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ACYL-CoA OXIDASE FROM *CANDIDA TROPICALIS*

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The preparation of a highly purified acyl-CoA oxidase from the cell extract of an *n*-alkane-utilizing yeast, *Candida tropicalis*, is described. It can be crystallized from ammonium sulfate solutions without an increase in specific activity, and is homogeneous on ultracentrifuge and disc electrophoresis. The enzyme is an octamer with approximately a 600,000 molecular weight, and has an isoelectric point of 5.5. It exhibits a typical flavoprotein spectrum with absorption maxima at 277, 365 and 445 nm, and contains 8 mol of FAD per mol of enzyme. The enzyme catalyzes the stoichiometric conversion of palmitoyl-CoA and O_2 into 2-hexadecenoyl-CoA and H_2O_2. It oxidizes acyl-CoAs with carbon chain lengths of 4 to 20, and is most active toward lauroyl-CoA, but acetyl- and succinyl-CoAs are not oxidized. The enzyme is sulfhydryl dependent and is inactivated by silver and mercury compounds.

Peroxisomal fatty acyl-CoA oxidase was first studied by Cooper and Beevers (1). The presence of this novel enzyme has been reported in castor bean endosperm (1), rat liver (2), *Tetrahymena* (3) and yeasts (4). Some properties of the enzyme have recently been found for the partially purified enzyme from rat liver (5,6). In *n*-alkane-utilizing yeasts, acyl-CoA oxidase is believed to play an important role in the β-oxidation of fatty acids formed from *n*-alkanes (4). We now have obtained the acyl-CoA oxidase from an *n*-alkane-utilizing yeast, *Candida tropicalis*, in a highly purified form. Some molecular and catalytic properties of this enzyme are reported here.

MATERIALS AND METHODS

Materials. Palmitoyl-CoA was purchased from PL-Biochemicals, Milwaukee. Other CoA esters were purchased from Sigma Chemical Co., St. Louis. Horse raddish peroxidase (EC 1.11.1.7) was purchased from Toyobo Co., Osaka. The *n*-alkane mixture (C_{13}-C_{16}) was the gift of Mitsui Petrochemical Industry Co., Tokyo. All other chemicals were commercially available and of analytical grade.

Cultivation. *Candida tropicalis* pK 233 AKU 4617 (4) was cultivated in a 2-liter flask containing 500 ml of a medium composed of 1 g *n*-alkane, 0.5

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g NH_4HPO_4, 0.25 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.002 g $FeCl_3$, 0.1 g corn steep liquor, and 0.02 g Tween 85, pH 5.2. Cultivation was carried out at 28°C for 40 hr with shaking.

Enzyme assay. Acyl-CoA oxidase activity was usually assayed by the method of Allain *et al.* (7). The reaction mixture contained, in 0.4 ml: 20 µmol potassium phosphate, pH 7.4; 0.33 µmol 4-aminoantipyrine; 4.24 µmol phenol; 0.004 µmol FAD; 0.04 µmol acyl-CoA; 12 IU peroxidase; and 0.5-5 µg enzyme. The reaction was carried out at 30°C, and the production of H_2O_2 was measured by following the increase in absorbance at 500 nm. The micromolar extinction coefficient of 6.39 $cm^2/\mu mol$ at pH 7.4 (5) was used. In some cases, enzyme activity was measured by following the increase in absorbance at 263 nm due to enoyl-CoA formation. The reaction conditions were the same as described above except that 4-aminoantipyrine, phenol, FAD, and peroxidase were omitted from the reaction mixture. The micromolar extinction coefficient of 6.70 $cm^2/\mu mol$ (8) was used.

Other methods. Protein determination was carried out by measuring the absorbance at 280 nm, unless otherwise stated. An E value of 15.6 for 10 mg/ml and 1-cm light path, determined by absorbance and dry weight measurements, was used. Spectrophotometric determinations were made with a Hitachi 200-10 spectrophotometer with a 1-cm light path. Polyacrylamide disc gel electrophoresis was carried out in Tris-glycine buffer, pH 8.3, according to the method of Davis (9). The molecular weight of a subunit(s) was determined by disc electrophoresis as described by Weber and Osborn (10) with 10% gels and an SDS-phosphate buffer system at pH 7.2. All ultracentrifugal analyses were carried out with a Hitachi 282 analytical ultracentrifuge at 20°C. Isoelectric focusing was carried out accoding to the method of Vesterberg (11).

RESULTS AND DISCUSSION

Purification of Acyl-CoA Oxidase

Cells (200 g wet weight) from 20 lots of 500 ml cultures were suspended in 0.01 M potassium phosphate, pH 7.4 (700 ml), then disrupted on an ultrasonic oscillator (19 KHz, 3 hr, 0-15°C). All subsequent procedures were carried out at 0-5°C. After the cell debris was removed by centrifugation (10,000 x *g*, 30 min), the supernatant was fractionated with ammonium sulfate (30-50% saturation), followed by dialysis against 0.01 M potassium phosphate, pH 7.4. The dialyzed solution was applied to a DEAE-cellulose column (6.5 x 30 cm) equilibrated with 0.01 M potassium phosphate, pH 7.4. After the column had been washed with 0.01 M potassium phosphate, pH 7.4, containing 0.08 M KCl (3,000 ml), the enzyme was eluted with 0.01 M potassium phosphate, pH 7.4, containing 0.25 M KCl. Active fractions were combined and concentrated by the addition of ammonium sulfate to 45% saturation. The precipitate was collected by centrifugation (16,000 x *g*, 30 min), then dissolved in a minimum volume of 0.02 M potassium phosphate, pH 7.4, This solution was

Table 1. Purification of acyl-CoA oxidase from *Candida tropicalis*. Enzyme activity was measured by following H_2O_2 formation as described in MATERIALS AND METHODS. Palmitoyl-CoA was used as the substrate. One unit of enzyme activity is defined as the amount of the enzyme which produces 1 μmol H_2O_2 per min. Specific activity is given as units per mg protein. Protein concentration was measured by the method of Lowry *et al.* (12) with bovine serum albumin as the standard. One mg of the enzyme is equivalent to 1.28 mg of Lowry protein.

Purification step	Total protein (mg)	Total units	Specific activity	Yield (%)
Cell-free extract	6,080	3,830	0.63	100
Ammonium sulfate fractionation	1,746	2,811	1.61	73.4
DEAE-cellulose chromatography	73.0	1,077	14.76	28.1
Sephadex G-200 gel filtration	42.4	811	19.13	21.1

passed through a Sephadex G-200 column (2.8 x 97 cm) equilibrated with the same buffer. Active fractions were combined and concentrated by ultrafiltration (Sartorius-Membranefilter GmbH, Göttingen). A summary of the typical purification procedures is given in Table 1.

Properties of the Acyl-CoA Oxidase

The purified acyl-CoA oxidase can be crystallized from a 30% saturated ammonium sulfate solution at 5°C, with no increase in specific activity. Yellow crystals appeared as fine rods (Fig. 1). The enzyme gave a single band on polyacrylamide gel electrophoresis and sedimented as a single symmetric peak with a sedimentation coefficient ($S_{20,w}$) of 16.7 S in the analytical ultracentrifuge (42,040 rpm). The molecular weight of the enzyme was determined to be 600,000 by sedimentation equilibrium (13) at 3000 rpm and by gel filtration with Sepharose 6B (14). The SDS-gel profile of the enzyme showed a single band. A molecular weight of 74,000 for the subunit(s) was estimated. The native enzyme probably consists of a molecule of 8 (possibly identical) subunits; its isoelectric point was 5.5. The enzyme showed absorption peaks in the visible spectrum at 365 and 445 nm ($E_{280\ nm}/E_{445\ nm} = 9.0$). The addition of palmitoyl-CoA to the enzyme under unaerobic conditions bleached the visible color (Fig. 2). The boiled extract

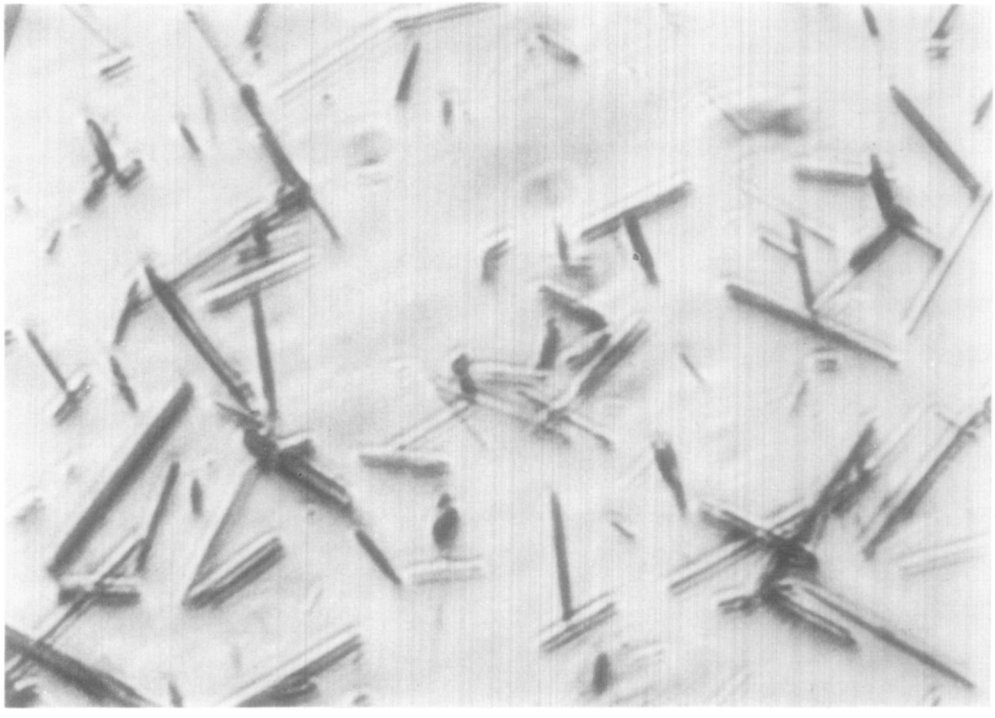

Fig. 1. Crystals of acyl-CoA oxidase.

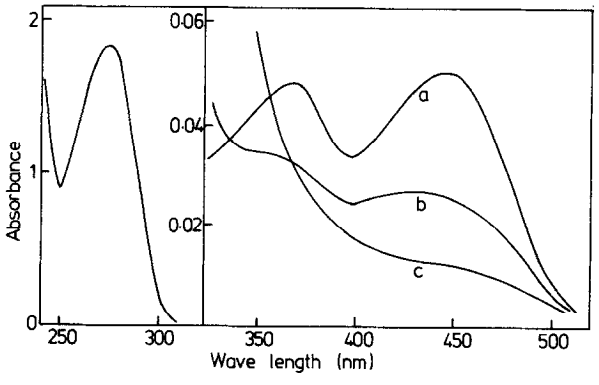

Fig. 2. Absorption spectra of acyl-CoA oxidase in 0.02 M potassium phosphate, pH 7.4. Spectra were recorded with 0.12% solution of the enzyme in the ultraviolet region and with 0.03% solution in the visible region. Curve (a) represents the native enzyme; curves (b) and (c) represent the reduced enzyme after the addition of 1 μmol palmitoyl-CoA and sodium dithionite, respectively, to 0.4 ml of the enzyme solution.

of the enzyme showed the typical absorption spectrum of flavin. The flavin derivative was identified as FAD by thin layer chromatography (15). The FAD content of the enzyme was calculated as 8.5 mol per mol of enzyme based on

the absorption coefficient of 11.3 $cm^2/\mu mol$ (16). Thus, the enzyme is thought to contain 8 mol of FAD per mol and possibly 1 mol of FAD in 1 mol of the subunit.

The oxidation of palmitoyl-CoA by acyl-CoA oxidase proceeded as follows:

Palmitoyl-CoA + O_2 ⟶ 2-Hexadecenoyl-CoA + H_2O_2

Consumption of O_2 and formation of the enoyl-CoA and H_2O_2 were found in stoichiometric amounts. The enzyme was active toward acyl-CoAs with carbon chain lengths of 4 to 20, but was inactive toward acetyl- and succinyl-CoAs. Table 2 shows the *Km* and *Vmax* values of the enzyme for some acyl-CoAs. Lauroyl-CoA was the preferred substrate for the enzyme. The enzyme had maximal activity at pH 8.0 and at 50°C. It was stable in the pH range of 5.5 to 9.0 at 35°C for 60 min. Almost full activity was retained after incubation at 50°C for 10 min, whereas at 65°C for 10 min the enzyme was completely inactivated. Sulfhydryl reagents such as $AgNO_3$, $HgCl_2$, mercuric acetate and *p*-chloromercuribenzoate markedly inhibited enzyme activity. The addition of glutathione protected the enzyme from inhibition.

Table 2. Chain length specificity of acyl-CoA oxidase for acyl-CoA substrates. Enzyme activity was measured by the H_2O_2 formation with various concentrations of acyl-CoAs. Apparent *Km* and *Vmax* values were determined from Lineweaver-Burk plots (17).

Fatty acyl-CoA[a)]	Chain length	*Km* (μM)	*Vmax* (μmol/min/mg)
Octanoyl-CoA	C-8	42.5	9.1
Decanoyl-CoA	C-10	24.0	55.5
Lauroyl-CoA	C-12	24.0 (22.5)[b)]	57.5 (60.4)[b)]
Myristoyl-CoA	C-14	29.0 (23.5)	28.6 (30.1)
Palmitoyl-CoA	C-16	33.5 (24.0)	22.2 (26.5)
Stearoyl-CoA	C-18	34.0	10.0
Oleoyl-CoA	C-18:1	46.0	37.0
Arachidoyl-CoA	C-20	33.5	4.1

a) Butyryl-CoA and hexanoyl-CoA were also oxidized by the enzyme, at the relative reaction rates of 5.4 and 1.8, respectively, in the comparison with lauroyl-CoA as 100.
b) Values in parentheses were determined from the data obtained by the enoyl-CoA formation.

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