Preliminary evidence for the existence of specific functional assemblies between enzymes of the β-oxidation pathway and the respiratory chain

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The electron-transferring flavoprotein (ETF) has been detected in two large soluble-protein complexes partially purified from sonicated porcine liver mitochondria. Size-exclusion chromatography and sucrose-density ultracentrifugation suggested molecular masses in the region of 390 to 420 kDa for the two complexes. Activities of medium-chain acyl-CoA dehydrogenase, sarcosine dehydrogenase and ETF:ubiquinone oxidoreductase were also detected. No evidence of oxidative-phosphorylation properties was obtained. Treatment with antimycin A inhibited the activity of both complexes. Pyridine haemochromogens, prepared from the partially purified species, show the presence of cytochrome proteins. The possible composition of these complexes and their relationship to the electron transport chain are discussed.

Key words: electron-transferring flavoprotein, medium-chain acyl-CoA dehydrogenase, multi-enzyme complexes, sarcosine dehydrogenase.

INTRODUCTION

The electron-transferring flavoprotein (ETF) is the physiological electron acceptor for at least nine mitochondrial matrix dehydrogenases [1]. As well as the four straight-chain acyl-CoA dehydrogenases (short, medium, long and very long), involved in the β-oxidation pathway [2–6], ETF serves as an electron acceptor for several dehydrogenases involved in amino acid catabolism (isovaleryl-CoA [7], glutaryl-CoA [8] and short/branched-chain acyl-CoA dehydrogenases [9]) and choline catabolism (sarcosine dehydrogenase [SDH] and dimethylglycine dehydrogenase [10]). In mammals the reducing equivalents from these dehydrogenases are transferred via ETF to ETF:ubiquinone oxidoreductase (ETF-QO) [11]. The electrons are further passed from ETF-QO into the electron transport chain via coenzyme Q (CoQ) to the $CoQH_2$-cytochrome *c* oxidoreductase (Complex III).

Physiologically, other major ports of entry to the electron transport chain, apart from ETF, are via NADH-CoQ oxidoreductase (Complex I) and succinate-CoQ oxidoreductase (Complex II). Complexes I–IV of the electron transport chain have been isolated and studied [12–15] as have larger assemblies, e.g. mitochondrial succinate:cytochrome *c* oxidoreductase (Complexes II and III), and the electron transport particle ('ETP') [16], an electron transfer unit containing Complexes I–IV.

The 'soluble' mammalian mitochondrial enzymes that catalyse fatty acid oxidation bind to the inner mitochondrial membrane [17–21] and thus locate partially in the insoluble fraction during cell fractionation. β-Hydroxyacyl-CoA dehydrogenase, catalysing the third step in β-oxidation, has also been found complexed to a protein responsible for its association with the inner mitochondrial membrane [20]. Sumegi and Srere [19] presented data showing that this protein also binds to Complex I, thus suggesting a ternary complex linking the β-oxidation pathway and the respiratory chain. An analogous complex between the acyl-CoA dehydrogenases, ETF, ETF-QO and Complex III was also suggested [21]. No ETF complexes with SDH or dimethylglycine dehydrogenase have been detected thus far, however. ETF has been detected in a complex with a short-chain acyl-CoA dehydrogenase, in extracts of the anaerobic bacteria *Megasphaera elsdenii* [22], but there has been no previous evidence of such complexes in mammalian systems.

The present investigation shows, for the first time, that mammalian ETF forms stable, soluble complexes with its partner dehydrogenases. It seems likely that these complexes are present also within the mitochondria and that ETF is an intrinsic, albeit easily detached, component of the electron transport chain.

MATERIALS AND METHODS

General materials

Butyryl-CoA, Coomassie Brilliant Blue R-250, dimethylglycine hydrochloride, Freund's adjuvant, molybdenum powder, molybdenum trioxide, nitrocellulose membrane, *p*-Iodonitrotetrazolium Violet, octanoyl-CoA, palmitoyl-CoA, phenazine methosulphate ('PMS'), phosphatidylcholine, sarcosine hydrochloride, Sephadex G150, Tris base, Triton X-100 and trypsin were purchased from Sigma Chemical Co. (Littlehampton, West Sussex, U.K). DEAE-cellulose 52 and Sephacryl S-300 gel filtration media were supplied by Whatman, and Pharmacia Biotech Ltd. respectively. Chloroform, glacial acetic acid, methanol, pyridine and sulphuric acid were purchased from BDH Chemicals Ltd (Poole, Dorset, U.K.). Octanoyl-*N*-acetyl cysteamine ('ONAC') was synthesized according to Kass and Brock [23]. Calibration proteins for gel filtration were: ferritin (450 kDa) from Boehringer Mannheim (Lewes, East Sussex, U.K.); β-amylase (200 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and cytochrome *c* (12.5 kDa) from Sigma Chemical Co.

Abbreviations used: BCD, butyryl-CoA dehydrogenase; ETF, electron transferring flavoprotein; ETF-QO, ETF:ubiquinone oxidoreductase; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain AD; SC, short-chain AD; SDH, sarcosine dehydrogenase; CoQ, coenzyme Q; Complex I, NADH-CoQ oxidoreductase; Complex II, succinate-CoQ oxidoreductase; Complex III, $CoQH_2$-cytochrome *c* oxidoreductase; Complex IV, cytochrome *c* oxidase; DCPIP, dichlorophenolindophenol.

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Purification of SDH

SDH was purified as reported previously [24].

Preparation of mitochondrial samples

Mitochondria prepared from pig liver (2 kg) [25], were on one occasion split into 40 aliquots for complex detection and on a separate occasion split into 8 aliquots for partial purification, in both cases using 10 mM potassium phosphate, pH 7.0, and stored at −20 °C. For use, each aliquot was thawed, diluted to 25 ml in 10 mM potassium phosphate, pH 7.0, at room temperature, hand homogenized in a Potter-Elvehjem homogenizer and sonicated using a ultrasonic processor (Milsonix Inc., Pittsburgh, PA, U.S.A.) at maximum setting 5, 50 % pulse cycle, for 30 s, cooled for 9 min and sonicated again for 20 s. The solution was centrifuged, at 30700 ***g*** for 20 min at 4 °C for complex detection, and 100000 ***g*** for 1 h at 4 °C for partial purification. The supernatant in both cases was termed the 'soluble protein fraction'.

Detection of ETF-containing complexes

The soluble protein fraction (368 mg in 8 ml) was diluted with 10 mM potassium phosphate, pH 7.0, alone or containing 100 mM sarcosine, 100 mM dimethylglycine or 10 mM octanoyl-*N*-acetyl cysteamine, and applied to a calibrated Sephadex G150 gel-filtration column (2.6 cm × 73 cm) equilibrated with 10 mM potassium phosphate, pH 7.0. For oxidized conditions the diluted extract was left stirring for 12–24 h at 4 °C prior to chromatography. The flow rate through the G150 column was 0.625 ml/min and 10 min fractions were collected.

Enzyme assays

Activities of ETF, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain AD (MCAD), short-chain AD (SCAD) and SDH

The activities of ETF, LCAD, MCAD, SCAD and SDH were determined spectrophotometrically at 25 °C (Uvikon 941 Plus; Kontron Instruments, Neufahrn, Germany) by measuring the reduction of dichlorophenolindophenol (DCPIP) at 600 nm (molar absorption coefficient of 21000 $M^{-1} \cdot cm^{-1}$ [26]) as reported previously [24,26–28]. To minimize non-specific DCPIP reduction [27] the blank contained buffer instead of the substrates.

SDH–ETF complex activity

To each 1 ml cuvette were added 200 mM Tris/HCl pH 8.3, 60 μM DCPIP, 5 mM KCN pH 8.0, an aliquot of enzyme and H_2O. A control with sarcosine instead of complex preparation was run concurrently. The assay was carried out at 25 °C.

ETF-QO activity

The activity of ETF-QO was determined spectrophotometrically at 25 °C (Uvikon 941 Plus; Kontron Instruments, Neufahrn, Germany) by measuring the reduction of Nitro Blue Tetrazolium to formazan at 530 nm (absorption coefficient of 19.8 $mM^{-1} \cdot cm^{-1}$), as reported previously [29,30].

Partial purification of two SDH–ETF complexes: Fractions A and B

A 5 ml aliquot of buffer A (10 mM potassium phosphate/125 mM sarcosine, pH 7.0) was added to the soluble protein fraction (one stored aliquot) which was then applied to a DEAE-cellulose 52 column (4 cm × 4.5 cm) equilibrated with buffer B (10 mM potassium phosphate/50 mM sarcosine, pH 7.0) and 10 ml fractions were collected. Fraction A eluted in the wash and was concentrated by ultrafiltration through a Diaflo fitted with a PM30 membrane (Amicon Instruments) to 15 ml, diluted to 20 ml with buffer A, and then applied to a calibrated Sephacryl-S-300 gel filtration column (2.5 cm × 100 cm) equilibrated in buffer B. Fractions of 13 ml were collected. For elution of fraction B, the DEAE-cellulose 52 column was washed with buffer B containing 0.5 M KCl, concentrated and treated similarly to fraction A.

Ultracentrifugation

For the determination of sedimentation coefficients a Beckman (Fullerton, CA, U.S.A.) L755 Ultracentrifuge with SW28 swing-out rotor buckets was operated at 50000 ***g*** and 4 °C using the 5–20 % (w/v) sucrose-density gradient method of Martin and Ames [31]. The sedimentation coefficient in the buffer (10 mM potassium phosphate/50 mM sarcosine, pH 7.0), $s_{T,b}$, was corrected to that at 20 °C in water, $s_{20,w}$, as described previously [31]. The protein concentration of loaded samples was 0.5 mg/ml. Catalase (250 kDa) was used as a control [31].

Preparation of pyridine haemochromogens

To 1 ml of complexes A and B were added 0.2 ml of acetone and 0.05 ml of 2.4 M HCl. After centrifugation at 30000 ***g*** for 15 min, the supernatant and pellet were stored separately. This was repeated three times. For cytochromes *a* and *b* the supernatants from three extractions were freeze-dried, reconstituted in pyridine/0.2 M KCl (1:1, v/v) and the absorption spectra were measured. No attempt was made to separate cytochromes *a* and *b*. For cytochromes *c* and c_1, the pellets from the three extractions were washed with 0.1 M KCl (cytochrome *c* is washed off the preparation with the KCl), combined, centrifuged, and the cytochrome c_1 was reconstituted in pyridine/0.2 M KCl (1:1, v/v).

Native PAGE and activity staining for SDH

The electrophoresis of complexes A and B on non-denaturing polyacrylamide gels was based on the denaturing discontinuous system of Laemmli [32]. Resolving gels of 7.5 % (w/v) were used with a 4.5 % (w/v) stacking gel in the absence of SDS. After electrophoresis, the gels were removed and one of them was stained with an aqueous solution containing 0.1 % Coomassie Brilliant Blue R-250, 40 % (v/v) methanol and 10 % (v/v) acetic acid for 5 min. The gel was destained in a solution of 40 % (v/v) methanol and 10 % (v/v) acetic acid. The other gel was added to an SDH activity stain (150 mM sarcosine pH 7.5, 200 mM Tris/HCl, pH 8.3, 5 mM KCN, pH 8.0, 15 mg *p*-Iodonitrotetrazolium Violet and water in a final volume of 50 ml) and left overnight in the dark at room temperature.

Preparation of rabbit anti-ETF and anti-MCAD sera

Human recombinant ETF, purified according to Husain and Steenkamp [33], was mixed with Freund's complete adjuvant and the ETF (0.3 mg) was injected into two rabbits. The first injections were followed by one booster of 0.3 mg after 4 weeks. The anti-ETF antibody was partially purified according to Cooper and Paterson [34]. The anti-(human MCAD) antibody was a gift from Dr. Peter Bross (Center for Medical Molecular Biology, Faculty of Health Science, University of Aarhus, Aarhus, Denmark).

Western blot analysis

Complexes A and B subjected to SDS/PAGE (12% gel) [32] were transferred on to nitrocellulose. ETF and MCAD were detected using the appropriate antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin.

Detection of phospholipids using TLC

Phospholipids were extracted from complexes A and B by mixing them with chloroform/methanol (2:1, w/v). The organic phase was evaporated overnight and the phospholipids were reconstituted in chloroform. Concentrated samples of chloroform-treated complexes A and B (5–10 μl), phosphatidylcholine (5 μl) and ETF (57 μM) were spotted on to a TLC plate (silica gel on aluminium). The solvent system contained chloroform/methanol/glacial acetic acid/water (25:15:2:2, by vol.). After 10 min the plates were removed and briefly sprayed with Molybdenum Blue reagent [35].

RESULTS

Purification of SDH and the detection of ETF-containing complexes

During the routine purification of SDH, activity eluted from the DEAE-cellulose column at two different salt concentrations (Figure 1). The same observation was made by Wittner and Wagner [24] who suggested the additional peak was due to separation of a second protein utilizing sarcosine as its substrate. ETF activity was not monitored. In the present study the first SDH peak co-eluted with ETF activity, suggesting that these fractions might contain an ETF–SDH complex.

Detection of ETF-containing complexes on gel filtration media

Figures 2(A) and 3(A) show that, during gel filtration of the mitochondrial extract, ETF, MCAD and SDH each elute as more than one peak of activity with the areas of activity overlapping. Peaks of SDH and ETF activity were found around 325 ml (peak 1) and MCAD at 288 ml, consistent with the molecular mass values for the free proteins, i.e. ETF, 60 kDa [25]; SDH, 100 kDa [24] and MCAD, 140–180 kDa [28]. Peaks of SDH and MCAD activity were also observed, however, around 135 ml (peak 2), implying a molecular mass of at least 300 kDa. ETF and ETF-QO activities were also detected in this fraction thus showing that at least part of the ETF exists in high-molecular-mass complexes, possibly with MCAD and SDH.

To study the possible effect of substrate reduction on formation of these complexes, sarcosine, dimethylglycine and octanoyl-*N*-acetyl cysteamine, a substrate analogue for MCAD, were added prior to gel filtration. Reduction of the extract with sarcosine or dimethylglycine led to the elution of two new peaks of SDH activity associated with ETF activity (Figures 2B and 2C, peaks 3 and 4) corresponding to molecular masses of 200 kDa and 158 kDa. ETF-QO activity was also detected in peak 3, suggesting the formation of SDH–ETF and SDH–ETF–ETF-QO complexes

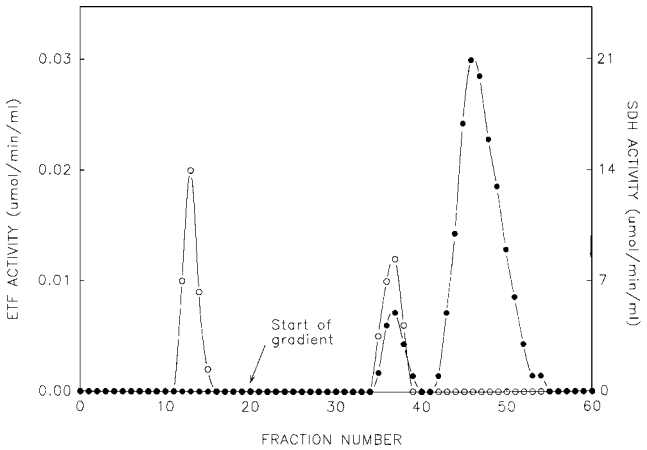

Figure 1 DEAE-cellulose chromatography of SDH

SDH was purified as reported previously [24]. The pooled fractions from a Sephadex G150 gel-filtration column were concentrated and applied to a DEAE-52-cellulose column (3 cm × 15 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM PMSF. After washing the column with equilibration buffer a 0–0.5 M gradient of KCl in equilibration buffer (1 litre total volume) was then applied. The column was run at 0.5 ml/min and fractions of approx. 10 ml were collected. Aliquots were withdrawn and assayed for ETF (○) and SDH (●) activities as described in the Materials and methods section.

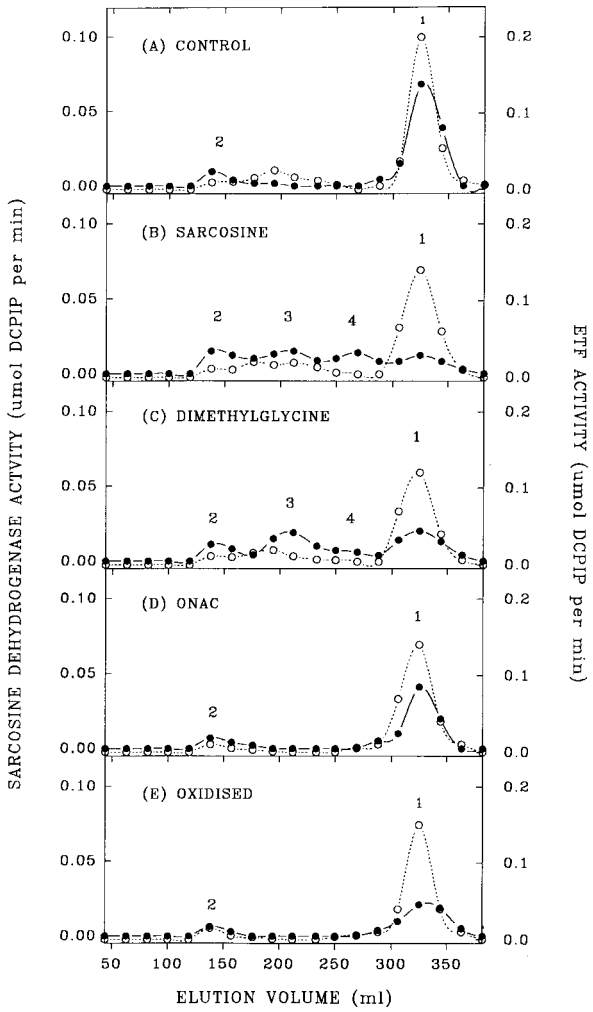

Figure 2 Detection of SDH–ETF complexes

The soluble protein fraction was diluted to 46 mg/ml in 10 mM potassium phosphate, pH 7.0, alone or containing reductants and applied to a calibrated Sephadex G150 gel filtration column (2.6 cm × 73 cm) equilibrated with 10 mM potassium phosphate, pH 7.0. For oxidized conditions the diluted extract was left stirring for 12–24 h at 4 °C. The flow rate was 0.625 ml/min and fractions were collected every 10 min. Aliquots were withdrawn and assayed for ETF (○), ETF-QO and SDH (●) activities.

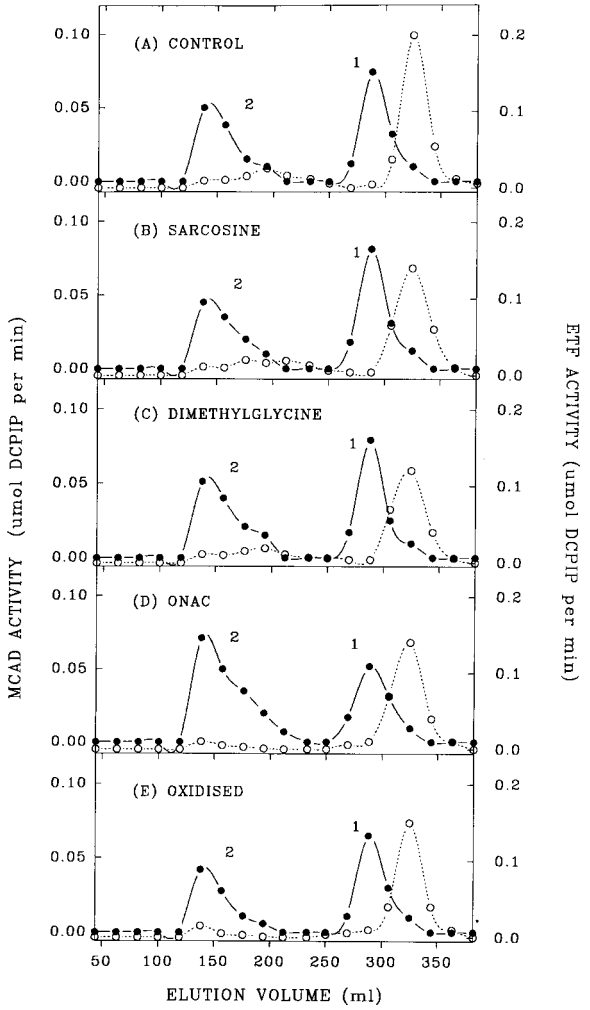

Figure 3 Detection of MCAD–ETF complexes

The soluble protein fraction was diluted to 46 mg/ml in 10 mM potassium phosphate, pH 7.0, alone or containing reductants and applied to a calibrated Sephadex G150 gel-filtration column (2.6 cm × 73 cm) equilibrated with 10 mM potassium phosphate, pH 7.0. For oxidized conditions the diluted extract was left stirring for 12–24 h at 4 °C. The flow rate was 0.625 ml/min and fractions were collected every 10 min. Aliquots were withdrawn and assayed for ETF (○), ETF-QO and MCAD (●) activities.

respectively. The ratio of activities of free SDH (peak 1) to high-molecular-mass SDH (peaks 2, 3 and 4) decreased from 7:1 to 0.5–0.9:1 in the presence of sarcosine. No new peaks of MCAD were detected (Figures 3B and 3C).

Excess octanoyl-*N*-acetyl cysteamine abolished all the higher-molecular-mass peaks of SDH activity except for peak 2 (Figure 2D), whereas the proportion of the MCAD activity in the fraction eluting at 135 ml increased. It is possible that the shoulder of MCAD activity (Figures 3A, 3B, 3C and 3E, volume 200 ml) is an MCAD–ETF complex (molecular mass 200 kDa). In the presence of octanoyl-*N*-acetyl cysteamine (Figure 3D) the shoulder appeared at 175 ml (molecular mass approx. 250 kDa) suggesting the formation of a larger complex under these conditions. Unlike the situation found with extracts of *M. elsdenii* [22], where the butyryl-CoA dehydrogenase–ETF complex dissociated readily under oxidizing conditions, here the ETF-containing complexes, once formed, appear to be relatively stable (Figures 2E and 3E). Peaks 3 and 4, however, corresponding to the SDH–ETF and SDH–ETF–ETF-QO complexes, disappeared during oxidation.

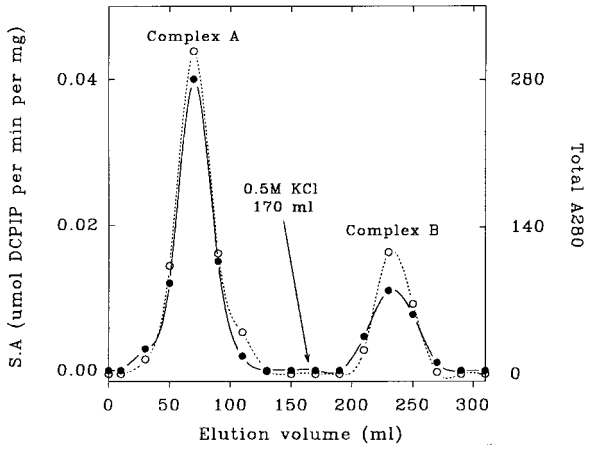

Figure 4 Elution profiles of complexes A and B from DEAE-cellulose 52

The soluble protein fraction was diluted with 5 ml of buffer A and applied to a DEAE-cellulose 52 column (4 cm × 5.5 cm) equilibrated with buffer B. The flow rate was approx. 3 ml/min and 10 ml fractions were collected. Aliquots were withdrawn and assayed for ETF–SDH complex activity (S.A.; ●) and protein (○).

Although there was a well-defined peak for free ETF in all the elution profiles, it is noteworthy that the peaks of dehydrogenase activity were also associated with ETF activity. Although this does not necessarily prove a physical association with the dehydrogenase in each case, it clearly indicates ETF forming part of a high-molecular-mass complex. Bearing this in mind it is worth noting that ETF interacts with ten electron donors and acceptors, of which only three were assayed for in this study. The activities of SCAD and LCAD were also measured, however, and behaved in a similar fashion to MCAD (results not shown). When measured at pH 6.0 or 8.0, instead of pH 7.0, elution profiles for ETF and the various dehydrogenases showed no significant difference (results not shown).

Partial purification of complexes A and B

Partial purification was attempted only on the higher-molecular-mass complexes (> 300 kDa) since, although all the activities detected may represent complexes in their own right, it could not be ruled out that they were in fact fragments of the larger complexes. Anion-exchange chromatography under reducing conditions yielded two ETF-containing fractions, one that did not bind to DEAE-cellulose 52 (Figure 4, complex A) and a second fraction that did bind (complex B). Both fractions contained SDH activity. It is of interest in this context that, unlike SDH [10,24], mammalian ETF does not bind to DEAE-cellulose [25,33] in Hepes, Tris/HCl or potassium phosphate buffers. Complexes A and B were subjected to size-exclusion chromatography on a Sephacryl S-300 column (Figure 5). Under the conditions used (10 mM potassium phosphate/50 mM sarcosine, pH 7.0) only two molecular mass markers eluted from the column, ferritin (450 kDa) and alcohol dehydrogenase (150 kDa), owing to the interaction of the others with the column, and therefore the inferred molecular mass values are to be treated with caution. Peaks of elution for the markers were 273 ml (ferritin) and 455 ml (alcohol dehydrogenase) and for complexes A and B, 325 ml and 299 ml respectively. The molecular masses of complexes A and B were accordingly estimated

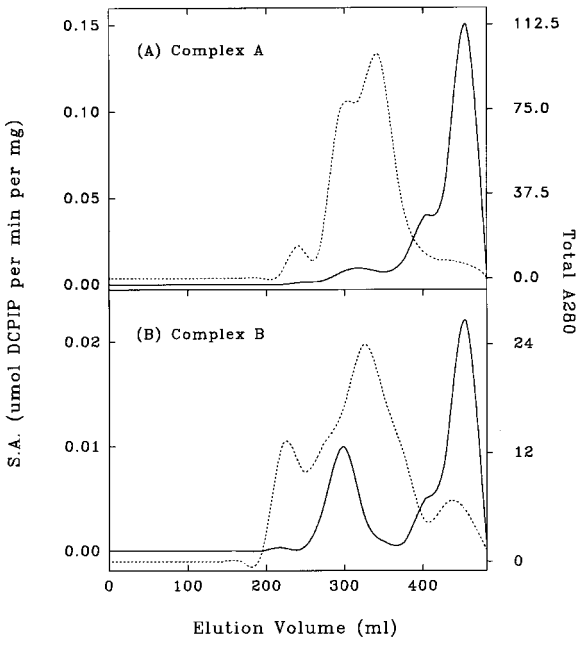

Figure 5 Elution of complex A and B from a Sephacryl-S-300 gel-filtration column

The sample was diluted from 15 to 20 ml with buffer A and applied to a Sephacryl S-300 gel-filtration column (2.5 cm × 100 cm) equilibrated in buffer B. The flow rate was 0.65 ml/min and 13 ml fractions were collected. Aliquots were withdrawn and assayed for ETF–SDH complex activity (solid line) and protein (dashed line).

to be 375 kDa and 410 kDa respectively. The shoulder of activity around 400 ml (molecular mass approx. 200–250 kDa) (Figure 5) contains the other SDH–ETF complexes detected on the Sephadex G150 gel-filtration column but separation of the lower-molecular-mass complexes was not attempted. The loss of ETF at each stage of purification in other complexes and as free protein resulted in a very low overall purification factor for both complexes A and B as highlighted by Wittner and Wagner [24] (see Table 1 in reference) and so purification was followed by both native and SDS/PAGE (results not shown).

Characterization of the ETF-containing complexes A and B

Western blot analysis, TLC and SDH activity staining: presence of SDH, MCAD, ETF and phospholipids

The specific immunological detection of ETF and MCAD in complexes A and B (Figure 6) showed that the activities measured were due to these enzymes and not to the promiscuity of the DCPIP assays [27]. The SDS gel showed impurities in the sample which were not part of the complexes. TLC showed evidence for the presence of phospholipids in the preparation with complexes A and B (results not shown). This would be consistent with the presence of membrane-bound components such as ETF-QO and Complex III. The control prepared from purified ETF gave a negative test for phospholipid. On native 7.5% PAGE gels (results not shown) activity staining for SDH revealed that all the activity in complexes A and B was associated with a band that had not entered the gel, consistent with complexes of high molecular mass.

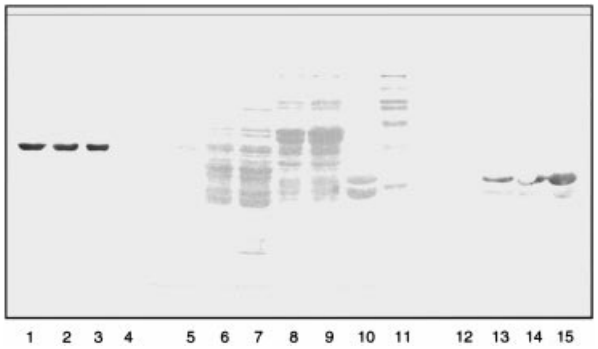

Figure 6 Immunoblots of ETF and MCAD

Complexes A and B were subjected to SDS/PAGE on 12% gels as described [32], and transferred on to a nitrocellulose membrane. ETF and MCAD were detected with the appropriate antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. MCAD immunoblot (lanes 1–4): lane 1, MCAD control; lanes 2 and 3, complexes A and B; lane 4, ETF control. Coomassie Blue-stained SDS gel (lanes 5–11): lane 5, MCAD control (10 μg); lanes 6 and 7, 5 μl and 10 μl complex A; lanes 8 and 9, 5 μl and 10 μl complex B; lane 10, ETF control (20 μg); lane 11, molecular mass markers (Sigma MW-SDS-200). ETF immunoblot (lanes 12–15): lane 12, MCAD control, lanes 13 and 14, complexes A and B; lane 15, ETF control.

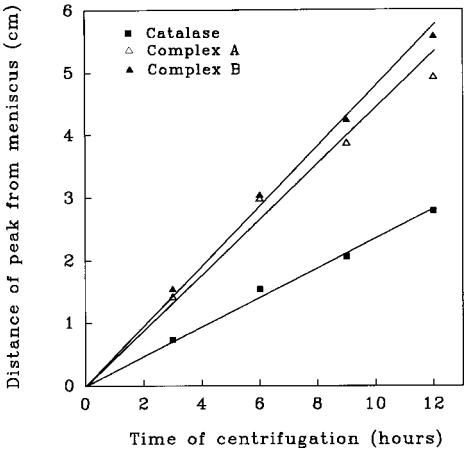

Figure 7 Ultracentrifugation of complexes A and B

Sucrose density gradient ultracentrifugation was performed at 50000 ***g*** and 4 °C. Distance from the meniscus was calculated by recording the activity of the ETF–SDH complex.

Specificity of the ETF-containing complexes

The pH and ionic strength of the buffers were maintained at 7.0 and 10 mM respectively, since the enzymes of the β-oxidation pathway have been shown to bind optimally and specifically in a complex formation using these conditions [17–21]. The low ionic strength minimizes non-specific self-assembly of proteins [36] and in this case does not affect the proven electrostatic interactions between individual members of complexes A and B [29,30,37]. The use of substrates in the buffers (e.g. sarcosine in the present case) has previously been shown to promote the specific association between enzymes of the citric acid cycle [38,39].

Estimation of molecular mass using ultracentrifugation

Figure 7 shows the results of sucrose-density-gradient ultracentrifugation for complexes A and B monitored by catalytic

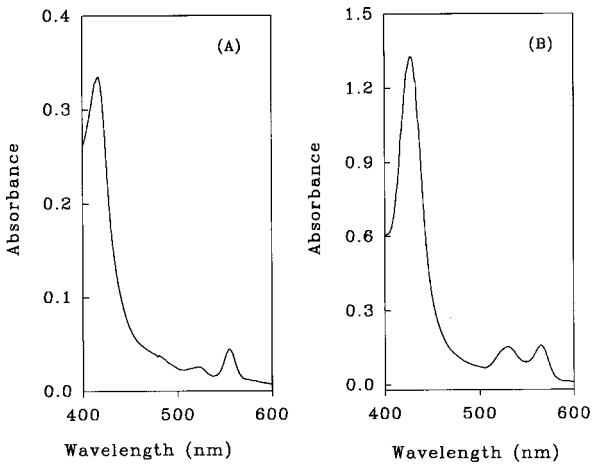

Figure 8 Pyridine haemochromogens of cytochromes *a* and *b* and c_1

To 1 ml of complex A and B were added 0.2 ml of acetone and 0.05 ml of 2.4 M HCl. After centrifugation, the supernatant and pellet were stored separately. (**A**) For cytochrome c_1, the pellet was washed with 0.1 M KCl (to remove cytochrome *c*), centrifuged and reconstituted in pyridine/0.2 M KCl (1:1, v/v) and the absorption spectra were measured. (**B**) For cytochromes *a* and *b*, the supernatant was freeze-dried, reconstituted in pyridine/0.2 M KCl (1:1, v/v) and the absorption spectra were measured.

assays for SDH–ETF activity. The sedimentation coefficients, $s_{20,w}$, determined for complexes A and B were 17.5 S and 18.6 S respectively. The $s_{20,w}$ for the catalase standard, molecular mass 250 kDa, was calculated to be 11.21 S [31]. Although ETF is heterodimeric [1], if we make the arbitrary assumption that the complexes are spherical and use the determined $s_{20,w}$ of 11.21 S for catalase, the approximate molecular masses of complexes A and B can be calculated to be 390 kDa and 415 kDa respectively, which is in close agreement with the molecular masses estimated from the calibrated Sephacryl-S-300 column. No other coloured bands were present in the gradients. Although lipids were detected, the composition was not quantified and so the density of the complex-associated lipid was not considered in the estimation of complexes A and B.

Pyridine haemochromogens: confirmation of cytochrome species present

The pyridine haemochromogens of complexes A and B also showed the presence of cytochromes in both preparations. The pyridine haemochromogen of cytochromes *a* and *b* for complex A (Figure 8) was mainly due to cytochrome *b*. Although trace amounts of cytochrome *a* may be present [cytochrome *c* oxidase (Complex IV); molecular mass 600 kDa] it is definitely not part of the SDH–ETF complex since the molecular mass does not exceed 1 MDa. The maxima of the Soret, β and α bands were at 428, 530 and 565 nm respectively. Cytochrome *a* contains shifted Soret and α bands at 439–443 and 600–604 nm respectively accompanied by the absence of the β band. The cytochrome c/c_1 spectrum is mainly due to c_1. No cytochrome *c* was present in the wash with 0.1 M KCl. It was therefore assumed that cytochrome *c* was not present, as described previously [16].

DISCUSSION

The β-oxidation of fatty acids involves the repetitive processing of a dwindling acyl chain through a cycle of four reactions involving oxidation, hydration, a second oxidation and then thiolysis, which provides acetyl-CoA and a ready primed acyl-CoA for the next turn of the reaction sequence. It has been suggested, from time to time, that substrate channelling [40] in such a cascade may occur and in this context it is of interest that in *Escherichia coli* some of the β-oxidation enzymes that are separate entities in mammalian mitochondria are in fact gene-fused to form a multifunctional enzyme [41]. This enzyme catalyses three of the four steps listed above. The one that is missing is the flavoprotein acyl-CoA dehydrogenase, which catalyses the first of the four steps and has an inescapable requirement for interaction with another protein, its electron acceptor, ETF. The question remains, however, how many components of the β-oxidation pathway are freely diffusible.

It has been reported that some metabolically sequential enzymes in the citric acid cycle [38,39] and in amino acid catabolism [42] are found in a specific association with one another. ETF, acyl-CoA dehydrogenases and the other proteins of the β-oxidation pathway, however, have always been regarded as soluble matrix proteins [43]. Nevertheless it has been shown that these enzymes form a specific association with both the inner mitochondrial membrane and Complexes I and III of the respiratory chain *in situ* [18–21]. Since the binding of the β-oxidation enzymes to the inner mitochondrial membrane is saturable [18] it is therefore reasonable to suppose that these enzymes exist as both bound complexes and free soluble proteins.

Proponents of substrate channelling would argue that even the acyl-CoA substrate is not freely diffusible. The reduced acyl-CoA dehydrogenase has to deliver its reducing equivalents via ETF to the electron transport chain and the surest way of doing this would be to have a stable complex between acyl-CoA dehydrogenase, ETF and Complex III. This, however, overlooks the role of ETF as an entry point to the electron transport chain for several other dehydrogenases. Unless ETF is present in sufficient excess to cater separately for all these dehydrogenases, committed, stable 1:1 complexes would block the action of some of them. It is interesting to note that Lehman and Thorpe have isolated two types of mammalian ETF [44]. Possibly the second type of ETF is in fact present in larger complexes while the normal type is present as the free soluble protein.

The acyl-CoA dehydrogenase molecule also has a remarkable adaptation that argues against the idea of a stable complex with ETF. Chemically reduced acyl-CoA dehydrogenase is readily auto-oxidizable, but substrate-reduced acyl-CoA dehydrogenase is extremely stable to oxygen [45]. This property can be seen as biologically essential if reduced acyl-CoA dehydrogenase has to travel through an aerobic environment to reach ETF. It seems redundant, however, if acyl-CoA dehydrogenase is tightly associated with ETF. A further argument comes from the mechanism of the acyl-CoA dehydrogenase reaction which requires the two electrons to be removed in two separate steps, i.e. steps probably involving two separate ETF molecules [46].

Coming to ETF, the question is whether it acts as a mobile carrier between various dehydrogenases and the electron transport chain or whether it is physically attached to the chain and awaits the arrival of the client dehydrogenase molecules. Whilst there has been no evidence offered on this point, it may be argued that the speed of the process would be severely diminished if it depended on two independent protein diffusion steps.

If one considers the possible composition of the complexes with MCAD and SDH, an arithmetically 1:1:1 complex of all three proteins would add up to a molecular mass of about 300 kDa but it is more likely that MCAD and SDH form separate complexes with ETF. ETF and SDH alone in a 1:1 complex would only add up to 160 kDa and therefore, with no other protein components present, a molecular mass of 320 kDa could only be accommodated by postulating a dimer of this 1:1

complex, for which there is no other evidence. On the other hand, the presence of ETF-QO activity offers an obvious alternative explanation of a high-molecular-mass complex, namely a functional complex of SDH, ETF and ETF-QO. These three proteins alone would give a molecular mass of 230 kDa. Although the electron transport particle, containing the membrane-bound Complexes III and IV, can be released via sonic oscillation [15], ETF-QO has not previously been successfully removed without the use of detergent [11,30]. However, it is not unreasonable to suggest that the ETF-QO activity in these fractions was also associated with other components of the electron transport chain.

Turning to the nature of a high-molecular-mass complex containing MCAD it has to be borne in mind that MCAD is a homotetramer. Although arithmetic combinations of one MCAD and three or four molecules of ETF may be prevented by steric hindrance, complexes of one dehydrogenase and one or two ETF molecules have been reported [22,47]. Here again, however, the presence of ETF-QO activity shows a ternary complex of MCAD, ETF and ETF-QO.

In summary our preliminary results show the partial purification of two macromolecular assemblies consisting of either SDH (100 kDa [24]) or MCAD (140–180 kDa [28]) together with ETF (60 kDa [25]), ETF-QO (64 kDa [30]), CoQ (1 kDa [48]) and Complex III, containing cytochrome *b* and c_1, (200 kDa [48]) with a molecular mass of approximately 430 kDa. It seems possible that these complexes represent fragments of the structures within which the oxidoreductases function physiologically in the mitochondrion.

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