Biochem. J. (1987) 241, 883–892 (Printed in Great Britain)

Reactions of electron-transfer flavoprotein and electron-transfer flavoprotein:ubiquinone oxidoreductase

Rona R. RAMSAY,*†§ Daniel J. STEENKAMP‡ and Mazhar HUSAIN*†

*Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, U.S.A., †Molecular Biology Division, Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A., and ‡Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa

Electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF-Q oxidoreductase) catalyses the re-oxidation of reduced electron-transfer flavoprotein (ETF) with ubiquinone-1 (Q-1) as the electron acceptor. A kinetic assay for the enzyme was devised in which glutaryl-CoA in the presence of glutaryl-CoA dehydrogenase was used to reduce $ETF_{ox.}$ and the reduction of Q-1 was monitored at 275 nm. The partial reactions involved in the overall assay system were examined. Glutaryl-CoA dehydrogenase catalyses the rapid reduction of $ETF_{ox.}$ to the anionic semiquinone ($ETF^{\cdot-}$), but reduces $ETF^{\cdot-}$ to the fully reduced form (ETF_{hq}) at a rate that is about 6-fold lower. $ETF^{\cdot-}$, but not ETF_{hq}, is directly re-oxidized by Q-1 at a rate that, depending on the steady-state concentration of $ETF^{\cdot-}$, may contribute significantly to the overall reaction. ETF-Q oxidoreductase catalyses rapid disproportionation of $ETF^{\cdot-}$ with an equilibrium constant of about 1.0 at pH 7.8. In the presence of Q-1 it also catalyses the re-oxidation of ETF_{hq} at a rate that is faster than that of the overall reaction. Rapid-scan experiments indicated the formation of $ETF^{\cdot-}$, but its fractional concentration in the early stages of the re-oxidation of ETF_{hq} is low. The data indicate that the re-oxidation of ETF_{hq} proceeds at a rate that is adequate to account for the overall rate of electron transfer from glutaryl-CoA to Q-1. An unusual property of ETF-Q oxidoreductase seems to be that it not only catalyses the re-oxidation of the reduced forms of ETF but also facilitates the complete reduction of $ETF_{ox.}$ to ETF_{hq} by disproportionation of the radical.

INTRODUCTION

ETF-Q oxidoreductase was discovered by Ruzicka & Beinert (1975), who isolated the enzyme by monitoring its characteristic e.p.r. signal during purification, and it was further characterized by Beckmann & Frerman (1985*a*,*b*). The physiological function of ETF and ETF-Q oxidoreductase is an important one, and a deficiency of these proteins results in the severe metabolic disorder, glutaricaciduria type II (Frerman & Goodman, 1985). As the catalyst for the re-oxidation of reduced forms of ETF, ETF-Q oxidoreductase is an essential component of the fatty acid-oxidation pathway. It is also necessary for the catabolism of the amino acids leucine, isoleucine, valine, lysine and tryptophan (Gholson *et al.*, 1962; Ikeda *et al.*, 1983), and for the operation of the mitochondrial 'one-carbon cycle' (Frisell & MacKenzie, 1962). The flavoprotein dehydrogenases of these pathways are oxidized by $ETF_{ox.}$, which is rapidly reduced first to $ETF^{\cdot-}$, and then, more slowly, to ETF_{hq} (Hall & Lambeth, 1980; Beckmann *et al.*, 1981; Steenkamp & Husain, 1982). It therefore seemed likely that only the oxidized and semiquinone forms of ETF contribute to catalytic turnover. The anionic semiquinone of ETF is comparatively stable under anaerobic conditions, disproportionating to an equilibrium mixture of the three redox forms after several days (Gorelick *et al.*, 1982). Remarkably, ETF-Q oxidoreductase catalyses this disproportionation (Steenkamp *et al.*, 1984; Beckmann & Frerman, 1985*a*).

In the present paper the rate of electron flux from an acyl-CoA dehydrogenase to Q-1 mediated by ETF and ETF-Q oxidoreductase was measured in an assay that mimics the physiological reaction. This overall rate was compared with the rate of disproportionation of $ETF^{\cdot-}$ by ETF-Q oxidoreductase, with the non-enzymic reoxidation of $ETF^{\cdot-}$ by Q-1 and with the rate of re-oxidation of ETF_{hq} in the presence of ETF-Q oxidoreductase and Q-1. Both steady-state and rapid-reaction studies indicate that ETF_{hq} formed as a result of disproportionation of the $ETF^{\cdot-}$ is a significant intermediate in the electron flux to Q-1.

This work has been presented in preliminary form at the 8th International Symposium on Flavins and Flavoproteins (Steenkamp *et al.*, 1984).

EXPERIMENTAL

Materials

Hepes, Mops, Tris, triethanolamine and CoA were purchased from Sigma Chemical Co. Glutaryl-CoA was synthesized by the anhydride method (Stadtman, 1957). Q-1 was kindly given to us by Eisai (USA) Ltd., Torrance, CA, U.S.A.

Abbreviations used: ETF, electron-transfer flavoprotein; $ETF_{ox.}$, oxidized ETF; $ETF^{\cdot-}$, one-electron-reduced ETF; ETF_{hq}, two-electron-reduced ETF; ETF-Q oxidoreductase, ETF:ubiquinone oxidoreductase; Q-1, ubiquinone-1 [2,3-dimethoxy-5-methyl-6-(3-methylbut-2-en-1-yl)-1,4-benzoquinone].

§ To whom correspondence should be addressed at Molecular Biology Division 151-S, Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A.

Enzymes

Glutaryl-CoA dehydrogenase was prepared from *Paracoccus denitrificans* that had been grown on glutaric acid as the sole source of carbon (Husain & Steenkamp, 1985). ETF from pig liver mitochondria was prepared as previously described (Husain & Steenkamp, 1983). ETF-Q oxidoreductase from pig liver mitochondria was prepared by a slightly modified version of the procedure developed by Ruzicka & Beinert (1977), starting from submitochondrial particles as suggested by Beckmann & Frerman (1983*b*). The pure oxidoreductase was dialysed against 20 mM-Mops/KOH buffer, pH 7.4, and 10% (v/v) ethylene glycol was added before storage. All enzymes were at least 90% pure, as judged by SDS/polyacrylamide-gel electrophoresis. On the basis of flavin content, protein concentration determined by the biuret method and an M_r of 73000 (Beckmann & Frerman, 1985*a*), the purity of ETF-Q oxidoreductase was 89%. All enzymes were free from spectral contaminants in the visible region.

The e.p.r. spectrum of ETF-Q oxidoreductase was the same as that shown in Fig. 1 of Ruzicka & Beinert (1975). The sample used in the present work contained, per mg of protein, 43.9 ng-atoms of Fe, 46.2 ng-atoms of acid-labile sulphide and 12.2 nmol of non-covalently bound flavin. The visible-region spectrum was same as that observed by Beckmann & Frerman (1985*a*). On the basis of the flavin content, absorption coefficients for the spectral maxima were calculated to be $\epsilon_{380} = 27.0\ mM^{-1}\cdot cm^{-1}$ and $\epsilon_{424} = 25.0\ mM^{-1}\cdot cm^{-1}$.

Methods

Iron was determined by the method of Brumby & Massey (1967), sulphur by the basic procedure of the semi-micro method described by Beinert (1983) and flavin by the riboflavin fluorescence method after trichloroacetic acid precipitation (Koziol, 1971). Protein was determined by the biuret method (Gornall *et al.*, 1949). The concentrations of flavoenzymes were calculated by assuming absorption coefficients of $13.5\ mM^{-1}\cdot cm^{-1}$ at 435 nm for ETF (Husain & Steenkamp, 1983) and $13.6\ mM^{-1}\cdot cm^{-1}$ at 445 nm for glutaryl-CoA dehydrogenase (Husain & Steenkamp, 1985). Q-1 oil was diluted in 40% (v/v) ethanol and the concentration was determined from the change in absorbance at 275 nm on the addition of $NaBH_4$ by using an absorption coefficient of $12.5\ mM^{-1}\cdot cm^{-1}$ (Crane & Barr, 1971). ETF was reduced by anaerobic titration with dithionite in 1 ml quartz cuvettes fitted with silicone-rubber stoppers. $Na_2S_2O_4$ solutions (5 mg in 8 ml) were made up anaerobically in 5 mM-K_2HPO_4. A gas-tight syringe was used to inject dithionite into a solution containing ETF, 30 mM-glucose, glucose oxidase (1 unit), catalase (24 units) and other ingredients as necessary in a volume of 0.8 ml. The silicone-rubber stopper permitted convenient additions to the cuvette, and no re-oxidation of ETF semiquinone was observed over 15 min.

Assays

ETF-Q oxidoreductase activity was measured at 25 °C in potassium phosphate buffer, pH 7.0 and I 0.02, which also contained 30 μM-glutaryl-CoA, 0.38 μM-glutaryl-CoA dehydrogenase, 1.4 μM-ETF and 100 μM-Q-1. The reaction was initiated by the addition of approx. 1 μg of ETF-Q oxidoreductase/ml of assay mixture, and the reduction of Q-1 was monitored at 275 nm in a Cary 219 spectrophotometer. Turnover numbers are based on the flavin content. The assay was performed aerobically. Rates determined under anaerobic conditions were identical, suggesting that direct interaction of any of the reduced intermediates with O_2 was insignificant under the conditions of the assay. In some experiments glutaryl-CoA and glutaryl-CoA dehydrogenase were replaced with octanoyl-CoA (10 μM) and general acyl-CoA dehydrogenase (0.78 μM) from pig kidney (kindly donated by Dr. Colin Thorpe, University of Delaware, Newark, DE, U.S.A.), as indicated in the Figure legends. Substitution of the primary dehydrogenase made no difference to the assay because it was used at concentrations such that the assay was independent thereof.

Routinely glutaryl-CoA in the presence of glutaryl-CoA dehydrogenase was used as the source of electrons. This enzyme can be isolated in good yield from *P. denitrificans* grown on glutaric acid, and it was shown to reduce mammalian and bacterial ETF with a similar apparent $V_{max.}$. The apparent K_m for mammalian ETF was lower than that for the bacterial ETF (Husain & Steenkamp, 1985). Moreover, the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA is essentially irreversible.

The difference molar absorption coefficient for the reaction:

$$\text{Glutaryl-CoA} + \text{Q-1} \rightarrow \text{crotonyl-CoA} + \text{Q-1H}_2 + \text{CO}_2$$

was calculated by subtraction of the value for the conversion of glutaryl-CoA into crotonyl-CoA from that for the reduction of Q-1, since these are of opposing magnitude. The difference molar absorption coefficient for the conversion of glutaryl-CoA into crotonyl-CoA, in turn, was estimated by comparing the rates of change in absorbance at 420 and 275 nm for the reaction:

$$\text{Glutaryl-CoA} + 2\,\text{Fe(CN)}_6^{3-} \rightarrow \text{crotonyl-CoA} + \text{CO}_2 + 2\,\text{Fe(CN)}_6^{4-}$$

in the presence of glutaryl-CoA dehydrogenase and ETF from *P. denitrificans*. The contribution of the reduction of ferricyanide to the absorbance change at 275 nm was subtracted to obtain the absorbance change due to the oxidative decarboxylation of glutaryl-CoA. The difference molar absorption coefficient estimated for the latter reaction was $5.1\ mM^{-1}\cdot cm^{-1}$ at 275 nm and was deducted from the value of $12.5\ mM^{-1}\cdot cm^{-1}$ for the reduction of Q-1 to obtain the value of $7.4\ mM^{-1}\cdot cm^{-1}$ applicable to the assay.

Since ETF is more stable at pH 7.8, both the overall and the partial reactions were studied at this pH. Reaction conditions are given in the Figure legends. The rate of reduction of ETF to $ETF^{\cdot -}$, catalysed by glutaryl-CoA dehydrogenase, was measured by monitoring the decrease in the fluorescence of ETF (excitation at 372 nm, emission at 490 nm). The activity was assayed in 25 mM-Hepes/KOH buffer, pH 7.8, containing 10% (v/v) ethylene glycol, 30 mM-glucose, glucose oxidase (1 unit), catalase (24 units), 30 μM-glutaryl-CoA, 6.75 nM-glutaryl-CoA dehydrogenase and 0.5–5 μM-ETF in a volume of 0.8 ml. The rate of reduction of $ETF^{\cdot -}$ to ETF_{hq} in the presence of glutaryl-CoA dehydrogenase was measured by monitoring the decrease in the absorbance at 372 nm (difference molar absorption coefficient $8.6\ mM^{-1}\cdot cm^{-1}$). The assay mixture was as

described above except that 4–20 μM-ETF was titrated to $ETF^{\cdot-}$ by addition of portions of dithionite before the reaction was started by the addition of glutaryl-CoA dehydrogenase to give 6.75 nM.

Stopped-flow experiments

Anaerobic stopped-flow spectrophotometry was carried out with a modified Aminco–Morrow stopped-flow system and photomultiplier transducer. The system was made anaerobic by overnight incubation with buffer containing 100 mM-glucose, 5 units of glucose oxidase/ml and 50 units of catalase/ml. The system was flushed with anaerobic reaction mixture without ETF before use. A stream of N_2 around the reaction chamber provided further protection from O_2. Rapid data acquisition and subsequent analysis employed an IBM–PC compatible set of hardware and software subsystems. Raw voltage proportional to percentage transmission was offset and expanded as necessary and digitized via a 12-bit IBM–PC bus analogue-to-digital converter (Tecmar, Cleveland, OH, U.S.A.) with an on-board crystal-controlled clock to generate precise sampling intervals. Data stored as disc-files were archived and analysed by a program that allows for non-linear least-squares analysis via a gradient-expansion re-iterative algorithm to a large number of integro-differential equations. The analysis part of the current program is a microcomputer-based descendent of an earlier program described by Morris *et al.* (1980).

The second-order rate constant for the oxidation of $ETF^{\cdot-}$ by Q-1 was determined from the apparent first-order rate constants at high concentrations of Q-1. $ETF^{\cdot-}$ (14.1 μM) was generated by titration with dithionite in a tonometer with a cuvette attached, and loaded into one syringe of the stopped-flow apparatus. The other syringe contained Q-1 at twice the desired concentration in the same buffer mixture. The solutions were mixed 1:1, and the decrease in absorbance at 372 nm was measured (difference molar absorption coefficient 5.5 $M^{-1} \cdot cm^{-1}$). The data were fitted to a first-order rate equation to obtain k, the pseudo-first-order rate constant.

Rapid-scan experiments

Spectral changes during the re-oxidation of ETF_{hq} were recorded with a Hewlett–Packard HP-8451 rapid-scan spectrophotometer. Oxidation of ETF_{hq} prepared by dithionite titration in the presence of ETF-Q oxidoreductase was initiated by the addition of Q-1 through the silicone-rubber stopper and the spectrum was recorded every 2 s for 1 min. A multicomponent analysis subroutine of the Hewlett–Packard HP-8451 spectrophotometer was used to calculate the contribution of each species to the experimental spectra by comparison with standard spectra for $ETF_{ox.}$, $ETF^{\cdot-}$, ETF_{hq}, Q-1 and Q-$1H_2$.

RESULTS AND DISCUSSION

Steady-state assay of ETF-Q oxidoreductase and kinetic parameters

ETF-Q oxidoreductase was assayed by a coupled assay that mimics the physiological pathway. Either the dehydrogenation of octanoyl-CoA by the general acyl-CoA dehydrogenase from pig kidney or the oxidative decarboxylation of glutaryl-CoA by glutaryl-CoA dehydrogenase from *P. denitrificans* provided the source of electrons for the reduction of ETF. Q-1 was the electron acceptor. The general acyl-CoA dehydrogenase or glutaryl-CoA dehydrogenase were used at concentrations such that the rate of the assay depended only on the concentrations of ETF, ETF-Q oxidoreductase and Q-1. The contribution of ETF to the total spectral change is minimal because of its low concentration and because the reduced forms approach steady state during turnover.

The effects of buffer composition, pH and ionic strength on the ETF-Q oxidoreductase assay system were examined (Fig. 1). At the substrate concentrations used, the highest activity observed over a range of pH values was in phosphate buffer (Fig. 1*a*). The pH optimum was

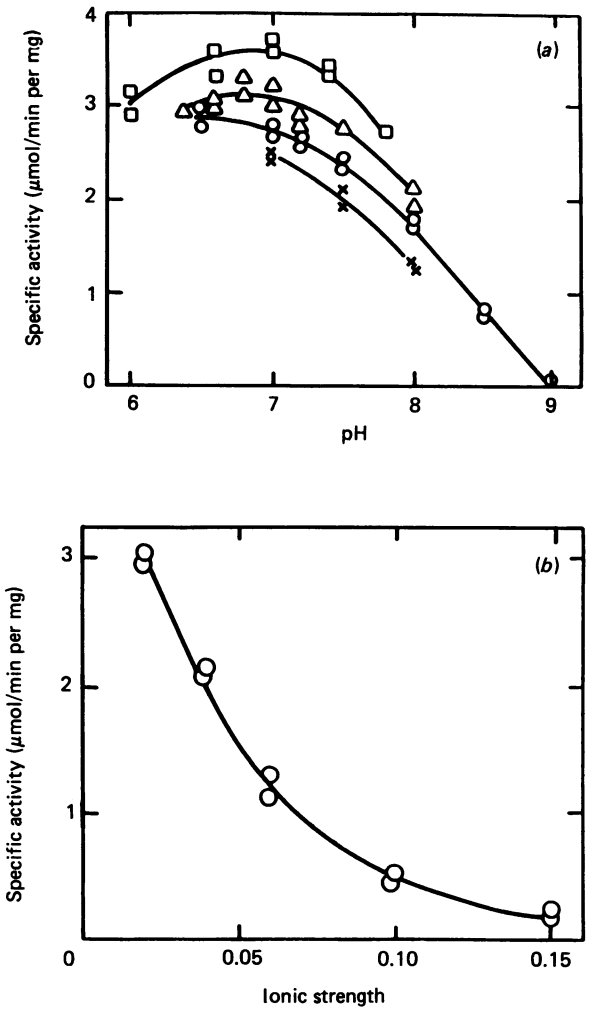


Fig. 1. Effect of pH and ionic strength on ETF-Q oxidoreductase activity

The assay is described in the Experimental section. Pig kidney acyl-CoA dehydrogenase and octanoyl-CoA were used to reduce ETF. (*a*) Effect of pH. The buffers, adjusted to ionic strength 0.05 with NaCl, were 50 mM-Tris/HCl (○), 50 mM-triethanolamine/HCl (×) and 50 mM-Mops/KOH (△); potassium phosphate buffer (*I* 0.05) (□) was made by mixing precalculated amounts of KH_2PO_4 and K_2HPO_4. (*b*) Effect of ionic strength. The buffer was 20 mM-Mops/KOH, pH 7.0, adjusted to each ionic strength with NaCl.

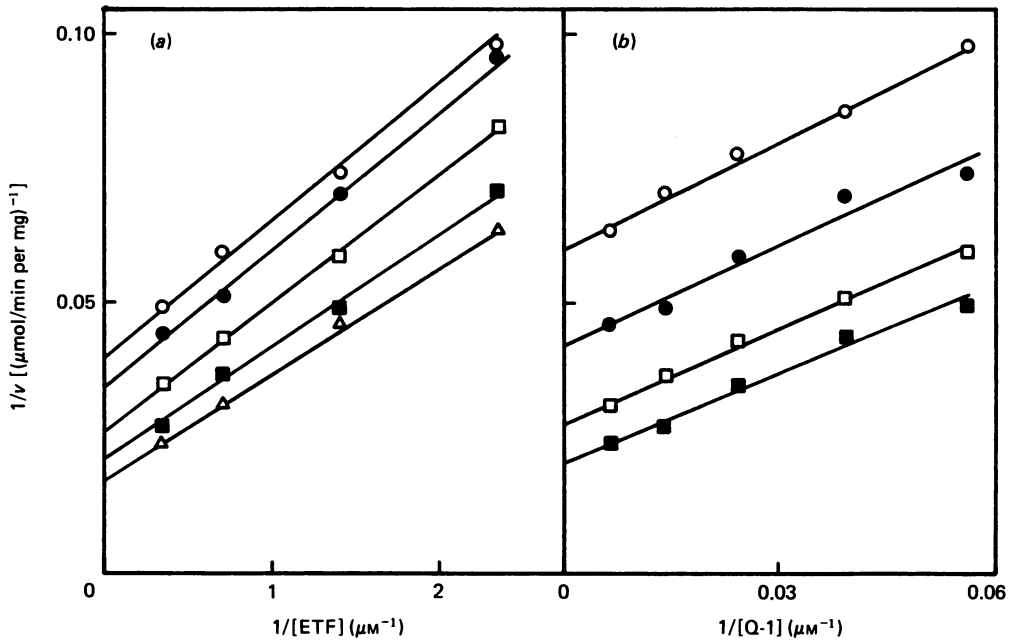


Fig. 2. Double-reciprocal plots for ETF-Q oxidoreductase with ETF and Q-1 as the varied substrates

The assays were done in 25 mM-Hepes/KOH buffer, pH 7.8, as described in the Experimental section. The concentration of ETF-Q oxidoreductase was 0.6 μg of protein/ml of assay mixture. (*a*) The concentrations of Q-1 were: ○, 17.5 μM; ●, 25.1 μM; □, 41.7 μM; ■, 72.1 μM; △, 157 μM. (*b*) The concentrations of ETF were: ○, 0.42 μM; ●, 0.7 μM; □, 1.4 μM; ■, 2.8 μM.

7.0 and was independent of the nature of the buffer. Anaerobiosis, which would prevent apparent inhibition due to a possible re-oxidation of QH_2 in aerobic alkaline solution, did not change the pH-dependence. The dramatic effect of ionic strength on the reaction is shown in Fig. 1(*b*) and is consistent with the reported ionic-strength-dependencies of the interactions of ETF with acyl-CoA dehydrogenase (Beckmann & Frerman, 1983*a*) and ETF-Q oxidoreductase (Beckmann & Frerman, 1985*b*).

Routinely, assays were carried out in potassium phosphate buffer at pH 7.0 and at an ionic strength of 0.02 as described in the Experimental section. The reaction rate was linearly dependent on the concentration of ETF-Q oxidoreductase when the other components were present in excess. The stability of the enzyme at different pH values was investigated by diluting a stock solution of ETF-Q oxidoreductase in 10 mM-Tris/HCl buffer, pH 7.6, 10-fold into 50 mM incubation buffer at 4 °C. The pH was then measured with a micro-electrode. Buffers used were a mixture of 20 mM-Mes, 15 mM-Pipes and 15 mM-Hepes or Tris or glycine, adjusted to an ionic strength of 0.05 with NaCl. There was no loss of activity after more than an hour between pH 7 and 9. After 1 h at pH 6 or 10 the activity of the enzyme had declined to 81% of the original value. At pH 5.5 activity was lost completely.

The steady-state experiments with $ETF_{ox.}$ and Q-1 as the varied substrates were carried out at pH 7.8, the pH at which the partial reactions were studied. The pattern of the kinetic plots is consistent with a Ping Pong mechanism (Fig. 2). The kinetic parameters calculated from Fig. 2 are $V_{max.}$ 84 μmol/min per mg (turnover number 115 s^{-1}), K_m (ETF) 1.97 μM and K_m (Q-1) 57.1 μM.

Partial reactions of ETF

Reduction by *P. denitrificans* glutaryl-CoA dehydrogenase. ETF is rapidly reduced to the anionic semiquinone by all ETF-dependent dehydrogenases so far investigated, and more slowly to the fully reduced form (Hall & Lambeth, 1980; Beckmann *et al.*, 1981; Steenkamp & Husain, 1982). Glutaryl-CoA dehydrogenase from *P. denitrificans* (Husain & Steenkamp, 1985) resembles the mitochondrial acyl-CoA dehydrogenases in this regard. The reduction of $ETF_{ox.}$ to $ETF^{\cdot-}$ was measured by monitoring the disappearance of the strong fluorescence of $ETF_{ox.}$, the reduced species ($ETF^{\cdot-}$ and ETF_{hq}) being essentially non-fluorescent. In determining the rate of conversion of ETF into $ETF^{\cdot-}$ as a function of ETF concentration, the assumption was made that the observed initial velocities were not significantly influenced by the subsequent reduction of $ETF^{\cdot-}$ to ETF_{hq}. Since the latter occurs at a much lower rate, this was a reasonable assumption. At pH 7.8, where $ETF^{\cdot-}$ is relatively stable, the apparent $V_{max.}$ for the formation of $ETF^{\cdot-}$ in the presence of 30 μM-glutaryl-CoA was 6.4 μmol/min per mg (turnover number 4.6 s^{-1}) and the apparent K_m for ETF was 1 μM. An attempt was also made to obtain satisfactory Lineweaver–Burk plots for the reduction to ETF_{hq} of $ETF^{\cdot-}$ generated by dithionite titration of $ETF_{ox.}$. Since $ETF^{\cdot-}$ is essentially non-fluorescent, the disappearance of $ETF^{\cdot-}$ was monitored by absorbance spectrophotometry. The $ETF^{\cdot-}$ concentrations required for measurement of the absorbance changes were higher than the concentrations required for determination of the K_m for $ETF^{\cdot-}$. The apparent $V_{max.}$ for the reduction of $ETF^{\cdot-}$ to ETF_{hq} was 1.3 μmol/min per mg (turnover number 0.9 s^{-1}).

Re-oxidation of $ETF^{\cdot-}$ by Q-1. Reactivity of the reduced forms of ETF with components present in the steady-state assay was investigated. Under anaerobic conditions ETF_{hq} did not react with Q-1. In contrast, $ETF^{\cdot-}$ was directly oxidized by Q-1 in a non-enzymic reaction. The reaction was pseudo-first-order at high concentrations of Q-1, and the apparent first-order rate constants calculated from stopped-flow experiments at various concentrations of Q-1 indicated a second-order rate constant of 1320 $M^{-1} \cdot s^{-1}$. In the assay of ETF-Q oxidoreductase activity, this direct re-oxidation of $ETF^{\cdot-}$ by Q-1 was observed as a significant but decreasing blank rate at low concentrations of glutaryl-CoA dehydrogenase (Steenkamp *et al.*, 1984). The blank rate was abolished by the addition of excess glutaryl-CoA dehydrogenase or by preincubation of ETF with glutaryl-CoA dehydrogenase in the presence of glutaryl-CoA before addition of Q-1. Under these conditions reduction of Q-1 in the absence of ETF-Q oxidoreductase is eliminated, because ETF is converted into ETF_{hq}, which does not react directly with Q-1.

The rate of reduction of Q-1 observed in the steady-state assays (the overall reaction) represents the sum of ETF-Q oxidoreductase-dependent and -independent reactions. The proportion of ETF present as ETF_{hq} during steady-state turnover would depend on its rate of formation relative to the rate at which it is re-oxidized by ETF-Q oxidoreductase. Even with excess glutaryl-CoA dehydrogenase, some small amount of $ETF^{\cdot-}$ must be present. If ETF-Q oxidoreductase utilized $ETF^{\cdot-}$ preferentially, a sigmoidal dependence of reaction velocity on ETF-Q oxidoreductase concentration would be expected. This, however, was not observed over a wide range of enzyme concentrations (0.02–0.6 μg/ml).

Disproportionation of $ETF^{\cdot-}$ by ETF-Q oxidoreductase. As previously reported, ETF-Q oxidoreductase catalyses the disproportionation of $ETF^{\cdot-}$ to an equilibrium mixture of $ETF_{ox.}$, $ETF^{\cdot-}$ and ETF_{hq} (Steenkamp *et al.*, 1984; Beckmann & Frerman, 1985*a*). The spectral changes shown in Fig. 3 are characterized by decreases in the absorbance at 372 nm and at 401 and 478 nm, the isosbestic points for the oxidation of $ETF^{\cdot-}$ to $ETF_{ox.}$, and by increases in the absorbance at 435 nm and in the region 300–336 nm.

When glutaryl-CoA dehydrogenase was added to $ETF^{\cdot-}$, very slow disproportionation (0.03 μmol/min per mg at 9.5 μM-ETF) was observed. This low rate was not reproducible, and it may be concluded that glutaryl-CoA dehydrogenase catalyses the disproportionation of ETF at a rate that is at least three orders of magnitude lower than that catalysed by ETF-Q oxidoreductase. This observation is in agreement with the slow formation of ETF_{hq} by the acyl-CoA dehydrogenases.

Difference molar absorption coefficients for the interconversion of the redox states of ETF at pH 7.8 were computed (Table 1) and were used to calculate the equilibrium concentrations of ETF, $ETF^{\cdot-}$ and ETF_{hq} from the spectral changes observed during disproportionation. The equilibrium constant for disproportionation:

$$K = \frac{[ETF][ETF_{hq}]}{[ETF^{\cdot-}]^2} \quad (1)$$

was estimated to be about 1 at pH 7.8, in agreement with $K = 1$ calculated from the redox potential data of Husain *et al.* (1984). Since the disproportionation of ETF could proceed with the net uptake of protons, according to the reaction:

$$2ETF^{\cdot-} + nH^+ \rightarrow ETFH_n + ETF_{ox.}$$

the equilibrium constant should be pH-dependent. Experiments (such as documented by Ghisla *et al.*, 1974) to determine whether the FAD moiety in ETF_{hq} is anionic or neutral have not been done. However, Beckmann & Frerman (1985*a*) examined the dependence of $K_{app.}$ on pH and found that $n = 1$, indicating the uptake of only one proton in the disproportionation reaction.

The disproportionation reaction followed simple Michaelis–Menten kinetics with $V_{max.}$ 172 μmol/min per mg (turnover number 235 s^{-1}) and an observed K_m of 1.47 μM at pH 7.8. In this reaction $ETF^{\cdot-}$ functions both as an electron donor to oxidized ETF-Q oxidoreductase and as the electron acceptor from the reduced form of the enzyme. The initial-velocity equation for a bi-substrate reaction, therefore, takes the form:

$$v = \frac{V[A]^2}{(K_a + K_{a'})[A] + [A]^2 + K_{ia}K_{a'}} \quad (2)$$

where v is the initial velocity, [A] is $ETF^{\cdot-}$ concentration, K_{ia} is the dissociation constant, and K_a and $K_{a'}$ are limiting Michaelis constants for $ETF^{\cdot-}$ functioning as an electron donor and acceptor respectively (Cleland, 1970). A double-reciprocal plot will be linear if $K_{ia}K_{a'}$ is zero, as in double-displacement mechanisms, or of small magnitude, as in sequential mechanisms.

Several earlier studies (Hall & Lambeth, 1980; Beckmann *et al.*, 1981; Steenkamp & Husain, 1982)

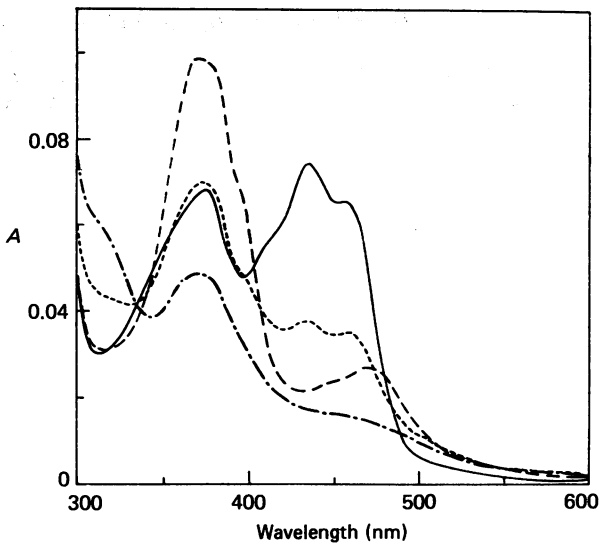


Fig. 3. ETF-Q oxidoreductase-catalysed disproportionation of $ETF^{\cdot-}$

$ETF_{ox.}$ (13.9 μM) in 0.8 ml of 25 mM-Hepes/KOH buffer, pH 7.8, containing 10% (v/v) ethylene glycol, 30 mM-glucose, glucose oxidase (1 unit) and catalase (24 units) was reduced to $ETF^{\cdot-}$ by titration with dithionite. Disproportionation was initiated by addition of 98 ng of ETF-Q oxidoreductase. Further titration with dithionite of the equilibrium mixture after disproportionation yielded ETF_{hq}. The spectra are: ——, $ETF_{ox.}$; – – – –, $ETF^{\cdot-}$; —·—, ETF_{hq}; ----, the equilibrium mixture after disproportionation.

Table 1. Experimental absorption coefficients for ETF

The absorbance values at each wavelength were determined from titrations of ETF as described for Fig. 4. The absorption coefficients are the average of values from five experiments with different concentrations of ETF (3.7–13.9 μM).

Species	Wavelength . . .	Experimental absorption coefficient ($mM^{-1} \cdot cm^{-1}$) 316 nm	372 nm	401 nm	435 nm
$ETF_{ox.}$		6.0	12.3	9.3	13.5
$ETF^{\cdot-}$		6.0	17.7	9.3	4.9
ETF_{hq}		10.7	8.7	5.7	3.1
Equilibrium mixture in the presence of ETF-Q oxidoreductase*		7.8	12.4	8.4	7.5
Difference ε for oxidation					
$ETF^{\cdot-} \rightarrow ETF_{ox.}$		0	−5.4	0	8.6
$ETF_{hq} \rightarrow ETF^{\cdot-}$		−4.7	8.9	3.6	1.8
$ETF_{hq} \rightarrow ETF_{ox.}$		−4.7	3.6	3.6	10.4
Difference ε for disproportionation†					
$2ETF^{\cdot-} \rightarrow ETF_{ox.} + ETF_{hq}$		2.3	7.2	1.8	3.2

* Note that at equilibrium all three species are present; $K_{eq.} = 1$.

† The values given are calculated from the observed ε for each species for complete disproportionation to $ETF_{ox.} + ETF_{hq}$.

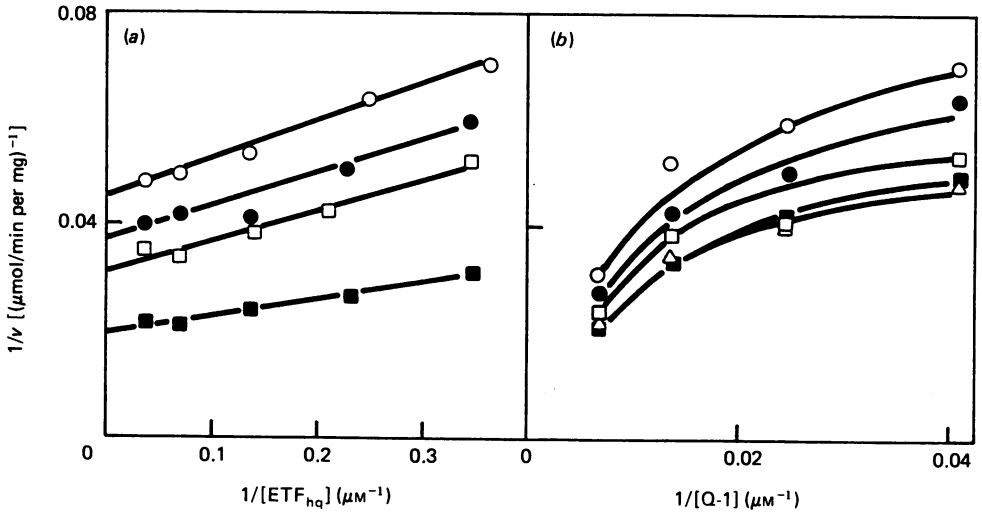


Fig. 4. Oxidation of ETF_{hq} by Q-1 catalysed by ETF-Q oxidoreductase

The rate of conversion of ETF_{hq} into $ETF_{ox.}$ was measured by monitoring the increase in absorbance at 435 nm (difference molar absorption coefficient = 10.4 $mM^{-1} \cdot cm^{-1}$). The assay mixture consisted of 25 mM-Hepes/KOH buffer, pH 7.8, 10% (v/v) ethylene glycol, 30 mM-glucose, glucose oxidase (1 unit), catalase (24 units), 2.9–27.7 μM-ETF_{hq}, 24.4–146 μM-Q-1 and 98 ng of ETF-Q oxidoreductase. The ETF_{hq} was generated by titration of ETF with dithionite in the presence of ETF-Q oxidoreductase. The reaction was started by the addition of Q-1. (*a*) The concentrations of Q-1 were: ○, 24.4 μM; ●, 40.5 μM; □, 71.6 μM; ■, 146 μM. (*b*) The concentrations of ETF_{hq} were: ○, 2.9 μM; ●, 4.2 μM; □, 7.3 μM; ■, 14.2 μM; △, 27.7 μM.

demonstrated that the reduction of ETF to $ETF^{\cdot-}$ by ETF-dependent dehydrogenases is rapid, whereas the further reduction of $ETF^{\cdot-}$ to ETF_{hq} by these enzymes is much slower and probably not physiologically significant. It seemed unlikely, therefore, that ETF_{hq} would be a prominent intermediate in the transfer of electrons from the acyl-CoA derivatives to the respiratory chain. The data presented here clearly indicate that the rate of electron transfer through ETF-Q oxidoreductase during the disproportionation of $ETF^{\cdot-}$ (which is measured as an electron transfer) is twice as fast during the overall reaction (which is a two-electron transfer), i.e. that the rate of transfer of one electron is the same in both. Consequently, ETF is continually regenerated for rapid reduction by the acyl-CoA dehydrogenases even in the absence of Q-1, and the combined action of the two enzymes results in the accumulation of ETF_{hq} during catalytic turnover. The slow step in the reduction of ETF to ETF_{hq} is thereby circumvented, and it becomes evident that ETF_{hq} may, indeed, be the predominant

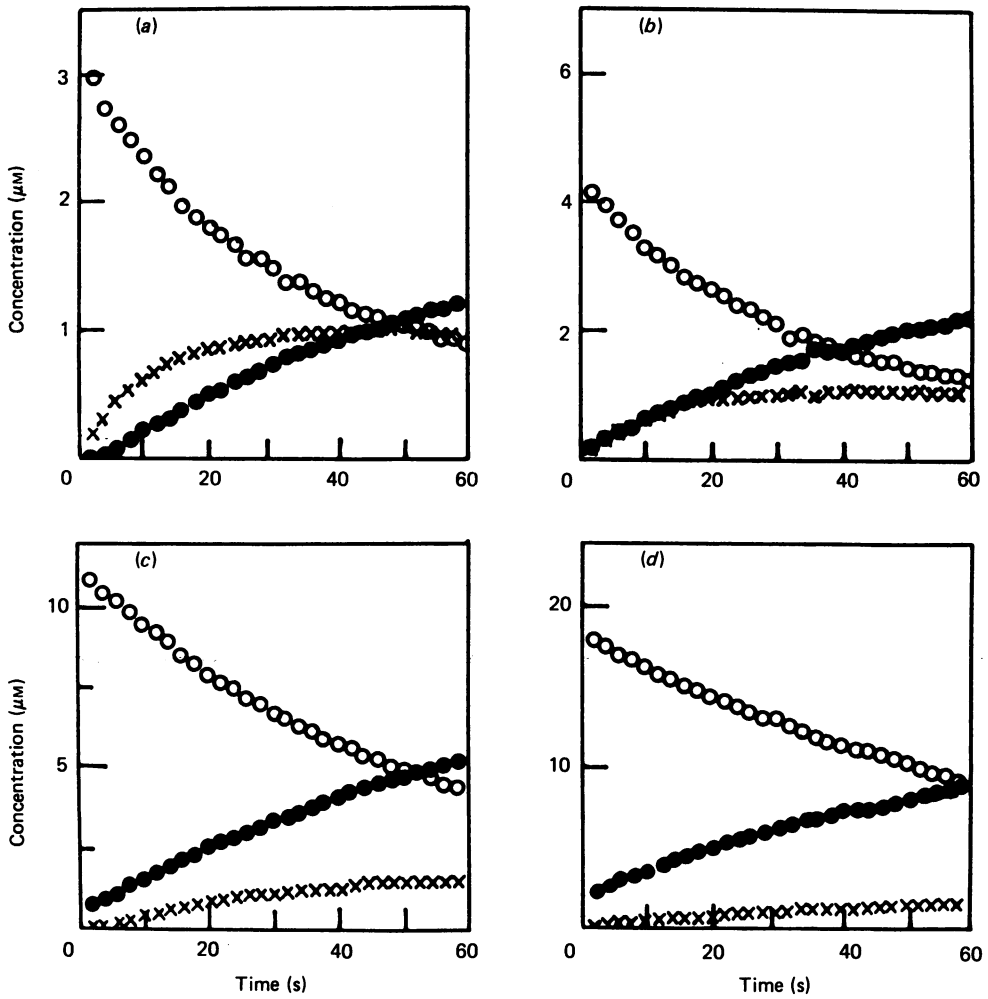


Fig. 5. Concentrations of ETF_{hq}, $ETF^{\cdot-}$ and $ETF_{ox.}$ as estimated by multicomponent analysis of spectra recorded during the re-oxidation of ETF_{hq} by Q-1 in the presence of ETF-Q oxidoreductase

Reaction conditions were as indicated in Fig. 4 legend. The molar concentrations of ETF_{hq} and Q-1 were varied in a fixed ratio of 1:6. The different ETF concentrations, representing the average sum of the three redox forms of ETF calculated by multicomponent analysis of the 30 spectra recorded at 2 s intervals for 1 min, were 3.15 μM (*a*), 4.49 μM (*b*), 11.24 μM (*c*) and 19.05 μM (*d*). The symbols denote ETF_{hq} (○), $ETF^{\cdot-}$ (×) and $ETF_{ox.}$ (●) concentrations at various times.

form of reduced ETF during catalytic turnover. The observation that ETF-Q oxidoreductase not only catalyses the re-oxidation of ETF_{hq}, but also facilitates the reduction of the radical to the fully reduced form by disproportionation, appears to be a unique property of this enzyme.

Re-oxidation of ETF_{hq} by Q-1 in the presence of ETF-Q oxidoreductase. In these experiments ETF_{hq} was generated by dithionite titration of $ETF_{ox.}$ in the presence of ETF-Q oxidoreductase. Since the reduction of $ETF^{\cdot-}$ by dithionite is very slow in the absence of ETF-Q oxidoreductase, the disproportionation reaction was used to facilitate formation of ETF_{hq}. Q-1 did not react directly with ETF_{hq} in the absence of ETF-Q oxidoreductase.

The interpretation of absorbance changes at a single wavelength during the re-oxidation of ETF_{hq} is complicated by the formation of both $ETF^{\cdot-}$ and $ETF_{ox.}$. The $ETF^{\cdot-}$ can be formed as a reaction intermediate or by comproportionation of $ETF_{ox.}$ and ETF_{hq}. Fig. 4 shows double-reciprocal plots for the initial rate of conversion of ETF_{hq} into $ETF_{ox.}$ measured by monitoring the increase in absorbance at 435 nm. The maximal velocity could not be determined because of the non-linearity of the double-reciprocal plot. The non-enzymic oxidation of $ETF^{\cdot-}$ by Q-1 could be partly responsible for the accelerated appearance of $ETF_{ox.}$ at high Q-1 concentrations, but its contribution could not be assessed because the concentration of $ETF^{\cdot-}$ was unknown.

To obtain information on the amounts of each of the three redox forms of ETF present in the course of re-oxidation of ETF_{hq} by Q-1 in the presence of ETF-Q oxidoreductase, the spectral changes during the re-oxidation reaction were recorded by rapid-scan spectrophotometry. Re-oxidation of ETF_{hq} was initiated by addition of Q-1 and the spectrum was recorded every 2 s for 1 min. The changes in concentrations of ETF_{hq}, $ETF^{\cdot-}$ and $ETF_{ox.}$ estimated by deconvolution of spectra are shown in Fig. 5. In these experiments the concentrations of ETF_{hq} and Q-1 were varied in a fixed ratio. At low concentrations of ETF_{hq} and Q-1, $ETF^{\cdot-}$

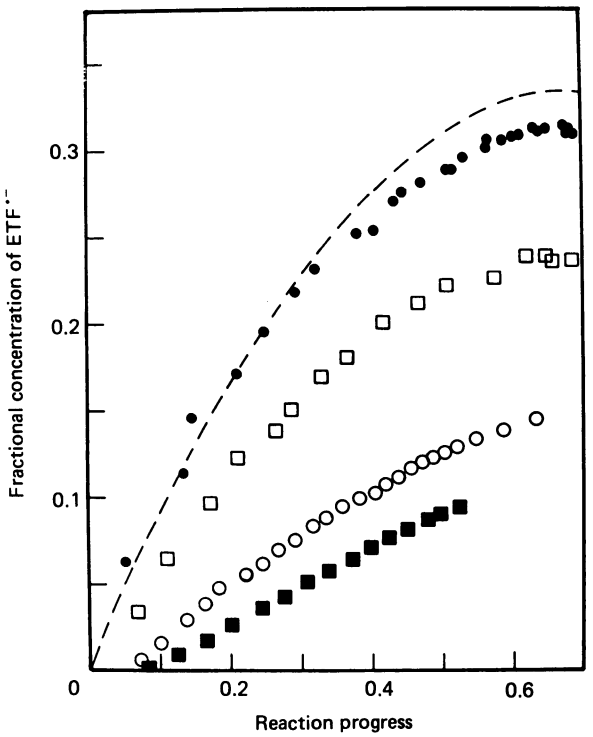


Fig. 6. Fractional concentration of ETF$^{\cdot-}$ as a function of reaction progress for the reactions shown in Fig. 5

The broken line represents the theoretical curve for a disproportionation equilibrium constant of 1.0. The symbols represent the different reactant (ETF) concentrations: ●, 3.15 μM; □, 4.49 μM; ○, 11.24 μM; ■, 19.05 μM.

increases before $ETF_{ox.}$, whereas at high reactant concentration the semiquinone appears more slowly. The amount of $ETF^{\cdot-}$ formed is a high proportion of the total at low reactant concentrations, but is a low proportion when reactant concentrations are high (Fig. 5). The decreasing fractional amounts of $ETF^{\cdot-}$ formed indicate increasing deviation from the disproportionation equilibrium. These observations are primarily a result of the relatively high Michaelis constant of ETF-Q oxidoreductase for Q-1 (57.1 μM, as compared with 1.97 μM for the K_m for ETF). At high concentrations, Q-1 competes more effectively for ETF-Q oxidoreductase, so that the stoichiometry of $ETF^{\cdot-}$ and $ETF_{ox.}$ formation changes. Since the difference molar absorption coefficient for the oxidation of ETF_{hq} to $ETF_{ox.}$ at 435 nm is much higher than for the oxidation of ETF_{hq} to $ETF^{\cdot-}$, the decreased proportion of $ETF^{\cdot-}$ formed at higher Q-1 concentrations is reflected in a downwards curvature of the double-reciprocal plots in Fig. 4.

Because the proportion of $ETF^{\cdot-}$ formed at different reactant concentrations varies, extrapolation of the observed initial velocities for the oxidation of ETF_{hq} in the experiment in Fig. 5 is also of doubtful validity. At an ETF concentration of 19.05 μM the observed reaction rate indicated a turnover of about 150 s^{-1} for a reaction that was predominantly a two-electron transfer. This value may be compared with the turnover of 115 s^{-1} for the overall reaction.

The contribution of direct oxidation of $ETF^{\cdot-}$ by Q-1 to the rate of oxidation of ETF_{hq} can be estimated from the $ETF^{\cdot-}$ formed at short times (Fig. 5). For example, at a total concentration of 19.05 μM-ETF the $ETF^{\cdot-}$ concentration at 10 s reaction time was only 0.267 μM. The corresponding non-enzymic rate of 2.4 μM-$ETF^{\cdot-}$/min represents a one-electron reaction and may be compared with the initial rate of disappearance of ETF_{hq}, which may be estimated at 12.8 μM/min. It is therefore clear that the contribution of the non-enzymic re-oxidation of $ETF^{\cdot-}$ to the observed initial velocity would be small and that both electron transfers in the two-electron oxidation of ETF_{hq} are catalysed by ETF-Q oxidoreductase.

The data also permitted assessment of the significance of the disproportionation reaction to the oxidation of ETF_{hq} to $ETF_{ox.}$. If the disproportionation equilibrium is maintained during the re-oxidation of ETF_{hq}, equation (1), written as a function of reaction progress, a, becomes:

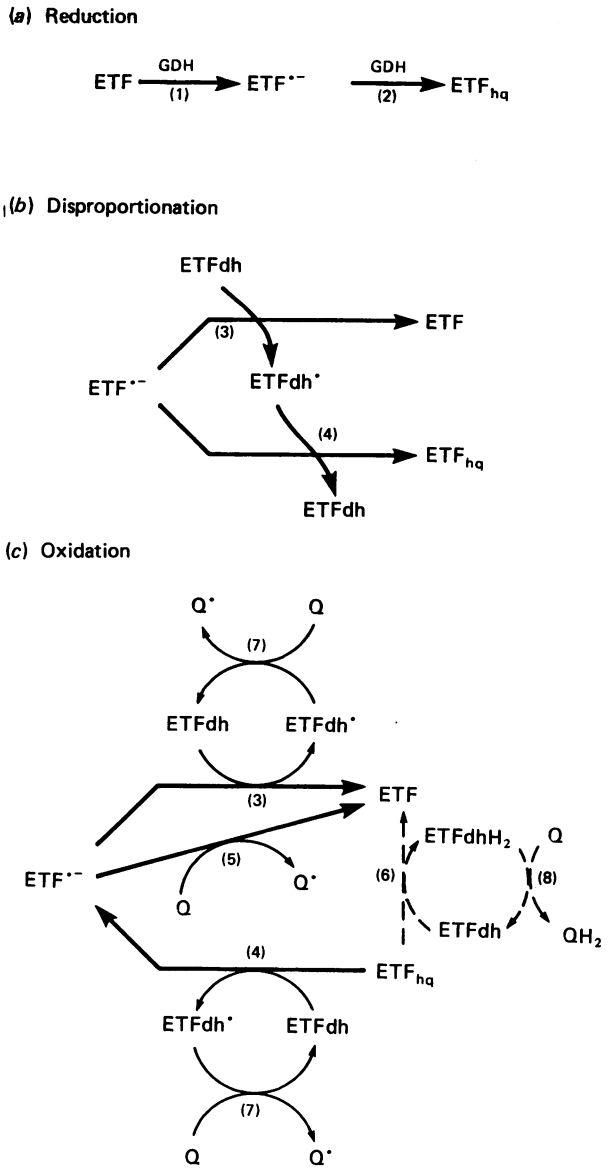
$$K = \frac{(1-a)(a-b)}{b^2} \quad (3)$$

where b is the fractional concentration of $ETF^{\cdot-}$ formed. Only one physically meaningful solution of b exists:

$$b = \frac{-1+\sqrt{[1+4Ka/(1-a)]}}{2K/(1-a)} \quad (4)$$

The fractional concentration of $ETF^{\cdot-}$ (b) as a function of a reaction progress (a) was computed for the equilibrium constant $K = 1$ (Fig. 6). It is evident that $ETF^{\cdot-}$ will constitute a significant fraction of the total ETF present even if the re-oxidation of ETF_{hq} proceeds by the transfer of electron pairs to Q-1. The disproportionation equilibrium, however, will only be maintained during the re-oxidation of ETF_{hq} if it represents a rapid-equilibrium segment in the overall reaction, i.e. if ETF-Q oxidoreductase transfers electrons to and from ETF molecules at a rate that far exceeds the rate of electron transfer to Q-1. The relatively high proportion of $ETF^{\cdot-}$ formed at low reactant concentrations indicates that the disproportionation equilibrium is approximately maintained (see Fig. 6). At higher reactant concentrations the fractional concentration of $ETF^{\cdot-}$ as a function of the reaction progress decreases significantly (Fig. 6), indicating a deviation from the disproportionation equilibrium. This is consistent with the similarity of the rates of electron transfer either to another ETF molecule or to Q-1.

The rate of reduction of Q-1 observed in the steady-state assays (the overall reaction) represents the sum of ETF-Q oxidoreductase-dependent and -independent reactions, the latter being due to the direct oxidation of $ETF^{\cdot-}$ by Q-1. It was previously shown that in assay mixtures containing glutaryl-CoA, ETF, Q-1 and low concentrations of glutaryl-CoA dehydrogenase the non-enzymic re-oxidation of $ETF^{\cdot-}$ by Q-1 can be eliminated by raising the glutaryl-CoA dehydrogenase concentration, thus converting ETF into ETF_{hq}, which does not react with Q-1 (Steenkamp *et al.*, 1984). When ETF-Q oxidoreductase is included in the reaction mixture the contribution of the non-enzymic reaction can also be observed at low concentrations of ETF-Q oxidoreductase and glutaryl-CoA dehydrogenase. The observed reaction rate reaches a maximum at about 12.5 nM-glutaryl-CoA dehydrogenase (or less, depending on the concentration of ETF-Q oxidoreductase) and decreases at higher concentrations of this enzyme. This



Scheme 1. Scheme for the redox reactions of ETF

ETFdh, ETFdh• and $ETFdhH_2$ represent oxidized, one-electron-reduced and two-electron-reduced ETF-Q oxidoreductase respectively, and GDH represents glutaryl-CoA dehydrogenase.

effect is most pronounced at lower ETF-Q oxidoreductase concentrations, both because the rate of the direct oxidation of $ETF^{\bullet-}$ by Q-1 is of comparable magnitude with the enzyme-catalysed rate and because disproportionation of $ETF^{\bullet-}$ by ETF-Q oxidoreductase proceeds at a lower rate. The contribution of the non-enzymic reoxidation of $ETF^{\bullet-}$ by Q-1 to the overall reaction was minimized by performing assays at relatively high concentrations of both ETF-Q oxidoreductase (0.6 μg of protein/ml) and glutaryl-CoA dehydrogenase (0.58 μM).

CONCLUSIONS

The data presented in this paper demonstrate significant formation of ETF_{hq} by the concerted action of glutaryl-CoA dehydrogenase and ETF-Q oxidoreductase and suggest that the re-oxidation of ETF_{hq} by ETF-Q oxidoreductase in the presence of Q-1 is sufficiently rapid to present a feasible pathway for the flux of reducing equivalents from glutaryl-CoA to Q-1.

The reactions that can occur during the oxidation and reduction of ETF are shown in Scheme 1. The uncertainties in this scheme are whether one-electron-reduced ETF-Q oxidoreductase can be re-oxidized by Q-1 (reaction 7) and whether ETF_{hq} can donate a hydride equivalent to ETF-Q oxidoreductase (reaction 6), questions that the present study was not designed to address. Not shown is the rapid disproportionation of putative $Q\text{-}1^{\bullet-}$. The main features of the scheme are: (i) a rapid reduction of $ETF_{ox.}$ to $ETF^{\bullet-}$ followed by a much slower reduction to ETF_{hq} by acyl-CoA dehydrogenase [Scheme 1(*a*), reactions (1) and (2)], (ii) rapid and reversible disproportionation of $ETF^{\bullet-}$ to an equilibrium mixture by ETF-Q oxidoreductase [Scheme 1(*b*), reactions (3) and (4)], (iii) ETF-Q oxidoreductase-catalysed re-oxidation of ETF_{hq} either by direct removal of a hydride equivalent or two successive one-electron transfers [Scheme 1(*c*), reactions (3), (4) and (6)], and (iv) non-enzymic reoxidation of $ETF^{\bullet-}$ by Q-1, which probably does not contribute significantly to the turnover rate [Scheme 1(*c*), reaction (5)].

Whether these reactions also occur *in vivo* is another question. The scheme presupposes mobile access of several ETF molecules to one ETF-Q oxidoreductase molecule or a permanent cluster of ETF molecules around an ETF-Q oxidoreductase molecule. The relative quantities of the two enzymes in mitochondria and their structural relationship to each other and to the ETF-dependent dehydrogenases are not known. Moreover, the role of the membrane environment and the effect of the presence of Q-10, the natural electron acceptor, cannot readily be predicted. It is evident from this study that all three redox states of ETF are involved as intermediates in the turnover of the system that transfers electrons from the ETF-dependent dehydrogenases into the electron-transport chain. The role of ETF-Q oxidoreductase in catalysing the production of fully reduced ETF_{hq} as well as its re-oxidation is unusual, with consequences for the efficiency and regulation of electron flow in the pathway.

This work was supported by Grant HL-16251 from the U.S. National Institutes of Health and Grant DMB-8416967 from the U.S. National Science Foundation. The technical assistance of Ann Friend and Ann Trason is appreciated. We are grateful to Dr. Steven C. Koerber for interfacing the stopped-flow apparatus to an IBM-PC computer and for assistance with data manipulation.

REFERENCES

Beckmann, J. D. & Frerman, F. E. (1983*a*) J. Biol. Chem. **258**, 7563–7569
Beckmann, J. D. & Frerman, F. E. (1983*b*) Fed. Proc. Fed. Am. Soc. Exp. Biol. **42**, 1761
Beckmann, J. D. & Frerman, F. E. (1985*a*) Biochemistry **24**, 3913–3921
Beckmann, J. D. & Frerman, F. E. (1985*b*) Biochemistry **24**, 3922–3925
Beckmann, J. D., Frerman, F. E. & McKean, M. C. (1981) Biochem. Biophys. Res. Commun. **102**, 1290–1292
Beinert, H. (1983) Anal. Biochem. **131**, 373–378

Brumby, T. E. & Massey, V. (1967) Methods Enzymol. **10**, 463–476
Cleland, W. W. (1970) Enzymes 3rd Ed. **2**, 1–61
Crane, F. L. & Barr, R. (1971) Methods Enzymol. **18C**, 137–165
Frerman, F. E. & Goodman, S. I. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4517–4520
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
Gholson, R. K., Nishizuka, Y., Ichiyama, A., Kawai, H., Nakamura, S. & Hayaishi, O. (1962) J. Biol. Chem. **237**, PC2043–PC2045
Gorelick, R. J., Mizzer, J. P. & Thorpe, C. (1982) Biochemistry **21**, 6936–6942
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) J. Biol. Chem. **177**, 751–766
Hall, C. L. & Lambeth, J. D. (1980) J. Biol. Chem. **255**, 3591–3595
Husain, M. & Steenkamp, D. J. (1983) Biochem. J. **209**, 541–545
Husain, M. & Steenkamp, D. J. (1985) J. Bacteriol. **163**, 709–715
Husain, M., Stankovich, M. T. & Fox, B. G. (1984) Biochem. J. **219**, 1043–1047
Ikeda, Y., Dabrowski, C. & Tanaka, K. (1983) J. Biol. Chem. **258**, 1066–1076
Koziol, J. (1971) Methods Enzymol. **18B**, 253–285
Morris, R. J., Saliman, G. & Dunn, M. F. (1980) Biochemistry **19**, 725–731
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
Stadtman, E. K. (1957) Methods Enzymol. **3**, 931–941
Steenkamp, D. J. & Husain, M. (1982) Biochem. J. **203**, 707–715
Steenkamp, D. J., Ramsay, R. R. & Husain, M. (1984) Flavins Flavoproteins Proc. Int. Symp. 8th 459–462

Received 9 July 1986/3 September 1986; accepted 15 October 1986