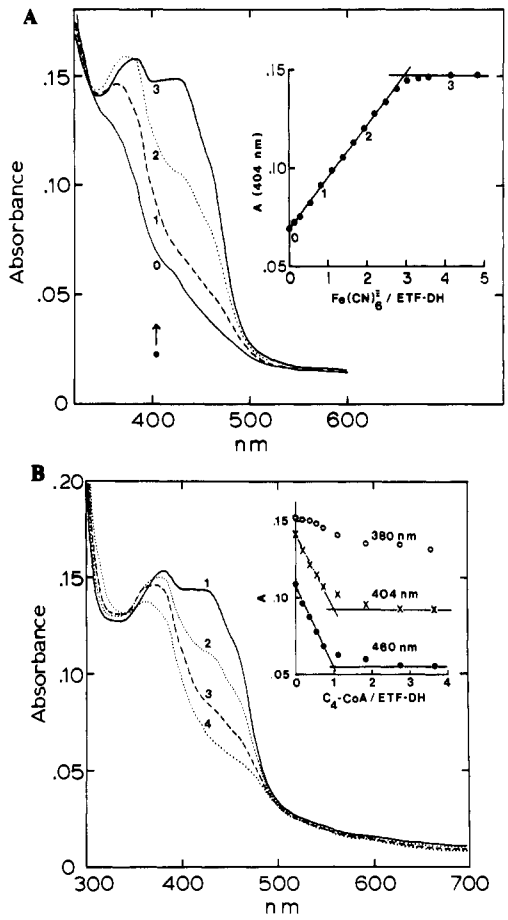

FIGURE 3: (A) Titration of fully reduced ETF-QO with ferricyanide. The anaerobic solution contained 6.2 μM enzyme in 0.70 mL of 20 mM Tris-HCl, pH 7.4, containing 1% glycerol, 1 mM EDTA, and 0.5 μM 5-deazaflavin. After complete photoreduction in the presence of 5-deazaflavin (spectrum 0), the solution was titrated with an anaerobic solution of 600 μM potassium ferricyanide. The inset shows the absorbance at 404 nm (arrow) vs. the molar ratio of titrant to enzyme. The numbered points in the inset correspond to the spectra, which were corrected for dilution. Intermediate spectra are omitted for clarity. (B) Anaerobic enzymatic reduction of ETF-QO. The anaerobic solution contained 5.9 μM ETF-QO, 0.1 μM G-AD, and 0.1 μM ETF in 0.70 mL of 20 mM Tris-HCl, pH 7.4, containing 7 mM CHAPS. The anaerobic oxidized sample (spectrum 1) was then titrated with butyryl-CoA. Spectra were recorded after 30 min of equilibration and are not corrected for dilution. The inset shows the corrected absorbance values vs. the mole ratio of titrant to ETF-QO. Spectra 2–4 correspond to mole ratios of 0.37, 0.74, and 3.7, respectively.

tryptophan. Assuming half of the aspartate and glutamate residues to be present as the carboxylates in the native protein, an average hydrophobicity of 1300 cal per residue is calculated (Nozaki & Tanford, 1971; Manavalan & Ponnuswamy, 1978a,b). This value is comparable to soluble proteins (Wirtz, 1982). Less than 0.05 mol of phospholipid was detected per mole of purified enzyme.

Spectral and Redox Properties of ETF-QO. For purposes of comparison, the absorption spectra of ETF-QO in various redox states are shown in Figure 3A. Spectra from a titration of fully reduced ETF-QO with ferricyanide are shown; comparable spectra are obtained during photochemical and enzymatic reduction. In these experiments, it was found that the absorbance at 404 nm is a linear function of ETF-QO redox state from fully oxidized to fully reduced (three electrons), with $\Delta\epsilon_{404nm} = 4.22$ mM^{-1} cm^{-1} per reducing equivalent. This parameter is used in subsequent calculations of the average redox state of ETF-QO. Absorbance changes at other

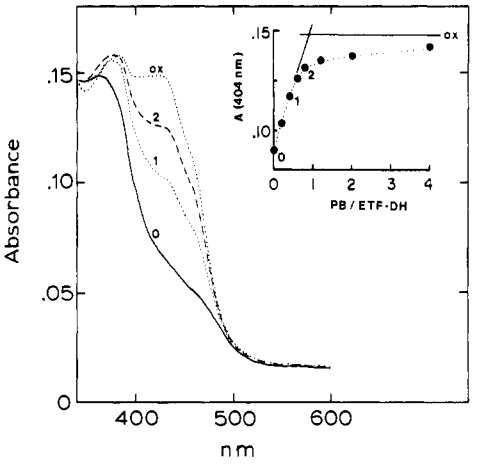

FIGURE 4: Titration of two-electron reduced ETF-QO with PB. The anaerobic oxidized sample (ox) contained 6.2 μM enzyme in 0.70 mL of 20 mM Tris-HCl, pH 7.4, containing 1% glycerol, 1 mM EDTA, and 0.5 μM 5-deazaflavin. The sample was then photoreduced to contain two electrons per enzyme (spectrum 0) and then titrated with anaerobic 0.87 mM PB. The numbers in the inset correspond to the numbered spectra. Intermediate spectra are not shown.

wavelengths were not found to vary linearily with enzyme redox state.

Reduced ETF has been shown to completely reduce the 4Fe-4S cluster of ETF-QO (Ruzicka & Beinert, 1977); however, the number of electrons transferred to the enzyme upon enzymatic reduction has not been determined. To address this question, a solution of anaerobic ETF-QO containing catalytic G-AD and ETF was titrated with butyryl-CoA (Figure 3B). Even with a 4-fold molar excess of substrate, it is apparent that ETF-QO accepts a maximum of two electron equivalents upon enzymatic reduction. This result was also obtained under anaerobic or aerobic conditions with a 30-fold molar excess of octanoyl-CoA, the optimal substrate of G-AD.

Since ETF-QO has been proposed to function as an ubiquinone reductase (Ruzicka & Beinert, 1977), it was of interest to quantitate electron equivalents transferred to this oxidant. Previous freeze–quench EPR data were not quantitative for all electron equivalents but only for those localized on the 4Fe-4S cluster. Figure 4 shows the titration of two-electron-reduced ETF-QO with the water-soluble Q_1 analogue, PB, and demonstrates the capability of the quinone to oxidize the enzyme. Although complete oxidation is approached, a large ratio of PB to enzyme is required. This result suggested the reaction to be reversible. Therefore, we investigated the reduction of ETF-QO by PB-H_2. As shown in Figure 5, ETF-QO is partially reduced by a 3.8-fold molar excess of PB-H_2, with about 11% reduction (two electrons) at a pH value of 7.3. A preliminary experiment demonstrated the extent of reduction to be pH dependent. Therefore, the reaction mixture (Figure 5) was titrated with anaerobic AMPD base to raise the pH, and the spectra were recorded 15 min after each addition to allow equilibration. Since the pH dependence of the two-electron redox potential of ubiquinone is known (Morrison et al., 1982), it was possible to calculate a redox potential of ETF-QO for two-electron reduction at each pH (see Appendix, part I). As shown in the inset of Figure 5, the redox potential varies dramatically with pH, decreasing by about 47 mV per pH unit. A potential of 38 mV is observed at a pH value of 7.3.

Catalytic Properties of ETF-QO. Although ETF-QO is able to catalyze the transfer of electrons from ETF to NBT (see Methods), this assay is not considered to be particularly meaningful. The NBT reductase activity was used only to

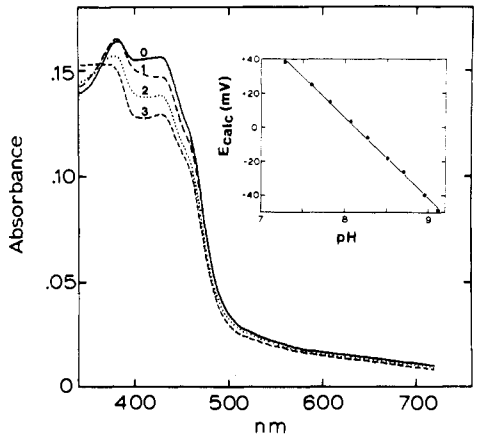

FIGURE 5: pH dependence of reduction of ETF-QO by PB-H_2. The anaerobic oxidized sample (0) contained 6.5 μM enzyme in 20 mM Tris-HCl, pH 7.3, containing 1% glycerol. A 3.8-fold molar excess of anaerobic PB-H_2 was then added, and the spectrum (1) recorded after equilibration at 25 °C. The solution was then titrated with 1 M AMPD base, and the corrected spectra were recorded after equilibration, which required about 15 min. The spectra numbered 2 and 3 were recorded at pH 8.27 and 9.11, respectively. Values of pH were obtained from a control titration of the reaction mixture that did not contain enzyme. Intermediate spectra are omitted for clarity. The inset shows the pH dependence of the calculated redox potential, E_{calcd}, of ETF-QO. The 404-nm absorbance values from the spectra were used to calculate the redox potentials (see Appendix, eq 5).

Table IV: Steady-State Rate Constants for Equilibration of ETF Redox States Catalyzed by ETF-QO at pH 7.4

reaction	TN (s^{-1})	$K_m^{ETF_{sq}}$ ($M \times 10^6$)	$K_m^{ETF_{ox}}$ ($M \times 10^6$)	$K_m^{ETF_{hq}}$ ($M \times 10^6$)
disproportionation	200 ± 10	7.7 ± 1.2[a]		
comproportionation	78 ± 18		0.32 ± 0.13	0.31 ± 0.03

[a] This value is an apparent K_m value that represents the sum of two K_m values in a Ping Pong Bi Bi mechanism that cannot be measured independently (Beckmann & Frerman, 1985).

measure relative amounts of enzyme during purifications and to assess the effects of various physical and chemical treatments on enzymatic stability. In this respect, the NBT-linked assay has been very useful.

To study the interaction between ETF and ETF-QO directly, we initially attempted to observe the anaerobic reoxidation of reduced ETF by ETF-QO, with ubiquinone as the terminal oxidant. However, it was observed that ETF semiquinone was rapidly disproportionated by ETF-QO to a mixture of all three ETF redox states. That is, after accepting an electron from one ETF semiquinone, the reduced ETF-QO readily transfers the equivalent to a second ETF semiquinone, forming both oxidized and hydroquinone ETF species as products. A typical disproportionation experiment is shown in Figure 6. The reaction may also be followed fluorometrically due to the formation of oxidized ETF (not shown). Single progress curves, as shown in the inset of Figure 6, were used to determine initial steady-state velocities at several different ETF semiquinone concentrations. The steady-state constants for the catalyzed disproportionation at pH 7.4 are given in Table IV. Reaction equilibrium constants may also be obtained from experiments as shown in Figure 6 (see Appendix, part II).

The disproportionation reaction is useful for some investigations; however, this approach is not very practical as a catalytic assay, since relatively large amounts of ETF are required per assay. Therefore, the reverse reaction, or comproportionation, has been investigated in considerable detail.

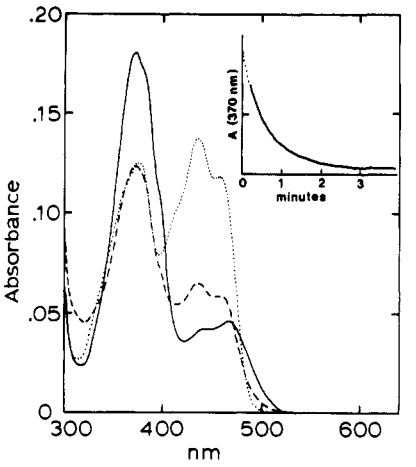

FIGURE 6: Disproportionation of ETF semiquinone catalyzed by ETF-QO. The anaerobic solution initially contained 10.2 μM oxidized ETF (dotted curve) in 20 mM Tris-HCl, pH 7.4, containing 1% glycerol. ETF semiquinone (solid curve) was generated by titration with dithionite. ETF-QO was then added to give a final concentration of 0.95 nM and the absorbance at 370 nm monitored continuously with time (inset, ordinate is identical with main diagram). After 10 min, the spectrum of the equilibrium mixture was recorded (dashed curve).

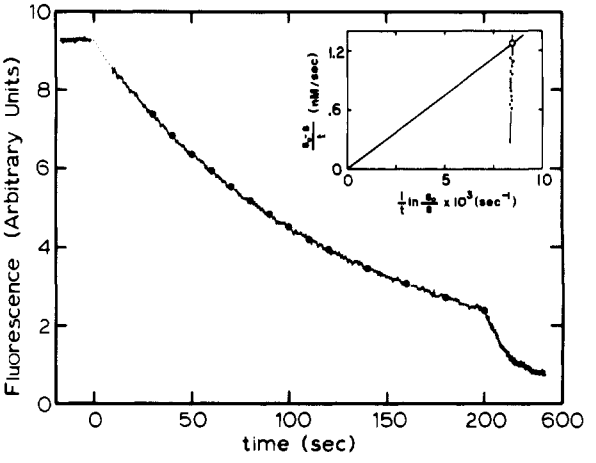

FIGURE 7: Representative steady-state fluorometric comproportionation assay of ETF-ubiquinone oxidoreductase. The assay contained 1.5 μM ETF hydroquinone, 0.15 μM oxidized ETF, and 52 pM ETF-QO in 0.70 mL of 20 mM Tris-HCl, pH 8.75, containing KCl to give an ionic strength of 0.017 M.

The objective of these experiments was to observe the decrease in oxidized ETF flavin fluorescence continuously with time, using ETF hydroquinone as the cosubstrate. Figure 7 shows a fluorescence decay single progress curve, in which a 10-fold molar excess of ETF hydroquinone over oxidized ETF was used at pH 8.75. The residual fluorescence of 8%, relative to oxidized ETF, corresponds to the residual fluorescence of the ETF semiquinone product and indicates completion of the reaction (Beckmann & Frerman, 1983a). The extent of the reaction is pH dependent since the reaction equilibrium is pH dependent (see below). The initial velocity is determined from progress curves. The positive slope of the integrated replot indicates product inhibition, which is consistent with the reversibility of the reaction. Initial velocities of comproportionation were directly proportional to the concentration of ETF-QO up to at least 100 pM. Steady-state constants for the catalyzed comproportionation at pH 7.4 are given in Table IV.

Redox Properties of ETF. The equilibration of ETF redox states has been previously observed (Gorelick et al., 1982);

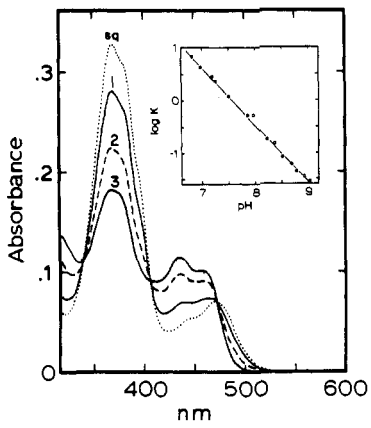

FIGURE 8: Effect of pH on ETF semiquinone disproportionation equilibrium. The sample initially contained 18.0 μM ETF semiquinone (dotted curve) in 2 mM dibasic potassium phosphate/9 mM AMPD-HCl, pH 9.03, containing 0.8% glycerol. Spectrum 1 was recorded after equilibration in the presence of 1 nM ETF-QO. The sample was then titrated with 1 M monobasic potassium phosphate, and the spectra (corrected for dilution) was recorded after equilibration. Spectra 2 and 3 correspond to pH values of 7.85 and 6.81, respectively. Intermediate spectra, and those resulting from the back-titration with AMPD, are omitted for clarity. The insert shows the effect of pH on the apparent equilibrium constant, K, that was calculated at each pH from the 370-nm absorbance value (Appendix, part II). The closed circles (●) correspond to decreasing pH with KH_2PO_4, and the open circles (○) were calculated from the back-titration with AMPD. The line is a linear least-squares fit to the closed symbols and has a slope of −1.1.

however, several days are required to achieve equilibrium in the absence of a catalyst. To determine the dependence of the equilibrium on pH, the experiment shown in Figure 8 was performed. ETF semiquinone was quantitatively formed by titration with dithionite at pH 9.1 and then disproportionated to equilibrium by ETF-QO. The mixture was then titrated with 1 M monobasic potassium to lower the pH, followed by titration with 0.5 M AMPD base. Spectra were recorded after equilibration at each addition of titrant (5–10 min), and the K_{app} values were determined as a function of pH. The inset of Figure 8 shows a plot of log K_{app} vs. pH, as determined from the data in the figure. The slope of −1.1 indicates $n = 1$ in the disproportionation reaction (see Appendix, part II). A K_{app} of about 1 is predicted at pH 7.6, which is in reasonable agreement with the value of 0.49 previously determined at this pH (Gorelick et al., 1982). That the pH effect is reversible indicates that the equilibration reaction is freely reversible.

Discussion

ETF-QO from pig liver appears comparable in all respects to the enzyme from beef heart (Ruzicka & Beinert, 1975, 1977). The protein has been further characterized in this investigation. The amino acid composition gives no indication of extreme hydrophobicity, which might be anticipated for a membrane protein. However, since ETF-QO reacts with the soluble matrix electron transferase, ETF, the protein may contain a relatively small hydrophobic domain. The acidic isoelectric point of 5.2 is of interest since the primary flavoprotein reductants of ETF are also acidic proteins (Noda et al., 1980; Ikeda et al., 1983). Surface lysine groups on ETF play a crucial role in the interaction of ETF with the anionic G-AD (Beckmann and Frerman, 1983). The accompanying paper (Beckmann & Frerman, 1985) demonstrates a similar interaction between ETF and ETF-QO.

In this investigation we have employed equilibrium spectrophotometric titrations in order to study the redox properties of ETF-QO. Although the enzyme is readily reduced photochemically or by dithionite (Ruzicka & Beinert, 1977) to the three-electron-reduced state, enzymatic reduction of ETF-QO by ETF proceeds only as far as two-electron reduction. Previous EPR measurements indicated the two-electron-reduced enzyme to consist primarily of a reduced 4Fe-4S cluster and FAD semiquinone (Ruzicka & Beinert, 1977). Although the two-electron-reduced enzyme is capable of transferring both equivalents to PB, it is not clear why the two electrons from acyl-CoA substrates should be split at the level of ETF and paired at the level of the presumed quinone oxidant of ETF-QO. However, it is possible that the actual electron acceptor may be ubisemiquinone or ubiquinone tightly associated with complex III (Trumpower, 1976, 1981). The function of the iron–sulfur cluster may be to efficiently donate one electron to an obligate one-electron acceptor. Such a model, however, does not necessarily rule out equilibration of ETF-QO with a mobile Q pool (Kroger & Klingenberg, 1973). The ability of ETF-QO to react directly with PB, and presumably ubiquinone, contraindicates the necessity of a separate Q binding protein in this region of the respiratory chain (Yu & Yu, 1981).

Previous observations with submitochondrial particles (Ohnishi et al., 1972) and with the procaryote *Paracoccus denitrificans* (Albracht et al., 1980) have indicated that the 4Fe-4S cluster of ETF-QO equilibrates with the NADH and succinate dehydrogenase systems. One mechanism for this occurrence would be via a mobile Q pool, and reverse electron flow from $Q\text{-}H_2$ to ETF-QO. Indeed, our data show this to be a reasonable possibility. The extent of reduction of ETF-QO by $Q\text{-}H_2$ in the membrane may be different than that with the solubilized components. However, this work shows that any difference is not due to alteration of the redox potential of the 4Fe-4S cluster by binding to the membrane, since our data with the soluble enzyme agree with previous determinations of the redox potential of the cluster in submitochondrial particles (see below). Preliminary experiments also show that incorporation of the solubilized enzyme into liposomes does not alter the steady-state kinetic constants in the comproportionation assay.[2] Our spectrophometrically determined redox potentials are in very good agreement with the values determined for the 4Fe-4S cluster in submitochondrial particles. For example, a redox potential of 40 mV at a pH value of 7.2 was obtained (Ohnishi et al., 1972), and a potential of 30 mV was observed at a pH value of 7.5 (Thayer et al., 1980). Our data indicate potentials of 43 and 29 mV at these two pH values, respectively. Therefore, the spectrophotometrically determined potentials appear to reflect the potential of the 4Fe-4S cluster. This observation can be explained if the ETF-QO flavin is reduced to the anionic semiquinone; anionic flavin semiquinones of flavoproteins can exhibit isosbestic absorbance with the oxidized state near 404 nm. The pH dependence of −47 mV per pH unit is interesting since $dE_0/d(pH)$ is expected to be $-60\ mV/n$ per pH unit, where n is the number of electrons transferred. Such non-Nernstian behavior has been observed with other iron–sulfur proteins (Stombaugh, 1976). Recent spin–echo experiments by Orme-Johnson et al. (1983) have ascribed this phenomenon in ferredoxin-type iron–sulfur clusters to a decrease in solvent accessibility to the redox center upon reduction; thus, some charge compensation must take place internally. This interpretation suggests that the redox state of the cluster governs the rate and number of protons available for exchange and infers a change in protein conformation. Such a model ex-

[2] D. B. Schowalter and F. E. Frerman, unpublished studies.

plains the observed behavior of ETF-QO, and the suggested conformational change would also explain the observation that fully reduced ETF-QO requires over 2 h for complete reoxidation in air (Beckmann & Frerman, 1983b).

A useful steady-state assay for the reaction of ETF with ETF-QO has been developed on the basis of the capacity of ETF-QO to catalyze equilibration of ETF redox states. One advantage of the ETF redox state equilibration reaction is that no other coupling enzymes or terminal electron acceptor is required as in the NBT coupled assay. The steady-state mechanism of the equilibration is addressed in the accompanying paper (Beckmann & Frerman, 1985). Although we have not generated any data regarding rates of electron flux from ETF to Q, the disproportionation rate constants do provide an estimate of a minimal second-order rate constant, TN/K_m, for the rate of reduction of ETF-QO by ETF semiquinone. This minimal rate of 5.4×10^7 M^{-1} s^{-1} is about 10-fold greater than the reduction of ETF to the semiquinone by G-AD (Beckmann & Frerman, 1983a). This may indicate that ETF functions as a one-electron carrier, since reoxidation catalyzed by ETF-QO can occur about 100 times faster than reduction of ETF to the hydroquinone state by G-AD (Reinsch et al., 1980; Hal & Lambeth, 1980).

The pronounced pH dependence on the equilibration of ETF redox states by ETF-QO is consistent with the known acid–base properties of flavin. The absorbance of ETF flavin semiquinone (Husain & Steenkamp, 1983; Gorelick et al., 1982) and EPR evidence (McKean et al., 1982) are consistent with its assignment as an anionic semiquinone. Therefore, the one-electron reduction of ETF should be pH independent. Also, the absorbance of ETF hydroquinone in the 360-nm region indicates the flavin to be an anion (Ghisla et al., 1974). Therefore, the anionic ETF flavosemiquinone binds only one proton upon subsequent reduction to the hydroquinone. This prediction is in accord with the data shown in Figure 8.

It is still unclear whether ETF functions in vivo as a one-, two-, or mixed-electron transferase. The ability of ETF-QO to equilibrate the redox states of ETF provides an indication that ETF hydroquinone may be formed within the mitochondrion. Although ETF is preferentially reduced to the semiquinone by the primary dehydrogenases (Beckmann et al., 1981; Hall & Lambeth, 1980), the stabilization of the radical will only be thermodynamic under basic conditions. Therefore, the disproportionation reaction may be physiologically significant. The rates of electron flux from ETF to ubiquinone have not been investigated. Measurements of this flux under different mitochondrial energy states may be necessary to determine how ETF functions in vivo.

Finally, we emphasize that the electron flow from the primary ETF-linked dehydrogenases into the main respiratory chain is not unidirectional. Rather, each reaction step is readily reversible under physiological conditions. It has been known for some time that the acyl-CoA dehydrogenases transfer electrons to ETF in a reversible manner, which is dependent upon the presence of an appropriate enoyl-CoA product (Beinert & Page, 1957). In this investigation, we have demonstrated for the first time reverse electron flow from purified ETF-QO to ETF. Furthermore, submitochondrial particles exhibit NADH–ETF oxidoreductase activity that is completely inhibited by antibodies directed against ETF-QO.[3] Since ETF semiquinone is known to be a potent product inhibitor of G-AD (Beckmann et al., 1981), it is reasonable to suggest that the rate of β-oxidation will be governed by the mitochondrial redox state. The ratio of oxidized to reduced ETF will be dependent upon the NADH/NAD ratio.

[3] J. D. Beckmann and F. E. Frerman, unpublished studies.

APPENDIX

(I) Calculation of Redox Potentials. Since the pH dependence of the two-electron-reduction potential of ubiquinone is known (Morrison et al., 1982), it is possible to calculate a redox potential of ETF-QO for two-electron reduction at each pH. Because the formation of ubisemiquinone is thermodynamically unfavored (Trumpower, 1981), this reaction is suggested:

$$\text{ETF-QO}_{ox} + \text{PB-H}_2 \rightleftharpoons \text{ETF-QO}_{2e^-} + \text{PB} \quad (1)$$

Since the mixture is observed at equilibrium

$$\Delta G = 0 = -nFE_t \quad (2)$$

Equation 1 can be split into two half-reactions for two-electron reduction (ETF-QO) and oxidation (PB), each with a definable reduction potential. From eq 1 and 2

$$E_t = E([\text{ETF-QO}_{ox}]/[\text{ETF-QO}_{2e^-}]) - E\frac{[\text{PB}]}{[\text{PB-H}_2]} = 0 \quad (3)$$

The pH dependence for two-electron reduction of Q has been determined to be (Morrison et al., 1982)

$$E_t\frac{[\text{PB}]}{[\text{PB-H}_2]} = 0.513 - \frac{0.059}{2}\log\frac{[\text{PB-H}_2]}{[\text{PB}]} - 0.059\text{pH} \quad (4)$$

The observed quantity is the concentration of ETF-QO_{2e}, from which all other reactant concentrations may be calculated, given that the initial concentrations of ETF-QO_{ox} and PB-H_2 are known. From the above initial conditions, and from eq 3 and 4, one may obtain

$$E([\text{ETF-QO}_{ox}]/[\text{ETF-QO}_{2e^-}]) = 0.513 - \frac{0.059}{2}\log\frac{[\text{PB-H}_2] - [\text{ETF-QO}_{2e^-}]}{[\text{ETF-QO}_{2e^-}]} - 0.059\text{pH} \quad (5)$$

Therefore, the redox potential for two-electron reduction may be calculated at each value of pH.

(II) ETF Semiquinone Disproportionation Equilibrium Calculations. The disproportionation of ETF semiquinone is described by the reaction

$$2\text{ETF}_{sq} + n\text{H}^+ \rightleftharpoons \text{ETF}_{ox} + \text{ETF}_{hq} \quad (6)$$

where sq, ox, and hq refer to the semiquinone, oxidized, and hydroquinone redox states, respectively. Since the initial concentration of $[\text{ETF}_{sq}]_0$ is known

$$[\text{ETF}_{sq}] = [\text{ETF}_{sq}]_0 - 2[\text{ETF}_{ox}] = [\text{ETF}_{sq}]_0 - 2[\text{ETF}_{hq}] \quad (7)$$

Using the extinction coefficients, ϵ, of the three redox states, the absorbance at 370 nm is given by

$$A = \epsilon_{ox}[\text{ETF}_{ox}] + \epsilon_{sq}[\text{ETF}_{sq}] + \epsilon_{hq}[\text{ETF}_{hq}] \quad (8)$$

Using eq 7 and 8, it is possible to obtain

$$[\text{ETF}_{ox}] = \frac{A - \epsilon_{sq}[\text{ETF}_{sq}]_0}{\epsilon_{ox} - 2\epsilon_{sq} + \epsilon_{hq}} \quad (9)$$

Therefore, all three species of ETF may be calculated from the absorbance at a single wavelength. Values for the extinction coefficients were as given (Husain & Steenkamp, 1983) and verified in preliminary experiments. Finally, the equilibrium constant for eq 6 may readily be calculated from

$$K = \frac{[\text{ETF}_{ox}][\text{ETF}_{hq}]}{[\text{ETF}_{sq}]^2[\text{H}^+]^n} = K_{app}(1/[\text{H}^+]^n) \quad (10)$$

Therefore

$$\log K_{app} = \log K - n(\mathrm{pH}) \quad (11)$$

Registry No. ETF-QO, 86551-03-3.

REFERENCES

Albracht, S. P. J., Van Verseveld, H. W., Hagen, W. R., & Kalkman, M. L. (1980) *Biochim. Biophys. Acta 593*, 173–186.

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Beckmann, J. D., & Frerman, F. E. (1983a) *J. Biol. Chem. 258*, 7563–7569.

Beckmann, J. D., & Frerman, F. E. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 17761 (Abstr).

Beckmann, J. D., & Frerman, F. E. (1985) *Biochemistry* (following paper in this issue).

Beckmann, J. D., Frerman, F. E., & McKean, M. C. (1981) *Biochem. Biophys. Res. Commun. 102*, 1290–1294.

Beinert, H. (1978) *Methods Enzymol. 54*, 435–445.

Beinert, H., & Page, E. (1957) *J. Biol. Chem. 225*, 479–497.

Bielski, B. H. J., Shiue, G. G., & Bajuk, S. (1980) *J. Phys. Chem. 84*, 830–833.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Chen, P. S., Jr., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Crane, F. L., Mii, S., Hauge, J. G., Green, D. E., & Beinert, H. (1956) *J. Biol. Chem. 218*, 701–716.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Flatmark, T., Ruzicka, F. J., & Beinert, H. (1976) *FEBS Lett. 63*, 51–56.

Fogo, J. K., & Popowsky, M. (1949) *Anal. Chem. 21*, 732–734.

Frisell, W. R., & MacKenzie, C. G. (1962) *J. Biol. Chem. 237*, 94–98.

Ghisla, S., Massey, V., Lhoste, J. M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589–597.

Goodman, S. I., & Frerman, F. E. (1984) *J. Inherited Metab. Dis. 7* (Suppl 1), 33–37.

Gorelick, R. J., Mizzer, J. P., & Thorpe, C. (1982) *Biochemistry 21*, 6936–6942.

Hall, C. L., & Lambeth, J. D. (1980) *J. Biol. Chem. 255*, 3591–3595.

Hauge, J. G., Crane, F. L., & Beinert, H. (1956) *J. Biol. Chem. 219*, 727–733.

Husain, M., & Steenkamp, D. J. (1983) *Biochem. J. 209*, 541–545.

Husain, M., Stankovich, M. T., & Fox, G. B. (1984) *Biochem. J. 219*, 1043–1047.

Ikeda, Y., & Tanaka, K. (1983) *J. Biol. Chem. 258*, 9477–9487.

Ikeda, Y., Dabrowski, C., & Tanaka, K. (1983) *J. Biol. Chem. 258*, 1066–1076.

Kroger, A., & Klingenberg, M. (1973) *Eur. J. Biochem. 34*, 358–368.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Manavalan, P., & Ponnuswamy, P. K. (1978a) *Nature (London) 275*, 673–674.

Manavalan, P., & Ponnuswamy, P. K. (1978b) *Nature (London) 275*, 830–833.

Massey, V., & Swoboda, B. E. P. (1963) *Biochem. Z. 338*, 474–484.

Massey, V., & Hemmerich, P. (1980) *Biochem. Rev. 8*, 246–257.

Massey, V., Brumby, P. E., Komai, H., & Palmer, G. (1969) *J. Biol. Chem. 244*, 1682–1691.

McKean, M. C., Frerman, F. E., & Mielke, D. (1979) *J. Biol. Chem. 254*, 2730–2735.

McKean, M. C., Sealy, R. C., & Frerman, F. E. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 614–617, Elsevier, New York.

Miller, G. L. (1959) *Anal. Chem. 31*, 964.

Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.

Morrison, L. E., Schelhorn, J. E., Cotton, T. M., Bering, C. L., & Loach, P. A. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 35–58, Academic Press, New York.

Noda, C., Rhead, W. J., & Tanaka, K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2646–2650.

Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.

Ohnishi, T., Wilson, D. F., Asakura, T., & Chance, B. (1972) *Biochem. Biophys. Res. Commun. 46*, 1631–1638.

Orme-Johnson, N. R., Mims, W. B., Orme-Johnson, W. H., Bartsch, R. G., Cusanovich, M. A., & Peisach, J. (1983) *Biochim. Biophys. Acta 748*, 68–72.

Orsi, B. A., & Tipton, K. F. (1979) *Methods Enzymol. 63*, 159–183.

Reinsch, J. W., Feinberg, B. A., & McFarland, J. T. (1980) *Biochem. Biophys. Res. Commun. 94*, 1409–1416.

Ruzicka, F. J., & Beinert, H. (1975) *Biochem. Biophys. Res. Commun. 66*, 622–631.

Ruzicka, F. J., & Beinert, H. (1977) *J. Biol. Chem. 252*, 8440–8445.

Schatz, G. (1967) *Methods Enzymol. 10*, 30–33.

Schleif, R. F., & Wensink, P. C. (1981) in *Practical Methods in Molecular Biology*, pp 74–75, Springer-Verlag, New York.

Schmidt, J., Beckmann, J., Frerman, F., & McFarland, J. T. (1983) *Biochem. Biophys. Res. Commun. 113*, 784–790.

Siegel, L. M. (1978) *Methods Enzymol. 53*, 419–429.

Siegel, L. M., Murphy, M. J., & Kamin, H. (1973) *J. Biol. Chem. 248*, 251–264.

Simpson, R. J., Neuberger, M. R., & Liu, T.-Y. (1976) *J. Biol. Chem. 251*, 1936–1949.

Stombaugh, N. A., Sundquist, J. E., Burris, R. H., & Orme-Johnson, W. H. (1976) *Biochemistry 15*, 2633–2641.

Thayer, W. S., Ohnishi, T., & Rubin, E. (1980) *Biochim. Biophys. Acta 591*, 22–26.

Trumpower, B. L. (1976) *Biochem. Biophys. Res. Commun. 70*, 73–80.

Trumpower, B. L. (1981) *J. Bioenerg. Biomembr. 13*, 1–24.

Trumpower, B. L., Aiyar, A. S., Opliger, C. E., & Olson, R. E. (1972) *J. Biol. Chem. 247*, 2499–2511.

Wan, Y.-P., & Folkers, K. (1978) *Methods Enzymol. 53*, 591–599.

Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3–27.

Wirtz, K. W. A. (1982) in *Lipid Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. 1, pp 151–231, Wiley-Interscience, New York.

Wrigley, C. W. (1971) *Methods Enzymol. 22*, 559–564.

Yu, C.-A., & Yu, L. (1981) *Biochim. Biophys. Acta 639*, 99–128.