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THE ACTIVITY OF 3-HYDROXYACYL-CoA EPIMERASE IS INSUFFICIENT TO ACCOUNT FOR THE RATE OF LINOLEATE OXIDATION IN RAT HEART MITOCHONDRIA. EVIDENCE FOR A MODIFIED PATHWAY OF LINOLEATE DEGRADATION

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The activities of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8), 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), and 2,4-dienoyl-CoA reductase, all of which have been proposed to function as auxiliary enzymes in the β-oxidation of polyunsaturated fatty acids, have been determined in mitochondria from rat heart and rat liver. In heart mitochondria the activity of 3-hydroxyacyl-CoA epimerase was lower, whereas that of 2,4-dineoyl-CoA reductase was higher than the observed rate of linoleate degration. This observation suggests that 2,4-dienoyl-CoA reductase and not 3-hydroxyacyl-CoA epimerase functions as an auxiliary enzyme in the metabolism of polyunsaturated fatty acids in heart. A modified pathway of linoleate degradation is presented.

The degradation of polyunsaturated fatty acids by β-oxidation is believed to require two auxiliary enzymes, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) (1). The former enzyme catalyzes the formation of trans-2-enoyl-CoA's from their cis-3-isomers which are formed by chain-shortening of unsaturated fatty acids with cis double bonds extending from odd-numbered carbons (2). Unsaturated fatty acids with cis double bonds extending from even-numbered carbons are presumably chain-shortened to cis-2-enoyl-CoA's which can be hydrated to D-3-hydroxyacyl-CoA's by enoyl-CoA hydratase (EC 4.2.1.17) (1). 3-Hydroxyacyl-CoA epimerase can then catalyze the isomerization of D-3-hydroxyacyl-CoA's to their L-isomers which are further degraded by β-oxidation. More recently, Kunau and Dommes (3) have identified an NADPH-dependent reductase that can reduce 2,4-dienoyl-CoA's derived from unsaturated fatty acids with double bonds extending from even-numbered carbons. 3-Enoyl-CoA's formed by the 2,4-dienoyl-CoA reductase can be completely degraded by the sequential actions of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and the enzymes of the β-oxidation cycle.

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Further evidence for a modified pathway by which unsaturated fatty acids may be degraded was provided by Cuebas and Schulz (4) who demonstrated that 2-trans,4-cis-decadienoyl-CoA, a presumed metabolite of linoleic acid, is not directly acted upon by the β-oxidation enzymes, but is reduced by the NADPH-dependent 2,4- dienoyl-CoA reductase. Thus, the complete degradation of linoleic acid apparently requires the auxiliary enzymes cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase, but not 3-hydroxyacyl-CoA epimerase. This situation has prompted us to measure the activities of 3-hydroxyacyl-CoA epimerase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase in rat heart and rat liver mitochondria and to compare them with the rates of oxidation of palmitoyl-CoA, oleoyl-CoA, and linoleoyl-CoA in the same mitochondria.

MATERIALS AND METHODS

NAD, NADPH, CoASH, and palmitoyl-CoA were purchased from P-L Biochemicals. Oleic acid and linoleic acid were obtained from Supelco, Inc. Sigma was the source of rotenone, pig heart 3-hydroxyacyl-CoA dehydrogenase as well as of all standard biochemicals. Beef liver crotonase (5) and pig heart 3-ketoacyl-CoA thiolase (6) were purified according to published procedures. The CoA derivatives of oleic acid, linoleic acid, 3-hydroxyoctanoic acid, 3-hydroxydodecanoic acid, 3-cis-octenoic acid, and 2-trans-4-trans-decadienoic acid were synthesized from the corresponding free acids and CoASH by the mixed anhydride method (7). During the preparation of oleoyl-CoA and linoleoyl-CoA all operations were carried out under nitrogen. The 3-hydroxy acids, 3-cis-octenoic acid, and 2-trans-4-trans-decadienoic acid were synthesized as previously described (4,8). The concentrations of the acyl-CoA's were determined by the method of Ellman (9) after cleaving the thioester bond with hydroxylamine at pH 7. Rat heart mitochondria were isolated by the procedure of Chappel and Hansford (10). Rat liver mitochondria were isolated in a similar fashion except that 0.25 M sucrose was used instead of 0.21 M mannitol plus 0.07 M sucrose and the treatment with Nagarse was omitted.

Oxygen uptake by mitochondria was measured at 25°C by use of a Clark oxygen electrode. A standard incubation mixture for heart mitochondria contained 0.11 M KCl, 33 mM Tris (pH 7.4), 2 mM KP_i (pH 7.4), 2 mM $MgCl_2$, 0.1 mM EGTA, 14.7 μM bovine serum albumin, 0.5 mM L-malate, 13 mM L-carnitine, and mitochondria (1 mg of protein per ml). An incubation mixture for liver mitochondria contained 0.1 M KCl, 20 mM Tris (pH 7.4), 4 mM KP_i (pH 7.4), 4 mM $MgCl_2$, 0.1 mM EGTA, 14.7 μM bovine serum albumin, 0.5 mM L-malate, 13 mM L-carnitine and mitochondria (2 mg of protein per ml). After preincubation of the mixture for about 1 min, the reaction was started by the addition of 1 mM ADP and 15 μM of either palmitoyl-CoA, oleoyl-CoA, or linoleoyl-CoA.

All enzymes were assayed spectrophotometrically at 25°C. 3-Hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase were measured by coupled assays in which the formation of NADH was followed at 340 nm as described in detail by Binstock and Schulz (8). An assay mixture for 3-hydroxyacyl-CoA epimerase contained 0.2 M KP_i (pH 8), 0.33 mM NAD, 60 μM CoASH, 52 μM DL-3-hydroxyoctanoyl-CoA or 52 μM DL-3-hydroxydodecanoyl-CoA, bovine serum albumin (0.2 mg/ml), 0.08% Triton X-100, pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.3 U/ml), pig heart 3-ketoacyl-

CoA thiolase (33 mU/ml). The reaction was allowed to proceed until the L-isomer of 3-hydroxyacyl-CoA was completely degraded. The epimerase assay was then initiated by the addition of mitochondria. When a mitochondrial extract was assayed for 3-hydroxyacyl-CoA epimerase in the absence of Triton X-100, 20 nM rotenone was added to prevent the oxidation of NADH. An assay mixture for cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase contained 0.2 M KP_i (pH 8), 0.33 mM NAD, 60 μM CoASH, 52 μM 3-cis-octenoyl-CoA, bovine serum albumin (0.2 mg/ml), 0.08% Triton X-100, beef liver crotonase (4 U/ml), pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.3 U/ml), and pig heart 3-ketoacyl-CoA thiolase (33 mU/ml). The reaction was initiated by the addition of mitochondria. 2,4-Dienoyl-CoA reductase was assayed as described in principle by Kunau and Dommes (3). An assay mixture contained 0.2 M KP_i (pH 7), 60 μM NADPH, 0.08% Triton X-100, and mitochondria. The reaction was initiated by the addition of 26 μM 2,4-decadienoyl-CoA. A unit of enzyme activity is defined as the amount that catalyzes the conversion of 1 μmol of substrate to product per minute.

RESULTS AND DISCUSSION

The activities of 3-hydroxyacyl-CoA epimerase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase were measured in mitochondria from rat liver and rat heart (see Table I). 3-Hydroxyacyl-CoA epimerase was assayed with both D-3-hydroxyoctanoyl-CoA and D-3-hydroxydodecanoyl-CoA as substrates because the former substrate is a hypothetical intermediate in the degradation of linoleic acid, whereas the latter one was used by Stoffel and Caesar (1) to assay the same enzymes. The values for 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase presented in Table I differ significantly from those reported by Stoffel and Caesar (1). Activities of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase reported by them for heart and liver are 52 mU/mg and 260 mU/mg, respectively, whereas we have determined activities of more than 700 mU/mg in both tissues. However, we assayed the enzyme at the more physiological pH of 8 in contrast to Stoffel and Caesar (1) who made their measurements at a pH of 9.5. Activities of 3-hydroxyacyl-CoA epimerase in liver of 85 mU/mg and 146 mU/mg observed by us and Stoffel and Caesar (1), respectively, are of the same order of magnitude. However, the epimerase activity of 3.4 mU/mg determined by us in heart mitochondria is dramatically lower than the activity of 64 mU/mg reported by Stoffel and Caesar (1). This difference in activity cannot be due only to a pH difference of 8 vs. 9.2 used by us and Stoffel and Caesar, respectively, to assay this enzyme.

We have previously measured the activities of other β-oxidation enzymes at pH 8 in rat heart mitochondria (11). The activities of carnitine

Table I - Activites of auxiliary enzymes of β-oxidation required for the degradation of polyunsaturated fatty acids in rat liver (RLM) and rat heart mitochondria (RHM).

Enzymes	Substrates[a]	Enzyme Activities[b] RLM	RHM
		nmol/min and mg of protein	
3-Hydroxyacyl-CoA epimerase	D-3HOC$_8$-CoA	79.3 ± 14.4(3)	2 ± 0.3(7)
	D-3HOC$_{12}$-CoA	84.9 ± 8.4(3)	3.4 ± 0.5(5)
cis-Δ^3-trans-Δ^2-Enoyl-CoA isomerase	3-cis-C$_8$-CoA	721 ± 134(3)	745 ± 93(3)
2,4-Dienoyl-CoA reductase	$\Delta^{2,4}$-C$_{10}$-CoA	73.6 ± 17(3)	27.4 ± 5(5)

[a] D-3-HOC$_8$-CoA, D-3-hydroxyoctanoyl-CoA; D-3-HOC$_{12}$-CoA, D-3-hydroxydodecanoyl-CoA; 3-cis-C$_8$-CoA, cis-3-octenoyl-CoA; $\Delta^{2,4}$-C$_{10}$-CoA, 2-trans, 4-trans-decadienoyl-CoA.

[b] Values are means ± S.E. Figures in parentheses are the number of observations with samples from different animals. Each sample was assayed a minimum of three times.

palmitoyltransferase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase were found to be similar but low as compared to those of other β-oxidation enzymes. The optimal activities of these three enzymes are sufficient to permit the oxidation of approximately 25 nmol of fatty acids per min and per mg of mitochondrial protein. When we compare this optimal value for the rate of the β-oxidation cycle with the activities of the auxiliary enyzmes of β-oxidation listed in Table I, it is obvious that in liver all three enzymes are more active than the cycle. However, in heart only cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase, but not 3-hydroxyacyl-CoA epimerase, are sufficiently active to match the optimal rate of the β-oxidation cycle. In order to determine the actual rates of β-oxidation in mitochondria, we have measured rates of respiration supported by fatty acid oxidation. The rates of respiration observed with coupled mitochondria from rat liver and rat heart in the presence of either palmitoyl-CoA, oleoyl-CoA or linoleoyl-CoA are presented in Table II. With both types of mitochondria the respiratory rates changed little with different fatty acyl-CoA's as substrates. If one assumes that in heart mitochondria fatty acids are completely oxidized to CO_2, the observed rates of respiration can be used to calculate rates of β-oxidation which are given in Table II. These values are minimal ones because of the incomplete oxidation of fatty

Table II - Rates of respiration supported by fatty acid oxidation in rat liver (RLM) and rat heart (RHM) mitochondria.

Substrate	Rates of respiration[a] RLM	RHM	Rates of β-oxidation RHM
	nanoatoms O_2/min and mg of protein		nmol/min and mg of protein
Palmitoyl-CoA	73.7 ± 9.7	167.1 ± 18.8	3.6 ± 0.4
Oleoyl-CoA	78.8 ± 8.9	174 ± 26.1	3.4 ± 0.5
Linoleoyl-CoA	73.9 ± 8.4	153.1 ± 23.9	3.1 ± 0.5

[a]Values are means ± S.E. The values are based on three assays each with three different preparations of mitochondria all of which had RCR values larger than 4.

acids and the concomitant accumulation of intermediates, especially those of the tricarboxylic cycle. However, even if underestimated, a rate of linoleate degradation of 3.1 nmol/min/mg is higher than the activity of 2 nmol/min/mg determined for 3-hydroxyacyl-CoA epimerase with D-3-hydroxyoctanoyl-CoA which is a postulated intermediate in the pathway of linoleate degradation according to Stoffel and Caesar (1). Since the activity of 3-hydroxyacyl-CoA epimerase is insufficient to account for the observed rate of linoleate degradation in rat heart mitochondria, we conclude that linoleic acid and other polyunsaturated fatty acids with double bonds extending from even-numbered carbons are degraded via a sequence of reactions which includes the NADPH-dependent 2,4-dienoyl-CoA reductase.

The pathway of linoleate degradation proposed by Stoffel and Caesar (1) is shown in Fig. 1A. A modified pathway of linoleate degradation supported by data of Kunau and Dommes (3) as well as Cuebas and Schulz (4) is presented in Fig. 1B. The only difference between these two pathways is the removal of the cis-12-double bond. According to Stoffel and Caesar (1) linoleoyl-CoA is chain-shortened to cis-2-octenoyl-CoA which is hydrated to D-3-hydroxyoctanoyl-CoA. The D-hydroxy intermediate is isomerized by 3-hydroxyacyl-CoA epimerase to L-3-hydroxyoctanoyl-CoA which is completely degraded via β-oxidation. According to Kunau and Dommes (3) as well as Cuebas and Schulz (4) linoleoyl-CoA is chain-shortened to cis-4-decenoyl-CoA followed by dehydrogenation to cis-4,-trans-2-decadienoyl-CoA which is reduced by the NADPH-

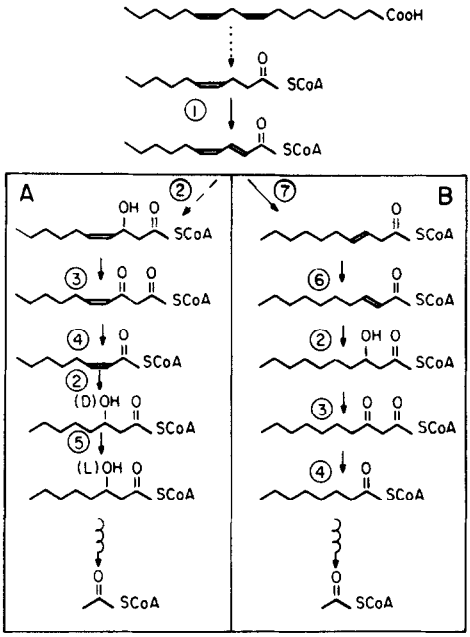

Fig. 1. Pathway of linoleic acid degradation. A, pathway proposed by Stoffel and Caesar (1). B, modified pathway supported by findings published by Kunau and Dommes (3) as well as Cuebas and Schulz (4). Reactions catalyzed by: (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase; (5) 3-hydroxyacyl-CoA epimerase; (6) cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase; (7) 2,4-dienoyl-CoA reductase.

dependent 2,4-dienoyl-CoA reductase to 3-decenoyl-CoA. After isomerization, the resulting 2-decenoyl-CoA can be completely β-oxidized. Acceptance of the modified pathway of linoleate degradation, however, raises the question as to the physiological function of 3-hydroxyacyl-CoA epimerase.

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