Acyl-CoA dehydrogenases, electron transfer flavoprotein and electron transfer flavoprotein dehydrogenase

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Introduction
Enzymic oxidation of acyl-CoA substrates to trans-2,3-enoyl-CoA thiolesters is a common reaction in the mitochondrial oxidation of fatty acids, branched-chain amino acids and lysine. These oxidations are catalysed by six flavoprotein dehydrogenases localized in the mitochondrial matrix. Electron transfer from the dehydrogenases to the ubiquinone pool is mediated by electron transfer flavoprotein (ETF) and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO, also referred to as ETF dehydrogenase). ETF is also the electron acceptor for the N-methyl dehydrogenases, dimethylglycine and sarcosine dehydrogenases. Thus, this electron transfer pathway is also significant in one-carbon metabolism (Wittwer & Wagner, 1981). The fatty acyl-CoA dehydrogenases, ETF and ETF-QO, were initially described by Helmut Beinert and his co-workers (Beinert, 1963a, b; Ruzicka & Beinert, 1977). The foundations laid in Beinert's laboratory have supported the recent advances in this system. Over the last several years, progress in this system has led to descriptions of the chemical mechanism of the primary flavoprotein dehydrogenases, characterizations of the electron transfer steps and chemistry of the interactions between the proteins, crystallographic description of the general acyl-CoA dehydrogenase (GAD) and, most recently, to cloning of the GAD gene and determination of its chromosomal location. These basic investigations have, in turn, permitted advances in the understanding of metabolic diseases in which these proteins are defective (Ferrman & Goodman, 1985; Hale et al., 1985; Ikeda et al., 1986).

Structural aspects
Mammalian acyl-CoA dehydrogenases are homotetramers containing one equivalent of FAD per subunit (Ikeda & Tanaka, 1983; Lenich & Goodman, 1986; Finocchiato et al., 1987). The dehydrogenases catalyse similar transformations of substrates, have similar quaternary structures and are all oxidized by ETF, but are immunologically distinct (Ikeda et al., 1985a). Subunit M, values of the mature mammalian dehydrogenases are in the range of 43,000 with the exception of human glutaryl-CoA dehydrogenase (M = 59,000). Lenich & Goodman, 1986). GAD is the most extensively investigated of these dehydrogenases. Crystallographic studies at 0.55 nm resolution show two pairs of distinct ellipsoidal monomers. The monomers are arranged head to tail and are displaced about 1.5 nm from each other along a crystallographic 2-fold axis (Kim, 1987). The flavin environment of GAD has been investigated by resonance Raman spectroscopy and by the substitution of analogues of FAD. From resonance Raman spectra, McFarland and colleagues have concluded that the flavin is extensively hydrogen bonded and that substrates bind in the region of N-5-C-4 = 0 (Benecky et al., 1979; Schmidt et al., 1981). Thorpe & Massey (1983) have substituted apoGAD with a number of FAD analogues, using these analogues as probes of the mechanism of dehydrogenation and the FAD environment. This latter approach indicates that the region of C-8 is sequestered from solvent and that the region of N-1-C-2 = 0 is able to stabilize negative charge such as develops in the one-electron reduced-enoyl-CoA dehydrogenase intermediate (Mizner & Thorpe, 1981; Gorelick et al., 1985). The GAD and isovaleryl-CoA dehydrogenase genes have been cloned and sequenced (Kelly et al., 1987; Matsubara et al., 1987) and the human GAD gene has been localized on chromosome 1 (Matsubara et al., 1987).

ETF is a heterodimeric electron transferase containing a single equivalent of FAD per dimer (Gorelick et al., 1982; Husain & Steenkamp, 1983; McKeen et al., 1983). The subunits, α and β, have M, values of 32,000 and 28,000, respectively. This electron transferase is unusual in that it contains dissimilar subunits and a single redox centre. Gorelick & Thorpe (1986) have reconstituted apoETF with FAD analogues to show that the region of C-8 is exposed to solvent. Photolyis of the 8-azido-FAD ETF derivative resulted in a low extent of covalent cross-linking (= 10%) of the flavin analogue to the protein. Approximately 80% of the label was associated with the β-subunit. The remainder of the analogue was cross-linked to the α-subunit. Steenkamp (1987) has used bifunctional cross-linking reagents to covalently cross-link preferentially the β-subunit to GAD. The covalent complex(es) were not isolated nor shown to have electron transfer activity. Interestingly, ETF from several patients deficient in ETF activity have defective α-subunits (Ferrman & Goodman, 1985; Ikeda et al., 1986).

ETF and the acyl-CoA dehydrogenases are isolated with the soluble fraction of disrupted mitochondria, but ETF-QO is an integral membrane protein. ETF-QO (M = 68,000) is an iron–sulphur flavoprotein containing one equivalent of
Electron transfer mechanisms

The generally accepted catalytic mechanism of the dehydrogenases involves proton abstraction from C-2 of the substrate and hydride transfer from C-3 to the N-5 position of the flavin. Evidence for the proton abstraction was obtained from studies with active-site-directed inhibitors, 3-acetylenic acyl-CoAs (Fremuran, 1980; Beckmann & Fremuran, 1982), from kinetic investigation with 2-deuterated acyl-CoA which yields a primary isotope effect ($k_{H}/k_{D} = 2.5$) (Pohl et al., 1986) and the exchange of deuterium (or tritium) from solvent $^2$H$_2$O (or $^3$H$_2$O) with one of the C-2 protons in the absence of turnover (Ghisla et al., 1984; Ikeda et al., 1985). No exchange at C-3 is detected with the mammalian enzymes, but exchange at C-3 is detectable with two bacterial enzymes (Pohl et al., 1986; Gomes et al., 1981). Hydride transfer from C-3 to the N-5 position of the flavin is inferred from experiments employing GAD reconstituted with 5-deaza-FAD. The flavin analogue was used since protons at N-5 in FAD exchange with solvent. The reconstituted dehydrogenase, reduced with NaB$_3$H$_4$, transfers $^3$H to the C-3 position of crotonyl-CoA, exclusively, in the reduction to butyryl-CoA. The reaction proceeds with a deuterium isotope effect of 5 (Ghisla et al., 1984). Reduction of GAD with perdeuter-butyryl-CoA yields a very large isotope effect ($k_{H}/k_{D} = 30-50$) (Reinsch et al., 1986). This has been interpreted to reflect concerted deprotonation and hydride transfer. However, an alternative mechanism involving an intermediate C-2 substrate anion has also been suggested (Reinsch et al., 1980).

Gorelick et al. (1985) have proposed a minimal mechanism for the oxidative half-reaction of GAD with ETF which involves electron transfer in two ternary complexes to yield two equivalents of ETF flavin semiquinone.

\[
\begin{align*}
S + DH_{ox} & \rightleftharpoons DH_{ox}^- + P \\
DH_{ox}^- + \text{ETF} &= DH_{ox}^- + P + \text{ETF} \\
DH_{ox}^- + \text{ETF} & \rightleftharpoons DH_{ox}^- + P + \text{ETF}
\end{align*}
\]

Reduction of ETF is clearly an obligatory one-electron transfer (see also Gorelick & Thorpe, 1986; Gustafson et al., 1986). The reaction between GAD and ETF is dependent on complementary charge interactions between the proteins (Fremuran, 1980; Beckmann & Fremuran, 1983). Modification of four carboxylate residues per GAD subunit and a similar number of lysine residues on ETF and the pH and ionic strength dependencies of the oxidative half-reaction provided evidence for this electrostatic interaction. The fact that the ETF$_{c}$ is an azoreducing flavin semiquinone may also promote dissociation from the acyl-CoA dehydrogenases which are all acidic proteins. It has been proposed that ETF functions as a shuttle between the primary dehydrogenases and ETF-QO and contains a single site for electron transfer (Beckmann & Fremuran, 1985). The mechanism of the oxidative half-reaction of ETF with ETF-QO is still open to some question beyond the observation that it is also dependent on electrostatic interaction between the proteins (Beckmann & Fremuran, 1985b). It is clear that ETF$_{c}$ is the product of the reductive half-reaction of the primary dehydrogenases. The minimum rate of electron transfer from ETF$_{c}$ to ETF-QO is estimated to be about 200 s$^{-1}$ based on the observation that ETF-QO can catalyse the equilibration of ETF redox states (Beckmann & Fremuran, 1985a):

\[
2\text{ETF}_{c} \rightleftharpoons \text{ETF}_{c^-} + \text{ETF}_{c^+}
\]

\[
K = 1, \text{pH} = 7.8
\]

Ramsay et al. (1987) have suggested that the disproportion of ETF$_{c}$ could be an obligatory step in the electron transport pathway from acyl-CoA substrates to Q, since the rate is at least as fast as the overall rate of Q$_r$ reduction by acyl-CoA; however, this conclusion may be affected by the nature of the assay system in which ETF had to be reduced to the two-electron reduced form to prevent direct reduction of Q$_r$. We have examined electron transfer in submitochondrial particles from NADH or succinate to ETF via ETF-QO (Beckmann, 1987). Ubiquinone depletion of the particles completely abolished NADH- and succinate-ETF reductase activities and reconstitution of the particles with ubiquinone quantitatively restored these activities, indicating that ETF-QO reacts with the pool of ubiquinone accessible to complex I and II. Furthermore, the sole reduced ETF product was identified as ETF$_{c^-}$. The principle of microscopic reversibility would indicate that electron transfer from ETF to ETF-QO is also a one-electron transfer from ETF$_{c^-}$. The redox states of ETF-QO during the steady state of electron transfer have not been explicitly determined. However, Beckmann & Fremuran (1985a) have shown that the protein can be enzymically reduced only to the two-electron reduced state, although the protein can accommodate a maximum of three electrons when chemically or photo-chemically reduced. These data imply that the protein oscillates between one- and two-electron reduced states.

The oxidation of ETF-QO has been investigated using soluble, short-chain ubiquinone analogues and homologues. Ramsay et al. (1987) reported a turnover number of 115 s$^{-1}$ for ETF oxidation and we have obtained similar values with a range of soluble ubiquinone analogues and Q$_r$ and Q$_c$ in the presence of detergent, which presumably renders these latter quinones available to ETF-QO (F.E. Fremuran, unpublished work). The oxidation-reduction potentials of the flavin and iron-sulphur cluster (Ruzicka & Beinert, 1987; Beckmann & Fremuran, 1985a) suggest that electrons enter ETF-QO at the flavin site and exit at the cluster to ubiquinone. In a separate experiment, we have resorted to the relatively harsh procedure of treating ETF-QO with p-chloromercuri-sulphonate to remove the cluster which is sequestered from solvent (Beckmann & Fremuran, 1985a). After this procedure, in which the flavin is quantitatively retained and the cluster is destroyed, quinone reductase activity is abolished, but the protein retains 20-30% of the capacity to completely reduce ETF$_{c}$ and ETF$_{c^-}$ (F.E. Fremuran, unpublished work). ETF-QO reduces quinones to the corresponding quinols without the requirement for a Q-binding protein as in the case of succinate-ubiquinone oxidoreductase (Ackrell et al., 1980; Yu & Yu, 1980). The demonstration that antimycin and myxothiazole inhibit reduction of cytochromes $b$ and $c_1$ by ETF-QO in submitochondrial particles (Fremuran, 1987) according to the same pattern observed for inhibition of NADH and succinate reduction of the cytochromes indicates that ubiquinol is the product of the oxidative half-reaction of ETF-QO according to current understanding of the Q-cycle in complex III (Rich, 1986).

Finally, the reversibility of electron transfer through this system of flavoproteins indicates that fatty acid $\beta$-oxidation...
as well as the other ETF-linked oxidations may be controlled by the redox poise of the ubiquinone pool.


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Comparative enzymology of β-oxidation


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Occurrence of β-oxidation

β-Oxidation is a common pathway for the degradation of saturated and unsaturated fatty acids in eukaryotes and prokaryocytes (Schulz, 1985). Much of the earlier information concerning molecular and enzymic properties of β-oxidation enzymes was derived from studies of mitochondrial fractions from higher animals (Greville & Tubbs, 1968). These investigations revealed the functional relationship between fatty acid degradation and ATP production in mitochondria. Thus, by extrapolation, β-oxidation has widely been considered as a pathway predominantly occurring in the mitochondria of all eukaryotic cells. On the other hand, during the last two decades one example of non-mitochondrial β-oxidation had been known to exist, namely in germinating oil seeds (Cooper & Beevers, 1969). Two striking discoveries drastically changed the seemingly simple cell biology of β-oxidation. In 1968 a second β-oxidation system of animal cells was discovered in peroxisomes which has attracted considerable attention during the last 10 years (Lazarow & de Duve, 1976). Recently, it was observed that inborn deficiencies of peroxosomal β-oxidation enzymes may lead to severe human diseases (Schüttgens et al., 1986).

Present knowledge suggests (Table 1) that mitochondrial β-oxidation is mainly restricted to the animal kingdom. Some exceptions have been reported, but require further direct evidence (Wood et al., 1986). Non-mitochondrial β-oxidation is more widely distributed and has been detected in animals (Hashimoto, 1982), plants (Tolbert, 1981) and eukaryotic micro-organisms (Tanaka et al., 1982; Kunau et al., 1987). Indirect evidence suggests that β-oxidation is also a widely distributed metabolic pathway in prokaryocytes. Surprisingly, only the β-oxidation system of Escherichia coli has so far been characterized in molecular (Yang & Schulz, 1983) and genetic terms (Nunn, 1986). A characteristic part of it is a protein complex comprising five enzyme activities on two different polypeptides. Very recently, we isolated complexes with subunit sizes similar to E. coli from Achromobacter calcoaceticus (Schröder, 1987) and five different Pseudomonas strains (C. Burmann & W. H. Kunau, unpublished work).

Experimental work in our own laboratory is aimed at the molecular structure of non-mitochondrial β-oxidation proteins in eukaryotic micro-organisms. Owing to their accessibility to genetic and recombinant DNA methodology, some of these organisms offer an excellent opportunity to study the biogenesis of microbodies. This paper discusses primarily our results on fungal β-oxidation systems.

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