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Purification of Heart and Liver Mitochondrial Carnitine Acetyltransferase

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Heart and liver mitochondrial, as well as liver peroxisomal, carnitine acetyltransferase was purified to apparent homogeneity and some properties, primarily of heart mitochondrial carnitine acetyltransferase, were determined. Hill coefficients for propionyl-CoA are 1.0 for each of the enzymes. The molecular weight of heart mitochondrial carnitine acetyltransferase, determined by SDS–PAGE, is 62,000. It is monomeric in the presence of catalytic amounts of substrate. Polyclonal antibodies against purified rat liver peroxisomal carnitine acetyltransferase precipitate liver and heart mitochondrial and liver peroxisomal carnitine acetyltransferase, but not liver peroxisomal carnitine octanoyltransferase. Liver peroxisomes, mitochondria, and microsomes and heart mitochondria all give multiple bands on Western blotting with the antibody against carnitine acetyltransferase. Major protein bands occur at the molecular weight of carnitine acetyltransferase and at 33 to 35 kDa.

Carnitine acetyltransferase (CAT),[1] an enzyme that reversibly transfers short-chain acyl groups between carnitine and CoASH, is prevalent in most, if not all, mammalian tissues (1,2). Although CAT is associated with mitochondria of all tissues, in organs such as liver, it is also located in peroxisomes and microsomes (3–5). Initial investigations into the properties and functions of this enzyme indicated a role in buffering the intracellular acetyl-CoA pool (6,7). Subsequent investigations demonstrated CAT has a high capacity to reversibly transfer several short-chain acyl groups between CoA and carnitine, which led to the demonstration that it is involved in detoxification of some non- or poorly metabolizable acyl residues and that it also modulates the acyl-CoA to CoA ratio in heart and liver mitochondria (8–12). Although CAT has been extensively studied by many investigators [see Refs. (2,13) for reviews], the structural/functional relationships between the various forms of CAT, i.e., mitochondrial, peroxisomal, and microsomal of liver, are not known. For example, CAT has been purified and sequenced from extracts of human liver (14), but its organelle origin was not firmly established. One report indicated CAT is a dimer (15), but subsequent investigations by others show that CAT can retain catalytic activity after proteolytic modification of the native monomer (2). Establishment of the functions and properties of individual CATs requires purification procedures that give adequate yields of high-quality enzymes. As part of ongoing investigations into the functions of CAT, we have purified CAT from rat heart and liver mitochondria and from rat liver peroxisomes. Their purification is reported herein.

[1] Abbreviations used: CAT, carnitine acetyltransferase; AG-CoA, agarose-coenzyme A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

MATERIALS AND METHODS

Isolation of Rat Heart Mitochondria

Rat heart mitochondria were isolated from male Sprague–Dawley rats weighing 150–200 g as described (16). Mitochondria were suspended in 5 ml of 225 mM mannitol, 75 mM sucrose, and 1 mM EGTA, pH 7.5 (buffer A), loaded into tubes containing 20 ml of 30% (v/v) Percoll in 225 mM mannitol, 1 mM EGTA, 25 mM Hepes, pH 7.5, and spun for 30 min at $100{,}000g_{max}$. The dense, brownish-yellow mitochondrial band was washed twice with buffer A by centrifuging for 10 min at $6500g_{max}$. Liver mitochondria were isolated as described in (9) and liver peroxisomes as described in (5).

Purification of Heart Mitochondrial CAT

(a) Extraction of CAT. Frozen rat heart mitochondria from 45 to 50 rats in buffer A (~400 mg) were thawed, diluted to 20 ml with buffer A containing 1 M

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KCl, 10% glycerol, 10 mM Hepes, and protease inhibitors (0.1 mM PMSF, 1 μg/ml of pepstatin, leupeptin, and bestatin), stirred for 30 min, and spun for 30 min at 100,000g_{max}. The pellet was reextracted, and about 49 U of CAT activity was diluted three-fold with 5 mM Hepes, 10% glycerol, and protease inhibitors, pH 7.5, and applied to a Sephacryl S-300 column.

(b) Sephacryl S-300 gel filtration. Sephacryl S-300 in degassed 5 mM Hepes, pH 7.5, was poured into a 2.5 × 100-cm column and equilibrated with 5 mM Hepes buffer, pH 7.5, containing 0.5 mM EDTA, 0.3 M KCl, 20% glycerol, and protease inhibitors. CAT was loaded and eluted with the equilibration buffer. Pooled CAT was concentrated to 20 ml or less by an Amicon concentrator, 10,000 cutoff, and subjected to cellulose phosphate chromatography.

(c) Cellulose phosphate column chromatography. A 2 × 20-cm column of cellulose phosphate in 0.02% NaN_3 was equilibrated with 5 mM Hepes, 0.5 mM EDTA, 20% glycerol, 0.02% NaN_3, 0.1 M KCl, and protease inhibitors, pH 7.5. CAT was loaded and the column was washed with two column volumes of the equilibration buffer and then eluted with 300 ml of a linear gradient of 0.1–0.5 M KCl in the equilibration buffer. Fractions containing CAT were pooled and concentrated to 5 ml.

(d) Agarose-CoA (AG-CoA type 5) chromatography. The concentrated CAT from cellulose phosphate column chromatography was loaded onto a 1.5 × 10-cm agarose-CoA column that had been equilibrated with 5 mM Hepes, 20% glycerol, 0.5 mM EDTA, 0.02% NaN_3, and protease inhibitors, pH 7.5. After washing with two column volumes of the equilibration buffer, CAT was eluted with 100 ml of a linear gradient of 0–0.5 M KCl in the equilibration buffer. Pooled CAT was concentrated to 3 ml in 0.3 M KCl.

Liver mitochondrial CAT was purified essentially as described above, and liver peroxisomal CAT was purified as described in (5). The specific activity was 14.6 U/mg protein for liver mitochondrial CAT and was 11.9 U/mg protein for peroxisomal CAT.

Enzyme and Protein Assays

CAT was assayed spectrally at room temperature in a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.5, 1.3 mM L-carnitine, 150 μM Dithiopyridine, and 200 μM acetyl-CoA or propionyl-CoA, instead of decanoyl-CoA, as described (16,17). Kinetic constants (K_m and Hill coefficients) were estimated from replots of the velocity versus acyl-CoA concentrations. Protein was determined by the modified Lowry method (18).

Sephadex G-100 Gel Filtration

Molecular sieving was performed as described (19), using Sephadex G-100. The column (2 × 60 cm), with a flow rate of 15–20 ml/h, was packed under gravity at 4°C. The column was equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 0.02% NaN_3, 0.2 M KCl, 20 μM acetyl-CoA, and 3.75 mM L-carnitine. CAT in 200 μM acetyl-CoA was added to 1 ml of equilibration buffer and applied to the column. The column was eluted with the equilibration buffer, and 2.9-ml fractions were collected and each was assayed for CAT. The column was calibrated using 1 mg/ml each of bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome C, and blue dextran (Pharmacia gel filtration kit). SDS–PAGE was performed by the procedure of Laemmli (20).

Preparation of Immune Serum

Rat liver peroxisomal CAT (34 U) in 0.5 ml of 150 mM NaCl and 10 mM potassium phosphate, pH 7.5, was emulsified with Freund's complete adjuvant and injected subcutaneously into rabbits. A second injection was made 3 weeks later by using 0.6 ml (30 U) of CAT emulsified with Freund's incomplete adjuvant. A booster injection was made 2 weeks later by using 1.5 ml (45 U) of CAT emulsified with Freund's incomplete adjuvant. Preimmune serum was prepared from blood taken prior to immunization. Antiserum was prepared from blood taken 7 days after the last booster injection. Both preimmune serum and immune serum were stored in 1-ml portions at −70°C.

The immunoglobulin fraction from rabbit antiserum was purified using a GammaBind-G prepack cartridge from GENEX by the protocol supplied by the company. After elution from the GammaBind-G column, the IgG was stored frozen at −70°C in 10 mM potassium phosphate, pH 7.0, 150 mM NaCl, at a protein concentration of 5.0 mg/ml.

Western Blot Analysis

Approximately 10–20 μg of carnitine acetyltransferase was subjected to SDS–PAGE. The protein was then electroblotted onto an Immobilon (polyvinylidene fluoride) membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0, at 90 V for 30 min. Staining of the gel with Coomassie brilliant blue indicated that peptides less than 120 kDa were completely transferred. The membrane was washed once with 50 mM Tris–HCl, pH 7.4, 500 mM NaCl buffer for 5 min. After drying, the membrane was incubated in a blocking solution consisting of 3% fatty acid-free bovine serum albumin in the washing buffer for 1 h at room temperature. The membrane was washed three times with a buffer containing 0.05% Tween 20, 50 mM Tris HCl, pH 7.4, 500 mM NaCl (buffer B) for 5 min and incubated overnight at room temperature in a 1:2000 dilution of the anti-liver peroxisomal CAT antibody in

TABLE 1

Purification of Heart Mitochondrial CAT

	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Mitochondrial extract	817	49	0.06	100	1
Sephacryl S300	183.6	27.5	0.15	56.2	2.5
Cellulose phosphate	6.3	13.6	2.16	27.7	36
Agarose-CoA (type 5)	0.46	5.5	11.9	11.2	198

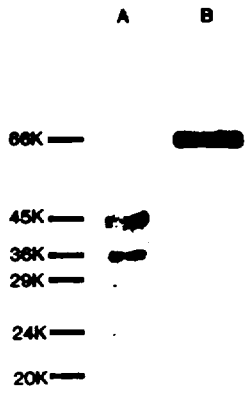

FIG. 1. SDS-PAGE of purified heart mitochondrial CAT. Lane A contains the molecular weight markers albumin, 66,000; ovalbumin, 45,000; G-3-P dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; and trypsin inhibitor, 20,100. Lane B contained 3 μg purified CAT. The gel was stained with silver nitrate.

buffer B plus 1% fatty acid-free bovine serum albumin. The membrane was washed three times with buffer B for 5 min and then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (a 1:2000 dilution in 1% bovine serum albumin in buffer B) for 1 h at room temperature. The membrane was then washed three times with buffer B for 5 min followed by a wash with 50 mM Tris, pH 7.5, 500 mM NaCl for 5 min. The alkaline phosphatase activity was detected by a colorimetric reaction at pH 9.8 with alkaline phosphatase color development reagent.

Materials

Most chemicals, including acyl-CoAs and CoASH, were purchased from Sigma Chemical Co. Chromatographic supports, Sephadex S-300, and agarose-CoA type 5 were from Pharmacia, and cellulose phosphate was from Sigma Chemical Co. L-carnitine was a gift from Sigma Tau (Rome, Italy). [*acetyl*-^{3}H]Acetyl-CoA (specific radioactivity, 9.3 Ci/mmol) was purchased from ICN Biochemicals, Inc.

RESULTS AND DISCUSSION

Purification of CAT

Initial attempts to purify heart mitochondrial CAT were unsuccessful due to loss of enzyme activity. Addition of 20% glycerol and 0.3 M KCl stabilized the enzyme. Inclusion of the anti-proteases PMSF, pepstatin, leupeptin, and bestatin was necessary throughout the purification. Failure to add bestatin produced CAT preparations that had more than one N-terminal amino acid (data not shown). Table 1 summarizes the purification of CAT from heart mitochondria. Figure 1, an SDS-PAGE gel, shows that CAT is essentially homogenous with an apparent molecular weight of 62 kDa. The enzymes remained stable for 6–12 months in 20% glycerol and 0.3 M KCl, but activity gradually decreased with complete loss of activity.

Characterization of CAT

The three CATs all exhibited non-Michaelis–Menten kinetics with acetyl- but not propionyl-CoA as substrate. The cause for the different kinetic behavior is not known and is currently under investigation. The enzymes exhibit Michaelis–Menten kinetics with propionyl-CoA, with Hill coefficients near 1.0 (see Table 2). The apparent $K_{0.5}$'s for propionyl-CoA are 3 and 2 μM for the heart and liver mitochondrial enzymes and 39 and 29 μM for acetyl-CoA, respectively (data not shown). In the presence of substrates for the forward reaction, CAT behaves as a monomer when subjected to molecular sieving in the presence of substrate (Fig. 2).

TABLE 2

Hill Coefficients for Propionyl-CoA

CAT source	Substrate	Hill *n*
Rat heart mitochondria	Propionyl-CoA	0.9
Rat liver mitochondria	Propionyl-CoA	1.0
Rat liver peroxisomes	Propionyl-CoA	1.1

Note. The purified enzymes were assayed spectrally and Hill *n*'s were estimated by plotting (log $V/V_{max} - V$)/(log[acyl-CoA]).

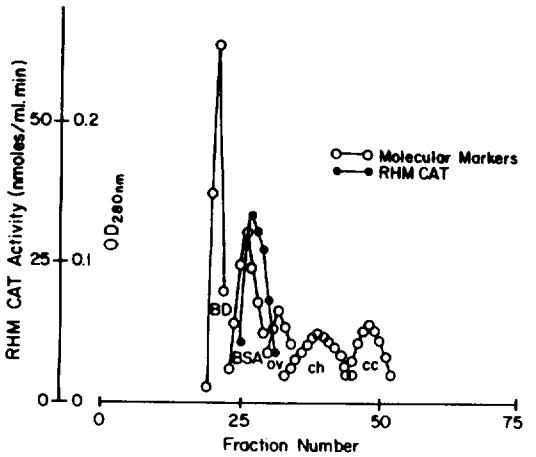

FIG. 2. Molecular weight of heart mitochondrial CAT in the presence of acetyl-CoA and carnitine. Molecular markers (○), blue dextran (BD), bovine serum albumin (BSA), ovalbumin (OV), chymotrypsinogen (Ch), and cytochrome C (CC), were detected by measuring absorbances at 280 nm, and CAT (●) was assayed as described under Materials and Methods. The eluting buffer contained 20 mM potassium phosphate, pH 7.5, 0.2 M KCl, 0.02% NaN_3, 20 μM acetyl-CoA, and 3.75 mM L-carnitine.

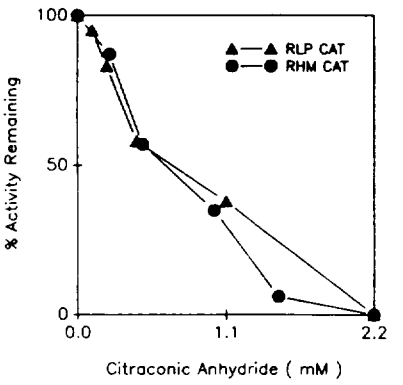

FIG. 3. Effect of citraconic anhydride on CAT activity. CAT was assayed as described under Materials and Methods in the presence of the concentrations of citraconic acid shown. RHM, heart mitochondrial CAT; RLP, liver peroxisomal CAT.

As discussed elsewhere (2), the dimeric form of CAT studied by Mittal and Kurup (15) was most likely due to proteolytic cleavage of CAT to give a catalytically active dimer. The molecular weight of their dimer was near 60,000 Da. No evidence for dimerization of purified CAT was obtained. Both peroxisomal CAT and heart mitochondrial CAT are completely inhibited by 2.2 mM citraconic anhydride (see Fig. 3), indicating a role for the epsilon amino group of lysine in catalysis or substrate binding.

Immunoprecipitation of CAT

Polyclonal antibodies were prepared against purified liver peroxisomal CAT. The antibody precipitated both mitochondrial CATs and peroxisomal CAT, but not peroxisomal carnitine octanoyltransferase (see Fig. 4). Western blot analyses were performed using liver and heart mitochondrial CAT, peroxisomal CAT, and solubilized rat liver microsomes [rat liver microsomes contain an unstable form of CAT (21)]. Figure 5 shows the results of the Western blot analysis. Multiple bands were obtained with each organelle. Major bands occurred near 60,000 and 34,000.

Numerous clinical investigations have shown that, in certain disease states in which short-chain acyl-CoA catabolism is compromised (i.e., propionic acidemia), considerable amounts of propionylcarnitine or other acylcarnitines, depending on the disease state, occur in human urine, thereby eliminating a potentially deleterious metabolite (2). The finding that purified mitochondrial CAT exhibits Michaelis–Menten-type behavior with

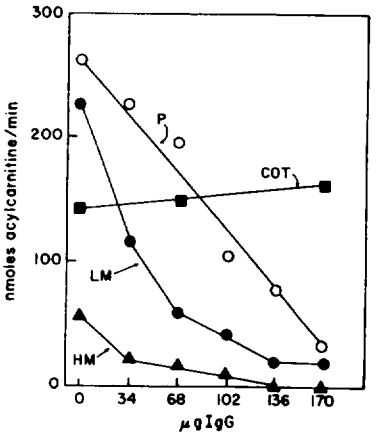

FIG. 4. Immunoprecipitation of different CATs by antibody against liver peroxisomal CAT. Purified heart mitochondrial, liver mitochondrial, and peroxisomal CAT, as well as partially purified peroxisomal carnitine octanoyltransferase, were incubated with increasing amounts of preimmune and immune serum in a final volume of 50 μl, containing 25 mM potassium phosphate and 150 mM NaCl, pH 7.5, at 4°C for 1 h. The samples were centrifuged at 10,000*g* for 10 min, and CAT in the supernatant fluids was assayed spectrophotometrically. The numbers are averages of two experiments. (△) Heart mitochondrial CAT, HM. (○) Liver peroxisomal CAT, P. (●) Liver mitochondrial CAT, LM. (■) Liver peroxisomal carnitine octanoyltransferase, COT.

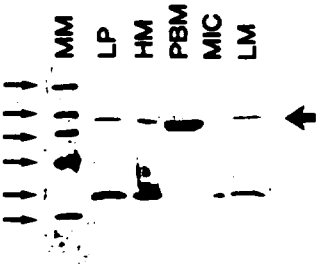

FIG. 5. Western blot analysis of CATs from different organelles. Extracts from liver peroxisomes (LP), liver mitochondria, heart mitochondria, pigeon breast muscle (PBM), and liver microsomes (MIC) were subjected to Western blot analyses using peroxisomal CAT antibody. Lane MM contains the molecular weight standards: phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); the standards were stained using Coomassie blue. Rat liver microsomes, prepared as described (22), were solubilized with 0.4 M KCl and 1% Triton X-100 for 18 h and centrifuged at 100,000*g* for 30 min at 4°C. Forty microliters of the supernatant fluid (50 μg of protein) was used for Western blot analysis. Rat heart and liver mitochondria were extracted with 1 M KCl, 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 10% glycerol, pH 7.5, and 0.1 M potassium phosphate buffer, pH 7.5, respectively, for 30 min at 4°C. The extracts were centrifuged at 100,000*g* for 30 min at 4°C, and 40 μl of heart mitochondrial supernatant fluid (50 μg of protein) was used. Forty microliters of liver mitochondrial extracts (50 μg of protein) was partially purified using Sephacryl S-300 and cellulose phosphate column chromatography. Rat liver peroxisomes were prepared and solubilized as described previously (5,23); the extract was partially purified using Blue-Sepharose CL 6B and CM-Sephadex C-50 column chromatography. Forty microliters of combined CAT fractions (50 μg of protein) was used for Western blotting and 20 μl of pigeon breast muscle CAT (5 μg of protein) from Boehringer-Mannheim Biochemicals) was used. The arrows show the position of standard molecular markers, beginning at the top with phosphorylase. The arrowhead indicates the position of CAT.

Hill coefficients near 1.0 when propionyl-CoA is the substrate is consistent with CAT being involved in the detoxification of propionyl-CoA when carboxylation of propionyl-CoA is compromised. Studies reported elsewhere (24) show that liver CAT also exhibits Michaelis–Menten kinetics with pivaloyl-CoA. Pivaloylcarnitine is a major carnitine-mediated detoxification product in humans (8).

It was shown previously that anti-mitochondrial CPT antibody does not cross-react with peroxisomal carnitine octanoyltransferase (16) and that anti-peroxisomal carnitine octanoyltransferase antibody does not cross-react with mitochondrial carnitine palmitoyltransferase (16). The polyclonal antibody against peroxisomal CAT cross-reacted with both liver and heart mitochondrial CAT and liver peroxisomal CAT, but not with peroxisomal carnitine octanoyltransferase. The multiple bands on Western blotting of extracts from liver microsomes, peroxisomes, and mitochondria and from heart mitochondria are very similar to the banding patterns reported for the antibody against human liver mitochondrial CAT (14). The major bands with molecular weights between 45 and 31 kDa for liver and heart mitochondria are similar to the subunit molecular weights of some of the β-oxidation enzymes [see Fig. 7 of Ref. (16)]. If so, this implies that the polyclonal antibody recognizes a structural region common to more than one enzyme. One possibility is the acyl-CoA binding region of some enzymes that utilize acyl-CoAs.

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