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Electron-transfer flavoprotein from anaerobic *Ascaris suum* mitochondria and its role in NADH-dependent 2-methyl branched-chain enoyl-CoA reduction

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Electron-transfer flavoprotein was purified to apparent homogeneity from mitochondria of the parasitic nematode, *Ascaris suum*. The native molecular weight of the enzyme was 70000, as estimated by gel filtration, and it migrated as two bands with apparent subunit molecular weights of 37000 and 31500 during sodium dodecylsulfate polyacrylamide gel electrophoresis. The enzyme exhibited an absorption coefficient for the bound FAD of 13.5 $mM^{-1} \cdot cm^{-1}$ at 436 nm and a protein/flavin (270 nm/436 nm) ratio of 5.6. While the ascarid enzyme is similar to its mammalian counterpart, physiologically it functions in the reverse direction, shuttling reducing power from the electron-transport chain to a soluble 2-methyl branched-chain enoyl CoA reductase. Indeed, when *A. suum* submitochondrial particles were incubated with NADH, 2-methylcrotonyl-CoA and purified *A. suum* 2-methyl branched-chain enoyl-CoA reductase, 2-methylbutyryl-CoA formation was proportional to the amount of electron-transfer flavoprotein added.

Introduction

The mitochondrial energy metabolism of the adult parasitic nematode, *Ascaris suum*, is predominantly anaerobic and relies on unsaturated organic acids, such as fumarate or 2-methyl branched-chain enoyl-CoA's, as terminal electron acceptors [1]. The 2-methyl branched-chain fatty acids, 2-methylbutyrate and 2-methylvalerate, are major end products of ascarid carbohydrate metabolism and are formed by a reversal of β-oxidation [2,3]. Many of the enzymes in this pathway have been identified and appear to differ from the corresponding mammalian enzymes [4,5]. Recently, the final reaction, the NADH-dependent reduction of 2-methylcrotonyl-CoA or 2-methyl-2-pentenoyl-CoA has been demonstrated, and a soluble 2-methyl branched-chain enoyl-CoA reductase has been purified to apparent homogeneity from *A. suum* mitochondria [6,7]. However, the purified reductase is capable of catalyzing the NADH-dependent reduction of 2-methylcrotonyl-CoA only with the addition of ascarid submitochondrial particles and a second soluble component, which appears to be electron-transfer flavoprotein [6,7].

ETF is a soluble protein located in the mitochondrial matrix. In mammalian mitochondria, it plays an important role in the β-oxidation of fatty acids and oxidative demethylation reactions by coupling several flavoprotein dehydrogenases to the electron-transport chain at the level of a membrane-bound, iron-sulfur, flavoprotein, ETF dehydrogenase [8,9]. In contrast, in *A. suum* mitochondria, it appears that ETF shuttles reducing equivalents in the opposite direction and couples rotenone-sensitive, NADH oxidation with the soluble 2-methyl branched-chain enoyl-CoA reductase [6,7]. The present study was designed to purify and characterize ETF from *A. suum* mitochondria and to assess its role in NADH-dependent branched-chain enoyl-CoA reduction.

Experimental Procedures

Materials

CoA and CoA esters were purchased from P-L Biochemicals. All other chemicals were of reagent grade and obtained from Sigma. 2-Methylbutyryl-CoA was synthesized by way of the L-acylimidizole [10] and the 2-methyl branched-chain enoyl-CoA reductase was purified from *A. suum* mitochondria as described previ-

Abbreviations: ETF, electron-transfer flavoprotein; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride.

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ously [7]. *A. suum* were obtained from Routh Packing Company, Sandusky, OH.

Methods

ETF was assayed spectrophotometrically at 30 °C using purified *A. suum* 2-methylbutyryl-CoA dehydrogenase and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT), as a terminal electron-acceptor [11]. The incubation mixture contained 50 mM Bicine (pH 7.5), 0.15% Triton X-100, 0.1 mM FAD, 0.1 mM 2-methylbutyryl-CoA, 10 μg *A. suum* 2-methylbutyryl-CoA dehydrogenase and ETF in a final vol of 1 ml. ETF and 2-methylbutyryl-CoA dehydrogenase were preincubated for 2 min prior to assay and the reaction was initiated by the addition of 2-methylbutyryl-CoA. Activity was constant for 2 min, proportional to the amount of ETF added, and expressed as nmol INT reduced per min, using an extinction coefficient for INT of 10.2 $mM^{-1} \cdot cm^{-1}$ at 492 nm [11]. During purification, ETF was monitored in column fractions by its intense yellow-green fluorescence.

Preparation of ETF. Mitochondria were isolated from about 400 g of *A. suum* body wall muscle as described previously and stored in 50 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA (about 10 mg protein/ml) at −20 °C until use [7,12]. All steps in the purification were carried out at 4 °C. The frozen mitochondria were thawed, homogenized with a teflon-coated homogenizer, and centrifuged at 155 000 × *g* for 1 h. The supernatant fraction was precipitated between 40% and 80% ammonium sulfate. The ammonium sulfate precipitate was immediately resuspended in 4 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol (w/v) and 0.5 mM EDTA (buffer A) and passed through a Sephadex G-25 column (2.5 × 25 cm) equilibrated with buffer A. The sample (5 mg protein per ml) then was applied to a DEAE-cellulose column (2.5 × 40 cm) and eluted with buffer A at a flow rate of 50 ml/h. ETF, which eluted in the void volume, was concentrated to 3 ml by ultrafiltration and 1.5 ml was immediately applied to a Sephadex G-100 column (1.5 × 90 cm) which had been equilibrated with 50 mM potassium phosphate buffer (pH 8.0), 10% glycerol (w/v) and 0.5 mM EDTA (buffer B). The column was eluted at a rate of 6 ml/h and 1 ml fractions were collected. Fractions containing ETF activity emerged immediately after the void volume and those containing absorbance ratios (270 nm/436 nm) less than 6.5 were pooled. A second Sephadex G-100 column was run with the remaining ETF and ETF-containing fractions from both were pooled and applied to a hydroxyapatite column (1.5 × 5 cm) equilibrated with buffer B. The column was washed with 100 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol (w/v) and 0.5 mM EDTA and ETF was eluted with 200 mM potassium phosphate buffer (pH 8.0), containing 10% glycerol (w/v) and 0.5 mM EDTA. Fractions containing ETF activity were pooled, concentrated to at least 5 mg/ml by ultrafiltration and stored at −20 °C for at least 1 month with little loss of activity. Purified ETF had a final specific activity of 0.24–0.46 μmol INT reduced per min per mg protein and a 270 nm/436 nm absorbance ratio of 5.6. Its recovery and stability was absolutely dependent on the presence of 10% glycerol (w/v) in all isolation buffers, as observed for the pig kidney ETF [13].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% gels according to Laemmli [14]. Gels were stained for 2 h in 0.3% Coomassie brilliant blue and destained in 10% acetic acid/10% methanol. Molecular weight standards were phosphorylase (97 400), bovine serum albumin (66 200), ovalbumin (42 700), carbonic anhydrase (31 000) and soybean trypsin inhibitor (21 500). Mol FAD/mol of enzyme and the absorption coefficient at 436 nm were determined by quantifying FAD released from the enzyme after treatment with 4 M guanidine hydrochloride [13]. Protein was estimated according to Bradford [15].

Amino acid analysis. Samples were analyzed in a Dionex D300 Amino Acid Analyzer equipped with a Dionex DC 5A column (Na^+ resin) following hydrolysis at 110 °C in 6 M HCl in sealed, evacuated tubes for 24, 48 and 72 h. Threonine and serine destruction were corrected for by extrapolation to 'zero time'. Alternatively, analyses were performed after precolumn derivatization of amino acids with phenylisothiocyanate [24].

Assay of 2-methylbutyrate formation. 2-Methylbutyrate formation was measured as described previously [7,12]. *A. suum* submitochondrial particles were prepared immediately prior to use according to Takamiya et al. [16]. Assays were conducted in duplicate in Warburg respirometers after flushing with nitrogen for 5 min. The final reaction mixture contained 240 mM sucrose, 10 mM potassium phosphate (pH 7.5), submitochondrial particles (4 mg protein), 40 μg 2-methyl branched-chain acyl-CoA dehydrogenase, 40 μg ETF, 0.1% (v/v) ethanol, 0.5 mM NAD^+, 10 μg alcohol dehydrogenase, 2 mM tiglyl-CoA and additions as indicated in a final vol of 2 ml. The reaction was initiated by the addition of tiglyl-CoA and NAD^+ from the side arms. Rotenone was added with the ethanol. After 10 min, the reaction was terminated by placing the reaction mixture in a boiling water bath for 5 min and 2-methylbutyrate formation was determined after CoA ester hydrolysis by ether extraction and gas-liquid chromatography as described previously [12]. Prior to boiling, 1 μmol of isobutyrate was added as a control and final 2-methylbutyrate values were corrected to 100% isobutyrate recovery.

Results

The purification scheme outlined above is similar to that reported for the isolation of ETF from mammalian

Fig. 1. PAGE with SDS of the purified electron-transfer flavoprotein. (a) Purified enzyme (15 μg protein); (b) *A. suum* 155000×*g* mitochondrial supernatant fraction (50 μg protein); (c) standards. Gels were electrophoresed as described in Methods.

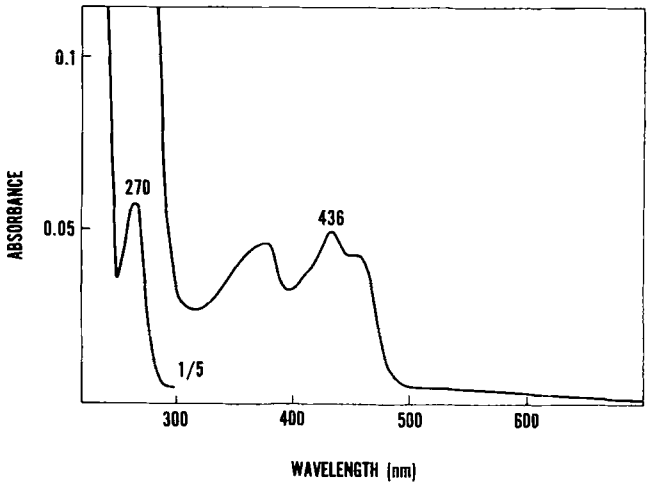

Fig. 2. Visible and ultraviolet absorption spectra of the purified *A. suum* electron-transfer flavoprotein. Spectra were recorded in 20 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol. The absorbance was scanned from 600 to 300 nm on the 0–0.1 scale and from 300 to 200 on the 0–0.5 scale.

tissues and yields about 8 mg of purified ETF from 400 g of *A. suum* body wall muscle [9,13]. Purified ETF eluted from a calibrated Sephadex G-200 column as a single, homogenous, peak with an apparent M_r of 70000 (data not shown). SDS-PAGE yielded two bands of similar staining intensity corresponding to apparent subunit M_r's of about 37000 and 31500, which were in good agreement with the apparent native M_r obtained by gel filtration (Fig. 1). The visible spectrum of the ascarid ETF had an absorbance maximum at 436 nm and a protein/flavin absorbance ratio (270 nm/436 nm) of 5.6 (Fig. 2). The extinction coefficient of the bound flavin at 436 nm was 13.5, as estimated by flavin release in 4 M guanidine-HCl. The flavin content of the purified ETF was constant from preparation to preparation and was not altered by the addition of free FAD to the isolation buffers. An amino acid analysis of the purified ETF is presented in Table I.

Many of the properties of the purified *A. suum* ETF appear to be similar to those reported for the mammalian ETF's, despite the altered role of ETF in *A. suum* mitochondrial metabolism, shuttling reducing power from the electron-transport chain to a soluble 2-methyl branched-chain enoyl-CoA reductase (Table II). When the purified ETF was incubated with *A. suum* submitochondrial particles, purified *A. suum* 2-methyl branched-chain acyl-CoA dehydrogenase, NADH and 2-methylcrotonyl CoA, the formation of 2-methylbutyrate was dependent on the presence of ETF (Fig. 3). The small amount of 2-methylbutyrate formation

TABLE I

Amino acid analysis of the A. suum electron-transfer flavoprotein

n.d., not determined.

Residue	Residues per mol of FAD		
	A. suum	pig kidney [a]	pig liver [b]
Asx	55	43	44
Thr	20	28	29
Ser	34	20	28
Glu	50	51	48
Pro	29	22	25
Gly	47	38	47
Ala	72	64	66
Val	68	53	53
Ile	27	28	28
Leu	50	50	53
Tyr	8	7	10
Phe	18	12	13
His	9	9	9
Lys	53	41	44
Arg	19	21	23
Try	n.d.	4	3
$\frac{1}{2}$cys	n.d.	15	12

[a] Taken from Gorelick et al. [13].
[b] Taken from Husain and Steenkamp [9].

TABLE II

Properties of electron-transfer flavoproteins

	Absorbance maximum (nm)	Absorption coefficient E_{436} ($mM^{-1} \cdot cm^{-1}$)	Protein/flavin absorbance ratio	M_r	
				native	subunits
Pig liver [a]	436	13.5–13.6	5.8	68000	38000
			(270 nm/436 nm)		32000
Pig kidney [b]	436	13.3	5.88	59000	33000
			(272 nm/436 nm)		30000
A. suum	436	13.5	5.6	70000	37000
			(270 nm/436 nm)		31500

[a] Husain and Steenkamp [9].
[b] Gorelick et al. [13].

observed in the absence of added ETF may be due to a small amount of ETF contaminating the preparations of submitochondrial particles. The role of electron transport and rotenone-sensitivity of this pathway has been observed previously [6,7]. The present results extend those studies and demonstrate that fumarate also dramatically inhibited 2-methylbutyrate formation (Table III). Fumarate inhibition was abolished by the addition of malonate, suggesting that fumarate functions as a terminal electron acceptor in these incubations and dissipates the reducing power necessary to drive ETF-dependent enoyl-CoA reduction. Indeed, significant amounts of succinate were formed in these incubations when fumarate was included (1.8 μmol per mg protein).

Discussion

The results of the present study indicate that ETF isolated from *A. suum* body wall muscle is similar to its mammalian counterpart despite the reversed direction of electron-flow in the ascarid organelle [7]. This observation is not surprising, since it has long been known that the mammalian acyl-CoA dehydrogenases transfer electrons to ETF in a reversible manner in the presence of a suitable enoyl-CoA acceptor [17]. Recently, reverse electron-flow from purified ETF dehydrogenase to ETF also has been demonstrated in mammalian systems, and it has become apparent that each step in this pathway is readily reversible under physiological conditions [18,19]. Beckman and Frerman [18,19] have suggested that the rate of β-oxidation is regulated by the mitochondrial redox state and that the ratio of oxidized to reduced ETF depends on the NADH/NAD^+ ratio. This situation appears to be taken to the extreme in anaerobic *A. suum* mitochondria which lack cytochrome oxidase and catalyze a rapid NADH-dependent enoyl-CoA reduction [7].

A number of other organisms catalyze an NADH-dependent reduction of enoyl CoA, but *A. suum* is unique

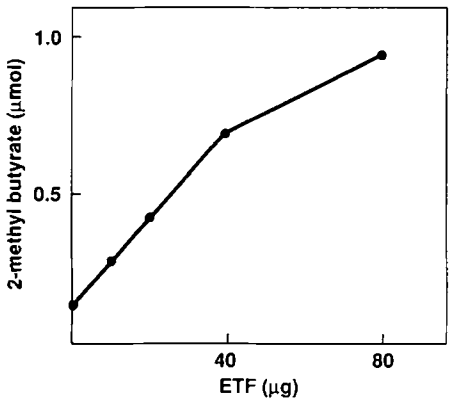

Fig. 3. Effect of varying electron-transfer flavoprotein concentration on 2-methylbutyrate formation. The complete assay system contained 240 mM sucrose, 10 mM potassium phosphate (pH 7.5), *A. suum* submitochondrial particles (4 mg protein), 2-methyl branched-chain acyl-CoA dehydrogenase (40 μg protein), ETF (40 μg protein), 0.1% ethanol (v/v), 1 mM NAD^+, 10 μg alcohol dehydrogenase, 2 mM tiglyl CoA and additions as indicated in a final vol of 2 ml. Fractions were incubated under nitrogen for 10 min and 2-methylbutyrate formation was assayed after CoA ester hydrolysis by gas chromatography as described in Methods.

TABLE III

ETF-dependent formation of 2-methylbutyrate in A. suum submitochondrial particles

The complete assay system contained 240 mM sucrose, 10 mM potassium phosphate (pH 7.5), *A. suum* submitochondrial particles (4 mg protein), 2-methyl branched-chain acyl CoA dehydrogenase (40 μg protein), ETF (40 μg protein), 0.1% v/v ethanol, 1 mM NAD^+, 10 μg alcohol dehydrogenase, 2 mM tiglyl CoA and additions as indicated in a final vol of 2 ml. Fractions were incubated in duplicate under nitrogen for 10 min and 2-methylbutyrate formation was assayed after CoA ester hydrolysis by gas-liquid chromatography as described in Methods.

Additions or deletions	2-Methylbutyrate (μmol)
Complete	1.0
Without ETF	0.1
With fumarate (5 mM)	0.2
With succinate (5 mM)	1.2
With fumarate (5 mM; malonate 20 mM)	0.7
With rotenone (0.2 μg)	0.1

in its apparent ability to couple this reduction to energy generation [7,12]. In rabbit heart mitochondria a unique enzyme, enoyl-CoA reductase, replaces acyl-CoA dehydrogenase and, since it reacts directly with NADH, shifts the equilibrium of the entire β-oxidation pathway in the synthetic direction [20,21]. In anaerobic butyrate-forming bacteria such as *Megasphera elsdenii,* ETF reacts directly with NADH, in contrast to ETF's isolated from eukaryotic sources, and elevated $NADH/NAD^+$ ratios drive the reduction of crotonyl CoA to butyryl CoA [22–25]. Neither the rabbit heart nor bacterial reductions are coupled directly to energy generation but theoretically the difference in potential between the $NADH/NAD^+$ and enoyl-CoA/acyl-CoA couples is large enough to make an electron-transport associated phosphorylation energetically feasible. This, indeed, may be the case in *A. suum*, based on the rotenone-sensitivity of this pathway [7,12]. It has been pointed out previously that the reversal of β-oxidation observed in the ascarid organelle theoretically does not increase its energy-generating capacity over that obtained with the formation of acetate and propionate [12]. Its primary role is probably the maintenance of mitochondrial redox balance by serving as a sink for excess reducing power [12]. The inhibition of NADH-dependent enoyl CoA reduction by fumarate observed in the present study would support this hypothesis and suggests that the fumarate reductase may have a competitive advantage over the ETF (dehydrogenase?) in competing for mitochondrially generated reducing power.

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