3-Hydroxyacyl-CoA epimerase is a peroxisomal enzyme and therefore not involved in mitochondrial fatty acid oxidation

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The subcellular location of 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) was studied by differential centrifugation and Percoll density gradient centrifugation of a rat liver homogenate. The enzyme was found to be associated with peroxisomes but not with mitochondria. This observation proves that 3-hydroxy-acyl-CoA epimerase does not function in mitochondrial β-oxidation of polyunsaturated fatty acids which are degraded by a modified pathway.

Rat liver *3-Hydroxyacyl-CoA epimerase* *Subcellular location* *Mitochondria* *Peroxisome* *β-Oxidation* *Polyunsaturated fatty acid*

1. INTRODUCTION

According to Stoffel and Caesar [1] 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) functions as an auxiliary enzyme in the mitochondrial oxidation of polyunsaturated fatty acids that have *cis* double bonds extending from even-numbered carbon atoms. More recently, Kunau and Dommes [2] have proposed an alternative pathway for the degradation of linoleic acid and other polyunsaturated fatty acids. According to their proposal an NADPH-dependent 2,4-dienoyl-CoA reductase catalyzes the reductive removal of *cis* double bonds extending from even-numbered carbon atoms. Evidence in support of this modified pathway has been obtained in several laboratories [2–5]. Perhaps most convincing is the observation that rat heart mitochondria, which rapidly oxidize linoleoyl-CoA, contain 2,4-dienoyl-CoA reductase, but are virtually devoid of 3-hydroxyacyl-CoA epimerase. However, the possibility remains that liver mitochondria, which have been reported to contain 3-hydroxyacyl-CoA epimerase [1], metabolize polyunsaturated fatty acids via both pathways. This possibility has prompted us to determine carefully the subcellular location of 3-hydroxyacyl-CoA epimerase in rat liver.

Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid

2. MATERIALS AND METHODS

DL-3-Hydroxyoctanoyl-CoA was synthesized from DL-3-hydroxyoctanoic acid and CoASH by the mixed anhydride method of Goldman and Vagelos [6]. Pig heart L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and Percoll were purchased from Sigma. Pig heart 3-oxoacyl-CoA thiolase (EC 2.3.1.16) was isolated by the procedure of Staack et al. [7].

Liver homogenates were prepared from adult male Sprague Dawley rats (250–350 g) which had been fed for 2 weeks either Purina rodent chow containing 0.3% (w/w) clofibrate or regular Purina rodent chow. Rats were starved 12 h before killing. Livers were quickly removed from the animals, immersed immediately in ice-cold isolation buffer containing 300 mM mannitol, 10 mM Hepes (pH 7.2) and 1 mM EGTA, rinsed several times with the same buffer, and finally cut into small pieces with a pair of sharp scissors. All subsequent operations were performed at 4°C. The liver pieces were suspended in 9 vols of isolation

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buffer and homogenized by 3 strokes in a loose-fitting Potter-Elvehjem type glass-Teflon homogenizer.

The homogenate was centrifuged at 2300 × *g* for 9 min to sediment nuclei and heavy mitochondria (fraction N + M). The supernatant was centrifuged at 12500 × *g* for 20 min to yield a pellet of light mitochondria (fraction L). The resulting supernatant was separated by centrifugation for 90 min at 33300 × *g* into a microsomal precipitate (fraction P) and supernatant (fraction S) containing soluble proteins.

Before being subjected to Percoll density gradient centrifugation the liver homogenate was centrifuged at 600 × *g* for 10 min to remove nuclei. The resulting supernatant was then centrifuged for 30 min at 32000 × *g* to pellet most of the mitochondria, peroxisomes, and lysosomes in addition to some microsomes (referred to as RLMP). This pellet was washed once with the isolation buffer and resuspended in the same buffer to give a protein concentration of 15 mg/ml. A sample of 1.5 ml of RLMP was layered onto 29 ml of a 50% (v/v) Percoll suspension containing 250 mM sucrose, 2 mM Mops (pH 7.2), 1 mM EGTA and 0.1% ethanol. Placed on top of the RLMP sample was 0.5 ml of 100 mM sucrose. Density gradient centrifugation was carried out at 63000 × *g* for 45 min in a swinging bucket rotor (SW 25.1). Ten fractions of 3 ml each were collected from the top of the density gradient and assayed for 3-hydroxyacyl-CoA epimerase and marker enzymes for several subcellular organelles.

Protein concentrations were determined by the method of Lowry et al. [8]. All enzyme assays were performed at 25°C. 3-Hydroxyacyl-CoA epimerase was assayed spectrophotometrically by measuring at 340 nm the formation of NADH in a coupled reaction described in principle by Binstock and Schulz [9]. The assay mixture contained 0.2 M potassium phosphate (pH 8.0), 0.33 mM NAD^+, 100 μM CoASH, 52 μM DL-3-hydroxyoctanoyl-CoA, bovine serum albumin (0.2 mg/ml), 0.08% Triton X-100, pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.3 U/ml), and pig heart 3-oxoacyl-CoA thiolase (33 mU/ml). The reaction was allowed to proceed until the L-isomer was completely degraded. The assay was initiated by the addition of 3-hydroxyacyl-CoA epimerase. Crotonase was assayed as described by Binstock and Schulz [9]. Glutamate dehydrogenase was assayed spectrophotometrically at 340 nm by a procedure similar to that of Beaufay et al. [10]. Succinate-cytochrome *c* reductase was assayed by the procedure of Stotz [11]. Urate oxidase was assayed as described by Schneider and Hogeboom [12]. Acid phosphatase was assayed with β-glycerophosphate as a substrate as described by Berthet and De Duve [13], and glucose-6-phosphatase was assayed by the method of De Duve et al. [14]. Both phosphatase assays were terminated by acidification with 8% trichloroacetic acid. The samples were heated for 2 min in a boiling water bath to aggregate Percoll which was removed by centrifugation at 6000 rpm for 10 min in an International table top centrifuge. The concentration of inorganic phosphate was determined by the method of Fiske and Subbarow [15] as modified by Dingle [16]. Blanks were prepared in an identical fashion except that enzyme fractions were heated for 2 min in a boiling water bath before being assayed. A unit of enzyme activity is defined as the amount that catalyzes the conversion of 1 μmol substrate to product per min.

3. RESULTS

The subcellular location of 3-hydroxyacyl-CoA epimerase, specifically its association with mitochondria and/or peroxisomes, was studied in rat liver. For this study a rat liver was homogenized and fractionated by differential centrifugation. The fraction obtained after removal of nuclei and soluble proteins from the homogenate was subjected to centrifugation on a Percoll density gradient. Fractions of the Percoll density gradient were assayed for 3-hydroxyacyl-CoA epimerase and for known marker enzymes of several subcellular organelles. The protein distribution pattern shown in fig.1 is indicative of the separation of the subcellular organelles into a low-density fraction (corresponding to fraction 2 with a density of 1.07 g/ml) and a high-density fraction (corresponding to fraction 9 and 10 with densities of 1.104 and 1.12 g/ml, respectively). Peroxisomes, detected by measuring urate oxidase, are almost exclusively associated with the low-density fraction which additionally contains most of the microsomes as revealed by the glucose-6-phosphatase

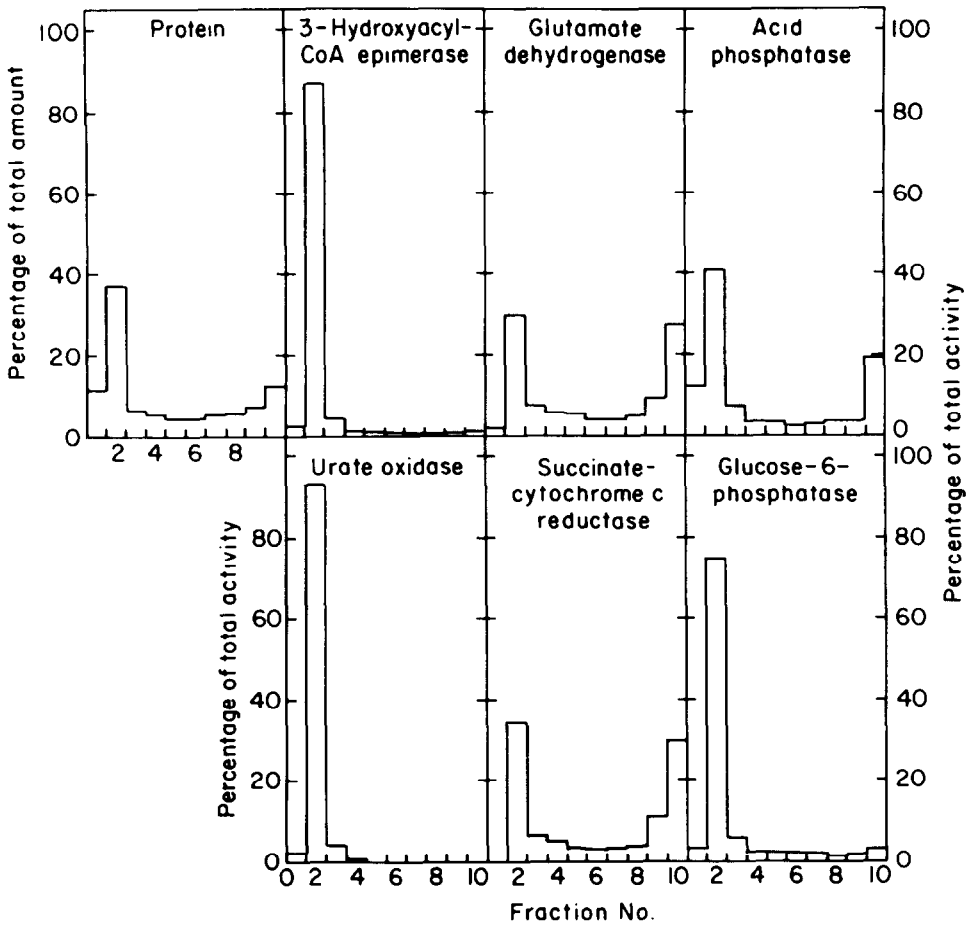

Fig.1. Separation by Percoll density gradient centrifugation of a rat liver fraction containing mitochondria, peroxisomes, lysosomes and microsomes.

activity (see fig.1). In contrast, mitochondria detected by the matrix enzyme glutamate dehydrogenase and succinate-cytochrome *c* reductase of the inner mitochondrial membrane are distributed equally between the low-density and high-density fraction. A similar distribution pattern was observed for the lysosomal marker enzyme acid phosphatase. Thus, the low-density fraction contains in addition to part of the mitochondria and lysosomes virtually all peroxisomes and most of the microsomes, whereas the high-density fraction contains only mitochondria and lysosomes. Since virtually all 3-hydroxyacyl-CoA epimerase activity was detected in fraction 2 (see fig.1 and table 1), this enzyme is associated with peroxisomes and/or microsomes. The near absence of 3-hydroxyacyl-CoA epimerase from fraction 10 (see table 1) prompted the question of whether this enzyme is absent from mitochondria. The residual epimerase activity in fraction 10 is most likely due to a peroxisomal contamination which was detected by its urate oxidase activity (see table 1). Unfortunately, the low specific activity of urate oxidase makes it difficult to assay this enzyme accurately in fractions where it is present at low concentrations. The epimerase activity applied to the Percoll density gradient was completely recovered after centrifugation (see table 1). Since crotonase, which is easily lost from damaged mitochondria [18], remained associated with fraction 10, the mitochondria present in this fraction are not extensively damaged. Altogether, these data lead us to con-

Table 1

Specific activities of 3-hydroxyacyl-CoA epimerase and marker enzymes associated with crude and partially purified fractions of peroxisomes and mitochondria

Sample	Protein (mg/ml)	Specific activity (nmol/min per mg)				
		3-Hydroxyacyl-CoA epimerase	Crotonase	Glutamate dehydrogenase	Succinate-cytochrome *c* reductase	Urate oxidase
RLMP[a]	13.6	457	6626	18.3	45.1	6.6
Fraction 2[b]	2.23	1229	12885	13.4	50	11.7
Fraction 10[b]	0.76	43	4130	36.3	127	0.2
Recovery (%) of total activity (amount) from 10 fractions	88	101	N.D.[c]	80	105	63

[a] A fraction containing rat liver mitochondria and peroxisomes obtained by differential centrifugation
[b] Fractions 2 and 10 (see fig.1) after Percoll density gradient centrifugation of RLMP
[c] Not determined

clude that epimerase is not a mitochondrial enzyme. The conclusion of Mizugaki et al. [19] that epimerase is present both in mitochondria and peroxisomes needs to be reevaluated especially because their short communication lacks detailed information about the epimerase assay and the separation of subcellular organelles.

The data shown in fig.1 were obtained with subcellular organelles isolated from livers of rats fed a diet containing clofibrate. Virtually identical results were obtained when livers of rats fed a diet devoid of clofibrate were used (not shown). However, clofibrate feeding caused a 3-fold increase in the specific activity of 3-hydroxyacyl-CoA epimerase. This degree of induction is similar to the reported induction of other enzymes of β-oxidation in response to feeding clofibrate [20]. Although the association of a soluble enzyme like 3-hydroxyacyl-CoA epimerase with microsomes seems unlikely, we wanted to prove this assumption. For this purpose a rat liver homogenate was fractionated by differential centrifugation and the resulting fractions were assayed for 3-hydroxyacyl-CoA epimerase, urate oxidase, and glucose-6-phosphatase (see fig.2). The distribution pattern of 3-hydroxyacyl-CoA epimerase is very similar to that of urate oxidase, but quite different from the pattern of glucose-6-phosphatase. We therefore conclude that 3-hydroxyacyl-CoA epimerase is a peroxisomal enzyme.

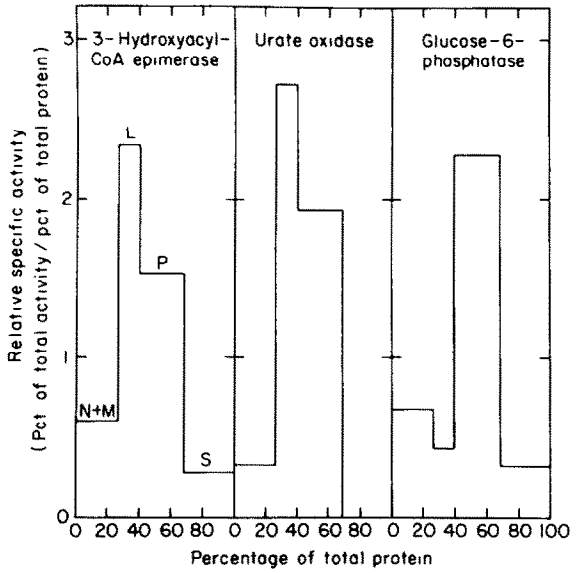

Fig.2. Separation of a rat liver homogenate by differential centrifugation. N + M, nuclei and heavy mitochondria; L, light mitochondria; P, microsomes; S, soluble proteins.

4. DISCUSSION

Our evidence strongly suggests that 3-hydroxyacyl-CoA epimerase is not present in liver mitochondria and hence does not function as an auxiliary enzyme in mitochondrial β-oxidation of linoleic acid and other polyunsaturated fatty acids. The pathway of linoleate degradation according to Stoffel and Caesar [1] must be revised to account for the absence of 3-hydroxyacyl-CoA epimerase from mitochondria. The modified pathway shown in fig.3 was originally proposed by Kunau and Dommes who discovered an NADPH-dependent 2,4-dienoyl-CoA reductase in mitochondria and suggested that it and not 3-hydroxyacyl-CoA epimerase functions as an auxiliary enzyme in the β-oxidation of linoleic acid. According to this modified pathway linoleoyl-CoA (I) passes 3 times through the β-oxidation cycle to yield 3-*cis*,6-*cis*-dodecadienoyl-CoA (II) which is isomerized by *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase to 2-*trans*,6-*cis*-dodecadienoyl-CoA (III). The latter compound yields after one additional β-oxidation cycle 4-*cis*-decenoyl-CoA (IV) which is dehydrogenated to 2-*trans*,4-*cis*-decadienoyl-CoA (V) by medium-chain acyl-CoA dehydrogenase. 2-*trans*,4-*cis*-Decadienoyl-CoA (V) cannot be directly β-oxidized [3], but is rapidly reduced to 3-*trans*-decenoyl-CoA (VI) by NADPH-dependent 2,4-dienoyl-CoA reductase [2]. 3-*trans*-Decenoyl-CoA (VI) is isomerized to 2-*trans*-decenoyl-CoA (VII) which is degraded to 5 mol acetyl-CoA by passing 4 times through the β-oxidation cycle.

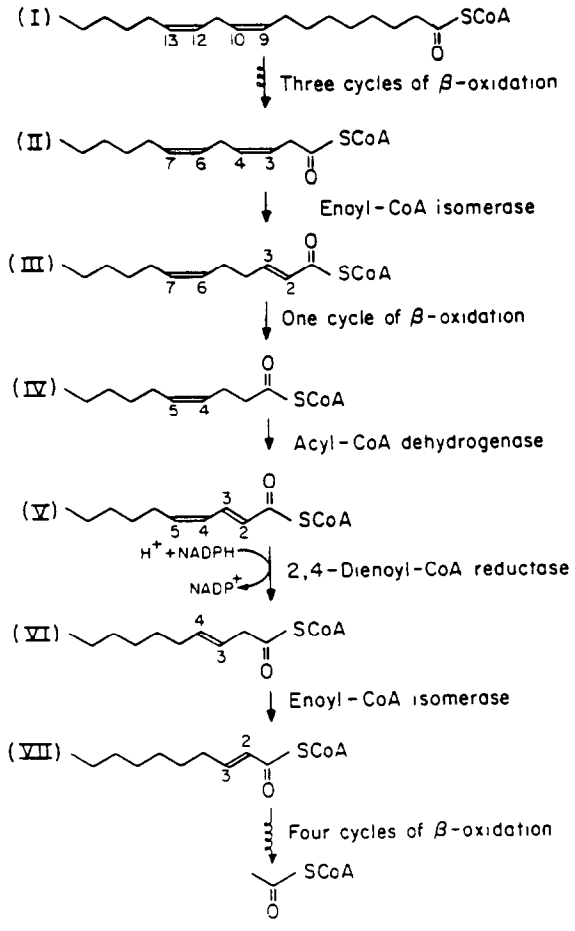

Fig.3. Pathway of linoleoyl-CoA degradation.

The association of 3-hydroxyacyl-CoA epimerase with peroxisomes and with the fatty acid oxidation complex from *E. coli* [21] suggests that it has a function in β-oxidation. Since both peroxisomes [22] and *E. coli* [23] contain additionally an NADPH-dependent 2,4-dienoyl-CoA reductase, it is possible that in more primitive organelles or cells linoleic acid may be degraded by both the original and the modified pathway. If not, 3-hydroxyacyl-CoA epimerase may have a yet undiscovered metabolic function in peroxisomes, *E. coli*, and possibly other simple organisms.

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REFERENCES

[1] Stoffel, W. and Caesar, H. (1965) Hoppe-Seyler's Z. Physiol. Chem. 341, 76–83.
[2] Kunau, W.H. and Dommes, P. (1978) Eur. J. Biochem. 91, 533–544.
[3] Cuebas, D. and Schulz, H. (1982) J. Biol. Chem. 257, 14140–14144.
[4] Hiltunen, J.K., Osmundsen, H. and Bremer, J. (1983) Biochim. Biophys. Acta 752, 223–232.
[5] Chu, C., Kushner, L., Cuebas, D. and Schulz, H. (1984) Biochem. Biophys. Res. Commun. 118, 162–167.

[6] Goldman, P. and Vagelos, P.R. (1961) J. Biol. Chem. 236, 6917–6922.
[7] Staack, H., Binstock, J.F. and Schulz, H. (1978) J. Biol. Chem. 253, 1827–1831.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[9] Binstock, J.F. and Schulz, H. (1981) Methods Enzymol. 71, 403–411.
[10] Beaufay, H., Bendall, D.S., Baudhuin, P. and De Duve, C. (1959) Biochem. J. 73, 623–628.
[11] Stotz, E. (1955) Methods Enzymol. 2, 740–744.
[12] Schneider, W.C. and Hogeboom, G.H. (1952) J. Biol. Chem. 195, 161–166.
[13] Berthet, J. and De Duve, C. (1950) Biochem. J. 50, 174–181.
[14] De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617.
[15] Fiske, C.H. and Subbarow, Y.J. (1925) J. Biol. Chem. 66, 375–400.
[16] Dingle, J.T. (1972) in: Lysosomes (Dingle, J.T. ed.) pp.111–113, American Elsevier, New York.
[17] Wakil, S.J. (1970) in: Lipid Metabolism (Wakil, S.J. ed.) p.6, Academic Press, New York.
[18] Schulz, H. (1974) J. Biol. Chem. 249, 2704–2709.
[19] Mizugaki, M., Nishimaki, T., Yamamoto, H., Sagi, M. and Yamanaka, H. (1982) J. Biochem. 92, 2051–2054.
[20] Bremer, J., Osmundsen, H., Christiansen, R.Z. and Borreback, B. (1981) Methods Enzymol. 72, 506–519.
[21] Pramanik, A., Pawar, S., Antonian, E. and Schulz, H. (1979) J. Bacteriol. 137, 469–473.
[22] Dommes, V., Baumgart, C. and Kunau, W.H. (1981) J. Biol. Chem. 256, 8259–8262.
[23] Mizugaki, M., Nishimaki, T., Yamamoto, H., Nishimura, S., Sagi, M. and Yamanaka, H. (1982) J. Biochem. 91, 1453–1456.