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# 2-METHYLBUTYRYL CoA DEHYDROGENASE FROM MITOCHONDRIA OF ASCARIS SUUM AND ITS RELATIONSHIP TO NADH-DEPENDENT 2-METHYLCROTONYL CoA REDUCTION

Richard Komuniecki, Steven Fekete, and Julia Thissen

Department of Biology, University of Toledo
Toledo, Ohio

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SUMMARY: Acyl CoA dehydrogenase and electron-transfer flavoprotein have been isolated and partially purified from mitochondria of the anaerobic nematode, Ascaris suum. Dehydrogenase activity was greatest with 2-methylbutyryl CoA and the relative substrate specificities of the ascarid dehydrogenase(s) differ greatly from their mammalian counterparts. It appears that the ascarid dehydrogenase functions physiologically as a reductase, catalyzing the final step in the synthesis of branched-chain fatty acids. In fact, incubations of A. suum mitochondrial membranes with electron-transfer flavoprotein, 2-methylbutyryl CoA dehydrogenase, 2-methylcrotonyl CoA and NADH resulted in a substantial, rotenone-sensitive, 2-methylbutyrate synthesis. These results suggest that the ascarid electron-transport chain and at least two soluble mitochondrial proteins are involved in the NADH-dependent reduction of 2-methylcrotonyl CoA.

The parasitic nematode, Ascaris suum, exhibits an anaerobic mitochondrial energy metabolism and uses unsaturated organic acids, instead of oxygen, as terminal electron acceptors (1,2). The tricarboxylic acid cycle is not functional and electron-transport is antimycin and cyanide insensitive (3,4). The branched-chain fatty acids, 2-methylbutyrate (2-MB) and 2-methylvalerate (2-MV) are major end products of carbohydrate dissimulation and are formed within the mitochondrion by the condensation of an acetate unit and a propionate unit or two propionate units, respectively, with the subsequent reduction of the condensation products (5,6). The first three enzymes in the pathway of 2-MB formation require CoA esters as substrates and appear to differ significantly from the corresponding enzymes of B-oxidation found in mammalian mitochondria (7,8). However, differences should be anticipated since the ascarid enzymes function physiologically in the direction of acyl CoA synthesis and not oxidation. The final reaction in the pathway of

Abbreviations: ETF, electron transfer flavoprotein; 2-MB, 2-methylbutyrate; 2-MV, 2-methylvalerate.

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2-MB formation, the NADH-dependent reduction of 2-methylcrotonyl CoA, requires both mitochondrial membrane-bound and soluble components and is rotenone-sensitive, suggesting the involvement of the ascarid electron-transport chain (9,10). In fact, recent studies with intact mitochondria have suggested that 2-methylcrotonyl CoA reduction is coupled to an electron-transport associated phosphorylation (11). Therefore, the present study was designed to identify the soluble components involved in the NADH-dependent reduction of 2-methylcrotonyl CoA by A. suum mitochondria.

## MATERIALS AND METHODS

CoA and CoA esters were obtained from P and L Biochemicals (Milwaukee, WI). 2-methylbutyrate was obtained from Aldrich Chemicals (Milwaukee, WI) and 2-methylvalerate from Pfaltz and Bauer, Inc. (Stamford, CT). All other chemicals were of reagent grade and purchased from Sigma Chemical Company (St. Louis, MO).

Ascaris suum body wall muscle strips were obtained by dissection within 6 hrs of helminth isolation and mitochondria were isolated as described previously (12,13). For the isolation of mitochondrial membrane and supernatant fractions, the mitochondria were resuspended in 50 mM potassium phosphate buffer (pH 7.4), containing 0.5 mM disodium EDTA and 2 mM dithiothreitol (Buffer A), frozen, thawed, and centrifuged at 155,000 x g for 60 min. The supernatant fraction was passed through a G-25 Sephadex column equilibrated with Buffer A prior to assay. For the separation of electron-transfer flavoprotein (ETF) and 2-MB CoA dehydrogenase, isolated A. suum mitochondria were stored for less than 2 wks at -70°C in 50 mM potassium phosphate buffer (pH 7.2), containing 0.5 mM EDTA (15 mg protein/ml). After thawing, the mitochondrial suspensions were rehomogenized and centrifuged at 155,000 x g for 60 min. The supernatant fraction was precipitated between 40 and 80% ammonium sulfate, resuspended in 4 mM potassium phosphate buffer (pH 7.2), containing 10% glycerol and 0.5 mM EDTA (5 mg protein/ml), and passed through a Sephadex G-25 column (2x10 cm) equilibrated with the same buffer. The sample then was applied to a DEAE-cellulose column (1.5x15 cm) which was eluted stepwise with 4 mM, 20 mM, 50 mM, and 300 mM potassium phosphate buffers (pH 7.2), containing 10% glycerol and 0.5 mM EDTA.

Assays for the various acyl CoA dehydrogenase activities were performed spectrophotometrically using Medola blue and INT as intermediate and terminal electron acceptors, respectively (14). The incubation mixture contained 200 mM Bicine buffer (pH 8.0), 0.15% Triton X-100, 0.1 mM acyl CoA, 0.25 mM INT, 0.1 mM Medola blue, 0.1 mM FAD, and enzyme in a final vol of 1 ml. INT reduction was monitored at 492 nm and was linear for 2 min. ETF was assayed in a similar assay system with ETF replacing Medola blue. After a 2-3 min lag, activity with ETF and 2-MB CoA dehydrogenase was linear for at least 10 min. 2-MB and 2-MV CoA esters were synthesized by way of 1-acylimidozoles and purified by high pressure liquid chromatography as described previously (15).

2-MB formation in incubations with isolated mitochondrial membranes and various soluble fractions was assayed anaerobically in Buffer A as described previously (11,12). The reaction was initiated by the addition of 2 mM 2-methylcrotonyl CoA and 4 mM NADH. The final reaction volume was 2 ml. After 30 min, the reaction was terminated by placing the reaction mixture in a boiling water bath for 5 min. Prior to boiling, 1 µmole (0.1 ml) of isobutyrate was added as an internal control. CoA esters were hydrolyzed by raising the pH to 11 with 3.5 N KOH and incubation for 20 min at 70°C. 2-MB was extracted with ether from acidified incubation media and assayed by

gas-liquid chromatography (12,16). Incubation media were centrifuged at 10,000 x g for 5 min and the supernatant fraction was acidified with 2N HCl, extracted with 1 ml of ether (2x), and concentrated to 0.2 ml for gas liquid chromatography. Volatile acids were identified by their relative retention times, as compared to standards, and quantified by calculation from their peak heights. Recoveries of 2-MB and 2-methylcrotonate were greater than 90%.

## RESULTS AND DISCUSSION

A. suum mitochondria catalyze a rotenone-sensitive, NADH-dependent, reduction of 2-methylcrotonyl CoA, as the final step in the pathway of branched-chain fatty acid synthesis (9-11). To determine which soluble components are involved in 2-methylcrotonyl CoA reduction, ETF and acyl CoA dehydrogenase activities were isolated from A. suum high-speed mitochondrial supernatant fractions by ammonium sulfate precipitation and were separated by chromatography on DEAE-cellulose (Fig. 1). The DEAE-cellulose column was eluted successively with 4, 20, 50, and 300 mM potassium phosphate buffers (pH 7.2). ETF eluted in the 20 mM peak and all of the acyl CoA dehydrogenase activity was found in the 300 mM peak. Recovery of 2-MB CoA dehydrogenase and ETF activities varied between 40 and 55% and was dependent on the presence of glycerol (10%w/v) in all isolation buffers. In mammalian systems, at least

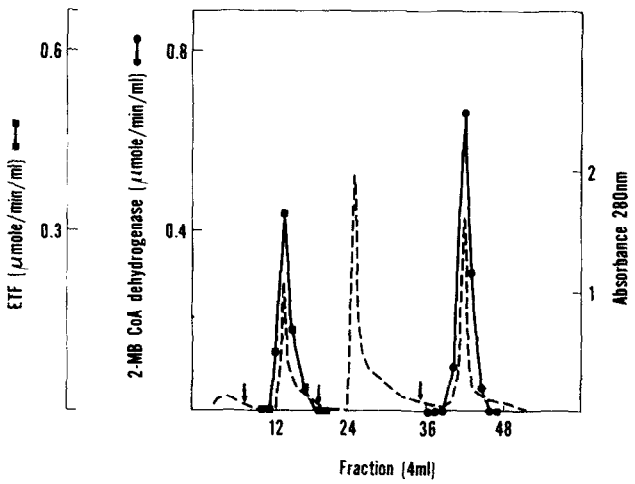


FIG 1. DEAE-cellulose chromatography of the ammonium sulfate precipitate fraction. The enzyme fraction (180mg protein, 25ml) from the ammonium sulfate fractionation step was applied to a DEAE-cellulose column (1.5 x 20cm) equilibrated with 4mM potassium phosphate buffer (pH 7.2) containing 10% glycerol and 0.5 mM EDTA as described in Methods. The column was eluted successively with 20mM, 50mM and 300mM potassium phosphate buffers (pH 7.2), containing 10% glycerol and 0.5 mM EDTA (at arrows). Fractions were assayed for ETF and various acyl CoA dehydrogenase activities using the dye reduction assay.

Table I. Relative activity of different acyl CoA's with the partially purified A. suum acyl CoA dehydrogenase(s)

| Substrate | Specific Activity | Relative Activity |
|---|---|---|
| | nmol INT reduced/ min/mg protein | % |
| 2-Methylbutyryl CoA | 104.3 | 100 |
| 2-Methylvaleryl CoA | 93.2 | 89 |
| n-Butyryl CoA | 14.2 | 14 |
| n-Valeryl CoA | 15.2 | 15 |
| n-Octanoyl CoA | 12.1 | 12 |
| n-Palmitoyl CoA | 0 | 0 |
| Isovaleryl CoA | 0 | 0 |
| Isobutyryl CoA | 0 | 0 |

1. Dehydrogenating activity was assayed by following INT reduction. The final reaction mixture contained 200 mM Bicine buffer (pH 8.0), 0.15% Triton X-100, 0.25mM INT, 0.1 mM Medola Blue, 0.1 mM FAD, 0.1 mM acyl CoA, and enzyme in a final vol of 1 ml.

five distinct acyl CoA dehydrogenses have been isolated: three specific for straight-chain acyl CoA esters of differing chain lengths and two specific for the branched-chain acyl CoA esters, isovaleryl CoA and 2-MB CoA (17-19). Interestingly, the relative substrate specificities of the acyl CoA dehydrogenase(s) isolated from A. suum appear to differ markedly from their mammalian counterparts (Table I). 2-MB CoA and 2-MV CoA were the most active substrates in the acyl CoA dehydrogenase assay system. No activity was detected with either palmitoyl or isovaleryl CoA's, suggesting that the long chain and isovaleryl CoA dehydrogenases are absent from A. suum mitochondria. In addition, no activity was detected using isobutyryl CoA. In contrast, this substrate was dehydrogenated at the same rate as 2-MB CoA by the 2-MB CoA dehydrogenase recently isolated from rat liver (19). Ratios of activities were similar if either the 40-80% ammonium sulfate precipitate or pooled DEAE-cellulose column fractions were used as the source of acyl CoA dehydrogenase activity.

To assess the role of 2-MB CoA dehydrogenase in the pathway of 2-methylcrotonyl CoA reduction, the partially purified 2-MB CoA dehydrogenase

Table II. 2-Methylbutyrate formation from NADH and 2-methylcrotonyl CoA by preparations from A. suum mitochondria

| Additions[1] | Protein | Rotenone | 2-methylbutyrate -FAD | 2-methylbutyrate +FAD |
|---|---|---|---|---|
| | mg | | μ mole ± SE(n=3) | |
| NONE | - | - | n.d. | 0 |
| SUPERNATE[2] | 5.2 | - | 0.95 ± 0.06 | 1.22 ± 0.07 |
| ETF[3] | 1.6 | - | n.d. | 0 |
| 2-MB D[3] | 1.0 | - | 0.21 ± 0.03 | 1.03 ± 0.14 |
| | 1.0 | + | n.d. | 0.83 ± 0.06 |
| 2-MB D + ETF | 1.0<br>1.6 | - | 0.93 ± 0.05 | 1.45 ± 0.16 |
| 2-MB D + ETF | 1.0<br>1.6 | + | 0.10 ± 0.01 | n.d. |

1. Fractions were incubated under nitrogen for 30 min at 30°C in 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM dithiothreitol, 4 mM NADH, 2 mM 2-methylcrotonyl CoA, 1 mM $MgCl_2$ and, where indicated, 0.2 mM FAD or 0.4 μg rotenone in a final vol of 2 ml. In addition, all incubations contained freshly isolated A. suum mitochondrial membranes (4.1 mg protein). 2-MB was assayed by ether extraction and gas-liquid chromatography as described in Methods.

2. Isolated A. suum mitochondria were suspended in 50 mM potassium phosphate buffer (pH 7.4), containing 0.5 mM EDTA, frozen, thawed, and the supernatant fraction (SUPERNATE) was isolated by centrifugation at 155,000 x g for 60 min.

3. After DEAE-cellulose chromatography, fractions containing either electron-transfer flavoprotein (ETF) or 2-MB CoA dehydrogenase (2-MB D) activity were pooled.
n.d. not determined.

was incubated anaerobically with freshly isolated A. suum mitochondrial membranes, 2-methylcrotonyl CoA, and NADH, as described previously (9,10). Only small amounts of 2-MB were formed, in contrast to results from similar experiments using high-speed mitochondrial supernatant fractions (Table II). However, when partially purified preparations of both ETF and 2-MB CoA dehydrogenase were included, the ability to form substantial amounts of 2-MB was restored. Interestingly, in addition to ETF, FAD also stimulated 2-MB formation by 2-MB CoA dehydrogenase in these incubations. FAD stimulation does not appear to result from the loss of FAD during purification of the enzyme, since it has only a slight stimulatory effect in the spectrophotometric dye reduction assay (data not shown). It seems more likely that, in these long-term incubations, $FADH_2$ is formed by the active NADH:FAD reductase present

in A. suum mitochondrial extracts. This $FADH_2$ then is able to react with the 2-MB CoA dehydrogenase directly, as has been observed previously (10). Indeed, stimulation of 2-MB formation by ETF is rotenone-sensitive, suggesting the involvement of the membrane-bound electron transport-chain, while stimulation by FAD is not (Table II). The physiological significance of FAD stimulation is unclear. In incubations containing A. suum mitochondrial membranes and either high-speed supernatant fractions or mixtures of partially purified ETF and 2-MB CoA dehydrogenase, 2-methylcrotonyl CoA oxidation also occurred and substantial amounts of acetate, propionate, and 2-MV accumulated as has been observed in earlier studies (9,10). However, in incubations containing only membranes, FAD, and the partially purified 2-MB CoA dehydrogenase, little oxidation occurred and the ratio of 2-methylcrotonyl CoA utilized to 2-MB formed was between 1.1 and 1.5 (data not shown). The results of these studies suggest that the ascarid electron-transport chain and at least two soluble mitochondrial proteins, ETF and 2-MB CoA dehydrogenase, are involved in the NADH-dependent reduction of 2-methylcrotonyl CoA. However, the site of coupling between these soluble components and the rotenone-sensitive portion of membrane-bound electron-transport chain remains to be elucidated.

REFERENCES

1. Saz, H.J. (1981) Ann. Rev. Physiol. 43, 323-341.
2. Kohler, P., and Bachman, R. (1980) Mol. Biochem. Parasitol. 1, 75-90.
3. Saz, H.J. (1971) Comp. Biochem. Physiol. 39B, 627-637.
4. Ward, C.W. and Fairbairn, D. (1970) J. Parasitol. 56, 1009-1012.
5. Saz, H.J. and Weil, A. (1960) J. Biol. Chem. 235, 914-918.
6. Saz, H.J. and Weil, A. (1962) J. Biol. Chem. 237, 2053-2056.
7. Suarez de Mata, Z., Saz, H.J., and Pasto, D. (1977) J. Biol. Chem. 252, 4215-4224.
8. Suarez de Mata, S., Zarranz, M.E., Lizardo, R., and Saz, H.J. (1983) Arch. Biochem. Biophys. 226, 84-93.
9. Komuniecki, R., Komuniecki, P.R., and Saz, H.J. (1981) J. Parasitol. 67, 601-608.
10. Komuniecki, R., Rioux, A., Thissen, J. (1983) Mol. Biochem. Parasit. (In Press)
11. Rioux, A. and Komuniecki, R. (1983) J. Comp. Physiol. (In Press).
12. Komuniecki, R., Komuniecki, P.R., and Saz, H.J. (1981) J. Parasitol. 67, 841-846.
13. Komuniecki, R., Komuniecki, P.R., and Saz, H.J. (1979) Biochim. Biophys. Acta 571, 1-11.
14. Dommes, V., and Kunau, W.H. (1976) Anal. Biochem. 71, 571-578.
15. Kawaguchi, A., Yoshimura, T., and Okuda, S. (1981) J. Biochem. 89, 337-339.
16. Rizzo, A.F. (1980) J. Clin. Microbiol. 11, 418-421.
17. Ikeda, Y., Dabrowski, C. and Tanaka, K. (1983) J. Biol. Chem. 258, 1066-1076.
18. Ikeda, Y., and Tanaka, K. (1983) J. Biol. Chem. 258, 1077-1085.
19. Ikeda, Y., and Tanaka, K (1983) J. Biol. Chem. 258, 9477-9487.