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ACYL-CoA OXIDASE OF RAT LIVER: A NEW ENZYME FOR FATTY ACID OXIDATION

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Acyl-CoA oxidase was purified from rat liver based on the palmitoyl-CoA-dependent H_2O_2-forming activity. Enoyl-CoA formation from palmitoyl-CoA by this enzyme was shown by the following observations; first, palmitoyl-CoA-dependent NAD^+ reduction in the presence of this enzyme, crotonase, and 3-hydroxyacyl-CoA dehydrogenase, and, second, palmitoyl-CoA-dependent increase in absorbance at 263 nm. Same amounts of enoyl-CoA and H_2O_2 were formed during the reaction. It is concluded that this enzyme catalyzes the following reaction: Palmitoyl-CoA + O_2 $\longrightarrow$ trans-2-Hexadecenoyl-CoA + H_2O_2. It was most active toward C_{12}-C_{18} acyl-CoAs. C_{20} and C_{22} acyl-CoAs were also oxidized, but C_4 and C_6 acyl-CoAs were hardly oxidized at all.

It has been reported that a fatty acyl-CoA oxidizing system is located in rat liver peroxisomes (1). The activity of this system is enhanced by the administration of various drugs or chemicals which exert hypolipidemic effects (1-3). In contrast to the mitochondrial fatty acyl-CoA oxidizing system, the peroxisomal system is insensitive to cyanide (1). A H_2O_2-producing enzyme, acyl-CoA oxidase, has been suggested to be involved in the initial step of the latter (1). However, this novel enzyme and the reaction catalyzed by it have not yet been characterized. In order to clarify the physiological role of the peroxisomal fatty acyl-CoA oxidizing system, we have tried to purify this enzyme. In the present study, using a purified preparation which is nearly homogeneous in sedimentation analysis, we observed a stoichiometric formation of

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trans-2-hexadecenoyl-CoA and H_2O_2 from palmitoyl-CoA. The chain length specificity for acyl-CoA substrates was also investigated.

MATERIALS AND METHODS

Materials —— Crotonase (enoyl-CoA hydratase; EC 4.2.1.17) was purified from bovine liver by the method of Steinman and Hill (4). Pig heart 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and horse radish peroxidase (EC 1.11.1.7) were purchased from Boehringer Mannheim GmbH. Acyl-CoAs were synthesized by mixed anhydride method (5). Longer chain acyl-CoAs were purified by precipitation with $HClO_4$ and by washing with acetone. CoA esters of C_8 and C_{10} acids were precipitated with $HClO_4$ in the presence of high concentration of NaCl (6). Those of C_4 and C_6 acids were used without further purification after extraction of free acids with ether. C_{20} and C_{22} CoA esters were dissolved in 1% Tween 20 after purification. Concentrations of C_4 and C_6 CoA esters were determined by measuring, with 5,5'-dithiobis-(2-nitrobenzoic acid), the thiol after the reaction with hydroxylamine (7). Other CoA esters were estimated based on absorbance at 260 nm. A molar extinction coefficient of 13,100 $M^{-1}cm^{-1}$ was used (8). NAD^+ and CoA were purchased from Kyowa Hakko Kogyo, Co., Tokyo. All other chemicals were of analytical grade.

Assay Method —— Acyl-CoA oxidase activity was assayed by measuring the palmitoyl-CoA-dependent H_2O_2 production by the method of Allain et al. (9). The reaction mixture contained, in a total volume of 1.0 ml, the following constituents: 50 mM potassium phosphate (pH 7.4), 0.82 mM 4-aminoantipyrine, 10.6 mM phenol, 10 μM FAD, 4 I.U. of horse radish peroxidase, 20 μM palmitoyl-CoA, and the enzyme. The reaction was carried out at 30°C, and the formation of H_2O_2 was measured by following the increase in absorbance at 500 nm. The molar extinction coefficient was 6,390 M^{-1} cm^{-1} at pH 7.4.

Preparation of Enzyme —— Male Wistar rats were fed with a diet containing 2% (w/w) di-(2-ethylhexyl)phthalate more than 2 weeks for the induction of peroxisomal fatty acyl-CoA oxidizing system (3). The liver (30 g) was homogenized with 150 ml of 50 mM Tris-HCl (pH 7.5) in a tissue disintegrator (Ultra-turrax). The resulting homogenate was centrifuged at 17,000 x g for 15 min. To the supernatant obtained was added FAD (final concentration of 25 μM), and the mixture was heated for 5 min in a water bath kept at 60°C. After rapid cooling to 4°C the precipitate formed was removed by centrifugation. The supernatant was fractionated with $(NH_4)_2SO_4$ and the protein precipitated between 30 and 45% saturation was collected by centrifugation and dissolved in 5 ml of 10 mM potassium phosphate (pH 7.8). This solution was passed through a Sephadex G-25 column (1.7 x 18 cm) with the same buffer for removal of the remaining $(NH_4)_2SO_4$, and then applied to a DEAE-cellulose column (1.7 x 12 cm) which had been equilibrated with the same buffer. The clear yellow protein fraction which passed through the column unretarded was collected. To this solution was added $(NH_4)_2SO_4$ (45% saturation), and the protein precipitated was collected and dissolved in 2 ml of 50 mM potassium phosphate (pH 7.4).

Determination of Protein —— Protein concentration was determined by the method of Lowry et al. (10).

Table 1. Purification of acyl-CoA oxidase from rat liver.

Step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)
1. 17,000 x g Supernatant	279	4,962	0.056	100
2. Heat treatment	230	1,254	0.18	82.4
3. 1st $(NH_4)_2SO_4$ (30-45%) precipitate	194	250	0.78	69.5
4. DEAE-Cellulose eluate	117	101	1.16	41.9
5. 2nd $(NH_4)_2SO_4$ (0-45%) precipitate	120	83.0	1.45	43.0

RESULTS AND DISCUSSION

A typical result of the purification of the enzyme is summarized in Table 1. The specific activity was raised by about 25-fold by these procedures. The 2nd $(NH_4)_2SO_4$ fraction was nearly homogeneous in sedimentation analysis. It did not contain crotonase, 3-hydroxyacyl-CoA dehydrogenase, or catalase. In the following experiments, this fraction was used as the enzyme. The results of the purification will be described elsewhere in detail.

In order to identify the product of the acyl-CoA oxidase reaction, it was tried to constitute a palmitoyl-CoA-dependent NAD^+-reducing system. As shown in Fig. 1, only in the presence of all of acyl-CoA oxidase, crotonase, and 3-hydroxyacyl-CoA dehydrogenase, NAD^+ was reduced by palmitoyl-CoA.

The formation of enoyl-CoA from palmitoyl-CoA was also shown by the increase in absorbance at 263 nm (Fig. 2). The absorbance was decreased by the addition of crotonase. The difference absorption spectrum of the reaction product in UV region, before and

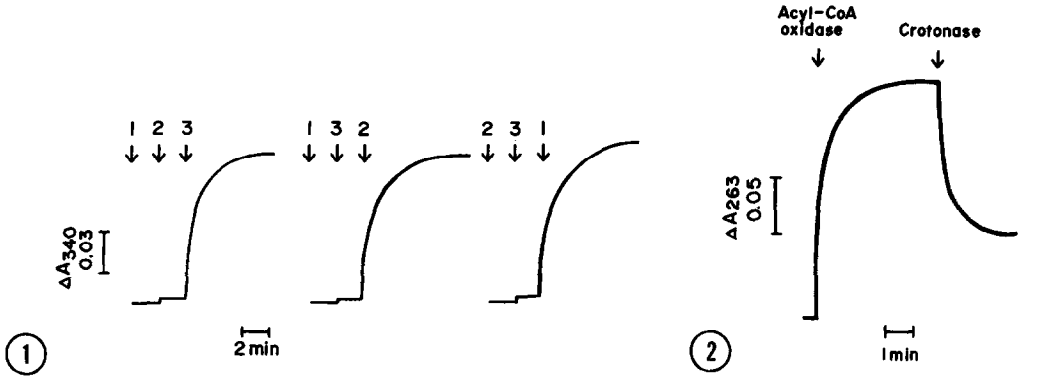

Fig. 1. Palmitoyl-CoA-dependent NAD^+ reduction in the presence of acyl-CoA oxidase, crotonase, and 3-hydroxyacyl-CoA dehydrogenase. The reaction mixture contained in a total volume of 1.0 ml, 90 mM boric acid-KCl-NaOH, pH 9.0, 1.0 mM NAD^+, 20 μM palmitoyl-CoA, and the enzymes. Acyl-CoA oxidase (12.4 μg), crotonase (52.5 μg), and 3-hydroxyacyl-CoA dehydrogenase (4.0 μg) were added at the time indicated by the arrows 1, 2, and 3, respectively, and the absorption change at 340 nm were followed.

Fig. 2. Enoyl-CoA formation from palmitoyl-CoA by acyl-CoA oxidase. The initial reaction mixture contained in a total volume of 1.0 ml, 50 mM potassium phosphate (pH 7.4), 20 μM palmitoyl-CoA, and acyl-CoA oxidase (18.6 μg). The reaction was started by the addition of acyl-CoA oxidase, and the absorption change at 263 nm was followed. Crotonase (10.5 μg) was added at the time indicated.

after treatment with NH_2OH, accorded well to that of crotonyl-CoA and trans-2-hexadecenoyl-CoA (data not shown). It had a peak at 262-263 nm, whereas the spectrum of palmitoyl-CoA had no characteristic peak around 263 nm. Though crotonase hydrates both trans and cis isomers of 2-enoyl-CoA and 3-enoyl-CoA, cis-2-enoyl-CoA has an absorption peak at 275 nm, but not at 263 nm, and 3-enoyl-CoA has no characteristic absorption peak around 263 nm (11). The above data indicate that trans-2-enoyl-CoA was formed from palmitoyl-CoA by acyl-CoA oxidase.

During the formation of enoyl-CoA, H_2O_2 was produced stoichiometrically (Table 2). This result excludes the possibilities of occurrence of any reaction other than the double bond formation

Table 2. Stoichiometric formation of 2-hexadecenoyl-CoA and H_2O_2 from palmitoyl-CoA by acyl-CoA oxidase. The reaction mixture contained in a total volume of 1.0 ml, 50 mM potassium phosphate (pH 7.4), palmitoyl-CoA of the amount indicated, and the enzyme (18.6 µg). The increase in absorbance at 263 nm was followed, and after 15 min, the reaction was stopped by the addition of $HClO_4$. The H_2O_2 formed during the reaction was determined by the method of Allain et al. (9) after neutralization of the solution with $KHCO_3$.

Palmitoyl-CoA added (nmol)	2-Hexadecenoyl-CoA formed (nmol)	H_2O_2 formed (nmol)
40.0	30.4	28.8
50.0	36.3	37.5
60.0	42.7	40.7

between C-2 and C-3 during acyl-CoA oxidase reaction. Therefore, the reaction can be represented by the following formula:

Palmitoyl-CoA + O_2 —— trans-2-Hexadecenoyl-CoA + H_2O_2

The chain length specificity for acyl-CoA substrates of the enzyme was studied (Fig. 3). The activity was highest toward the C_{12}-C_{18} substrates. C_{20} and C_{22} acyl-CoAs were also oxidized, though in lower rates. However, the activity toward C_6 acyl-CoA was only about 5% of that toward C_{16} acyl-CoA and no detectable activity was observed toward C_4 acyl-CoA. In pig liver mitochondria, there are three acyl-CoA dehydrogenases which have different chain length specificities (12). Acyl-CoA oxidase, however, seems to be more active to longer acyl-CoAs than any of those three enzymes. In this regard Bremer has suggested a possibility that the role of peroxisomal fatty acid oxidizing system is oxidation of very long chain acids which can scarcely be oxidized by the mitochondrial system (13).

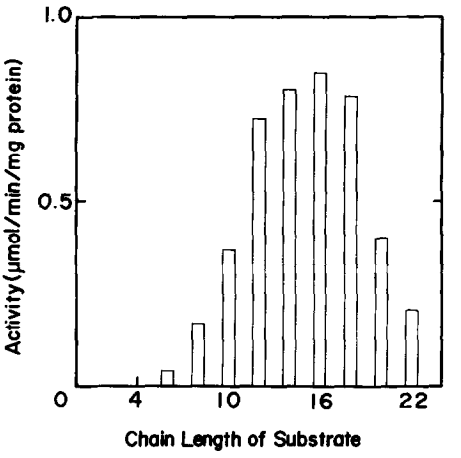

Fig. 3. Chain length specificity of acyl-CoA oxidase for acyl-CoA substrates. The activity was measured as described in "MATERIALS AND METHODS" except that 10 μM various acyl-CoAs were used as the substrates.

It was found that acyl-CoA oxidase is localized in peroxisomes and that its activity is enhanced by the administration of di-(2-ethylhexyl)phthalate (Osumi,T., unpublished observations). The present results suggest that in peroxisomes fatty acyl-CoA is first oxidized to the corresponding trans-2-enoyl-CoA by acyl-CoA oxidase, and then processed as it is in mitochondria. Further studies are now in progress on the catalytic and molecular properties of acyl-CoA oxidase.

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