Expression of human electron transfer flavoprotein-ubiquinone oxidoreductase from a baculovirus vector: kinetic and spectral characterization of the human protein

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Electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) is an iron–sulphur flavoprotein and a component of an electron-transfer system that links 10 different mitochondrial flavoprotein dehydrogenases to the mitochondrial bc_1 complex via electron transfer flavoprotein (ETF) and ubiquinone. ETF-QO is an integral membrane protein, and the primary sequences of human and porcine ETF-QO were deduced from the sequences of the cloned cDNAs. We have expressed human ETF-QO in Sf9 insect cells using a baculovirus vector. The cDNA encoding the entire protein, including the mitochondrial targeting sequence, was present in the vector. We isolated a membrane-bound form of the enzyme that has a molecular mass identical with that of the mature porcine protein as determined by SDS/PAGE and has an N-terminal sequence that is identical with that predicted for the mature holoenzyme. These data suggest that the heterologously expressed ETF-QO is targeted to mitochondria and processed to the mature, catalytically active form. The detergent-solubilized protein was purified by ion-exchange and hydroxyapatite chromatography. Absorption and EPR spectroscopy and redox titrations are consistent with the presence of flavin and iron–sulphur centres that are very similar to those in the equivalent porcine and bovine proteins. Additionally, the redox potentials of the two prosthetic groups appear similar to those of the other eukaryotic ETF-QO proteins. The steady-state kinetic constants of human ETF-QO were determined with ubiquinone homologues, a ubiquinone analogue, and with human wild-type ETF and a *Paracoccus*–human chimaeric ETF as varied substrates. The results demonstrate that this expression system provides sufficient amounts of human ETF-QO to enable crystallization and mechanistic investigations of the iron–sulphur flavoprotein.

Key words: EPR, glutaric acidemia type 2, iron–sulphur cluster, respiratory chain.

INTRODUCTION

Electrons from at least nine mitochondrial flavoprotein dehydrogenases are transferred to the respiratory chain via electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETF-QO) [1–4]. ETF-QO is an integral membrane protein that contains FAD and a $[4Fe4S]^{2+,1+}$ cluster [1,2]. Despite its central role in the oxidation of fatty acids and some amino acids, relatively little is known about ETF-QO. A cDNA encoding the human protein was cloned by Goodman et al. [3] and expressed, albeit at low levels, in *Saccharomyces cerevisiae*. Ramsay et al. [5] and Watmough et al. [6] determined steady-state kinetic constants of porcine ETF-QO with the water-soluble ubiquinone analogue, Q_1, and with porcine ETF as varied substrates. Beckmann and Frerman [2,7] demonstrated that porcine ETF-QO also catalyses the equilibration of ETF redox states:

$$2ETF_{1e} \rightleftharpoons ETF_{ox} + ETF_{2e^-} \quad (1)$$

and determined kinetic constants for the disproportionation and comproportionation reactions. Ramsay et al. [5] suggested that the disproportionation of ETF semiquinone (eqn. 1) catalysed by ETF-QO might be physiologically significant since it is faster than the overall rate of electron transfer from a primary dehydrogenase to ubiquinone. They proposed that ETF_{2e^-} is the physiological reductant of ETF-QO rather than ETF_{1e^-}, which is the product of the oxidative half-reactions of the acyl-CoA dehydrogenases [5]. However, the hypothesis is based on the kinetics of reduction of the water-soluble ubiquinone homologues, Q_1 rather than Q_{10}, which is confined to a membrane phase. The mechanism of reduction of ETF-QO by ETF remains unclear.

The potentials of porcine ETF-QO for the transfer of the first and second electrons to the flavin are $+0.028$ and -0.006 V, respectively, and $+0.047$ V for the iron–sulphur cluster, indicating that the flavin semiquinone is thermodynamically stabilized [8]. However, the potentials were determined by EPR spectroelectrochemistry at the temperature of liquid helium, so that re-equilibration of electrons on freezing is possible. Nonetheless, the values are in reasonable agreement with the overall potential for the two-electron transfer in the equilibration with the 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone couple at 15 °C [2]. Spin–spin coupling between the flavin radical and reduced cluster of ETF-QO is too small to be detected from changes in the EPR lineshape for the cluster [9]. This is in contrast to the substantial lineshape changes that are observed due to coupling between FMN semiquinone and the reduced $[4Fe4S]^{2+,1+}$ cluster that are separated by 4 Å (1Å ≡ 0.01 nm) in trimethylamine dehydrogenase [10,11].

Deficiency of ETF-QO is the cause of glutaric acidemia type II, often a fatal metabolic disorder [12]. An expression system for ETF-QO is desirable to be able to investigate the effects of some

Abbreviations used: ETF, electron transfer flavoprotein; ETF-QO, ETF-ubiquinone oxidoreductase; DTT, dithiothreitol; PFO, perfluoro-octanoic acid; TPCK, tosyl-L-phenylalanine-chloromethyl ketone; DQ, decylubiquinone.

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pathogenic mutations on electron-transfer reactions with ETF and ubiquinone and the structural changes involved. Similarly, it is desirable to pursue mechanistic investigations using site-directed mutagenesis with the prospect of correlating changes in activity with any changes in structure. In this paper we describe an expression system for human ETF-QO in insect cells using a baculovirus vector. We report catalytic and spectral properties of the recombinant protein and show that the recombinant protein provides a good system for investigating structure–function relationships in ETF-QO.

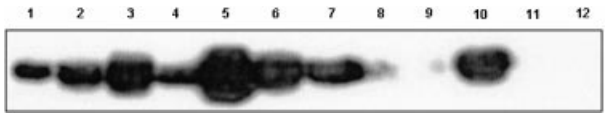

Figure 1 Expression of ETF-QO antigen by Sf9 cells infected with baculovirus recombinants

Sf9 cells (5×10^5) infected with recombinant baculoviruses (lanes 2–11) were assayed for ETF-QO antigen by Western blotting following SDS/PAGE. Sf9 cells in lane 12 are an infected control. Lane 1, 50 ng of porcine ETF-QO.

EXPERIMENTAL

Materials

Human medium-chain acyl-CoA dehydrogenase and human ETF were expressed and purified as described previously [13,14]. The expression vector for human medium-chain acyl-CoA dehydrogenase was provided generously by Dr Arnold Strauss (Vanderbilt University, Nashville, TN, U.S.A.). A chimaeric *Paracoccus denitrificans*–human ETF was prepared by the method of Herrick et al. [15]. In this chimaeric ETF, the first 83 residues of the β subunit contain the bacterial sequence; the remaining β subunit and the entire α subunit have the human sequence. FAD is located, for the greatest part, in the II domain of the α subunit, although the dimethyl benzene ring of the isoalloxazine ring contacts the β subunit. In the 83 residues, there is 64% sequence identity and about 70% identity/similarity between the bacterial and human sequences. The difference in charge of the *Paracoccus* sequence compared with the human sequence is -3 [15]. The *Paracoccus* component of the chimaeric β subunit is largely exposed on the 'top' of the subunit and at the *re* face of the flavin [16,17]. In *Paracoccus* ETF, Phe-38β and Tyr-13β occupy the same positions as Phe-41β and Tyr-16β in human ETF. These are the only residues in the β subunit that contact the flavin in both proteins and do so at the 7-methyl and 8-methyl groups of the flavin, respectively.

The water-soluble ubiquinone homologues, Q_1, Q_2 and Q_4, and the analogue decylubiquinone (DQ) were purchased from Sigma (St. Louis, MO, U.S.A.). Detergents were purchased from Anatrace (Maumee, OH, U.S.A.) and Calbiochem (San Diego, CA, U.S.A.). 5-Deazaflavin was a generous gift from Dr Betty Sue Masters (Department of Biochemistry, University of Texas, San Antonio, TX, U.S.A.). Perfluoro-octanoic acid (PFO) was purchased from Aldrich (Milwaukee, WI, U.S.A.). All other compounds were of reagent grade or better and were obtained from commercial sources.

Enzyme assays

ETF-QO was assayed routinely as a ubiquinone reductase in reaction mixtures containing 10 mM Hepes-K^+, pH 7.4, 1 μM medium-chain acyl-CoA dehydrogenase, 1 μM human ETF, 100 μM octanoyl-CoA and 70 μM Q_1 at 25 °C. The quinones were dissolved in ethanol and added to the assay mixtures such that the volume of ethanol added never exceeded 4% of the assay mixture [5,6]. The reaction was initiated by the addition of ETF-QO and monitored by the decrease of absorbance at 275 nm, ($\Delta\epsilon = 7.4 \times 10^3\ M^{-1} \cdot cm^{-1}$) due to ubiquinone reduction to the dihydroquinone [5,6]. ETF-QO was also assayed by disproportionation of ETF_{1e^-} at 25 °C [2,7]. In the latter assay, activity is expressed as ETF_{1e^-} converted/s. Steady-state kinetic constants and their S.E.M. values were calculated using Origin version 6.1 software.

EPR spectroscopy

Continuous-wave EPR spectra of substrate-reduced ETF-QO were recorded at 9.482 GHz on a Bruker E580E spectrometer with a split-ring resonator and an Oxford 935 liquid helium cryostat. The operating conditions were: 16 K, 0.5 G modulation amplitude at 100 GHz, and a microwave power of 25 μW. Spectra were computer simulated using software written in-house. ETF-QO (74 μM) was reduced with 0.5 mM octanoyl-CoA in the presence of 1 μM medium-chain acyl-CoA dehydrogenase and 1 μM ETF in 0.1 M Tris/HCl, pH 7.8, containing 8 mM CHAPS and 30% glycerol. Two-electron reduction of ETF-QO was estimated from the absorption spectrum (see below). The sample was promptly transferred to an EPR tube that had been evacuated and back-filled with helium. After transfer, the upper portion of the EPR tube was flushed with helium. After incubation under the helium atmosphere for 20 min, the sample was frozen and put into the cryostat.

Expression of human ETF-QO

Human ETF-QO cDNA in pHEDH22 [3] was amplified with *Taq* (Promega, Madison, WI, U.S.A.) and *Pfu* (Stratagene, La Jolla, CA, U.S.A.) polymerases (10:1), using the following primers: 5′-GCAATGGTGGTGCCGCTAGCCAAG-3′ and 5′-CTTACATTCCATTGTAAGCAGG-3′. The amplified sequence was cloned into pBlueBac4.5/V5 TOPO TA vector (Invitrogen, Carlsbad, CA, U.S.A.), and the insert in the resulting plasmid was sequenced; no errors were found. The insert contains the entire coding sequence for human ETF-QO, including the 33 amino acids at the N-terminus that are proposed to contain the mitochondrial targeting sequence [3]. This sequence is cleaved after translocation of the precursor into the mitochondrion. Recombinant viruses were constructed as described in [18], and screened by Western-blot analyses of virus-infected *Spodoptera fugiperda* (Sf9) insect cells (Figure 1). ETF-QO antigen was not detected in uninfected cells or in the soluble fraction of cells producing the membrane-associated antigen. In all succeeding experiments the cloned virus shown in lane 5 of Figure 1 was used.

Sf9 cells were grown routinely at 27 °C in oxygenated Grace's medium as described in [19]. Small-scale preliminary experiments were conducted to establish time of infection, growth temperature and concentrations of iron and riboflavin to give optimum conditions for ETF-QO expression. These experiments also established conditions for detergent solubilization of ETF-QO from a membrane fraction of Sf9 cells. After establishing optimum conditions, the cells were grown in 5 litre bioreactors and the medium was supplemented with 4 μM Fe^{2+}, provided as the 8-hydroxyquinoline complex, and 1.3 μM riboflavin. For the

production of enzyme, cells were infected with virus at a density of about 1.6×10^6 cells/ml with a multiplicity of infection of 1.0. Growth was continued for an additional 40 h at 27 °C to a density of about 2.3×10^6 cells/ml. Cells were harvested by centrifugation at 3000 ***g*** for 15 min, washed with PBS, and stored at −80 °C until needed.

Purification of human ETF-QO

The cell pellet from a 5 litre bioreactor was suspended in 25 mM Tris/HCl, pH 7.4, 50 mM KCl and 0.1 mM dithiothreitol (DTT) (50 ml) containing the protease inhibitors Pefablock (1 mM), aprotonin (1 μM), pepstatin (1 μM) and tosyl-L-phenylalanine-chloromethyl ketone (TPCK; 50 μg/ml). All procedures were conducted at 4 °C. The cells were homogenized and broken with a French pressure cell and the membrane fraction sedimented by centrifugation (100000 ***g***, 60 min). The membrane fraction was resuspended to a protein concentration of 10 mg/ml in the buffer described above containing the protease inhibitors and 0.1 mM DTT. The buffer containing protease inhibitors, DTT and 30 mM CHAPS was added slowly to the membrane fraction to a final concentration of 15 mM. The preparation was stirred for at least 6 h at 4 °C and then centrifuged at 100000 ***g*** for 1 h. The Tris/HCl concentration of the resulting supernatant was adjusted to 100 mM and loaded on to a column (2.5 cm × 20 cm) of DEAE-Sepharose (fast flow) that was equilibrated with 100 mM Tris/HCl, pH 7.4, 0.1 % Triton X-100 and 0.1 mM DTT. The column was washed with 400 ml of the equilibrating buffer and then eluted with a 1 litre linear gradient of 100–400 mM Tris/HCl, pH 7.4, containing 0.1 % Triton X-100 and 0.1 mM DTT. Fractions containing the majority of ETF-QO catalytic activity were pooled, concentrated by membrane ultrafiltration and dialysed against three changes of 20 vol. of 5 mM potassium phosphate, pH 7.4, 0.1 % Triton X-100 and 0.1 mM DTT. The protein was applied to a column of hydroxyapatite-Ultrogel (2.5 cm × 10 cm) equilibrated with the dialysis buffer and washed with 100 ml of the starting buffer with 24 mM *n*-octyl-β-D-glucopyranoside. The column was eluted with a 500 ml linear gradient from 5 to 80 mM potassium phosphate, pH 7.4, containing 24 mM *n*-octyl-β-D-glucopyranoside and 0.1 mM DTT. Fractions containing ETF-QO catalytic activity were pooled, concentrated and dialysed against 20 mM Tris/HCl, pH 7.4, and 0.1 mM DTT. The protein was loaded on a MonoQ column (0.5 cm × 5 cm) equilibrated with the dialysis buffer and washed with 20 ml of the equilibration buffer containing 24 mM *n*-octyl-β-D-glucopyranoside. Human ETF-QO was eluted with a 28 ml linear gradient of 0.24–0.4 M KCl in 20 mM Tris/HCl, pH 7.4, containing 0.1 mM DTT and 24 mM *n*-octyl-β-D-glucopyranoside. Fractions were analysed by determination of absorption spectra. Those fractions containing the typical absorption spectrum of ETF-QO, with a constant $A_{270}/A_{380}/A_{430}$ proportion of 6.0:1.06:1.0, were pooled, concentrated and the detergent removed on a column (1 cm × 2 cm) of Bio-Beads SM2 in 20 mM potassium phosphate, pH 7.4. After dialysis against 20 mM potassium phosphate, pH 7.4, the enzyme was concentrated to about 1.5 mg/ml, glycerol was added to 20 % and the protein was frozen at −80 °C.

PAGE and Western blotting

SDS/PAGE was carried out on 10 % gels by the procedure of Laemmli [20]. PFO/PAGE was carried out as described in [21]. For PFO/PAGE, mouse mitochondrial membranes and membranes derived from a crude mitochondrial fraction of Sf9 cells were extracted with 2.5 % PFO for 6 h at 4 °C. The preparation was centrifuged at 100000 ***g*** for 1 h and subjected to PFO/PAGE on 6–14 % gradient gels. Proteins were transferred from gels and ETF-QO was detected using anti-porcine ETF-QO [3] and the ECL® enhanced chemiluminescence kit from Amersham Bioscience (Piscataway, NJ, U.S.A.)

Other methods

When reactions were to be analysed spectrophotometrically under anaerobic conditions, 4 mM protocatechuate was added to the incubation mixtures. Cuvettes were sealed with silicone rubber stoppers and subjected to 10–12 cycles of alternating evacuation and purging with argon. Protocatechuate dioxygenase (0.4 units) was then added to scavenge residual oxygen, as described in [22]. The dioxygenase was a gift from Dr David Ballou (University of Michigan, Ann Arbor, MI, U.S.A.). Photochemical reduction of ETF-QO was carried out anaerobically in the presence of 1 mM EDTA and 0.5 μM 5-deazaflavin [23].

Purified human ETF-QO was subjected to SDS/PAGE and transferred to Immobilon P membrane. The protein was visualized by staining with 0.1 % Ponceau S in 5 % acetic acid, and the membrane washed with 1 % acetic acid. The band was excised and subjected to N-terminal sequence analysis on an Applied Biosystems 476a protein sequencer.

Mouse liver mitochondria were prepared as described in [24]. The mitochondria were disrupted by sonication and the preparation was centrifuged at 100000 ***g*** for 1 h. The sedimented membranes were resuspended in 10 mM Tris/HCl, pH 7.4, and were stored frozen at −80 °C in aliquots until needed. A crude mitochondrial fraction of Sf9 cells was obtained as described by Knecht et al. [18].

The concentration of purified human ETF-QO was estimated assuming an ϵ_{430} value of 2.4×10^4 $M^{-1} \cdot cm^{-1}$ [2]. The assumption is reasonable given the similarity of human and porcine ETF-QOs [2,3]. Protein concentrations were determined by the method of Miller [25].

RESULTS

Purification of human ETF-QO

In preliminary experiments, we found that zwitterionic detergents solubilized ETF-QO from Sf9 membranes most efficiently. A large amount of antigen that cross-reacts with anti-ETF-QO remained with the cell membranes that were sedimented at

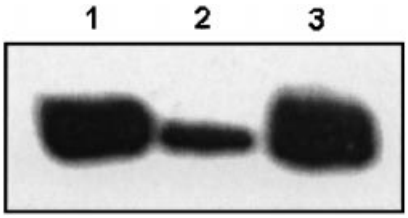

Figure 2 Solubilization of human ETF-QO from Sf9 cell membranes with CHAPS

Membranes (10 mg/ml protein) from Sf9 cells were suspended in 25 mM Tris/HCl, pH 7.4, 50 mM KCl and 0.1 mM DTT containing 1 mM Pefablock, 1 μM aprotonin, 1 μM pepstatin and 50 μg/ml TPCK. An equal volume of 30 mM CHAPS in the same buffer was added and the mixture was stirred for 6 h at 4 °C. The preparation was centrifuged at 100000 ***g*** and 50 μg of the particulate fraction was subjected to SDS/PAGE (lane 3). An equivalent amount of the soluble detergent extract based on the initial volume was also subjected to SDS/PAGE (lane 2). Lane 1 is 50 ng of porcine ETF-QO. Following electrophoresis, the ETF-QO antigens were detected by Western blotting.

Table 1 Purification of recombinant human ETF-QO expressed in Sf9 cells

One unit of activity is equivalent to 1 μmol of Q_1 reduced/min at 25 °C in the absence of detergent. HA-Ultrogel, hydroxyapatite-Ultrogel.

Fraction	Activity (total units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
Detergent extract	149	275	0.5	100
DEAE-Sepharose	71	24	3.0	48
HA-Ultrogel	41	7.4	7.5	28
Mono Q	26	1.5	17.6	17

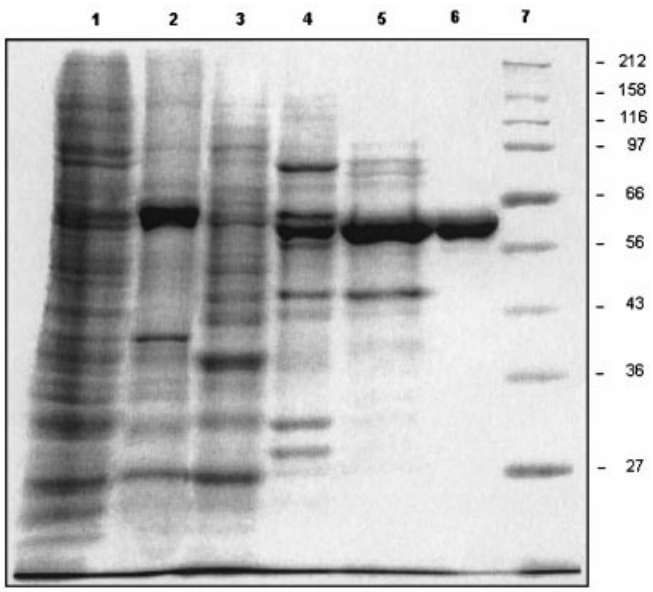

Figure 3 Purification of human ETF-QO expressed in Sf9 cells

Fractions generated during the purification of human ETF-QO were subjected to SDS/PAGE on 10% gels, and proteins were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, total membrane proteins (30 μg); lane 2, total membrane proteins (pellet) after detergent extraction and centrifugation (30 μg); lane 3, soluble detergent extract (supernatant) following extraction with 15 mM CHAPS and centrifugation (30 μg); lane 4, DEAE-Sepharose-purified ETF-QO (15 μg); lane 5, hydroxyapatite-Ultrogel-purified ETF-QO (5 μg); lane 6, Mono-Q-purified ETF-QO (5 μg); lane 7, protein molecular-mass standards.

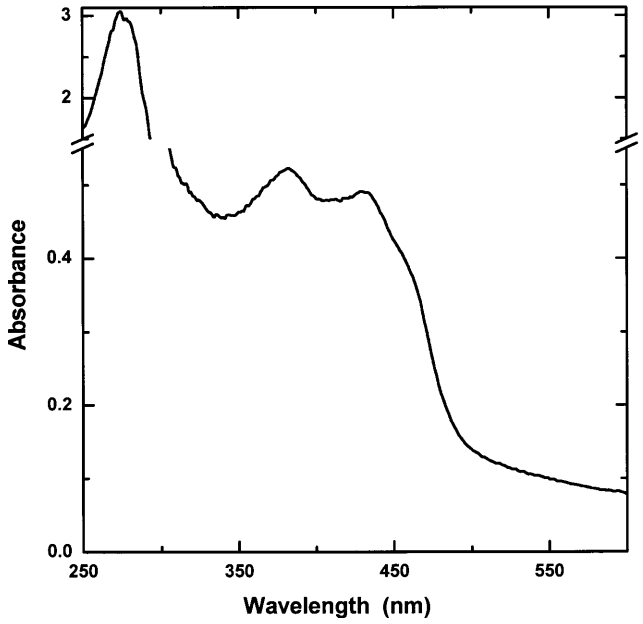

Figure 4 Absorption spectrum of purified, oxidized human ETF-QO

The absorption spectrum of purified human ETF-QO was determined at 25 °C in 20 mM potassium phosphate, pH 7.4, containing 20% glycerol.

100000 ***g*** following extraction with CHAPS, *N*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide or Zwittergent 3-14. Multiple extractions, combinations of detergents and alteration of ionic strength during solubilization did not extract the residual antigen (Figure 2). We did not attempt to localize the extractable antigen to the mitochondrial membrane. We have not established whether this non-extractable antigen contains the prosthetic groups and whether it may be aggregated due to disulphide cross-links. It may simply represent an aggregated form due to overexpression. Subcellular fractionation of Sf9 cells is relatively inefficient. The extractable antigen co-migrates with the mature form of porcine ETF-QO on SDS/PAGE. The mature form of the human protein is easily separated from the precursor that is predicted to be 33 amino acids longer than the mature ETF-QO [3]. Furthermore, the N-terminal sequence of the purified, expressed protein was determined to be SSTSTVPRITTHYTIYPRDK. This sequence is identical with the N-terminal sequence predicted from the deduced primary sequence of human ETF-QO [3] and the motif in the ETF-QO precursor recognized by the mitochondrial processing protease [26]. Therefore, it is reasonable to assume that the detergent-extractable antigen is processed by mitochondria, and localized in the mitochondrial inner membrane. The non-extractable antigen has the same molecular mass as the mature protein, based on its mobility on SDS/PAGE.

Table 1 shows the results of a typical purification using the quinone reductase assay, with Q_1 as the terminal electron acceptor to monitor the purification. The concentrations of ETF and medium-chain acyl-CoA dehydrogenase were each 1 μM in these assays; the specific activity of the preparation was 17.6 units/mg of protein. However, the optimum concentrations of the dehydrogenase and ETF were subsequently found to be 3 μM. At these concentrations, the specific activity of the preparation in Table 1 increased to 22.1 ± 2.6 units/mg of protein. This is similar to the value obtained for the porcine ETF-QO [3]. Figure 3 shows the results of SDS/PAGE of the preparations after each step in the purification. The data show that highly purified recombinant human ETF-QO can be obtained in good yield by a simple, three-step purification.

Spectra of human ETF-QO

Figure 4 shows the absorption spectrum of purified human ETF-QO. The spectrum is indistinguishable from those of the corresponding porcine and bovine proteins [1,2]. In the experiment

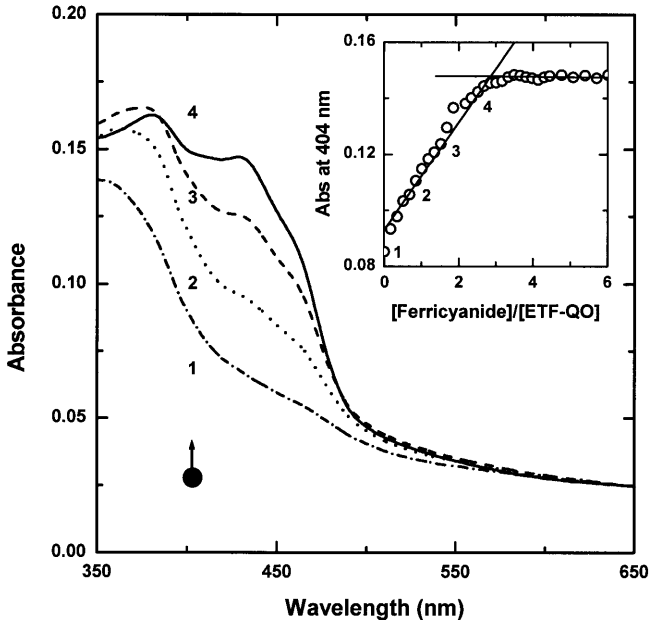

Figure 5 Anaerobic oxidative titration of fully reduced human ETF-QO with $K_3[Fe(CN)_6]$

Human ETF-QO (6.2 μM) in 20 mM buffer, pH 7.4, was fully reduced by irradiation in the presence of 0.5 μM 5-deazaflavin and 1 mM EDTA. The fully reduced iron–sulphur flavoprotein was then titrated with an anaerobic solution of $K_3[Fe(CN)_6]$. The inset shows the increase in absorbance at 404 nm as a function of the ferricyanide/ETF-QO molar ratio. The numbered points in the inset correspond to the numbered spectra which show the spectra at molar ratios (ferricyanide/ETF-QO) of 0 (spectrum 1), 1 (spectrum 2), 2 (spectrum 3) and 3 (spectrum 4). All spectra were corrected for dilution and some spectra are omitted for clarity. The arrow indicates 404 nm.

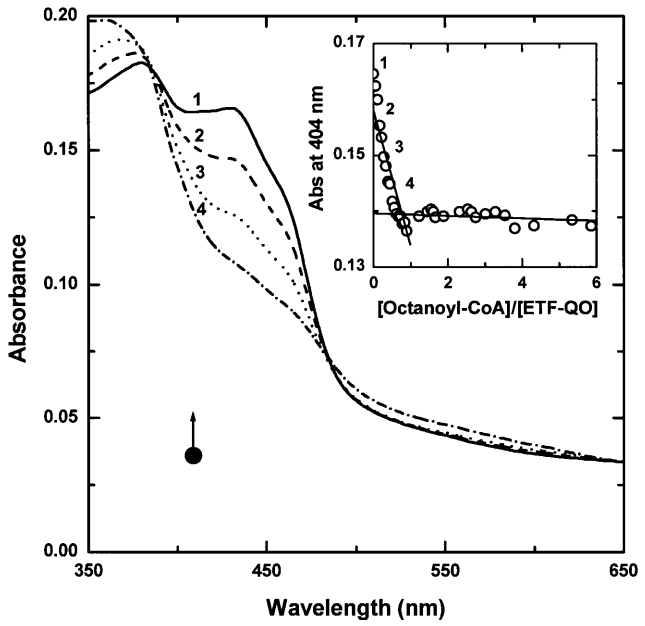

Figure 6 Anaerobic reductive titration of human ETF-QO with octanoyl-CoA

ETF-QO (6.9 μM) was titrated under anaerobic conditions with an anaerobic solution of octanoyl-CoA in the presence of 1 μM each of medium-chain acyl-CoA dehydrogenase and ETF. Spectra were determined after each addition and the decrease in absorbance at 404 nm was plotted against the octanoyl-CoA/ETF-QO ratio (inset). The numbered points in the inset correspond to the numbered spectra which show the spectra at molar ratios (octanoyl-CoA/ETF-QO) of 0 (spectrum 1), 0.25 (spectrum 2), 0.5 (spectrum 3) and 1.0 (spectrum 4). Some spectra are omitted for clarity. The arrow indicates 404 nm.

shown in Figure 5, the protein was fully reduced by irradiation in the presence of 1 mM EDTA and 0.5 μM 5-deazaflavin under anaerobic conditions and the reduced protein was then titrated with $K_3[Fe(CN)_6]$ while monitoring the spectra. The inset in Figure 5 shows the oxidative titration with ferricyanide monitored at 404 nm. The protein was fully oxidized by a 2.9 molar excess of ferricyanide, consistent with reduction of the flavin to the dihydroquinone state by the low-potential reductant, and one-electron reduction of the cluster. From the titration data, it is possible to demonstrate all four oxidation states of the protein. The data refer to the overall redox state of the protein because, in the one-electron reduced state, electrons equilibrate between the two redox centres [1,2]. Figure 6 shows an anaerobic titration of 6.9 μM ETF-QO with octanoyl-CoA in the presence of 1 μM human medium-chain acyl-CoA dehydrogenase and 1 μM human ETF. The titration shows that when ETF-QO is reduced with substrate, a maximum of two electrons are transferred to ETF-QO. After opening the cuvette to air, the reduced protein is stable in air for at least 2 h. Further, the spectrum of the protein in the presence of a 1:1 molar ratio of octanoyl-CoA is very similar to the absorption spectrum of the two-electron reduced protein generated by re-oxidation of fully reduced ETF-QO by $K_3[Fe(CN)_6]$ (Figure 5). These data are also consistent with the EPR spectrum obtained upon reduction of the recombinant ETF-QO with a 10-fold molar excess of octanoyl-CoA in the presence of catalytic concentrations of human medium-chain acyl-CoA dehydrogenase and human ETF as described previously [2,9]. The EPR spectrum of the enzymically reduced protein shows signals due to the flavin radical, $g = 2.004$, and the iron–sulphur cluster, $g_x = 1.886$, $g_y = 1.940$ and $g_z = 2.086$. These values are in good agreement with literature values for pig ETF-QO: 1.885, 1.939 and 2.085 respectively [1,9]. The additional signal with $g = 2.004$ was assigned to the flavin semiquinone. The EPR signals for the iron–sulphur cluster broadened as the temperature was increased above 20 K due to increasing rates of electron spin relaxation, which is also consistent with previous reports [1,9].

Kinetic characterization of ETF-QO

Figure 7 shows the dependence of the rate of Q_1, Q_2 and Q_4 reduction on the concentration of CHAPS. Maximum rates were obtained in the vicinity of the critical micelle concentration and then the rates progressively decreased as the concentration of detergent increased. The decrease is probably due to the formation of detergent/quinone mixed micelles. Detergent stimulation of activity is exerted at the level of quinone solubility because the detergent actually has a small inhibitory effect on the disproportionation reaction, suggesting that the interaction between ETF and ETF-QO could be affected by the detergent or the increased ionic strength due to the zwitterionic detergent [7]. The specific activity, expressed as an apparent turnover, was $24.1 \pm 2.8\ s^{-1}$ in the absence of CHAPS and $17.9 \pm 0.8\ s^{-1}$ in the presence of CHAPS. Table 2 shows the kinetic constants for the ubiquinone reductase activity of ETF-QO using the water-soluble homologues, Q_1 and Q_2, as substrates in the presence of 6 mM CHAPS. The kinetic constants with Q_4 as the varied substrate were determined with 11 mM CHAPS. We also investigated DQ as an alternate substrate. This compound differs from ubiquinone homologues by the presence of a 10-carbon

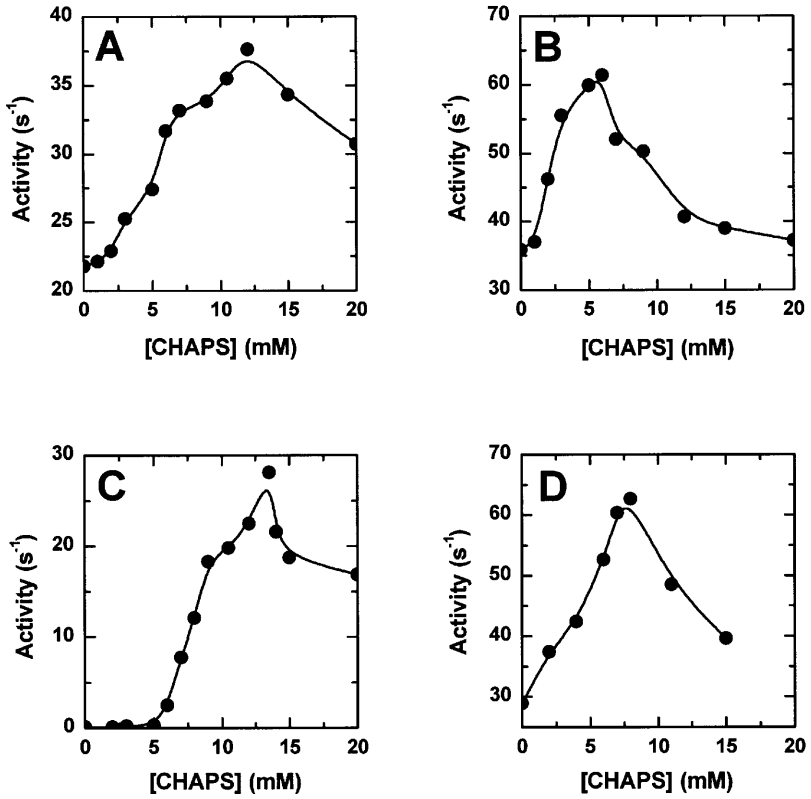

Figure 7 Effect of CHAPS on the rate of quinone reduction

ETF-QO was assayed in the quinone reductase assay with Q_1 (**A**), Q_2 (**B**), Q_4 (**C**) and DQ (**D**) as a function of CHAPS concentration. Each quinone was present at 50 μM in the assay mixtures. Activities are expressed as turnover numbers.

Table 2 Kinetic constants of human ETF-QO assayed by ubiquinone reduction and by disproportionation of ETF semiquinones

The concentrations of medium-chain acyl-CoA dehydrogenase and ETF were both 3 μM when the quinone homologues, Q_1 and Q_2, were the varied substrates and CHAPS was present at 6 mM. When DQ or Q_4 was the varied substrate the CHAPS concentration was increased to 8 or 11 mM, respectively. When ETF was the varied substrate, the concentration of medium-chain acyl-CoA dehydrogenase was 3 μM and the concentration of Q_1 was 70 μM; the reactions were run in the absence of detergent.

Varied substrate	Turnover number (s^{-1})	K_m (μM)
Ubiquinone reduction		
Q_1	33.5 ± 1.3	8.1 ± 1.0
Q_2	78.8 ± 3.2	4.9 ± 0.7
Q_4	35.5 ± 1.3	14.8 ± 1.4
DQ	74.3 ± 1.4	8.4 ± 0.8
ETF_{ox}	21.4 ± 0.6	0.13 ± 0.01
Disproportionation of ETF_{1e^-}		
ETF_{1e^-}	81.4 ± 3.3	15.4 ± 1.5
Chimaeric ETF_{1e^-}	4.4 ± 0.4	19.1 ± 3.5

linear alkyl side chain at C-5 rather than a 10-carbon isoprene side chain. The turnover of the enzyme with this alternate substrate is very similar to that obtained with Q_2; however, the K_m is similar to that of Q_1. Substitution of the hydrocarbon side chain with an alkyl group has only a modest effect on the apparent affinity of human ETF-QO for quinone substrate. In any case, the enzyme does not require a quinone containing isoprene side chain at C-5. The effect of CHAPS is not specific; rather, stimulation of catalytic activity is a general effect of detergents. Activity of ETF-QO with quinones as electron acceptors was optimal in the presence of 20 mM *n*-octyl-β-D-glucopyranoside, which is near the critical micelle concentration of this detergent. K_m values were very similar to those determined in the presence of CHAPS; the values of k_{cat} were 41.6 ± 1.6 and 88.3 ± 2.2 s^{-1} with Q_1 and Q_2, respectively, as the varied substrates.

The K_m for ETF_{ox} in the quinone reductase assay is 0.13 ± 0.02 μM with the saturating medium-chain acyl-CoA dehydrogenase (3 μM) and 70 μM Q_1, in the absence of detergent.

ETF-QO catalyses the disproportionation of ETF_{1e^-}, in which the semiquinone functions as both electron donor and acceptor. The kinetic constants for the disproportionation of human ETF_{1e^-} by human ETF-QO are also given in Table 2. The turnover number and K_m for ETF_{1e^-} of ETF-QO are 81.4 ± 3.3 s^{-1} and 15.4 ± 1.5 μM, respectively. The turnover with wild-type human ETF is about 50 % of that determined from experiments with porcine ETF and ETF-QO [2,5]. The reason for this difference is not known. A comparison of the three-dimensional structures of human and *P. denitrificans* ETFs and their different reactivities suggested that the region including Lys-56 and Lys-59 in the β subunit of ETF may contribute to the interaction of ETF with ETF-QO [16]. The steady-state kinetic constants with the chimaeric ETF semiquinone substrate show that K_m is not significantly changed but that k_{cat} is reduced about 20-fold (Table 2). The results suggest that this region of the β subunit plays a role in the reaction of human ETF with human ETF-QO.

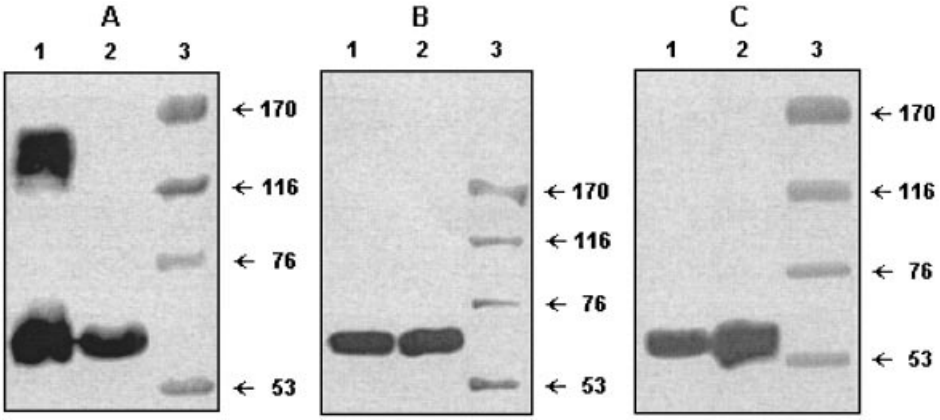

Figure 8 Western blotting of ETF-QO in crude Sf9 mitochondrial membranes and mouse mitochondrial membranes after PFO/PAGE and SDS/PAGE

(**A**) Western blot of PFO/PAGE of the soluble fraction from a 2.5% PFO extract of Sf9 mitochondrial membranes (lane 1), purified human ETF-QO (lane 2) and protein molecular-mass markers (lane 3). (**B**) Western blot of an SDS/PAGE gel of Sf9 mitochondrial membranes (lane 1), purified human ETF-QO (lane 2) and protein molecular-mass markers (lane 3). (**C**) Western blot of PFO/PAGE of the soluble fraction from a 2.5% PFO extract of mouse mitochondrial membranes (lane 1), purified human ETF-QO (lane 2) and molecular-mass markers (lane 3). The gels in all panels are 6–14% polyacrylamide gradient gels. Molecular-mass markers were stained with 0.1% Ponceau S in 5% acetic acid; excess dye was removed by washing with 1% acetic acid.

Functional state of ETF-QO

ETF-QO contains only one type of polypeptide; however, its functional quaternary structure in the mitochondrial inner membrane has not been defined. Baer and colleagues [21,27,28] have recently employed a novel gel-electrophoresis method to ascertain the oligomeric state of several membrane proteins. A mitochondrial fraction was isolated from Sf9 cells expressing human ETF-QO and a membrane fraction was prepared. The membranes were extracted with 2.5% PFO and PFO/PAGE gels were run as described in [21]; ETF-QO was detected by Western blot. Figure 8(A) shows that two proteins that cross-react with anti-ETF-QO serum were detected by Western blotting following PFO/PAGE. The first had the mass of the ETF-QO monomer ($\approx$ 64000 Da) and the second had approximately the mass of an ETF-QO dimer, which is dissociated by SDS. In contrast, only one protein was detected when the preparation from Sf9 cells was subjected to SDS/PAGE (Figure 8B). This result suggests that ETF-QO might function as a dimer; however, the result was investigated further in a naturally expressing system. Mouse liver submitochondrial particles were extracted with 2.5% PFO, subjected to PFO/PAGE and ETF-QO was detected by Western blotting. The results in Figure 8(C) show clearly only one protein band in PFO extracts of mouse mitochondrial membranes. Identical results were obtained when the concentration of PFO was increased incrementally to a maximum concentration of 4%. It is likely that the presence of the apparent dimer is an artifact of the over-expression from the baculovirus vector. Identical results were obtained when ETF-QO extracted from mouse liver submitochondrial particles were analysed with native blue gels [29] (M. Mueller and F. E. Frerman, unpublished work). We conclude that ETF-QO probably functions as a monomer in the mitochondrial membrane.

DISCUSSION

Several mitochondrial membrane proteins have been expressed in insect cells using baculovirus vectors [18,30–32]. We had attempted to express human ETF-QO in *Escherichia coli* and *Pichia pastoris* without success, and although we were able to express the human protein in *S. cerevisiae* [3], the level of expression was very low. This precluded expression of the protein in *Saccharomyces* for crystallization and mechanistic investigations involving site-directed mutagenesis. However, as demonstrated in this paper, expression of the protein in insect cells (Sf9) from a baculovirus vector provides reasonable yields of the protein for more detailed investigation.

Sf9 cells express human ETF-QO, and yield 0.3–0.5 mg of purified protein/l of culture. The protein was expressed from a full-length cDNA that included the coding sequence of the 33-amino acid mitochondrial targeting sequence that we presumed would be cleaved following Trp-33 [3] according to the motif recognized by the mitochondrial processing protease [26]. We expressed the complete protein with the rationale that if the protein was translocated into mitochondria, the processing and insertion of prosthetic groups could most likely be accomplished in the organelle. Yeast mitochondria carry out these steps in the maturation of the holoprotein [3]. The molecular mass of the expressed protein determined by SDS/PAGE is very similar to that of the mature porcine protein, $\approx$64 kDa [2], and the proposed structure of mature human ETF-QO deduced from the cDNA [3]. Direct determination of the N-terminal sequence of the expressed protein is consistent with the idea that the full-length protein is expressed, translocated into mitochondria and that maturation to the holoprotein occurs in the organelle. Among the mitochondrial membrane proteins that were expressed previously, β-hydroxybutyrate dehydrogenase was expressed without a targeting sequence and was inserted into a cellular membrane, although there was no direct evidence that it was inserted into a mitochondrial membrane [32]. Active forms of dihydro-orotate dehydrogenase, glycerol phosphate acyl transferase and ferrochelatase, three other mitochondrial membrane proteins, were also expressed in Sf9 cells from cDNAs that encoded mitochondrial targeting sequences [18,30,31]. The expression system that we established also provides a means for examining the interaction of ETF-QO with phospholipids, and the effect of phospholipid on catalytic activity.

The recombinant human ETF-QO exhibits kinetic properties that are similar to those of the porcine protein, although the

turnover numbers are about half those of the porcine protein [2,5,6]. The reason for this difference is not known. Comparing the results with Q_1 and Q_2, there is a 40 % decrease in K_m and a 2-fold increase in k_{cat} when the isoprene side chain is increased by five carbon atoms. Based on the change in K_m, the additional five carbon atoms in the isoprene side chain of Q_2 could contribute an additional 1.2 kcal/mol to the binding energy of the quinone. However, it is difficult to arrive at anything more than a qualitative interpretation of the data given the hydrophobic nature of the quinone substrates. Nonetheless, it was desirable to determine these values and the effects of detergents because we anticipate using quinone inhibitors and site-directed mutagenesis of the quinone site in structure–function investigations. Also, the chemistry of quinone reduction by this protein is somewhat novel in that ubiquinone is reduced by a complex redox protein with no requirement for a 'Q-binding' protein such as those that occur in complexes II and III [33,34].

Recent experiments using small-angle X-ray scattering demonstrated the dynamic solution structure of human and *Paracoccus* ETFs [35]. Those experiments suggested that a larger region of the ETF β subunit than originally suggested [17] could participate in the docking of ETF with dehydrogenase electron donors and the electron acceptor, ETF-QO. The dimensions of the docking site are influenced by the motion of the αI subunit domain relative to the αII domain of ETF in which the flavin is located [16,17]. The experiment with recombinant ETF-QO and the chimaeric ETF begins to map regions of ETF-QO that may function in docking with the β subunit.

The absorption spectrum of oxidized human ETF-QO is indistinguishable from those of oxidized bovine and porcine ETF-QOs, and the visible absorption spectrum is identical with that of the oxidized form of partially purified *P. denitrificans* ETF-QO [1,2,36]. This observation, and the fact that the recombinant protein catalysed the same reactions as eukaryotic and prokaryotic counterparts, suggested that recombinant human ETF-QO contained functional flavin and iron–sulphur cluster prosthetic groups. The demonstration that the protein takes up three electrons when fully reduced by the low–potential reductant, 5-deazaflavin, is also consistent with the prosthetic group content of the protein, i.e. a two-electron reduced flavin and a one-electron reduced iron–sulphur centre. The EPR spectrum of ETF-QO demonstrates clearly the presence of substrate-reducible flavin and an iron–sulphur cluster and their involvement in the enzymic reduction. Moreover, reduction of ETF-QO by octanoyl-CoA to a two-electron reduced form containing a flavin radical suggests that the oxidation reduction potentials are likely to be very similar to those of the porcine protein, and that the flavin semiquinone is likely to be thermodynamically stabilized [8]. The flavin dihydroquinone of human and porcine ETF-QO is thermodynamically inaccessible by substrate reduction, but is accessible when chemically reduced by low-potential reductants such as 5-deazaflavin or dithionite (results not shown).

To summarize, we have demonstrated the first system for heterologous expression of ETF-QO on a scale that will permit mechanistic and structural studies. We also showed that the system faithfully expresses the human iron–sulphur flavoprotein with the catalytic and redox properties of ETF-QO proteins isolated from primary sources. Finally, these experiments provide data to confirm our earlier hypothesis regarding the site for cleavage of the ETF-QO precursor by the mitochondrial processing protease after translocation of ETF-QO precursor into mitochondria.

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