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PURIFICATION OF THE PEROXISOMAL FATTY ACYL-CoA OXIDASE FROM RAT LIVER

Nibaldo C. Inestrosa, Miguel Bronfman and Federico Leighton

Department of Cell Biology, Universidad Católica de Chile,
Casilla 114-D, Santiago, Chile

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SUMMARY

Fatty acyl-CoA oxidase, the rate limiting enzyme of the peroxisomal fatty acid oxidizing system, has been purified from rat liver to near homogeneity by a procedure involving affinity chromatography of its apoenzyme on flavin adenin dinucleotide-Sepharose. The oxidase presents an absolute requirement for the dinucleotide which is weakly bound to the apoenzyme (K_D, 0.6 μM). The highest specific activity obtained was 27 units/mg protein. The purified enzyme has two major polypeptides with apparent molecular weights of 45,000 and 22,000. These results suggest that the enzyme is a flavoprotein with non covalently bound flavin adenin dinucleotide composed of four subunits, two of 45,000 m.w. and two of 22,000 m.w.

INTRODUCTION

The peroxisomal fatty acyl-CoA oxidizing system from rat or human liver catalyzes fatty acid β-oxidation by a cyanide insensitive sequence of reactions similar to that of mitochondria (1,2). The desaturation of the acyl-CoA derivative, mediated in mitochondria by a dehydrogenase tightly coupled to the electron transport chain (3), is catalyzed in peroxisomes by fatty acyl-CoA oxidase an enzyme active mainly on C_{12}-C_{16} fatty acids (4,5,6). This oxidase has been partially characterized as a globular flavoprotein of 136,000 m.w. and 7.7 S (7). It catalyzes the apparent rate limiting step of the system in human liver (2) as well as in the liver from normal rats (6,8,9) or from rats in which the system has been enhanced either 8-fold by hypolipidemic drugs (6,8) or 2.4-fold by feeding a diet rich in saturated fatty acids (10). In this communication we report the purification and some properties of the fatty acyl-CoA oxidase from rat liver. In addition, a model for the subunit organization of the enzyme is proposed.

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MATERIALS AND METHODS

The fatty acyl-CoA oxidase activity was measured polarographically from palmitoyl-CoA dependent O_2 consumption (6). The assay medium, at 37°, contained 100 mM Tris-HCl pH 8.3, 0.6 mg/ml bovine serum albumin (fraction V), 1 mM KCN, 170 μM CoA, 50 μM FAD and 37 μM palmitoyl-CoA. One unit of enzyme activity corresponds to one μmol O_2 utilized per minute. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard, and by the Kalb and Bernlohr procedure (12), for column effluents.

Batches of 550 g of livers from Nafenopin treated male Sprague-Dawley rats (6) were homogenized with a teflon-glass homogenizer in 0.25 M sucrose containing, at pH 7.4, 20 mM potassium phosphate, 1 mM mercaptoethanol and 0.3 mM EDTA in a final volume of 800 ml. The supernatant fraction, after centrifugation at 45,000 g for 30 min, was made 1 mM with zinc sulphate pH 6.6 and recentrifuged. The supernatant thus obtained was made 25 μM in FAD and then incubated 10 min at 55°. After removing the precipitated proteins, the new supernatant was fractionated with ammonium sulphate and the proteins precipitating between 35 and 45% saturation were dissolved in 31 ml of 20 mM potassium phosphate pH 7.4, 1 mM mercaptoethanol, 0.3 mM EDTA (buffer A) with 0.1 M potassium chloride and then applied to a Sepharose 6B column equilibrated with buffer A-0.1 M KCl. The proteins from the peak of activity were concentrated by ammonium sulphate precipitation, 30-45%, dissolved and dialyzed against buffer A, and applied to a phosphocellulose column previously equilibrated with the same buffer but at pH 6.2. Elution was performed by a stepwise increase of the concentration of potassium phosphate up to 0.5 M. The fractions containing the peak of activity were immediately pooled and concentrated by ammonium sulphate precipitation, dissolved in 4 ml of Tris-HCl, 10 mM, pH 8.3, 0.3 mM EDTA, 1 mM mercaptoethanol, 0.1 M KCl (buffer B) and applied to a 74x1.7 Sephadex G-200 column equilibrated in buffer B-0.01% sodium azide. The proteins from the peak of activity were concentrated by ammonium sulphate precipitation, dissolved in 1.7 ml of Tris-HCl, 50 mM, pH 8.3, 1 mM ammonium sulphate, dissolved in 17 ml of Tris-HCl 50 mM pH 8.3, 1 mM EDTA (buffer B) and applied to a FAD-Sepharose column for affinity chromatography. The adsorbent was prepared attaching FAD to amino hexyl Sepharose by a diazo coupling procedure. The details of the method for the preparation and for the evaluation of the column with apo D-aminoacid oxidase, will be described elsewhere. The FAD-Sepharose column (1.2 x 4.0 cm) was washed with buffer B, either alone or supplemented with 0.1 M or 0.5 M KCl and finally, the oxidase was eluted with buffer B containing 0.5 M KCl and 15 mM FAD.

The oxidase obtained from affinity chromatography was subjected to electrophoresis on polyacrylamide gel under both denaturing (13) and nondenaturing conditions (14). The following molecular weight markers were used: bovine liver catalase 60,000, pig kidney D-aminoacid oxidase 37,000 and yeast RNA polymerase I (kindly provided by Dr. P. Bull from our Department) with 11 subunits (15), 185,000; 137,000; 48,000; 44,000; 41,000; 36,000; 28,000; 24,000; 20,000; 14,500 and 12,000.

The apparent dissociation constant of FAD in the fatty acyl-CoA oxidase was calculated from the activities observed after exposing the apoenzyme to FAD at various concentrations, a method previously used with D-amino acid oxidase (16) and L-aminoacid oxidase (17).

Table I. PURIFICATION OF FATTY ACYL-CoA OXIDASE FROM RAT LIVER

Purification step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (times)	Yield (%)
1. Homogenate	79,360	3360	0.042	1.0	100
2. Supernatant (Zn^{+2})	11,040	2815	0.26	6.1	84
3. Precipitation 10 min at 55° 35-45% $(NH_4)_2SO_4$	1,327	2108	1.59	37.8	66
4. Sepharose 6B	356	1535	4.30	102.4	46
5. Phosphocellulose	64.7	600	9.28	221	18
6. Sephadex G-200	11.9	264	22.25	530	8
7. FAD-Sepharose	4.5	124	27.20	648	3.7

RESULTS AND DISCUSSION

The procedure employed to isolate the fatty acyl-CoA oxidase, as illustrated in Table I, results in an enzyme preparation purified over 600-fold with a specific activity of 27 U/mg protein. Osumi and Hashimoto, for an enzyme preparation they found to be homogeneous by sedimentation analysis (5), reported a specific activity of 1.45 U/mg protein and only 25-fold purification from a high speed supernatant.

The enzyme obtained after the Sephadex G-200 chromatography step appears homogeneous in native polyacrylamide gel electrophoresis, Fig 1a. After SDS-polyacrylamide gel electrophoresis, Fig 1b, three major bands with apparent molecular weights of 22,000; 45,000 and 77,000 are detected. The first two bands account for 75% of the Coomassie blue staining material. The molar ratio calculated densitometrically for the subunits was 1.00; 1.09 and 0.15 for the bands with 22,000; 45,000 and 77,000 m.w., respectively.

The additional purification following affinity chromatography, Fig 2, is accounted for by the elimination of minor protein bands and a selective decrease of the 77,000 m.w. band. After this step, 95% of

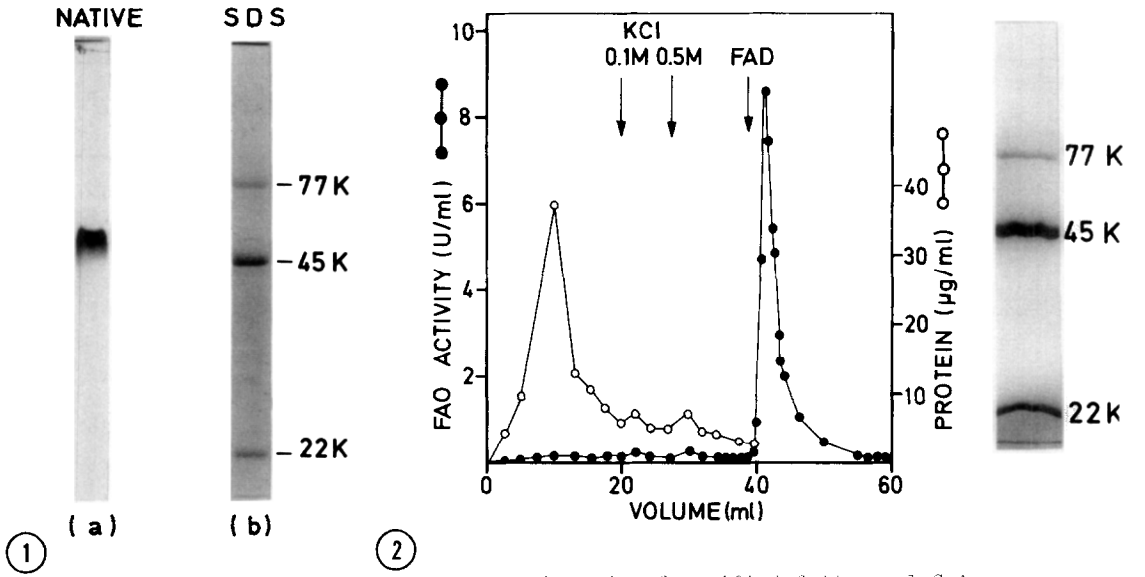

Fig. 1. Polyacrylamide gel electrophoresis of purified fatty acyl-CoA oxidase after the Sephadex G-200 step. (a) 4 µg of protein, under non denaturing conditions, in 5% acrylamide. (b) 4 µg of protein under denaturing conditions, 9% acrylamide in the presence of 0.1% sodium dodecyl sulphate and 5% β-mercaptoethanol.

Fig. 2. Affinity chromatography of fatty acyl-CoA oxidase on FAD-Sepharose. The column, 4 x 1.2 cm, was equilibrated with 50 mM Tris-HCl pH 8.5 with 1 mM EDTA. The enzyme, applied in the same buffer, was eluted by the addition of 15 mM FAD to the buffer already supplemented with 0.5 M KCl. 3 µg of protein from the peak of activity, after dialysis against the same buffer, were analyzed by slab gel electrophoresis at 9% acrylamide concentration in the presence of 0.1% sodium dodecyl sulphate and 5% mercaptoethanol. The molecular weight estimations derive from simultaneously run standards.

the Coomassie stained material is accounted for by the 45,000 and 22,000 m.w. peptides. The final molar ratio of the preparation was 1.00; 1.00 and 0.06 for the bands with 22,000; 45,000 and 77,000 m.w., respectively.

The non stoichiometric relation of the 77,000 m.w. peptide, together with our previous determination of a molecular weight of 136,000 for the oxidase (7) indicate that probably the enzyme is composed of 2 subunits of 22,000 m.w. and 2 subunits of 45,000 m.w. The 77,000 m.w. peptide, also a minor component of whole peroxisomes from normal rats (18), appears to be the peptide described by Reddy and Kumar (19) which is induced by hypolipidemic drugs and apparently

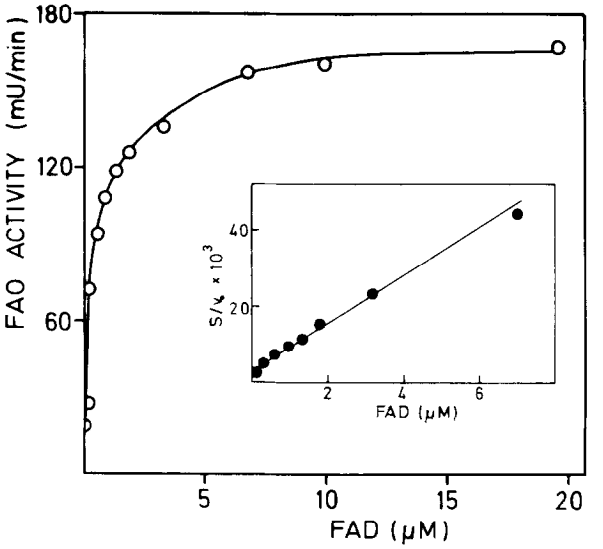

Fig. 3. Reactivation of purified fatty acyl-CoA oxidase by FAD. Enzyme with a specific activity of 22 U/mg protein was assayed at various FAD concentrations. FAD was measured spectrophotometrically from its absorbance at 455 nm, using an extinction coefficient of 11.3 mM^{-1} cm^{-1}. The apparent dissociation constant (K_D) for FAD was calculated from an S/v_o versus S graph, where S represents FAD concentration and v_o initial velocity. By extrapolation, a K_D value of 0.6 μM was obtained.

contains enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (20). These facts, together with the stimulatory effect of NAD^+ on the oxidase activity of crude preparations (6), and the parallelism of the activities of the oxidase and the peroxisomal fatty acid oxidizing system in various subcellular fractions (6), raise the possibility of a peroxisomal fatty acid oxidizing multienzyme complex.

The purified fatty acyl-CoA oxidase, known to be a flavoprotein with weakly bound FAD (7), is present mainly as apoenzyme after Sephadex G-200 chromatography. As shown in Fig 3, a 10-fold increase in activity is observed following the addition of FAD. The situation is reminiscent of the behaviour of D-aminoacid oxidase, another flavoprotein with weakly bound FAD, that could be purified as holoenzyme only after stabilization with benzoate (21). In fact, as shown in Fig. 3, an apparent dissociation constant of 0.6 μM was calculated for

FAD in the fatty acyl CoA oxidase, a value similar to that obtained for D-amino acid oxidase (21), for which also a fast exchange reaction between free and bound FAD has been described (22). These properties are in accordance with the behaviour of the apoenzyme on Sepharose-FAD and should allow us to develop simpler isolation procedures for the fatty acyl-CoA oxidase.

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