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Characterization of a Medium/Long-Chain Carnitine Acyltransferase Associated with Rat Liver Endoplasmic Reticulum

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Kathleen Lilly

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CHARACTERIZATION OF A MEDIUM/LONG-CHAIN CARNITINE ACYLTRANSFERASE ASSOCIATED WITH RAT LIVER ENDOPLASMIC RETICULUM

By

Kathleen Lilly

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ABSTRACT

CHARACTERIZATION OF A MEDIUM/LONG-CHAIN CARNITINE ACYLTRANSFERASE ASSOCIATED WITH RAT LIVER ENDOPLASMIC RETICULUM

By

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Rat liver contains carnitine acyltransferase activity with a medium to long acyl-chain length specificity located in both rough and smooth endoplasmic reticulum that is strongly inhibited by malonyl-CoA, although the existence of medium/long-chain carnitine acyltransferase activity in the endoplasmic reticulum has been disputed. This thesis reports characterization of a microsomal medium/long-chain carnitine acyltransferase that is membrane bound. It is designated microsomal carnitine octanoyltransferase (COT) because it has a higher specific activity with medium-chain acyl-residues such as decanoyl-CoA than with palmitoyl-CoA. Microsomal carnitine octanoyltransferase is not immunoprecipitated by antibody prepared against mitochondrial carnitine palmitoyltransferase and it is only slightly immunoprecipitated by antibody prepared against peroxisomal carnitine octanoyltransferase. This demonstrates it is antigenically distinct from either of the other liver carnitine acyltransferases with medium to long acyl-chain length specificity. This characterization provides evidence that microsomal COT is distinct from mitochondrial and peroxisomal medium/long-chain carnitine acyltransferases .

The concentration of malonyl-CoA required for 50% inhibition is 5.3 μM; in the

presence of 17 μM decanoyl-CoA and 1.7 mM L-carnitine respectively. Microsomal carnitine octanoyltransferase is also inhibited by etomoxiryl-CoA, with 0.6 μM etomoxiryl-CoA producing 50% inhibition. Although palmitoyl-CoA is a substrate at low concentrations, the enzyme is strongly inhibited by high concentrations of palmitoyl-CoA; 50% inhibition is produced by 11 μM palmitoyl-CoA. Microsomal COT is inhibited competitively with respect to L-carnitine by DL-aminocarnitine; 0.5 mM DL-aminocarnitine produces 50% inhibition.

Microsomal COT follows Michaelis-Menten kinetics with Hill coefficients of 0.91 for decanoyl-CoA and 0.96 for L-carnitine. The kinetics constants for microsomal COT show the $K_{0.5}$ for L-carnitine is 0.42 mM and 1.9 μM for decanoyl-CoA. The kinetic characteristics of microsomal COT can be used to distinguish it from mitochondrial CPT and peroxisomal COT.

To my Grandmothers
Mary Nicolls and Kathryn Lilly

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

CAT	carnitine acetyltransferase (acetyl-CoA:-L-carnitine O-acetyltransferase, EC 2.3.1.7)
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]1-propane sulfonate
CoA	coenzyme A
CoASH	reduced coenzyme A
COT	carnitine octanoyltransferase (octanoyl-CoA:L-carnitine O-octanoyltransferase)
CPT	carnitine palmitoyltransferase (palmitoyl-CoA:L-carnitine O-palmitoyltransferase, EC 2.3.1.21)
CPT_i	form of carnitine palmitoyltransferase in contact with the matrix of mitochondria
CPT_o	malonyl-CoA sensitive form of carnitine palmitoyltransferase in contact with the cytosol
DTBP	4,4'-dithio-bispyridine
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	(ethylenediamine)-tetra-acetic acid
Etomoxiryl-CoA	(B877-38), (R)-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxyl-coenzyme A ester
HPLC	high performance liquid chromatography
I_{50}	the inhibitor concentration required for 50% inhibition under given assay conditions

IMV CPT	carnitine palmitoyltransferase activity of inner mitochondrial membrane enriched vesicles
K_i	inhibition constant
$K_{0.5}$	Hill constant, $K_{0.5}$ is equal to K_m with Hill coefficient (n) equal to 1
K_m	Michaelis-Menten constant
munit	nmole min^{-1}
OMV CPT	carnitine palmitoyltransferase activity of outer mitochondrial membrane enriched vesicles
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TDGA-CoA	2-tetradecylglycidyl-coenzyme A ester
Tris	tris-(hydroxymethyl)aminomethane
Tween-20	polyoxyethylene sorbitan monolaurate
unit	μmole min^{-1}
V_{max}	maximum velocity

Introduction

Carnitine acyltransferases catalyze the reversible transfer of acyl-groups between L-carnitine and coenzyme A. Carnitine acyltransferase activity with medium-chain and long-chain acyl-groups as substrate has been reported to be located in mitochondria, peroxisomes, and endoplasmic reticulum in rat liver, although the existence of medium/long-chain carnitine acyltransferase activity in the endoplasmic reticulum has been disputed. This thesis reports the characterization of a carnitine acyltransferase in smooth and rough endoplasmic reticulum of rat liver with medium-chain to long-chain acyl-group specificity that is completely inhibitable by malonyl-CoA. This medium/long-chain carnitine acyltransferase is named microsomal carnitine octanoyltransferase (COT). The characterization provides evidence that this enzyme is distinct from mitochondrial and peroxisomal enzymes.

Chapter 1 is a review of literature about medium/long-chain carnitine acyltransferase activity of rat liver microsomes. Microsomal medium/long-chain carnitine acyltransferase activity is compared with the two other carnitine acyltransferases in rat liver that use medium-chain and long-chain acyl-groups as substrate which are mitochondrial carnitine palmitoyltransferase (CPT) and peroxisomal carnitine octanoyltransferase (COT). The inhibition of microsomal COT, mitochondrial CPT, and peroxisomal COT by malonyl-CoA, palmitoyl-CoA, etomoxiryl-CoA, and aminocarnitine is compared.

Chapter 2 presents data which show that microsomal COT is strongly inhibited by malonyl-CoA. Low contamination of the microsome fraction by mitochondria and peroxisomes is established using organelle marker enzymes. The inhibition of microsomal COT by palmitoyl-CoA and etomoxiryl-CoA is shown. The data show that malonyl-CoA sensitive microsomal COT is antigenically different than either mitochondrial CPT or peroxisomal COT.

Chapter 3 presents data showing the kinetic characterization of membrane-bound microsomal COT. Kinetic constants $K_{0.5}$ and V_{max} for decanoyl-CoA as the varied substrate and for L-carnitine as the varied substrate are determined. The I_{50} for inhibition of microsomal COT by DL-aminocarnitine, decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine and the effect of DL-aminocarnitine on the $K_{0.5}$ and V_{max} with L-carnitine as varied substrate are shown. The effect of pH on membrane bound microsomal COT activity is also presented.

Chapter 4 is a summary of the attempted purification of microsomal COT. Purification of detergent solubilized COT was attempted by column chromatography. Gel filtration, anion exchange, dye affinity, and hydrophobic interaction chromatography were not successful in increasing the specific activity of the detergent solubilzed microsomal COT. Results of column chromatography are discussed. A prospectus for future purification is presented.

Chapter 5 is a summary and conclusion of the microsomal COT data presented in Chapters 2, 3 and 4. Microsomal COT is compared to literature reports of mitochondrial CPT and peroxisomal COT to establish that microsomal COT is a distinct enzyme from

mitochondrial CPT or peroxisomal COT. The data show that there is more than one malonyl-CoA sensitive medium/long-chain carnitine acyltransferase in rat liver. A prospectus for future research is presented.

Materials and Methods

Isolation of Microsomes

Male, fed, Sprague-Dawely rats weighing 150-200 g were stunned lightly in CO_2 and decapitated. Livers were immediately collected, immersed in and coarsely minced in 0.25 M sucrose containing 25 µg/ml PMSF, 0.5 µg/ml Pepstatin A, and 0.05 µg/ml Leupeptin (homogenization solution). Livers were finely minced and rinsed with solution and homogenized at 4°C with 4 volumes of buffer using 4 passes of a loose-fitting Potter-Elvehjem homogenizer.

Rough and smooth microsomes were prepared using differential centrifugation. The liver homogenate was centrifuged at 400 x g for 5 min followed by centrifugation of the 400 x g supernatant fluids at 6,000 x g for 10 min Centrifugation of the 6,000 x g supernatant fluids at 10,000 x g for 10 min was followed by ultracentrifugation of the 10,000 x g supernatant fluids at 100,000 x g for 1 hour. The 6,000 x g pellet was resuspended in 0.25 M sucrose and the 100,000 x g pellet was resuspended in 10 mM potassium phosphate pH 7.5, 1 mM EDTA containing 20% glycerol. The microsomal membranes were stored frozen in aliquots at -20°C.

Rough and smooth microsomes were also prepared using $CaCl_2$ precipitation (1). The liver homogenate was centrifuged at 12,000 x g for 15 min. and the supernatant fluid was filtered through a loose plug of glass wool and centrifuged again at 12,000 x g for 15 min. The supernatant fluid was diluted with 4 volumes of 12 mM mannitol, 1 mM EDTA, and 8

mM $CaCl_2$ and kept on ice for 1 hour. The diluted supernatant fluid was centrifuged at 1,500 x g for 15 min. and the microsomal pellet resuspended in 0.25 M mannitol, 25 mM MOPS pH 7.4, 1 mM EDTA and stored frozen in aliquots at -20°C.

Rough microsomes were prepared using CsCl-sucrose density gradient centrifugation (2). The liver homogenate was centrifuged at 10,000 x g for 30 min. and the supernatant fluid made 15 mM in CsCl with the addition of 1 M CsCl. Ten ml of the 15 mM CsCl supernatant fluid was layered over 15 ml of 1.3 M sucrose, 15 mM CsCl in a 26.3 ml Beckman polycarbonate ultracentrifuge tube and centrifuged at 100,000 x g for 2 hours in a Beckman Ti70 rotor. The pellet containing rough microsomes was resuspended in 10 mM potassium phosphate pH 7.5, 1 mM EDTA and stored frozen in aliquots at -20°C.

Detergent solubilization was done by making the microsomes 8 mM in CHAPS, incubating on ice for 1 hour, and storing frozen at -20°C for at least 12 hours. They were thawed at room temperature and centrifuged at 100,000 x g for 30 min. at 4°C in a Beckman Airfuge. Trichloroacetic acid extraction of microsomes was done by making the microsomes 30% (w/v) in trichloroacetic acid and then the sample was centrifuged 5 min. in an Eppendorf centrifuge. The pH of the supernatant fluids was adjusted to ~7 with the addition of potassium hydroxide. Extraction of microsomal lipids was done as described (3). Microsomes were extracted with CHAPS as described above and the 100,000 x g pellet was resuspended in 100 mM potassium phosphate pH 7.5. The pellet was weighed and homogenized with 17 volumes (ml/g) of chloroform/methanol (2/1, v/v). The sample was centrifuged at 1,000 x g for 15 min. and the supernatant fluids were filtered through a sintered glass funnel. The pellet was extracted again and the supernatant fluids combined.

Enzyme and Protein Assays

Microsomal COT activity was assayed spectrally (at room temperature) at 324 nm in 50 mM potassium phosphate, pH 7.5, 50 mM potassium chloride, 150 μM dithiopyridine, 17 μM decanoyl-CoA, 1.7 mM L-carnitine (E_{324} = 19,600 M^{-1} cm^{-1}). Values were corrected for carnitine independent CoASH release (4). Malonyl-CoA inhibition was determined with 17 μM malonyl- CoA. Long-chain carnitine acyltransferase activity was assayed with 17 μM palmitoyl-CoA. Microsomal COT was assayed spectrally unless otherwise indicated. For some studies as indicated, microsomal COT was also assayed (at room temperature) using a radiochemical, isotope forward assay (5) in 100 μl with 17 μM [1-^{14}C]-decanoyl-CoA, 150 μM dithiopyridine, 1.7 mM L-carnitine in 50 mM potassium phosphate pH 7.5 and 50 mM KCl. The reaction was started with the addition of L-carnitine and stopped by the addition of 400 μl ice-cold methanol. The [1-^{14}C]-decanoylcarnitine was separated from the unreacted [1-^{14}C]-decanoyl-CoA (5). For Figures 1 and 4 in Chapter 2, the amount of [1-^{14}C]-decanoylcarnitine was determined using a combined HPLC-Flo Scint-β-counter described previously (6). The buffer system was 5 mM butanesulfonic acid, 5 mM ammonium acetate, pH 3.4 (A) and methanol (B). The flow rate was 1.0 ml min^{-1} and the gradient was as follows: at zero time, 20% A, 80% B; at 10 min., 0% A, 100% B; at 30 min., 20% A, 80% B. The retention time of [1-^{14}C]-decanoylcarnitine was 8.3 min.

The following marker enzymes were assayed: glucose-6-phosphatase, monoamine oxidase, urate oxidase, cytochrome *c* oxidase, NADPH-cytochrome *c* reductase. Samples assayed for monoamine oxidase, urate oxidase, and cytochrome *c* oxidase were stored overnight at -70°C, thawed, and solubilized in 1% Tween 20. Glucose-6-phosphatase (EC 3.1.3.9) was assayed in a 0.5 ml volume at room temperature containing 14 mM glucose-6-

phosphate, 25 mM histidine, 1 mM EDTA pH 7.0 and the amount of inorganic phosphate measured at A_{660} using the Fiske-SubbaRow reagent with a potassium phosphate standard curve (10-100 nmole inorganic phosphate) (7). Urate oxidase (EC 1.7.3.3) was assayed spectrally at room temperature in 20 mM borate pH 9.5 with 40 μM uric acid and the decrease in A_{293} was measured (E_{293} = 12,600 M^{-1} cm^{-1}) (8). Cytochrome *c* oxidase (EC 1.9.3.1) was assayed spectrally at room temperature in 200 mM potassium phosphate pH 6.0, 1 mM EDTA with 16 μM reduced cytochrome *c* and the decrease in A_{550} was measured (E_{550} = 19,600 M^{-1} cm^{-1}) (9). NADPH Cytochrome *c* reductase (EC 1.6.2.4) was assayed spectrally at room temperature in 50 mM potassium phosphate pH 7.7, 0.1 mM EDTA with 36 μM cytochrome *c* (Sigma Type III), 91 μM NADPH and the increase in A_{550} was measured (E_{550} = 21,000 M^{-1} cm^{-1}) (10). Monoamine oxidase activity was determined using an end point assay by measuring the production of 4-hydroxy-quinoline. Each assay was incubated 90 min. at 37°C in a 1 ml volume containing 70 mM potassium phosphate pH 7.5, and 0.31 mM kynuramine dihydrobromide (11). The A_{330} was measured and the activity calculated as described in (12).

Protein was determined by the modified Lowry method (13).

Immunoprecipitation

Antiperoxisomal-COT antibody (14), purified mouse liver peroxisomal COT (15), and anti-beef heart mitochondrial CPT IgG (16) prepared previously were used. The IgG fraction was purified from the anti-peroxisomal COT rabbit serum using a GammaBind™ G-PrePack™ cartridge from GENEX with the protocol supplied by the company. After elution from the GammaBind™-G column, the IgG was stored frozen at -20° in 10 mM sodium phosphate, pH 7.0, 150 mM sodium chloride, at a protein concentration of 4.1 mg/ml.

The effect of antiperoxisomal-COT and antimitochondrial-CPT antibodies on microsomal COT was determined by immunoprecipitation. Samples were incubated at 4°C in 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.15 mM sodium chloride. Antiperoxisomal-COT IgG and the antimitochondrial-CPT IgG were added as indicated (see Chapter 2, Figure 5); varying amounts of bovine serum albumin were added to the antiperoxisomal-COT IgG samples to keep the amount of added protein constant. The samples were centrifuged at 10,000 x g for 10 min. and the supernatant fluids assayed immediately for COT. CPT in the 6,000 x g fractions was assayed with 50 μM decanoyl-CoA, 25 mM L-carnitine, 150 μM dithiopyridine in 50 mM potassium phosphate, 0.1% Triton X-100, pH 7.5.

Labeling of Liver Microsomes with ^{3}H-Etomoxir

A 540 μg sample of rat liver microsomes was incubated for 15 min. at room temperature with 5 mM ATP, 5 mM $MgCl_2$, 50 μM CoASH, 50 mM potassium phosphate, 50 mM potassium chloride, pH 7.5, and 5 μM ^{3}H-etomoxir (specific radioactivity 40 Ci/mmole) in a final volume of 100 μl. After 15 min., the reaction was diluted with 1 volume of cold 50 mM potassium phosphate, 50 mM potassium chloride, pH 7.5, and centrifuged at 100,000 x g for 30 min. in a Beckman airfuge. The microsomal membrane pellet was rinsed three times with 200 μl of 50 mM potassium phosphate, 50 mM potassium chloride, pH 7.5, and the pellet was resuspended in 100 μl of electrophoresis sample buffer. For the pulse chase experiments, after the 15 minute incubation, etomoxiryl-CoA (unlabeled) was added to a final concentration of 50 μM and the reaction mixture was incubated for an additional 5 min. at room temperature.

Microsome samples (50 μl, 5.4 mg/ml)were extracted with 5.0 ml of cold hexane/isopropanol (3/2, v/v) by vortexing for 1.0 minute, followed by centrifugation for 15

min. at 3,000 x g. The pellet was extracted with 200 µl of water plus 5.0 ml of cold chloroform/methanol (3/2, v/v) and centrifuged, and the protein pellet was then dissolved in 300 µl 7% SDS, and aliquots were subjected to SDS-PAGE according to Laemmli (17). After electrophoresis, the gel was sliced into 2 mm slices, and the gel slices were treated with 0.5 ml of distilled water by shaking overnight and the radioactivity determined.

[^{3}H]-Etomoxir labeled microsomal and mitochondrial proteins were separated isocratically on a Dupont G-250 gel filtration column (9.4 mm ID x 250 mm) in 100 mM Tris-Cl, pH 6.8, containing 10% glycerol and 0.5% SDS at a flow rate of 1 ml min^{-1}. The A_{280} was recorded using a Water's Model 441 absorbance detector and Model 740 data module and 300 µl fractions were collected beginning at 5.6 min. A 50 µl aliquot of each fraction was mixed with 10 ml of scintillation fluid (Safety Solve, RPI) and counted in a Packard Tricarb 1900 CA. Peaks containing radioactivity were pooled and a 50 µl aliquot extracted with 1.0 ml of chloroform/methanol (3/2, v/v). The radioactivity remaining after extraction and the radioactivity in the chloroform/methanol phase was determined with scintillation counting.

Determination of Kinetic Constants

Membrane bound microsomal COT was assayed using a radiochemical, isotope forward assay with [1-^{14}C]decanoyl-CoA (5). [1-^{14}C]decanoyl-CoA was synthesized as described (18). Synthesis conditions were 2 mM [1-^{14}C]decanoic acid, 20 mM ATP, 10 mM CoASH, 20 mM $MgCl_2$, 2 mM DTT, 100 mM Mops-NaOH pH 7.5, 0.1% Triton x-100 (w/v) and 2.5 units Acyl-CoA Synthetase in a 5 ml volume and the mixture was stirred at 35°C for 2 hours. The mixture was applied to a Prep-Sep C-18 extraction column (Fisher Scientific, Fair Lawn, NJ) equilibrated in 100 mM MOPS-NaOH pH 7.5 and the column was washed with 1 ml of 50%

methanol (v/v) and the [1-^{14}C]decanoyl-CoA was eluted with 20 ml of methanol. The solvent was evaporated to dryness using a rotary evaporator and the residue dissolved in 50 mM potassium phosphate pH 5.3. [1-^{14}C]Decanoyl-CoA was purified using HPLC with a Water's μ-Bondapak C18 reverse phase column with an isocratic buffer of 50 mM potassium phosphate pH 5.3 and 32% acetonitrile at a flow rate of 1 ml min^{-1}. [1-^{14}C]Decanoyl-CoA was detected by absorbance at 254 nm and the fractions containing radioactivity with the retention time of authentic decanoyl-CoA were collected and dried under vacuum using a rotary evaporator. The specific activity of the [1-^{14}C]decanoyl-CoA was 19870 dpm/nmole.

The radiochemical isotope forward assay of membrane bound microsomal COT was done in a 100 μl volume, at 30°C for 30 sec in 50 mM potassium phosphate pH 7.5, 50 mM potassium chloride, and 150 μM DTBP, and 20-30 μg of microsomes. Substrate concentrations were 6 mM L-carnitine, 16 μM [1-^{14}C]decanoyl-CoA or were varied as indicated. The assay was linear with 30 μg of microsomal protein up to 60 seconds; less than 15% of the decanoyl-CoA was consumed in 30 seconds. The reaction was stopped with the addition of 400 μl ice cold methanol. [1-^{14}C]decanoylcarnitine was separated from unreacted [1-^{14}C]decanoyl-CoA using a 0.5 x 3.0 cm DE-52 column equilibrated in H_2O and the [1-^{14}C]decanoylcarnitine eluted with 1.0 ml of 80% methanol (v/v). The eluant was mixed with 10 ml of Safety-Solve cocktail and the radioactivity determined with a Packard 1900 CA liquid scintillation analyzer. A control, minus-carnitine, assay was done at each [1-^{14}C]decanoyl-CoA concentration to correct for background.

Ammonium Sulfate Fractionation of Detergent Solubilized Microsomal COT

Membrane bound microsomal COT was made 1% in Tween-20, incubated on ice for 1 hour, and centrifuged at 100,000 x g for 60 min. at 4°C. Aliquots of the supernatant fluids containing 100 munits of COT were made 0-60% saturation in ammonium sulfate with the addition of saturated ammonium sulfate, pH 7.0. The samples were incubated on ice for 1 hour then centrifuged at 15,000 x g for 15 min. in an Eppendorf centrifuge. The samples were pipeted into a pasteur pipet plugged with glass wool and the supernatant collected. The pellet retained by the glass wool was eluted with 1 ml of 5 mM potassium phosphate, 1 mM EDTA, pH 7.5 containing 0.5% Tween-20.

Column Chromatography

HPLC gel filtration of solubilized COT was done at room temperature using a Dupont G-250 gel filtration column (9.4 mm ID x 250 mm) equilibrated in 200 mM ammonium acetate pH 8.0 containing 20% glycerol. The A_{280} was recorded using a Water's Model 441 absorbance detector and Model 740 data module. The column was run isocratically at a flow rate of 1 ml min^{-1} and 1 ml fractions were collected and assayed for COT activity.

Gel filtration of solubilized microsomal COT was also done using a 2.5 cm x 80 cm Biogel P100 column with a column volume of 400 ml. The column was run at 4°C at a flow rate of 1.1 ml $min.^{-1}$ and 4 ml fractions were collected and assayed for COT activity and the A_{280} determined.

HPLC anion exchange chromatography was done using a Synchropak AX300 (4.1 mm x 250 mm) column with an average pore size of 300 Å equilibrated in 10 mM potassium phosphate, pH 7.5, 2 mM CHAPS containing 20% glycerol (buffer A). The A_{280} was recorded

using a Water's Model 441 absorbance detector and model 740 data module. The column was washed at a flow rate of 0.5 ml $min.^{-1}$ with buffer A until the A_{280} was ~0. Proteins were eluted with a linear gradient to buffer A containing 1 M potassium chloride and 1 ml fractions were collected and assayed for COT activity.

Hydroxylapatite chromatography was done using a Bio-gel HTP column with a 6 ml column volume equilibrated in 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 2 mM CHAPS, and 20% glycerol (buffer A). The column was washed until the A_{280} was ~0 and proteins were eluted with a linear gradient to buffer A containing 500 mM potassium phosphate, pH 7.5. Fractions were collected and assayed for COT activity and the A_{280} determined.

Materials

Most chemicals, including acyl-CoA, CoASH, and [1-^{14}C]-decanoic acid sodium salt with a specific activity of 10.6 mCi/mmole were purchased from Sigma Chemical Company (St. Louis MO). L-Carnitine was a gift from Sigma-Tau (Rome, Italy). DL-Aminocarnitine, decanoyl-DL-aminocarnitine, and palmitoyl-DL-aminocarnitine were a gift from Dr. Owen Griffith (Cornell University Medical School, New York, NY). Etomoxiryl-CoA (B877-38) and [^{3}H]-etomoxir with a specific activity of 40 Ci/mmole were a gift from Byk Gulden (D-7750 Konstanz, Fed. Rep. of Germany). Pigeon breast muscle carnitine acetyltranferase, Acyl-CoA synthetase, *aus mikroorganismen*, and CHAPS were from Boehringer Mannheim Biochemicals (Indianapolis, IN). DE 52, diethyl aminoethyl cellulose, was from Whatman (Hillsboro, OR). Safety-solve was from Research Products International Corp. (Mount Prospect, IL). Bio-Gel P100 and Bio-Gel HTP were from Bio-Rad (Richmond, CA). Blue Sepharose Cl-6B was from

Pharmacia (Piscataway, NJ). All other reagents were of analytical grade.

Chapter 1 Literature Review

Microsomal Medium\Long-Chain Carnitine Acyltransferase Activity in Rat Liver

Carnitine acyltransferases catalyze the reversible transfer of acyl groups between L-carnitine and coenzyme A (19). They are classified according to their acyl-chain length specificity into short-chain and medium/long-chain carnitine acyltransferases. Short-chain carnitine acyltransferases are commonly called carnitine acetyltransferase (acetyl-CoA:L-carnitine O-acetyltransferase, EC 2.3.1.7, CAT) while medium/long-chain transferases are further divided into carnitine octanoyltransferase (octanoyl-CoA:L-carnitine O-octanoyltransferase, COT) and carnitine palmitoyltransferase (palmitoyl-CoA:L-carnitine O-palmitoyltransferase, EC 2.3.1.21, CPT). COT and CPT both catalyze the transfer of medium-chain and long-chain acyl groups. COT has a higher activity towards medium-chain and CPT (with physiological concentrations of carnitine) towards long-chain acyl groups. CAT, COT, and CPT activities are located in mitochondria, peroxisomes and endoplasmic reticulum.

The mitochondrial medium/long-chain carnitine acyltransferase is named carnitine palmitoyltransferase (CPT). CPT is a membrane bound, oligomeric enzyme that facilitates the transfer of long-chain acyl residues through the inner mitochondrial membrane for subsequent ß-oxidation (5,19,20). CPT_o is located outside the inner membrane of mitochondria and promotes the formation of acylcarnitines using cytosolic acyl-CoAs. The carnitine/acylcarnitine translocase acts to transfer acylcarnitines into the matrix of mitochondria (21). CPT_i is located

inside the inner membrane of mitochondria and promotes the formation of acyl-CoAs using acylcarnitines. It has not been established if CPT_o and CPT_i are the same enzyme with different locations and regulatory properties or if CPT_o and CPT_i are two distinct enzymes. Results from some investigators have supported the view that CPT_o and CPT_i are distinct enzymes with CPT_o being more detergent labile than CPT_i (22-24). Other investigators have supported the view that CPT_o and CPT_i are the same protein (16). The topographical distribution of CPT_o and CPT_i within the mitochondria has also not been established. The results of some investigators have shown that CPT_i is located on the inner side of the inner mitochondrial membrane and CPT_o on the outer side of the inner mitochondrial membrane (25-28). The results of other investigators have suggested that while CPT_i is on the inner side of the inner mitochondrial membrane, CPT_o is on the inner side of the outer mitochondrial membrane (11,29-32).

The peroxisomal medium/long-chain carnitine acyltransferase is named carnitine octanoyltransferase (COT) (14,33-35) and could function to shuttle peroxisomal ß-oxidation-shortened acyl chains out of the peroxisome (36). Peroxisomal COT is easily solubilized by freeze\thaw treatment of peroxisomes (15,34,37). Peroxisomal COT is a soluble enzyme located in the matrix of peroxisomes (38,39). Recently, it has been reported that the malonyl-CoA sensitivity and ratio of decanoyltransferase activity to palmitoyltransferase activity of peroxisomal COT is altered when COT is solubilized from the peroxisome (40).

The endoplasmic reticulum (ER) also contains a medium/long-chain carnitine acyltransferase (37,39,41). It has a higher activity with decanoyl-CoA compared to palmitoyl-CoA; herein this enzyme is referred to as microsomal COT. Characterization of microsomal COT has been hindered by the instability of the enzyme and the difficulty in solubilizing it

from the membrane (37,39). Microsomal COT is tightly associated with the outer side of the endoplasmic reticulum membrane (42). The function of microsomal COT is not known.

Microsomes contain carnitine acyltransferase activity both with octanoyl-CoA as substrate (COT) and with acetyl-CoA as substrate (CAT) with approximately equal initial velocities (37). The initial velocity with both octanoyl-CoA and acetyl-CoA present is the sum of that obtained with each substrate alone (37). Microsomal CAT has different solubility characteristics, regulatory characteristics and substrate affinities than microsomal COT, and microsomal CAT has been purified free of medium\long-chain transferase activity. On the basis of these observations it is concluded that microsomal CAT is a different enzyme than microsomal COT (37,39).

Microsomes are sealed vesicles derived from the disruption of the ER which maintain the same cytoplasmic side out sidedness as does the ER; they vary in size, density, and surface charge (43). The ER of rat liver contains 19% of total cellular protein, 48% of total cellular phospholipid, and 58% of total cellular RNA (43). The microsomal membrane is composed of 60-70% protein and 30-40% phospholipid (43). The predominant microsomal membrane phospholipid is phosphatidylcholine (55%) followed by phosphatidylethanolamine (20-25%), phosphatidylserine (5-10%), phosphatidylinositol (5-10%), and sphingomyelin (4-7%) (43). The microsomal membrane contains at least 38 polypeptides (44). The microsomal membrane is permeable to uncharged molecules with MW < 600 daltons; it is impermeable to charged molecules > 90 daltons and to macromolecules (43).

Assay Methods for Carnitine Acyltransferases

Reports of microsomal carnitine acyltransferase activity in the literature have been complicated by the use of different assay methods which can give varying measurements of carnitine acyltransferase activity. The reaction is represented below.

Acyl-CoA + L-carnitine ↔ Acylcarnitine + CoASH

In the forward direction the enzymes can be assayed spectrally by directly measuring the disappearance of acyl-CoA by the decrease in absorbance at 232 nm (45,46) or indirectly by measuring the appearance of free CoASH using a thiol-trapping agent like DTNB, (5,5'-dithio-bis(2-nitrobenzoic acid), (4) or DTBP, (4,4'-dithio-bispyridine), (47). In the reverse direction the production of acyl-CoA can be measured using hydroxylamine and quantitating the acylhydroxamate formed (48). Carnitine acyltransferases can be assayed radiochemically in the forward or reverse direction using a radioactively labelled carnitine or acyl group and separating labelled product and substrate (49,50). The forward radiochemical assay can be done using fatty acid as substrate and coupling it with fatty acid synthetase, ATP-Mg^{2+},and CoASH to generate acyl-CoA (51). Carnitine acyltransferases can also be assayed using an isotope exchange method (46); in the forward direction the incorporation of radiolabeled carnitine into radiolabeled acylcarnitine is measured. These assay methods are summarized in Table 1.

Table 1. Summary of Assay Methods for Carnitine Acyltransferases.

Acyl-CoA + L-carnitine ↔ Acylcarnitine + CoASH
Spectrophotometric Continuous Rate Assays
Forward direction only: Measure CoASH with sulfhydryl trapping reagent such as DTNB (E_{412}=13,800 $M^{-1}cm^{-1}$) (4) or DTBP (E_{324}=19,600 $M^{-1}cm^{-1}$) (47). Need to correct for carnitine independent CoASH release. DTNB and DTBP may inhibit reactive thiol groups in the enzyme essential for catalysis. Forward or reverse direction: Measure the disappearance (forward) or appearance (reverse) of acyl-CoA directly by the A_{232} of the thioester bond (45,46). Need to correct for carnitine independent CoASH release. Usually used for nearly homogeneous enzymes because of high background A_{232} and low extinction coefficient (E_{232}= 4500 $M^{-1}cm^{-1}$). Reversibility of reaction hinders initial rate measurements.
End Point Assays
Forward or Reverse direction: Use radioactively labeled carnitine or acyl-group. Need to separate labeled product from labeled substrate (49,50). Need to ensure initial rate measurement. Forward direction only: Can be used with free fatty acid as substrate coupled to fatty acid synthetase to generate acyl-CoA (51). Reverse direction only: Use hydroxylamine to measure the production of acyl-hydroxymate from acyl-CoA (48).
Isotopic Exchange Assays
Both forward and reverse direction: Measure the rate of incorporation of radioactivity from carnitine fraction into the acylcarnitine faction or vice versa (46). Cannot be used for initial rate measurements since run at or near equilibrium. Rate underestimated in the presence of acyl-CoA hydrolase.

Distribution of Medium/long-chain Carnitine Acyltransferase Activity in Rat Liver

Microsomal long-chain carnitine acyltransferase (CPT) activity was first reported in 1962 by Bremer, et. al. who showed the production of [^{14}C]-palmitoylcarnitine from [^{14}C]-carnitine, palmitate, ATP-Mg^{+}, and CoASH in rat liver microsomes and mitochondria using an endpoint isotope forward assay (52). The microsomal CPT activity was estimated to be 70% of the mitochondrial CPT activity. In 1967 though, Norum and Bremer reported that CPT activity was exclusively localized in liver mitochondria (53). The mitochondrial CPT activity accounted for 65% and the microsomal activity for only 8% of the total rat liver homogenate CPT activity measured using an exchange assay in which the incorporation of radioactivity into [^{14}C]-palmitoylcarnitine was measured from unlabeled palmitoylcarnitine, CoASH, and [^{14}C]-carnitine. They proposed that the microsomal fraction isolated in 1962 had been contaminated by mitochondria leading to the putative microsomal CPT activity. An alternative explanation is that the different assay conditions used in 1962 and 1967 gave different estimates of CPT activity. The exchange assay used in 1967 (53) can underestimate CPT activity in the presence of palmitoyl-CoA hydrolase (4) and microsomes contain higher levels of palmitoyl-CoA hydrolase than do mitochondria (54,55).

Van Tol and Hulsmann in 1969 also reported a dual localization of CPT in both mitochondria and microsomes and that measurement of microsomal CPT activity is dependent on the assay method used (51). They assayed CPT using both an exchange assay and an isotope forward assay measuring the production of [^{3}H]-palmitoylcarnitine from [^{3}H]-carnitine, palmitate, $MgCl_2$, ATP, and CoASH. They found that ~50% of the total homogenate CPT activity was in the mitochondria when either assay was used. The percentage of total homogenate CPT activity in the microsomal fraction was dependent on how the CPT was

assayed; the exchange assay gave 12% and the isotope forward assay gave 28% as the percentage of total homogenate CPT in the microsomal fraction.

Hoppel and Tomec in 1972 reported the intracellular distribution of CPT in rat liver using different assay methods (27). Using an isotope forward end point assay, 12% of the total homogenate CPT activity was located in the microsome fraction. Using an isotope reverse end point assay, it was only 7% and using a hydroxamate assay, it was 5%. The mitochondrial CPT activity, in contrast, was 50% of the total homogenate CPT activity using the isotope forward assay, 66% using the isotope reverse assay, and 71% using the hydroxamate assay.

In 1973, the subcellular distribution of long-chain carnitine acyltransferase (CPT) activity in rat liver was studied by Markwell, et. al. (41). Microsomal CPT activity assayed spectrally using DTNB with 37.5 μM palmitoyl-CoA was ~10% of the mitochondrial CPT activity. Microsomal CPT activity can be underestimated by this spectral assay because assay conditions included high concentrations of palmitoyl-CoA and the presence of 0.1% Triton X-100 both of which inhibit the microsomal enzyme. Also, it was reported that a high background rate for palmitoyl-CoA hydrolase (carnitine independent CoASH release) was present in the microsomal fraction which could have masked low CPT rates. In rat liver, microsomal palmitoyl-CoA hydrolase has a higher specific activity than does the mitochondrial palmitoyl-CoA hydrolase (54,55).

The distribution of medium-chain carnitine acyltransferase (COT) activity in rat liver microsomes was also reported in 1973 by Markwell et al (41). The intracellular distribution of carnitine acyltransferase activity using octanoyl-CoA as substrate (COT activity) showed COT was 20% peroxisomal, 52% mitochondrial, and 28% microsomal. COT activity was assayed with DTNB and 100 μM octanoyl-CoA. The intracellular distribution was determined

using fractions isolated by isopynic sucrose density gradient centrifugation.

Further studies by Markwell, et. al., showed COT was present in a microsome fraction prepared using differential and zonal centrifugation that was free of marker enzymes for golgi and plasma membranes (37). COT activity was found in both rough and smooth microsomes of rat liver, and the specific activity of COT was two fold higher in smooth microsomes than in rough microsomes (37). Valkner and Bieber in 1972 reported that microsomal COT activity is located exclusively with the cytoplasmic face of the endoplasmic reticulum (42).

In 1974, Van Tol reported that CPT was located in both mitochondria and microsomes of rat liver, and that different assay conditions give varying measurements of microsomal CPT activity (56). The presence of enzymes and substrates for fatty acid activation and an ATP regenerating system (complete assay) were necessary for maximum CPT activity. A simplified assay procedure using palmitoyl-CoA as substrate in an isotope forward assay gave <50% of the microsomal CPT activity as did the complete assay procedure. When the complete assay was used the microsomal CPT activity was estimated to be ~35% of the mitochondrial CPT activity.

Kahonen in 1976 showed the intracellular distribution of CPT in rat liver fractions isolated by isopynic sucrose density gradient centrifugation (57). CPT was assayed spectrally. Microsomes contained 11% and the mitochondria contained 81% of the CPT activity of the total homogenate. The microsome fraction, though, contained high levels of acyl-CoA hydrolase activity. Kahonen also reported COT activity in microsomes (57). The subcellular distribution of COT was done using isopynic sucrose density gradient fractionation. COT was assayed spectrally using DTNB in an assay that contained 0.1% Triton X-100 and 100 μM octanoyl-CoA. The distribution of COT was 9% peroxisomal, 77% mitochondrial,

6% microsomal, and 9% soluble.

Literature reports of medium-chain carnitine acyltransferase (COT) and long-chain carnitine acyltransferase (CPT) activity in the endoplasmic reticulum have been contradictory (27,37,39,41,42,51-53,56,57). The use of different assay methods has contributed to the problem. For instance, the use of palmitoyl-CoA as substrate to assay medium/long-chain carnitine acyltransferase activity underestimates the contribution of the microsomal enzyme. Contamination of organelle fractions of liver homogenates is well documented (58,59) and could also contribute to contradictory reports of the distribution of COT and CPT activity in rat liver. For example, electron microscopic studies have shown association between rough ER and mitochondria and putative rough-ER\mitochondria complexes have been isolated (60). It has also been shown that in rat liver there is a subfraction of ER associated with mitochondria that sediments at 10,000 x g (61). It was proposed that this mitochondrial-ER fraction is involved in lipid transfer (61). Indeed, reports that CPT_o is located in outer mitochondrial membrane vesicles could result from contamination of outer membrane preparations with ER. Outer membrane preparations can contain as much as 14% ER (58).

Tissue Distribution of Microsomal Medium\long-chain Carnitine Acyltransferase Activity

The subcellular distribution studies of COT discussed above used rat liver. These studies showed that COT/CPT activity in rat liver is located in mitochondria, microsomes, and peroxisomes (37,39,41,51). In 1978, Fogle and Bieber reported the presence of COT and CPT in rat heart microsomes (62). Microsomes from rat heart were prepared by differential centrifugation and $CaCl_2$ precipitation. The distribution of COT in heart was 10.5% microsomal and 32.6% mitochondrial of the total COT activity of the homogenate. The

distribution of CPT in heart was 13.2% microsomal and 29.6% mitochondrial of the total CPT activity of the homogenate. Marker enzyme data showed the microsomes were <10% contaminated by mitochondria. The ratio of C10/C16 activity of rat heart microsomes is 1.75.

Effect of Drug Treatment and Feeding\Fasting on Medium\Long-chain Carnitine Acyltransferase Activity of Liver Microsomes

Markwell et al reported in 1977 that treatment of rats with clofibrate (ethyl-p-chlorophenoxy isobutyrate), a hypolipidemic drug, increases the specific activity of COT in microsomes from male, fed rats isolated by isopynic sucrose gradient centrifugation from 8.0 to 20.9 nmole min^{-1} mg^{-1} protein (63). Treatment with phenobarbital, a proliferator of smooth ER, did not change the specific activity of microsomal COT (63).

Kahonen in 1976 reported the effect of clofibrate treatment on the specific activity and percent distribution of microsomal COT and CPT in a membrane fraction in rats (57). The specific activity of microsomal COT in clofibrate treated rats was increased 4.5 fold compared to normal while the percentage microsomal COT in the membrane fraction compared to total homogenate COT was 5.8% in normal rats and 5.0% in clofibrate treated rats. The specific activity of microsomal CPT increased 2.5 fold in clofibrate treated rats compared to normal rats. The percentage microsomal CPT in the membrane fraction compared to total homogenate CPT was 10.6% in normal and 5.1% in clofibrate treated. Carnitine acyltransferase activity was assayed spectrally in the forward direction with octanoyl-CoA or palmitoyl-CoA as substrate using DTNB.

Van Tol in 1974 reported the effect of fasting on microsomal and mitochondrial CPT activity. Microsomal CPT activity from 48 hour fasted rats increased approximately 2 fold

from 6.4 in fed to 14.0 nmole min^{-1} mg^{-1} protein in fasted rats (56). In contrast, mitochondrial CPT increased only 1.1 fold from 16.7 in fed to 18.7 nmole min^{-1} mg^{-1} protein in fasted rats (56). A recent report in abstract form by Ramsay showed carnitine acyltransferase activity with both decanoyl-CoA and palmitoyl-CoA as substrate increased in gradient purified microsomes from fasted as compared to fed rats (64).

Kinetics of Medium\Long-Chain Carnitine Acyltransferase Activity in Microsomes

Van Tol in 1974 (56) reported that the $K_{0.5}$ for the microsomal medium\long-chain carnitine acyltransferase is 7.1μM for palmitoyl-CoA and 0.18mM for L-carnitine. Assays were done using a radiochemical isotope forward assay with [^{3}H]-carnitine. The $K_{0.5}$ of the microsomal medium\long-chain carnitine acyltransferase for palmitoyl-CoA and for L-carnitine were not significantly altered with fasting (56).

Solubility and Stability Characteristics of Microsomal Medium\long-chain and Short-chain Carnitine Acyltransferase Activity

COT and CAT of rat liver microsomes are membrane bound and require detergent for solubilization (39). Freeze\thaw treatment of microsomes solubilizes <10% of COT (39). Microsomal COT can be completely solubilized in 1% Triton X-100, 0.4M KCl but the activity is not stable (39). Conditions such as 0.4M KCL, or 1% Triton X-100 which solubilized and stabilized CAT caused a complete loss of COT activity (37). Microsomal CAT can be selectively solubilized be treatment of microsomes with 0.3M sucrose in 0.1M pyrophosphate pH 7.5 which solubilizes 80-90% of CAT activity while 81-83% of microsomal COT remains with the pellet fraction (39).

Palmitoyl-CoA Inhibition of Medium\long-chain Carnitine Acyltransferase Activity in Rat Liver

No studies have reported palmitoyl-CoA inhibition of microsomal COT, but studies have shown that varying estimates of microsomal COT activity with palmitoyl-CoA as substrate are obtained with different assay methods. The assay methods have varied in the concentration of palmitoyl-CoA, and the presence of bovine serum albumin. Indirectly these studies show that high concentrations of palmitoyl-CoA as substrate could underestimate microsomal COT activity. For example when palmitoyl-CoA is generated in the assay using palmitate, CoASH, ATP-MG^{+}, and fatty acyl-CoA synthetase the COT activity is higher than when the same preparation is assayed using palmitoyl-CoA directly as substrate (56).

CPT activity of outer mitochondrial membrane vesicles is inhibited by palmitoyl-CoA (29). The CPT activity versus palmitoyl-CoA concentration curve shows inhibition of CPT activity above 15μM palmitoyl-CoA (29). The inhibition was not reversed with addition of bovine serum albumin or with lowering the palmitoyl-CoA concentration (29). It was also shown that the CPT activity of inner mitochondrial membrane vesicles was not inhibited by concentrations of palmitoyl-CoA up to 150μM (29). This agrees with previous studies which showed that CPT activity versus palmitoyl-CoA concentration curves for CPT_o of intact rat liver mitochondria are sigmoid (32,65,66).

Aminocarnitine as Substrate and Inhibitor of Medium\long-chain Carnitine Acyltransferase Activity in Rat Liver

DL-Aminocarnitine (3-amino-4-trimethylaminobutyric acid) inhibition of mitochondrial carnitine palmitoyltransferase (CPT) was first reported by Jenkins and Griffith in 1985 (67).

DL-Aminocarnitine and acetyl-DL-aminocarnitine both inhibited rat liver mitochondrial CPT with 5μM DL-aminocarnitine inhibiting 64% and 5μM acetyl-DL-aminocarnitine inhibiting 15% of total CPT activity of Triton X-100 treated mitochondria (67). CPT was assayed spectrally in the forward direction using DTNB (67). In 1986, Jenkins and Griffith reported that decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine also inhibited mitochondrial CPT (68). CPT was assayed spectrally in Triton X-100 treated mitochondria and 5μM decanoyl-DL-aminocarnitine inhibited 75% and palmitoyl-DL-aminocarnitine 99% of total CPT activity (68). DL-Aminocarnitine was not acylated by palmitoyl-CoA by rat liver mitochondrial CPT (67).

The inhibition of mitochondrial CPT_o by L-aminocarnitine was reported in 1989 by Kanamaru and Okazaki (69). Acetyl-L-aminocarnitine, propionyl-L-aminocarnitine, and butryl-L-aminocarnitine were isolated from a culture filtrate of *Emericella quadrilineata* IFO 5859 in the process of screening for long-chain fatty acid oxidation inhibitors. L-Aminocarnitine was named emeriamine. The I_{50} for L-aminocarnitine for CPT_o of intact rat liver mitochondria assayed using a radiochemical, isotope forward assay with L-[^{3}H]carnitine was 62.5 μM (see Figure 6 of ref. 69). The I_{50} for palmitoyl-L-aminocarnitine was 2.2μM. Unlike the report by Jenkins and Griffith in 1985 (67), Kanamaru and Okazaki (69) report that palmitoyl-L-aminocarnitine is formed by liver mitochondria from L-aminocarnitine and palmitoyl-CoA.

The two medium\long-chain carnitine acyltransferases of mitochondria (CPT_o and CPT_i) and the medium\long-chain carnitine acyltransferase of peroxisomes (COT) have been compared in their inhibition by L-aminocarnitine and their ability to use L-aminocarnitine as substrate using a radiochemical isotope forward assay with [1-^{14}C]palmitoyl-CoA or [1-^{14}C]decanoyl-CoA as substrate (70). The I_{50} for L-aminocarnitine of CPT_o of outer

mitochondrial membrane vesicles is approximately 250μM while the I_{50} for L-aminocarnitine of CPT_i of inner mitochondrial membrane vesicles and purified mitochondrial CPT is 25μM. The I_{50} for L-aminocarnitine of medium\long-chain carnitine acyltransferase activity of intact peroxisomes is approximately 250μM (70) and purified peroxisomal COT is not inhibited by 2mM L-aminocarnitine. L-Aminocarnitine was not acylated with palmitoyl-CoA as cosubstrate by mitochondrial CPT_i, mitochondrial CPT_o or by peroxisomal COT in agreement with Jenkins and Griffith (67). L-Aminocarnitine was acylated, though, with octanoyl-CoA as cosubstrate by CPT_o of outer mitochondrial membrane vesicles; the rate with 20mM L-aminocarnitine was 34% of the rate with 5mM L-carnitine for CPT_o of outer mitochondrial membrane vesicles. CPT_i of inner membrane vesicles and purified mitochondrial CPT used L-aminocarnitine with octanoyl-CoA as cosubstrate at 20% of the rate with L-carnitine.

Malonyl-CoA Regulation of Medium\long-chain Carnitine Acyltransferase Activity in Rat Liver

Malonyl-CoA, the first committed intermediate in fatty acid synthesis acts in the coordinate regulation of fatty acid synthesis and degradation via inhibition of the outer form of carnitine palmitolytransferase (CPT_o) of mitochondria. Malonyl-CoA inhibition of mitochondrial CPT_o was first reported by McGarry et al in 1978 (71,72). Malonyl-CoA regulation of mitochondrial CPT_o allows inhibition of fatty acid oxidation via CPT_o when fatty acid synthesis is occurring preventing a futile cycle of synthesis and oxidation (30). Malonyl-CoA does not inhibit the inner mitochondrial CPT (CPT_i) (71,72). It has not been established if malonyl-CoA sensitive CPT_o and malonyl-CoA insensitive CPT_i are the same protein or if they are two different proteins (19). It has been proposed that CPT_o and CPT_i are different proteins (22-24,70,71,72) and that CPT_o is destroyed by detergent solubilization (22,23,73).

Recently it has been reported that malonyl-CoA sensitive CPT can be solubilized in octylglucoside (16). Bergseth et al have shown that rat liver mitochondrial CPT solubilized from the membrane can be separated from a malonyl-CoA binding protein(s) and have proposed that CPT is sensitive to malonyl-CoA inhibition through association with a regulatory protein (74). Zammit et al (75) have reported different molecular weights for malonyl-CoA sensitive CPT_o and the inner CPT_i and Zammit et al (76) have reported different molecular weights for CPT_o and malonyl-CoA binding.

Malonyl-CoA inhibition of mitochondrial CPT_o has been extensively studied. In intact mitochondria from fed rats the K_i for malonyl-CoA inhibition of CPT_o is 1.5μM (77). The K_i for malonyl-CoA inhibition increases to 5.0μM in mitochondria from 42 hour fasted rats (77). Experimental conditions which cause substrate depletion, malonyl-CoA depletion, or high acyl-CoA concentrations can influence the determination of malonyl-CoA sensitivity (77).

The medium\long-chain carnitine acyltransferase (COT) activity of intact rat liver peroxisomes isolated by centrifugation through iso-osmotic Nycodenz solution is inhibitable by malonyl-CoA (40). The concentration of malonyl-CoA required to inhibit 50% of COT activity of intact peroxisomes is 2.2μM (40). The ratio of C10\C16 activity for COT of intact peroxisomes is 2.1 (40). The COT activity of intact peroxisomes is approximately 20% of the COT activity of the liver homogenate (40). The specific activity of COT of intact peroxisomes assayed with an isotope forward assay using L-^{3}H-carnitine is 4.9 ± 0.43 nmole min^{-1} mg^{-1} with palmitoyl-CoA as substrate and 10.5 ± 1.3 nmole min^{-1} mg^{-1} with decanoyl-CoA as substrate (40). Seventy percent of the COT activity of intact peroxisomes can be released by sonication but is only 20% inhibited by 10μM malonyl-CoA compared with 90% inhibition of intact peroxisomal COT by 10μM malonyl-CoA (40).

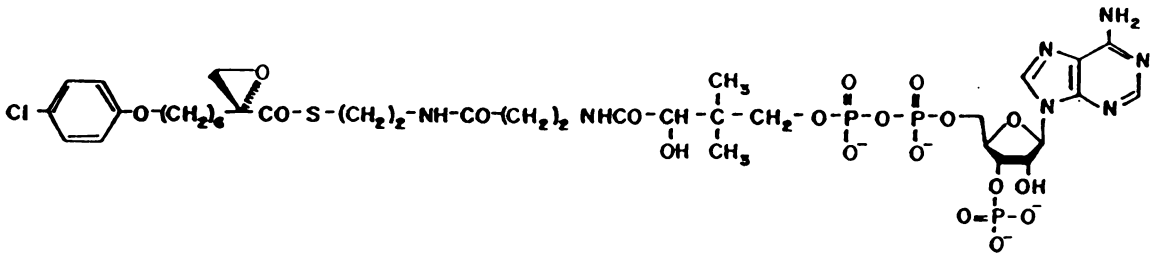
The effect of malonyl-CoA on the COT activity of microsomal medium\long-chain carnitine acyltransferase activity has not been reported. It has been shown though that the COT activity of microsomes assayed with palmitoyl-CoA as substrate is increased with fasting (56). The concentration of malonyl-CoA in rat liver is decreased in the fasted state. The malonyl-CoA content of liver from fed rats is 7.5 nmole g^{-1} wet weight and from 24 hour fasted rats it is 1.7 nmole g^{-1} wet weight (78).

Etomoxiryl-CoA Inhibition of Medium\long-chain Carnitine Acyltransferase Activity in Rat Liver

The regulation of medium\long-chain carnitine acyltransferase activity by malonyl-CoA has been studied using epoxy containing fatty acid derivatives such as TDGA (2-tetraglycidic acid) and etomoxir (ethyl-2-[6-(-chlorophenoxy)hexyl]oxirane-2-carboxylate) (22,23,79-81). These derivatives are activated *in vivo* via conjugation to coenzyme A. The structure of etomoxiryl-CoA is shown in Fig. 1. Etomoxiryl-CoA is also called B827-33. Etomoxiryl-CoA is a specific inhibitor of mitochondrial CPT_o (82). Etomoxiryl-CoA has been proposed to be an active-site directed, irreversible inhibitor of mitochondrial CPT_o (22,23); TDGA-CoA has been characterized as an active-site directed, irreversible inhibitor of CPT_o (79). In rat liver mitochondria ^{3}H-etomoxir forms a covalent adduct to an approximately 94,000 MW protein while in rat skeletal muscle mitochondria ^{3}H-etomoxir forms a covalent adduct to an approximately 86,000 MW protein. It has been proposed that these labeled proteins are CPT_o (22,23).

Lopaschuck et al have used etomoxir in studying heart function in fatty acid perfused ischemic rat hearts (83,84). It was proposed that etomoxir can protect hearts from fatty acid

induced ischemic injury by inhibiting CPT_o and decreasing myocardial long-chain acylcarnitine levels. Lopaschuck et al showed that etomoxir protects hearts from fatty acid induced ischemic injury independent of changes in long-chain acylcarnitine and long-chain acyl-CoA (83). They concluded that the protective effect of etomoxir could have resulted from a stimulation of glucose oxidation and that etomoxir at micromolar concentrations can inhibit both CPT_o and the inner mitochondrial $CPT_{i.}$ (84).



Etomoxiryl - CoA

Figure 1. Structure of Etomoxiryl-CoA. The structure of etomoxiryl-CoA (ethyl-2-[6-(-chlorophenoxy)hexyl]oxirane-2-carboxyl-coenzyme A ester) is shown.

Chapter 2. Malonyl-CoA Inhibition of Medium\long-chain Carnitine Acyltransferase Activity of Rat Liver Microsomes

The data show that rough and smooth endoplasmic reticulum of rat liver contain a medium\long-chain carnitine acyltransferase (COT) that is strongly inhibited by malonyl-CoA. The average percent inhibition for 25 preparations is 87.4 ± 11.7, with nine preparations showing 100% inhibition; the concentrations of decanoyl-CoA and L-carnitine were 17 μM and 1.7 mM, respectively. The concentration of malonyl-CoA required for 50% inhibition is 5.3 μM. Microsomal COT is also strongly inhibited by etomoxiryl-CoA, with 0.6 μM etomoxiryl-CoA producing 50% inhibition. Detergent solubilized microsomal COT is not inhibitable by malonyl-CoA while 1.5 μM etomoxiryl-CoA inhibits 50% of detergent solubilized microsomal COT. [^{3}H]-Etomoxir forms a covalent adduct with two proteins in rat liver microsomes with molecular weights of ~87,000 and ~51-57,000 daltons. Palmitoyl-CoA is a substrate of microsomal COT at low concentrations and palmitoyl-CoA strongly inhibits microsomal COT at higher concentrations. The concentration of palmitoyl-CoA required for 50% inhibition of microsomal COT is 11 μM. Microsomal COT is stable to freezing at -70°C. The microsomal medium\long-chain carnitine acyltransferase is not immunoprecipitated by antibody prepared against mitochondrial carnitine palmitoyltransferase and it is only slightly inhibited by antibody prepared against peroxisomal carnitine octanoyltransferase.

Introduction

Medium/long-chain carnitine acyltransferase activity is found in mitochondria, peroxisomes and endoplasmic reticulum in rat liver (19,41). The mitochondrial medium/long-chain carnitine acyltransferase (CPT) has been extensively studied (19,30,85). The peroxisomal medium/long-chain carnitine acyltransferase (COT) has also been characterized (14,15,19), but the microsomal medium/long-chain carnitine acyltransferase is poorly characterized. Literature reports of microsomal carnitine acyltransferase activity have been contradictory (27,37,39,41,42,51-53,56,57) and characterization of the membrane bound enzyme has been hindered by the instability of the enzyme and difficulty in solubilizing it from the microsomal membrane (37,39). Contamination of organelle fractions of rat liver prepared by differential centrifugation is common (35,58,59,61) and can make interpretation of data difficult with enzyme activity that has a multi-organelle distribution in liver. To address this problem we have studied the distribution of medium/long-chain carnitine acyltransferase activity in fractions of a rat liver homogenate isolated by differential centrifugation has been studied using organelle marker enzymes.

The outer form of mitochondrial carnitine palmitoyltransferase that is in contact with the cytosol (CPT_o) is inhibited by malonyl-CoA (30,71,72,85). It has also recently been reported that medium/long-chain carnitine acyltransferase activity of intact rat liver peroxisomes is inhibitable by malonyl-CoA (40). This indicates malonyl-CoA can coordinately

regulate both fatty acid synthesis and oxidation. Since microsomal COT uses medium-chain as well as long-chain acyl-CoA's as substrate it has the capacity to generate cytosolic acylcarnitines which could enter the mitochondria for ß-oxidation. Uncontrolled production of cytosolic acylcarnitines by microsomal COT could bypass regulation of CPT_o by malonyl-CoA. To clarify this issue we investigated the distribution of malonyl-CoA sensitivity of COT in rat liver fractions was also determined and the effect of malonyl-CoA concentration on microsomal COT studied.

The regulation of mitochondrial carnitine palmitoyltransferase by malonyl-CoA has been studied using epoxy-containing fatty acids such as etomoxir. Etomoxir is an epoxy-containing fatty acid that is a potent inhibitor of the malonyl-CoA sensitive carnitine palmitoyltransferase of mitochondria (CPT_o) (22,82,86,87). ^{3}H-Etomoxir specifically labels one protein in rat liver mitochondria with a molecular weight of ~90,000 daltons (88). The inhibition of malonyl-CoA sensitive microsomal COT by etomoxiryl-CoA was studied. The labeling of microsomal proteins by ^{3}H-etomoxir is presented and compared to the results from rat heart and liver mitochondria. The data show that microsomal COT is strongly inhibited by malonyl-CoA and etomoxiryl-CoA and that microsomal COT does not cross react with antimitochondrial-CPT or antiperoxisomal-COT.

Results

Distribution of Malonyl-CoA Sensitive Medium\long-chain Carnitine Acyltransferase Activity in Rat Liver

Rat liver contains medium\long-chain carnitine acyltransferase activity inhibitable by malonyl-CoA in addition to malonyl-CoA sensitive microsomal COT. The malonyl-CoA inhibition of mitochondrial CPT_o. has been well documented (30,71,72,85). Also, it has recently been reported that medium\long-chain carnitine acyltransferase activity associated with intact peroxisomes is inhibitable by malonyl-CoA (40). Since malonyl-CoA sensitive microsomal COT activity could result from mitochondrial or peroxisomal contamination of the microsomal fraction, experiments were done to determine the distribution of malonyl-CoA sensitive COT activity and organelle marker enzymes in fractions of a rat liver homogenate. The fractions were not washed so that percent recovery of enzyme activity could be determined.

Fractions of a rat liver homogenate isolated by differential centrifugation were assayed for COT activity, percent inhibition of COT by malonyl-CoA, and for marker enzymes for endoplasmic reticulum (NADPH cytochrome *c* reductase), peroxisomes (urate oxidase), and for the inner membrane (cytochrome *c* oxidase) and outer membrane (monoamine oxidase) of mitochondria. Three experiments were done. The data are given in Table 1. All of the fractions contain COT activity with a specific activity at least equal to that of the 400 x g supernatant fraction demonstrating the multiple organelle distribution of COT activity. The

Table I. Distribution of COT Activity, Mg

Table 1. Distribution of COT Activity, Malonyl-CoA Sensitivity of COT, and Marker Enzymes in Fractions of a Rat Liver Homogenate Isolated by Differential Centrifugation. The data are the means of three experiments ± SEM. ND, not detected. Fractions prepared as described under Materials and Methods. The % distribution is the percentage of total units of enzyme present in the 400 x g supernatant fluid. The total units (μmole/min) of COT, cytochrome *c* oxidase, monoamine oxidase, urate oxidase, and NADPH cytochrome *c* reductase in the 400 x g supernatant fluid are 15.3 ± 1.4, 136.7 ± 8.2, 12.9 ± 0.7, 12.1 ± 0.63, and 44.9 ± 4.4, respectively. The % inhibition by malonyl-CoA was determined with 17μM malonyl-CoA.

Fraction	COT munits/mg (% Distribution)	% Inhibition by Malonyl-CoA	Cytochrome c Oxidase munits/mg (% Distribution)	Monoamine Oxidase munits/mg (% Distribution)	Urate Oxidase munits/mg (% Distribution)	NADPH Cyto-chrome c Reductase munits/mg (% Distribution)
400 x g supernatant fluid	5.3 ± 0.5 (100)	15.7 ± 1.3	50.7 ± 8.0 (100)	4.5 ± 0.4 (100)	4.2 ± 0.35 (100)	15.1 ± 0.6 (100)
6,000 x g pellet	10.6 ± 0.47 (62.8)	37 ± .8	94.4 ± 5.8 (64.1)	9.4 ± 0.52 (66.6)	7.7 ± 0.2 (58.7)	6.0 ± 0.88 (12.1)
10,000 x g pellet	6.8 ± 2.1 (6.6)	17.3 ± 7.6	37.7 ± 1.9 (5.2)	2.4 ± 0.58 (7.2)	1.9 ± 0.66 (25.1)	20.8 ± 2.1 (8.2)
100,000 x g supernatant fluid	7.3 ± 1.6 (49.0)	4.6 ± 2.7	0.31 ± 0.09 (0.21)	0.47 ± 0.19 (5.1)	N.D.	4.7 ± 0.07 (10.5)
100,000 x g pellet	5.6 ± 0.47 (14.8)	76.7 ± 3.1	7.2 ± 0.69 (2.4)	0.67 ± 0.21 (1.7)	1.4 ± 0.43 (9.6)	51.4 ± 3.1 (44.7)

COT activity in the 400 x g supernatant fraction is 16% inhibited by malonyl-CoA. Malonyl-CoA inhibits 37% of the COT activity of the 6,000 x g pellet, the fraction most enriched in mitochondrial marker enzyme activity. The 6,000 x g pellet contains 64% of the cytochrome *c* oxidase, 67% of the monoamine oxidase, 58% of urate oxidase, as well as 12% of the microsomal marker, NADPH cytochrome *c* reductase. In contrast, the COT activity of the 100,000 x g pellet is 77% inhibited by malonyl-CoA. The 100,000 x g pellet is the fraction most enriched in the microsomal marker; it contains 45% of NADPH cytochrome *c* reductase, and it only contains 2.4% of cytochrome *c* oxidase, 1.7% of monoamine oxidase, and 10% of urate oxidase. The COT activity in the 100,000 x g pellet fraction cannot be accounted for by mitochondrial or peroxisomal contamination of this fraction.

Malonyl-CoA and Etomoxiryl-CoA Inhibition of Microsomal COT

The medium-chain carnitine acyltransferase (COT) activity of microsomes isolated from rat liver homogenates using three different methods is shown in Table 2. Microsomes derived from rough and smooth endoplasmic reticulum (ER) prepared by differential centrifugation and $CaCl_2$ precipitation as well as microsomes derived from rough ER prepared by centrifugation through a discontinuous CsCl-sucrose gradient contain COT activity averaging 4-6 nmole min^{-1} mg^{-1} protein that is >87% inhibitable by malonyl-CoA. Nine out of the 25 preparations isolated by differential centrifugation are 100% inhibited by malonyl-CoA. In these assays microsomal COT was assayed spectrally using a rate forward assay. Since microsomes contain high acyl-CoA hydrolase activity which contributes to large blank values due to carnitine independent CoASH release, a series of experiments was done to confirm that the COT activity measured spectrally by the rate forward assay is producing the expected product,

Table 2. Malonyl-CoA Inhibition of COT Activity of Microsomes Isolated by Three Different Procedures. COT was assayed spectrally with 17 μM decanoyl-CoA, 1.7 mM L-carnitine, 150 μM DTBP in 50 mM potassium phosphate, pH 7.5. Malonyl-CoA was 17 μM. Data presented as mean ± SEM. n = number of preparations assayed.

Isolation Procedure	n	COT Activity munit/mg protein	%Inhibition by Malonyl-CoA
Differential Centrifugation	25	6.2 ± 2.7	87 ± 11.7
CsCl-Sucrose Gradient	3	3.7 ± 0.3	98 ± 1.3
$CaCl_2$ Precipitation	3	4.8 ± 0.04	82 ± 5.3

decanoylcarnitine, and that decanoylcarnitine formation is inhibited by malonyl-CoA.

Microsomes were incubated with [1-^{14}C]-decanoyl-CoA and L-carnitine and the [1-^{14}C]-decanoylcarnitine formed was separated by HPLC and the dpm in the [1-^{14}C]-decanoyl-carnitine peak determined. Figure 1A demonstrates the production of [1-^{14}C]-decanoylcarnitine by microsomes representing a COT activity of 5.7 nmole min^{-1} mg^{-1} protein and figure 1B shows inhibition of [1-^{14}C]-decanoylcarnitine production by malonyl-CoA. In this experiment, 100% inhibition of [1-^{14}C]-decanoylcarnitine production occurred. The same microsomal preparation was also assayed spectrally using identical conditions with unlabeled decanoyl-CoA; the COT activity assayed spectrally was 4.6 nmole min^{-1} mg^{-1} protein with 92% inhibition by malonyl-CoA.

The effect of the concentration of malonyl-CoA and etomoxiryl-CoA on membrane bound microsomal COT activity is shown in Figure 2. When microsomal COT is assayed spectrally with 17 μM decanoyl-CoA and 1.7 mM L-carnitine, the concentration of malonyl-CoA required to inhibit 50% of microsomal COT is 5.3 ± 0.43 μM; no preincubation of microsomal COT with malonyl-CoA is required. When microsomal COT is assayed either spectrally or radiochemically, the concentration of etomoxiryl-CoA required for 50% inhibition of microsomal COT is 0.58 ± 0.17 μM; membrane bound microsomal COT was incubated with etomoxiryl-CoA for two minutes prior to assay.

The effect of the concentration of malonyl-CoA on detergent solubilized microsomal COT activity is shown in Figure 3. Microsomal COT was solubilized with the zwitterionic detergent CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate). The specific activity of membrane bound microsomal COT is 7.9 ± 1.1 nmole min^{-1} mg^{-1} . The specific activity of the CHAPS-solubilized microsomal COT is 4.7 ± 0.6 nmole min^{-1} mg^{-1} .

Figure 1. Effect of Malonyl-CoA on the Production of [1-^{14}C]-Decanoylcarnitine. The production of [1^{14}C]-decanoylcarnitine by microsomal bound COT was determined with a radiochemical assay using [1-^{14}C]-decanoyl-CoA as described in Materials and Methods. Panel A shows the HPLC separation of the [1-^{14}C]-decanoylcarnitine produced; the COT activity was 5.73 nmole min^{-1} mg^{-1} protein. Panel B shows the effect of the addition of 17 μM malonyl-CoA to the assay.

Figure 1.

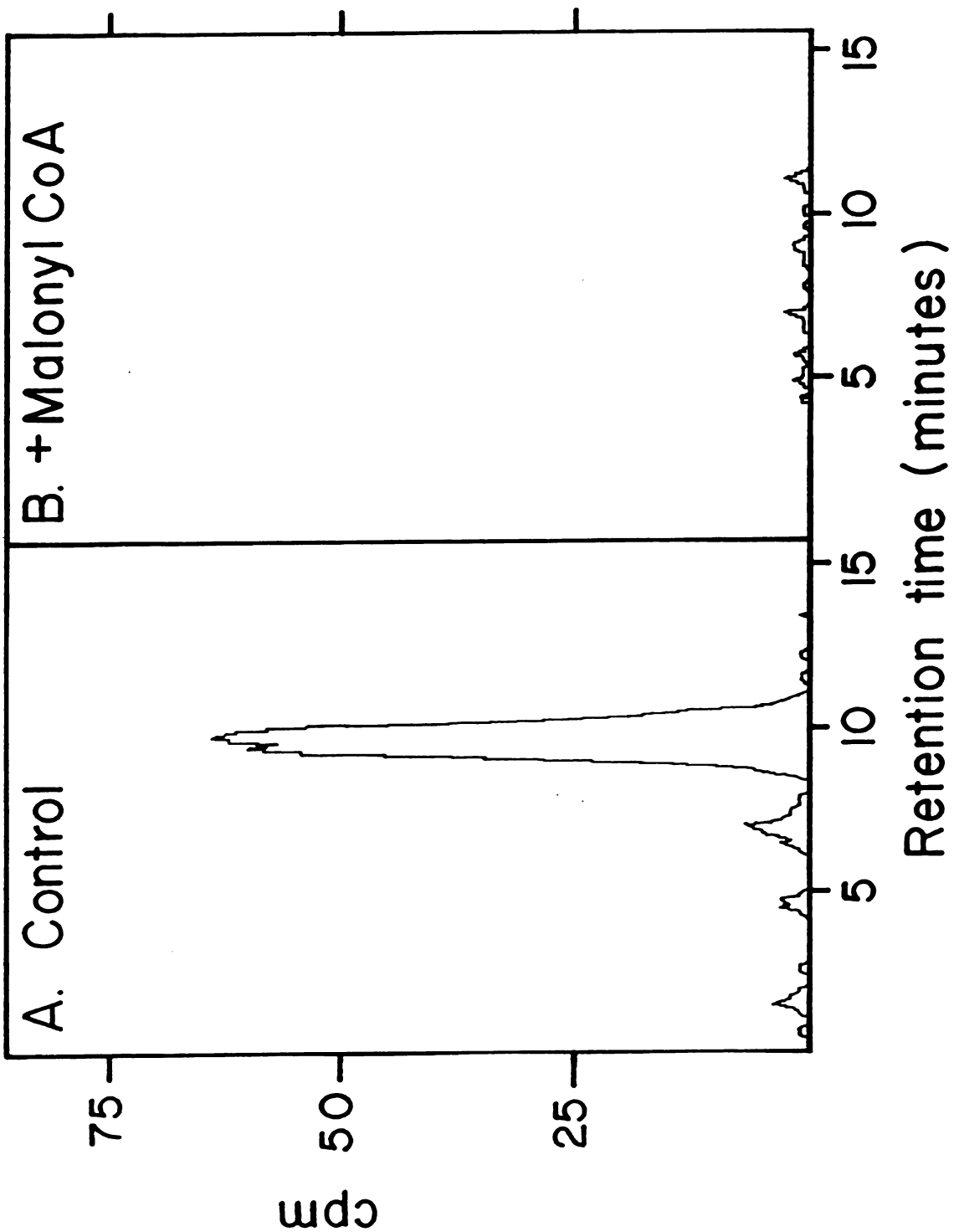

A. Control
B. + Malonyl CoA
cpm
75
50
25
5
10
15
5
10
15
Retention time (minutes)

Figure 2. Malonyl-CoA and Etomoxiryl-CoA Inhibition of Microsomal COT. Microsomes were prepared by differential centrifugation. COT was assayed spectrally as described in Materials and Methods in the presence of the concentrations of malonyl-CoA indicated. The values were corrected for carnitine independent release of CoASH at each malonyl-CoA concentration (n=3). The concentration of malonyl-CoA required to inhibit 50% of the COT activity is 5.3 ± 0.43 μM. COT was assayed radiochemically using [1-^{14}C]-decanoyl-CoA as described in Materials and Methods in the presence of the concentrations of etomoxiryl-CoA indicated (n=2). Microsomes were preincubated with etomoxiryl-CoA and [1-^{14}C]-decanoyl-CoA for 2 minutes. The concentration of etomoxiryl-CoA required to inhibit 50% of the COT activity is 0.58 ± 0.17 μM. COT was also assayed spectrally in the presence of the indicated concentrations of etomoxiryl-CoA and the I_{50} was identical to the I_{50} from the radiochemical assay, within experimental error. Initial COT activity (100%) was 10.9 ± 2.9 nmole min^{-1} mg^{-1} protein for malonyl-CoA and 7.9 ± 0.80 nmole min^{-1} mg^{-1} protein for etomoxiryl-CoA. The data are presented as the mean ± SEM.

Figure 2.

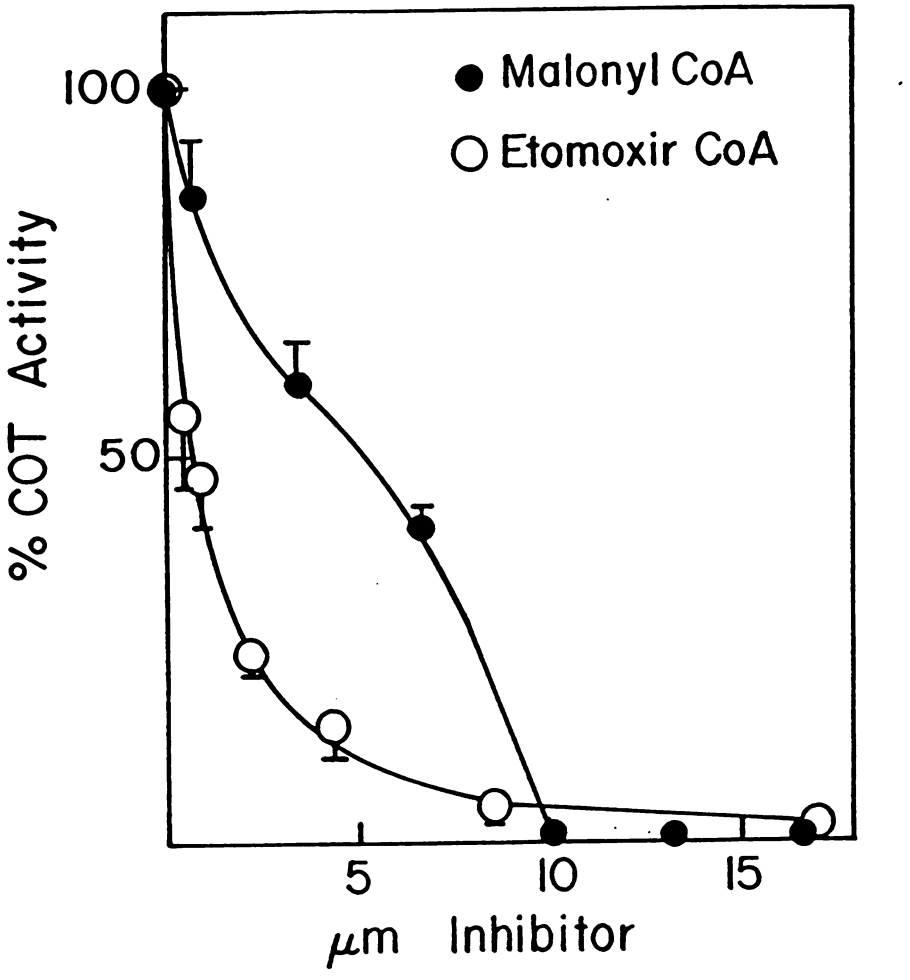
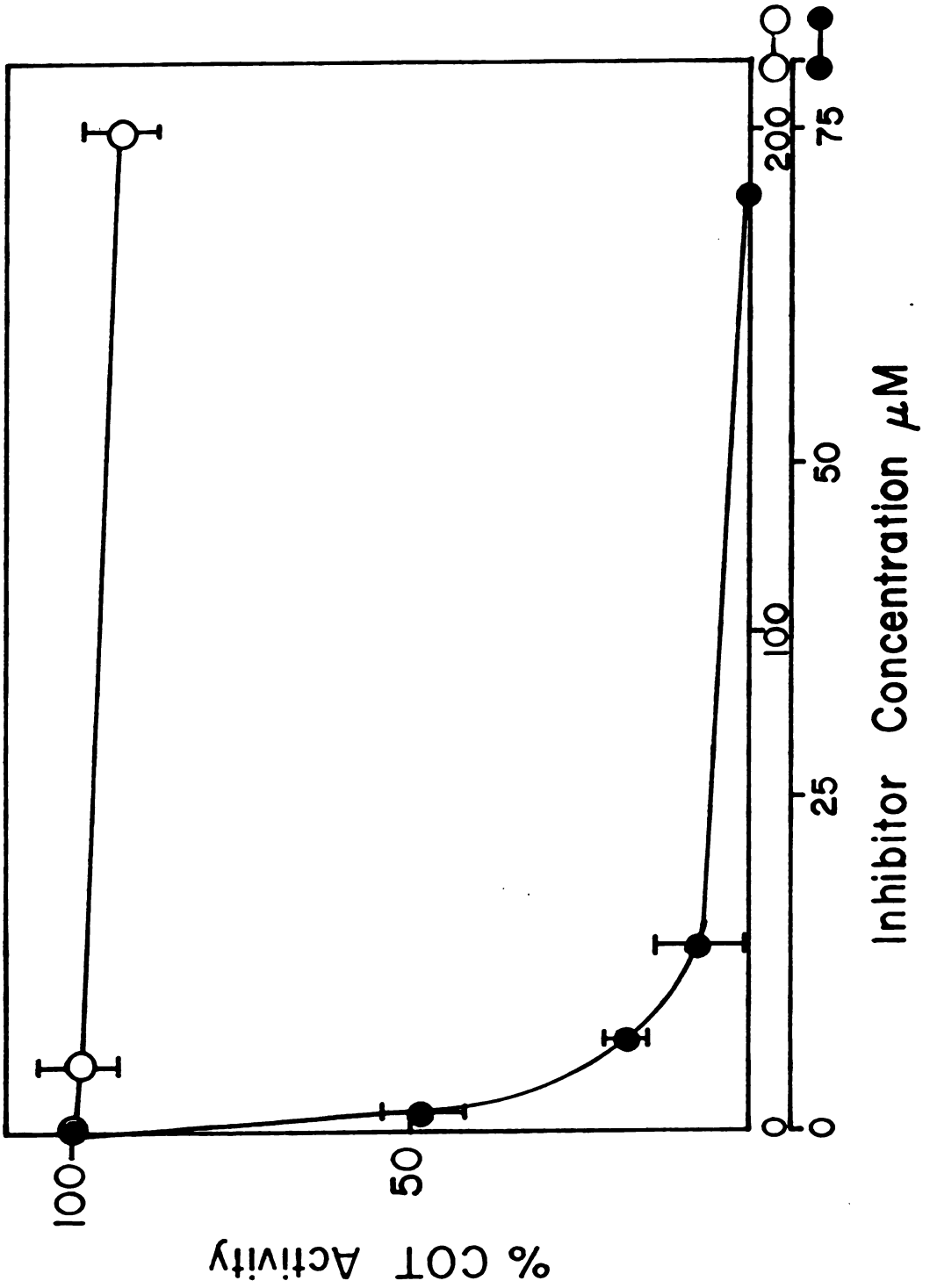

Malonyl CoA
Etomoxir CoA
100
50
% COT Activity
5
10
15
μm Inhibitor

Figure 3. Effect of Etomoxiryl-CoA and Malonyl-CoA on CHAPS-Solubilized Microsomal COT. CHAPS-solubilized microsomes were prepared and COT was assayed spectrally as described in Materials and Methods. COT was determined in the presence of the indicated concentrations of etomoxiryl-CoA with a 2 minute preincubation (closed circles) or malonyl-CoA with no preincubation (open circles). COT activity was corrected for carnitine independent CoASH release at each inhibitor concentration. The concentration of etomoxiryl-CoA required for 50% inhibition of COT activity is 1.5 μM. The initial (100%) COT activity is 4.7 ± 0.6 nmole min^{-1} mg^{-1} protein. The data are plotted as the mean ± SEM (n=2).

Figure 3.


% COT Activity
100
50
0
0
25
100
50
200
75
Inhibitor Concentration μM

COT was assayed spectrally with no preincubation for malonyl-CoA and with a 2 minute preincubation for etomoxiryl-CoA. The upper curve (o) shows the effect of malonyl-CoA. CHAPS-solubilized microsomal COT is only inhibited 11% by 200μM malonyl-CoA. The lower curve (•) shows the effect of etomoxiryl-CoA. The concentration of etomoxiryl-CoA required to inhibit 50% of CHAPS-solubilized microsomal COT activity is 1.5 μM. CHAPS solubilized microsomal COT is inhibited 11% by 200 μM malonyl-CoA and 78% by 14 μM etomoxiryl-CoA. When both 200 μM malonyl-CoA and 14 μM etomoxiryl-CoA are added to the assay, CHAPS solubilized microsomal COT is inhibited 67%, less than the inhibition with only etomoxiryl-CoA.

The inhibition of microsomal COT by etomoxiryl-CoA is reversible. When membrane bound microsomal COT is incubated with 40 μM etomoxiryl-CoA for 1 hour, the COT activity is completely inhibited. When these etomoxiryl-CoA inhibited microsomes are solubilized in CHAPS and the CHAPS-solubilized supernatant passed over a Biogel P6 desalting column, the COT activity is completely restored. Two experiments gave an average recovery of 98%. Experiments were tried to reverse etomoxiryl-CoA inhibition of membrane bound microsomal COT with washing, but microsomal COT activity was not stable to multiple washing steps.

Palmitoyl-CoA Inhibition of Microsomal COT

Membrane bound microsomal COT was assayed spectrally with decanoyl-CoA as substrate in the presence of increasing concentrations of palmitoyl-CoA. The data are shown in Figure 4. The concentration of palmitoyl-CoA required to inhibit 50% of microsomal COT activity is 10.9 ± 0.46 μM. Palmitoyl-CoA is also a substrate of microsomal COT at lower concentrations. The ratio of decanoyltransferase to palmitoyltransferase activity of microsomes

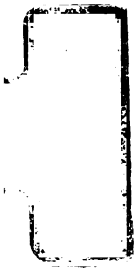
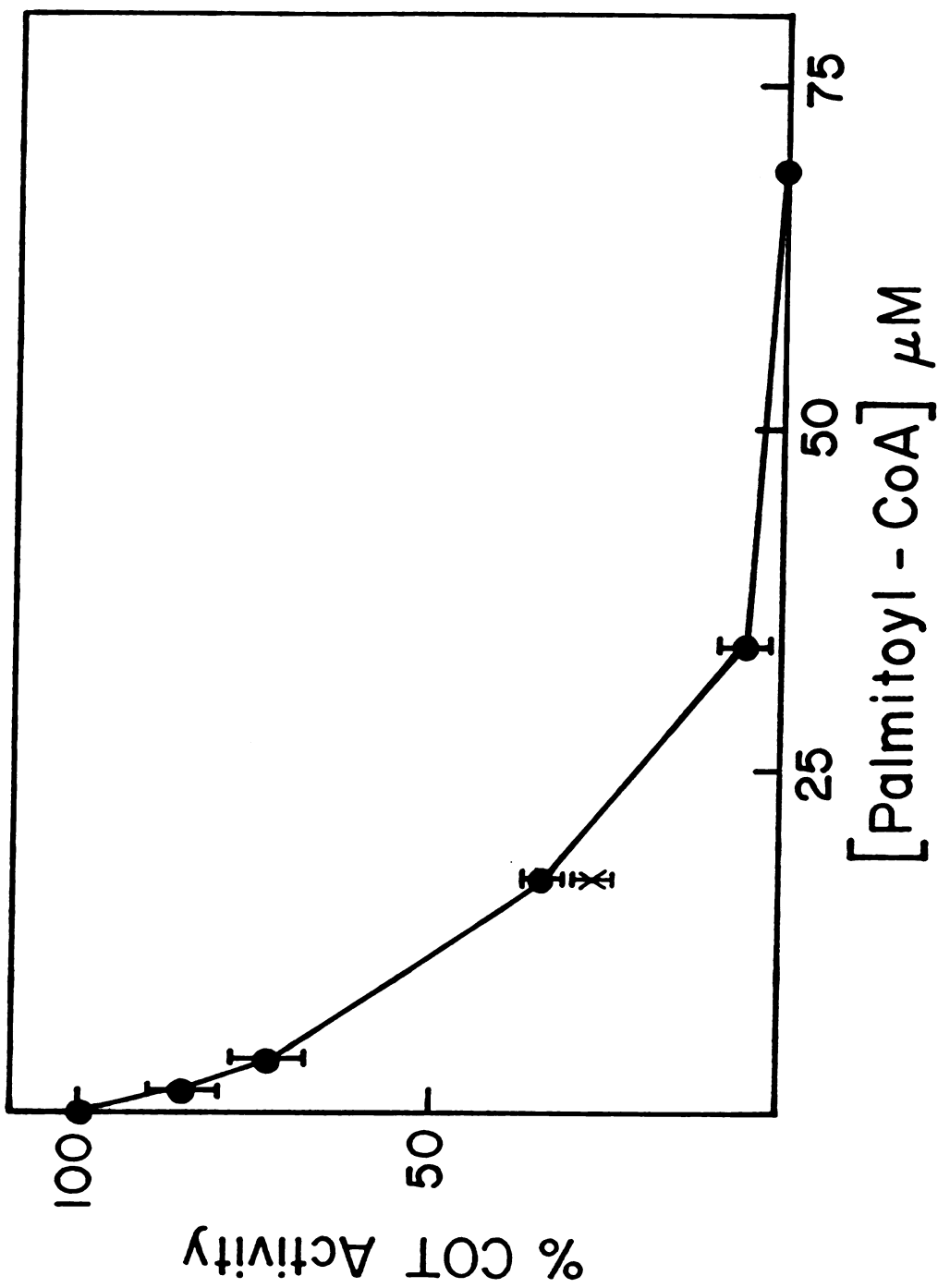


Figure 4. Palmitoyl-CoA Inhibition of Microsomal COT. Microsomes were prepared using differential centrifugation and COT was assayed spectrally as described in the Materials and Methods. Palmitoyl-CoA was added to the assay at the concentrations indicated. The values were corrected for carnitine independent CoASH release at each palmitoyl-CoA concentration. The initial COT activity (100%) was 12.9 ± 2.4 nmole min^{-1} mg^{-1} protein. The data are plotted as the mean of three experiments ± SEM. The concentration of palmitoyl-CoA required to inhibit 50% of the COT activity was 10.9 ± 0.46 μM. The effect of palmitoyl-CoA on microsomal COT was also determined using a radiochemical assay as described in Materials and Methods. The %[1-^{14}C]-decanoylcarnitine formation in the presence of 17 μM palmitoyl-CoA is shown by the X (n=2); initial COT activity (100%) was 14.6 ± 3.5 nmole min^{-1} mg^{-1} protein.

Figure 4.



is approximately 10. Palmitoyltransferase activity of microsomes is completely inhibitable by malonyl-CoA. Rat liver microsomes also contain acetyltransferase activity but it is not inhibitable by malonyl-CoA. For a representative microsome sample, the decanoyltransferase activity is 5.9 nmole min^{-1} mg^{-1} with 97% inhibition by 17 μM malonyl-CoA, the palmitoyltransferase activity is 0.74 nmole min^{-1} mg^{-1} with 100% inhibition by 17 μM malonyl-CoA, and the acetyltransferase activity is 2.2 nmole min^{-1} mg^{-1} with 0% inhibition by 17 μM malonyl-CoA.

The effect of palmitoyl-CoA on microsomal COT activity was also assayed radiochemically. Microsomes were incubated with 17 μM [1-^{14}C]-decanoyl-CoA and 1.7 mM L-carnitine and [1-^{14}C]-decanoylcarnitine formation quantitated using HPLC. The addition of 68 μM palmitoyl-CoA produced 99% inhibition of [1-^{14}C]-decanoylcarnitine formation (n=2, data not shown). The addition of 17 μM palmitoyl-CoA produced 72% inhibition of [1-^{14}C]-decanoylcarnitine formation as shown in Figure 4 by the (x).

Immunoprecipitation of Microsomal COT with Antiperoxisomal- COT and Antimitochondrial-CPT

The effect of antiperoxisomal-COT and antimitochondrial-CPT on microsomal COT activity is shown in Figure 5. Mouse liver peroxisomal COT purified to homogeneity was used previously to generate polyclonal antibodies (14). This antiperoxisomal-COT does not cross react with mitochondrial CPT (14). An IgG fraction of the antiperoxisomal-COT serum was purified using Protein-G chromatography and used for immunoprecipitation. Figure 5A shows the immunoprecipitation of microsomal COT activity with antiperoxisomal-COT IgG. The lower curve (▲) shows a control experiment with purified mouse liver peroxisomal COT;

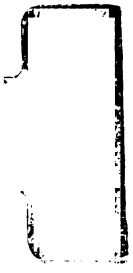
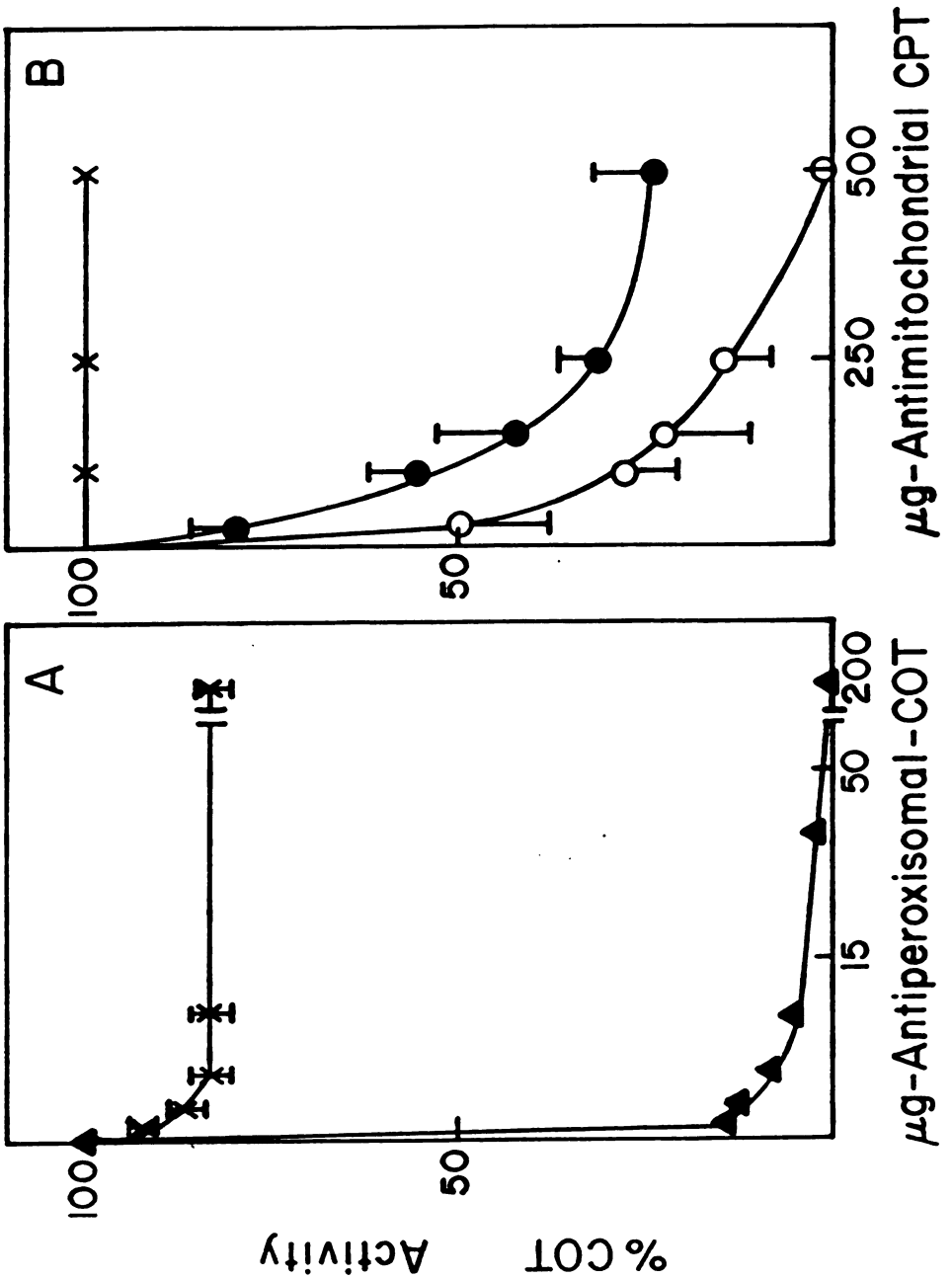


Figure 5. Immunoprecipitation of Microsomal COT with Antiperoxisomal-COT and Antimitochondrial-CPT. The microsome enriched fraction (100,000 x g pellet) and the mitochondrial enriched fraction (6,000 x g pellet) were prepared as described in Materials and Methods. They were solubilized on ice for 1 hour with 8 mM CHAPS and 1% Triton X-100 respectively, followed by centrifugation at 100,000 x g for 10 minutes. The supernatant fluids and purified peroxisomal COT were assayed spectrally for COT; 10 munits of COT were incubated with the antiperoxisomal-COT for 2 hours (Panel A) and 40 munits of COT were incubated with the antimitochondrial-CPT overnight (Panel B). The COT activity remaining in the supernatant after immunoprecipitation was determined. The x's represent COT activity remaining in the microsome enriched fraction (n=3, Panels A + B). The triangles (▲) represent the COT activity remaining in the purified peroxisomal COT (n=2, Panel A). The closed circles (•) represent the COT activity remaining in the mitochondrial enriched fraction (n=3, Panel B). The open circles (o) represent the COT activity after the total mitochondrial COT activity was corrected for antiperoxisomal-COT inhibitable activity (n=2, Panel B). Data plotted as mean ± SEM.

Figure 5.


% COT Activity
A
B
100
50
15
50
200
250
500
μg-Antiperoxisomal-COT
μg-Antimitochondrial CPT

it is completely immunoprecipitated by antiperoxisomal-COT IgG. The upper curve (x) shows the effect of antiperoxisomal-COT IgG on solubilized microsomal COT activity. Seventeen percent of the solubilized microsomal COT is immunoprecipitated by antiperoxisomal-COT IgG. Since the detergent present in solubilized microsomal COT could interfere with immunoprecipitation, a control experiment was done which showed that when equal amounts of purified peroxisomal COT and solubilized microsomal COT are mixed, 62% of the combined COT activity is immunoprecipitated by antiperoxisomal-COT. This demonstrates detergent did not interfere with immunoprecipitation. It was shown previously that Triton X-100 (0.1%) did not interfere with the immunoprecipitation of purified peroxisomal COT with antiperoxisomal-COT serum (14).

Beef heart mitochondrial CPT was purified to homogeneity and previously used to generate polyclonal antibodies (16). The antimitochondrial-CPT does not cross react with peroxisomal COT (16). Antimitochondrial-CPT serum was purified using Affiblue chromatography (16). The effect of the antimitochondrial CPT on solubilized microsomal-COT activity is shown in the upper curve (x) of Figure 5B. No microsomal COT activity is immunoprecipitated by antimitochondrial-CPT. The middle curve (•) shows the effect of antimitochondrial-CPT on the COT activity of the 6,000 x g pellet of a rat liver homogenate. This was the fraction most enriched in mitochondrial markers (see Table 1). Seventy five percent of the COT activity of the 6,000 x g pellet can be immunoprecipitated by the antiperoxisomal-COT. The lower curve (o) shows the effect of antimitochondrial-CPT on the COT activity of the 6,000 x g pellet after it was corrected for antiperoxisomal-COT inhibitable activity. All of the COT activity of the 6,000 x g pellet is immunoprecipitated by either antimitochondrial-CPT IgG or antiperoxisomal-COT IgG. The data show microsomal COT

is antigenically different than either mitochondrial CPT or peroxisomal COT.

Solubilization and Stability of Microsomal COT

The COT activity of rat liver microsomes is membrane bound and requires detergents for solubilization (37,39) and the detergent solubilized COT is not stable (37,39). The effect of detergent on microsomal COT activity is shown in Table 3. The activity is stable in 8 mM CHAPS and 1% Tween 20 although the specific activity of the solubilized COT is less than that of the membrane bound enzyme. COT activity is not stable in 0.5% Triton X-100. No CAT activity was found in the 8 mM CHAPS solubilized microsomes.

The COT activity of membranes is stable to freezing at -70°C for at least 6 months; however, a decrease in malonyl-CoA sensitivity occurs in some preparations. As shown in Figure 6, the malonyl-CoA inhibition of frozen-thawed microsomes can be increased by preincubation of frozen-thawed microsomes with 1 mM reduced glutathione. Although the COT activity of the frozen-thawed and glutathione-treated microsomes apparently increased compared to control values, this difference was not statistically significant at $p < 0.05$. Addition of 100 mM KCl to freshly isolated microsomes doubles the amount of COT activity, as well as the amount of malonyl-CoA inhibitable COT (n=4; data not shown). The cause for the effect of KCl has not been determined.

[^{3}H]-Etomoxir Labeling of Rat Liver Microsomal Proteins

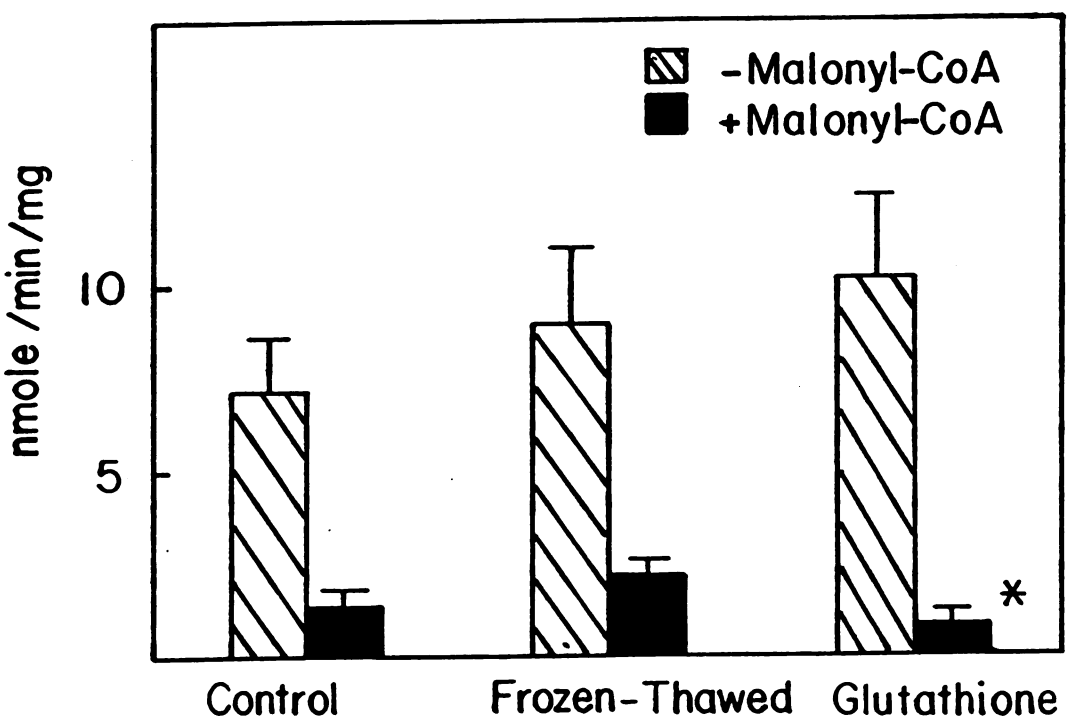
Etomoxir is a clorophenoxy-epoxy containing fatty acid derivative that is conjugated *in vivo* to Coenzyme A similar to tetradecylglycidic acid (TDGA). [^{3}H]-TDGA-CoA produces

Table 3. Detergent Solubilization of Microsomal COT. Microsomes were prepared by differential centrifugation and stored frozen prior to solubilization. Microsomes were thawed at room temperature and assayed for COT; initial COT activity of thawed microsomes 11.1 ± 1.4 nmole min^{-1} mg^{-1} protein. Detergent was added as indicated and samples were kept on ice for 1 hour prior to centrifugation at 100,000 x g for 1 hour at 4°C in a Beckman ultracentrifuge. The 100,000 x g supernatant fluids were assayed for COT activity and protein concentration.

	n	Microsomal COT nmole min^{-1} mg^{-1}
8 mM CHAPS	3	3.5 ± 0.61
0.5% Triton X-100	3	1.7 ± 0.26
1% Tween-20	2	2.5 ± 0.98

Figure 6. Effect of Reduced Glutathione on the Activity and Malonyl-CoA Sensitivity of Microsomal COT after Freeze-Thawing. Microsomes were prepared using differential centrifugation and COT was assayed spectrally as described in Materials and Methods. Malonyl-CoA was 17 μM. Control microsomes were assayed immediately following preparation. Frozen-thawed microsomes were stored frozen for a minimum of one week at -70°C and then thawed at room temperature prior to assay. Glutathione microsomes were frozen-thawed microsomes that were made 1 mM in reduced glutathione and then incubated on ice for 2 hours prior to assay. The data are plotted as the mean of four experiments ± SEM. Statistical significance was determined using a One Way ANOVA with Randomized Blocks Design and a Post Hoc Tukey's Test. *Significantly different from frozen-thawed at $p < 0.05$.

Figure 6.


-Malonyl-CoA
+Malonyl-CoA
nmole /min/mg
10
5
*
Control
Frozen-Thawed
Glutathione

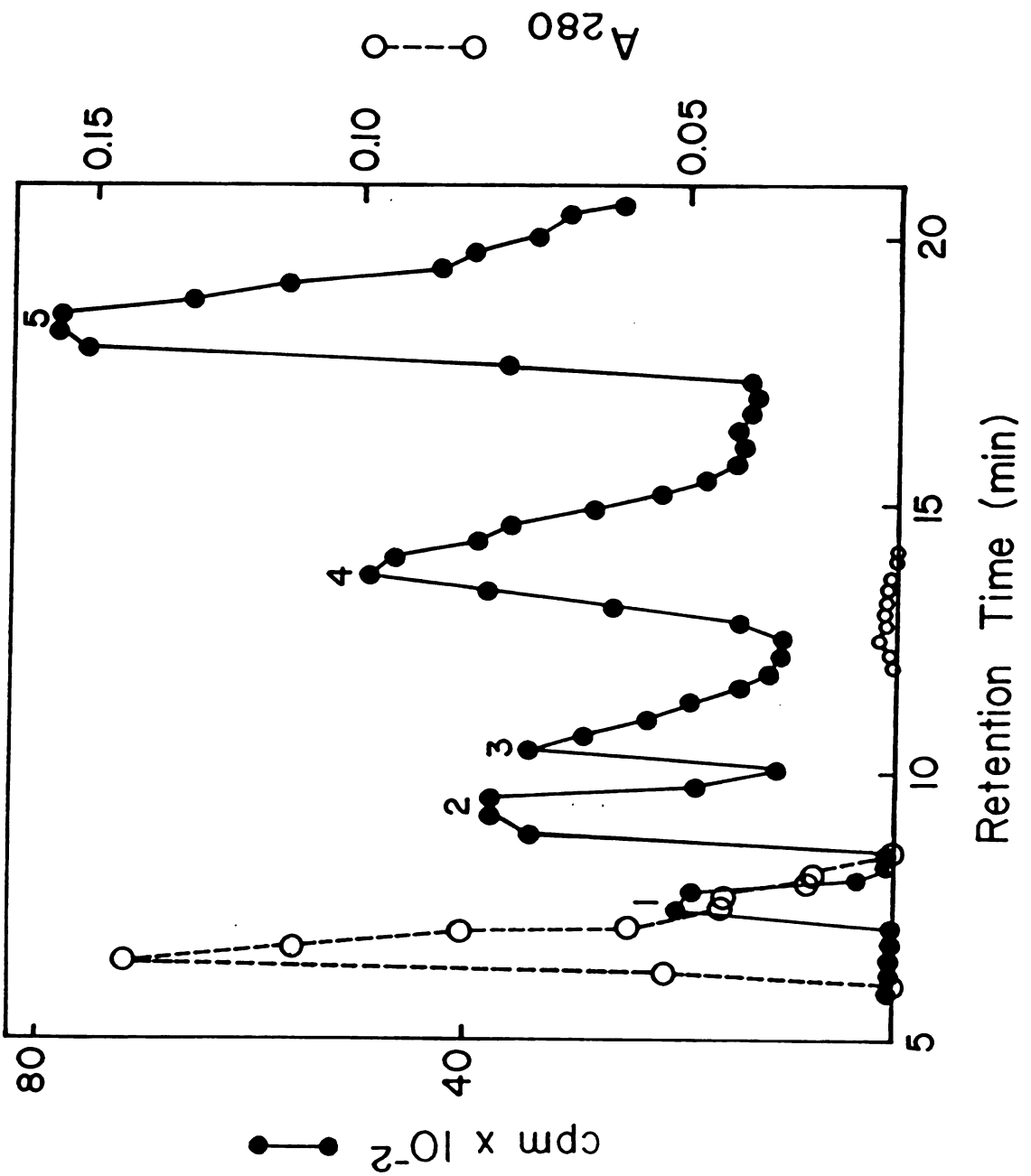
a covalent adduct with an ~94,000 dalton mitochondrial protein in liver that is thought to be involved in the inhibition of CPT_o by malonyl-CoA (22,23). [^{3}H]-Etomoxir was incubated with rat liver microsomal proteins in the presence of ATP-Mg^{++} and CoASH to generate [^{3}H]-etomoxiryl-CoA to determine if specific proteins were labeled. The [^{3}H]-etomoxir labeled proteins were solubilized in 1% SDS. An HPLC gel filtration separation of the solubilized [^{3}H]-etomoxir labeled microsomal proteins is shown in Figure 7. There was a large incorporation of radioactivity into the detergent solubilized protein. Five peaks of radioactivity were found, of which only peak one coincided with protein. All of the radioactivity in peaks 2-5 could be extracted by chloroform/methanol (3/2, v/v).

When the detergent solubilized [^{3}H]-etomoxir labeled proteins were extracted by hexane/isopropanol or separated by SDS-PAGE, <2% of the total radioactivity was protein bound. Figure 8 shows the separation of [^{3}H]-etomoxir labeled proteins on by SDS-PAGE. There were two peaks of labeled protein in microsomes with molecular weights of ~51-57,000 daltons and ~87,000 daltons. The major protein labeled in liver microsomes is ~51-57,000 daltons and incorporation of label into this peak is decreased ~60% by a chase incubation with unlabeled etomoxiryl-CoA as shown by the (o) in Figure 8A. There is also a minor protein labeled in microsomes with a molecular weight of ~ 87,000 daltons and the incorporation of radioactivity into this peak was not decreased by a chase incubation with etomoxiryl-CoA.

The effect of pretreatment with malonyl-CoA on the [^{3}H]-etomoxir labeled proteins is shown in Figure 8B. Incorporation of radioactivity into the ~51,000 dalton protein was only slightly decreased by malonyl-CoA pretreatment. In contrast, incorporation of radioactivity into the ~87,000 dalton protein was decreased ~80% by the malonyl-CoA pretreatment.

Figure 7. HPLC Gel Filtration of [^{3}H]-Etomoxir labeled Microsomal Proteins. Rat liver microsomes were incubated for 15 minutes with 5 μM [^{3}H]-etomoxir, ATP-Mg^{++}, and CoASH as described in Materials and Methods. The labeled proteins were separated isocratically on a Dupont G250 gel filtration HPLC column in 100 mM Tris-Cl, pH 6.8, containing 10% glycerol and 0.5% SDS at a flow rate of 1 ml min^{-1}. Three hundred μl fractions were collected beginning at 5.6 minutes. The radioactivity in each fraction was determined by scintillation counting and is plotted as closed circles (•). The counting efficiency for [^{3}H] is 66%. The open circles (o) are the absorbance at 280 nm of each fraction.

Figure 7.


A280
0.15
0.10
0.05
cpm x 10-2
80
40
5
10
15
20
Retention Time (min)
1
2
3
4
5

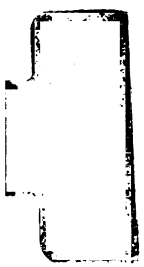
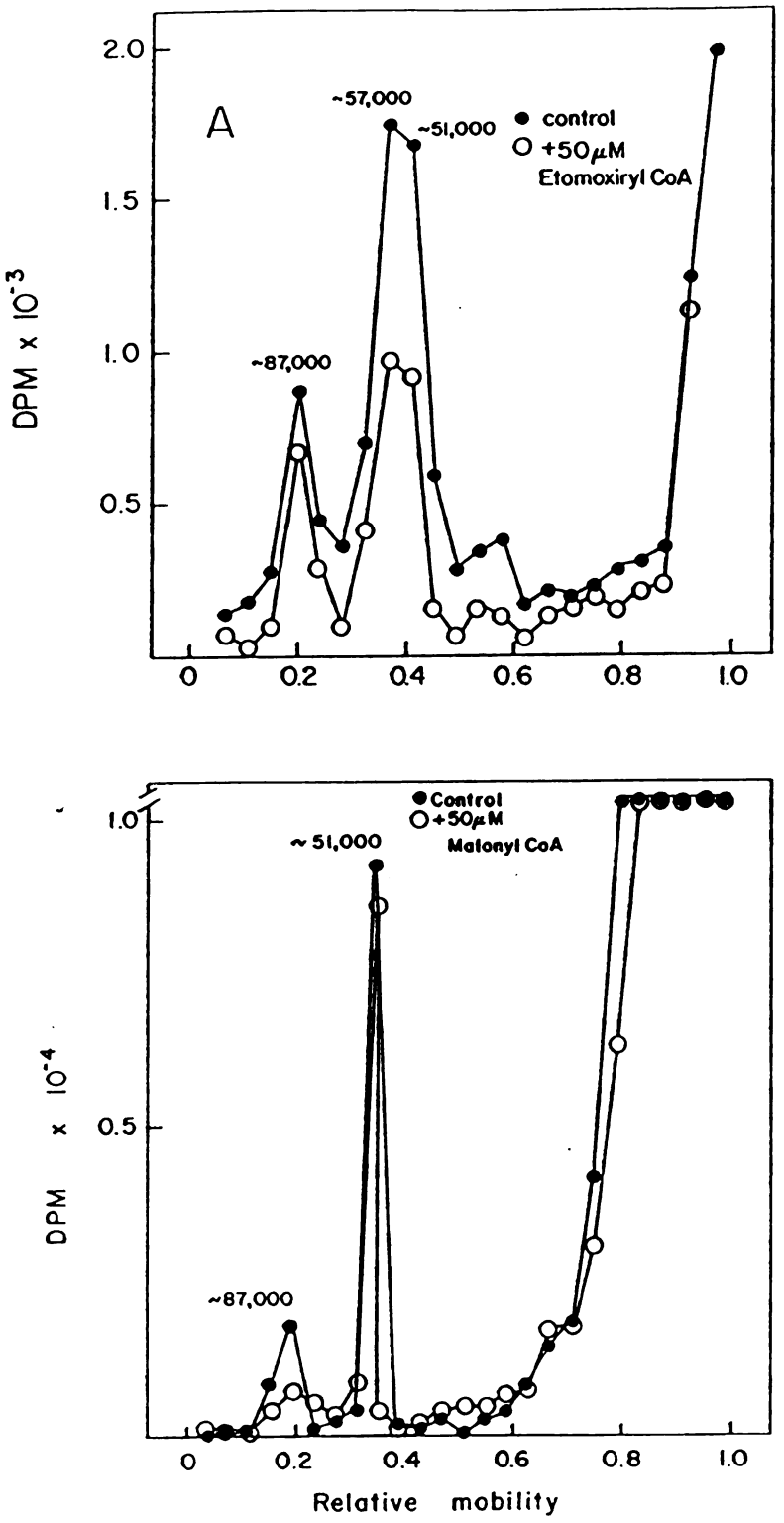


Figure 8. SDS-PAGE of Microsomal Proteins After Incubation with [^{3}H]-Etomoxir. Rat liver microsomes prepared by differential centrifugation were incubated for 15 minutes with 5 μM [^{3}H]-etomoxir, ATP-Mg^{++}, and CoASH as described in Materials and Methods, and the proteins separated by SDS-PAGE. The gel was sliced into 0.2 cm pieces and the radioactivity (•) plotted versus the relative mobility. Molecular weight markers were run on the same gel and silver stained. Panel A shows the effect of a 5 minute chase incubation with unlabeled 50 μM etomoxiryl-CoA (o). Panel B shows the effect of a 5 minute preincubation with 50 μM malonyl-CoA (o). The microsomes sample in Panel A was extracted with hexane/isopropanol and chloroform/methanol prior to SDS-PAGE. The microsome sample in Panel B was not extracted. The molecular weight range of the labeled proteins from four separate gels was 87,500 ± 986, and 57,00 ± 577 and 51,600 ± 1,250.

Figure 8.


A
~57,000
~51,000
~87,000
control
+50μM
Etomoxiryl CoA
DPM x 10⁻³
2.0
1.5
1.0
0.5
0
0.2
0.4
0.6
0.8
1.0
Control
+50μM
Malonyl CoA
~ 51,000
~87,000
DPM x 10⁻⁴
1.0
0.5
Relative mobility

DISCUSSION

Localization of Malonyl-CoA Sensitive COT

The data presented herein show that rat liver contains more than one malonyl-CoA sensitive medium-chain/long-chain carnitine acyltransferase. In addition to the malonyl-CoA sensitive CPT_o associated with mitochondria, marker enzyme distribution studies show that there is a malonyl-CoA sensitive COT associated with microsomes. This enzyme has been tentatively designated a medium-chain transferase (COT) because both microsomes and a partially purified preparation show a higher activity with decanoyl-CoA than with palmitoyl-CoA as substrate. Recent studies indicate the COT activity of intact rat liver peroxisomes is also inhibitable by malonyl-CoA (40). If so, all of the carnitine acyltransferases of liver that exhibit medium-chain and long-chain carnitine acyltransferase activity and which are in contact with cytosolic pools of acyl-CoAs can be inhibited by malonyl-CoA.

Surprisingly, the activity in the 100,000 x g pellet, the fraction enriched in microsomes, is more inhibited by 17 μM malonyl-CoA than the activity in the 6,000 x g fraction which contains primarily CPT_o and peroxisomal COT. For the experiment shown in Table I, COT in the microsomal enriched fraction was 77% inhibited by malonyl-CoA, while the mitochondrial enriched fraction (6,000 x g pellet) was only 37% inhibited. This degree of inhibition is less than that shown for purer mitochondrial preparations (42); it seems likely that the low percent inhibition is due to the presence of solubilized peroxisomal COT and also to damaged mitochondria, thereby exposing CPT_i (CPT-II). The medium-chain and long-chain

carnitine acyltransferase activities of microsomes are both inhibited by malonyl-CoA and palmitoyl-CoA, suggesting these activities result from a single enzyme. Microsomal CAT activity is not inhibited by malonyl-CoA, which suggests it is due to a different enzyme as has been proposed (39).

The potential contribution of mitochondria and peroxisomes to the microsome enriched fractions used for these studies has been determined. The specific activity of the malonyl-CoA sensitive microsomal COT (see Table II) is comparable to the specific activity of malonyl-CoA sensitive COT in intact density gradient purified peroxisomes (9.32 munits/mg) (40), while the percent recovery of urate oxidase in the microsome enriched fraction is < 10% (see Table II). Thus, the COT in our microsome preparations could not result entirely from contamination of the 100,000 x g pellet with peroxisomes. This conclusion is confirmed by the finding that only 17% of the COT activity of microsomes is immunoprecipitated by anti-peroxisomal COT using conditions that completely inhibit peroxisomal COT (see Fig. 5). Recent reports indicate CPT_o is associated with the outer mitochondrial membrane (11,29,32). Contamination of microsomes by outer membrane fragments seems plausible, so the percent recovery in the microsome enriched fraction of both cytochrome c oxidase, an inner mitochondrial membrane marker, and monoamine oxidase, an outer mitochondrial membrane marker, was determined. They both represent < 3.0% of the total marker activity (see Table I). Anti-CPT that inactivates both CPT_o and CPT_i of mitochondria (16), and that reacts to a single peptide on Western blots of rat liver mitochondria (see Fig. 5 of ref. 89) had no detectable effect on microsomal COT (see Fig. 5B). Thus, the microsome enriched fractions used for these studies were not significantly contaminated with either peroxisomes, or mitochondrial inner or outer membranes.

The microsomal COT has properties similar to the malonyl-CoA sensitive CPT that

occurs in preparations enriched with outer mitochondrial membranes of liver. These include a tight association with the membrane and a high degree of malonyl-CoA sensitivity; some of our rough and smooth endoplasmic reticulum preparations were completely inhibited by 17 μM malonyl-CoA, such as the preparation shown on Fig. 1. The malonyl-CoA sensitivity of outer mitochondrial membranes and of our preparations is quite labile, sensitive to some detergents, and the I_{50}'s are low micromolar. The microsomal COT is quite stable in CHAPS and Tween-20, but the activity is rapidly lost in Triton X-100 (data not shown). However, the titration curves for malonyl-CoA inhibition of COT are different for liver microsomes (see Fig. 2) than those shown for rat liver mitochondria (65,66,71). Abrupt breaks in the titration curve for mitochondria were not found. Microsomal COT is strongly inhibited by concentrations of palmitoyl-CoA > 11 μM. The inhibition curve is almost identical to the one reported for inhibition of the CPT activity of the outer membrane enriched preparations reported by Murthy and Pande (29); compare Figure 3 to Figure 2 of Murthy and Pande (29). In contrast, the velocity versus [palmitoyl-CoA] curves for CPT_o of intact rat liver mitochondria appear sigmoid (see Fig. 1 of Saggerson *et al.* (66), Fig. 1 of Cook *et al.* (32), and Fig. 2 of Grantham and Zammit (65). The inhibition of microsomal COT by increasing concentrations of palmitoyl-CoA provides a method for differentiating CPT_o of rat liver mitochondria from microsomal COT.

Etomoxiryl-CoA inhibits CPT_o of intact rat liver mitochondria with an I_{50} value of approximately 3 nm (22). The I_{50} of membrane bound microsomal COT for etomoxiryl-CoA is approximately 600 nm (see Figure 2). There is a 200-fold difference in the etomoxiryl-CoA I_{50} of membrane bound microsomal COT and mitochondrial CPT_o. Octyl glucoside solubilized CPT activity in outer mitochondrial membrane enriched vesicles (OMV CPT) is inhibited 41%

by 0.2 μM etomoxiryl-CoA (88). The I_{50} of detergent solubilized microsomal COT is approximately 1.5 μM (see Figure 3). The concentration of etomoxiryl-CoA required to inhibit OMV CPT and microsomal COT is similar. [^{3}H]-Etomoxir also labels proteins with similar molecular weights in outer mitochondrial membrane enriched vesicles and in microsomes. In microsomes [^{3}H]-etomoxir forms a covalent adduct to a major protein with a molecular weight of approximately 51-57,000 daltons and to a minor protein with a molecular weight of 87,000 daltons (see Figure 8). In octyl glucoside solubilized OMV CPT eluted from a hydroxyapatite column [^{3}H]-etomoxir forms a covalent adduct to a major protein with a molecular weight of approximately 90,000 daltons and to a minor protein with a molecular weight of approximately 45,000 daltons (88).

The microsomal COT has properties similar to those reported for the malonyl-CoA sensitive medium-chain carnitine acyltransferase of intact peroxisomes. In addition to comparable specific activities, the concentration of malonyl-CoA required to inhibit 50% of COT activity is 5.3 ± 0.43 μM in microsomes (see Fig. 2) and is 2.2 μM in intact peroxisomes (40). Although both enzymes show a higher V_{max} with C10-CoA than C16-CoA, the ratio of C10\C16 activity for the microsomal COT is approximately 10 and the ratio for the medium-chain transferase of intact peroxisomes is 2.1 (40). The data in Table I show that the COT activity of microsomes represents at least 15% of the total COT activity of the liver homogenate. The COT activity of intact peroxisomes is approximately 20% of the COT activity of the liver homogenate (40).

While the microsomal COT and the peroxisomal COT show similar substrate specificities and kinetic properties, the data in Fig. 5 clearly show that the microsomal COT is antigenically different than purified peroxisomal COT. The solubilization characteristics of

microsomal-bound COT and the COT activity of intact peroxisomes are strikingly different. Sonication releases at least 70% of the COT activity of intact peroxisomes (40). Previously, it was shown that freeze/thaw treatment of peroxisomes completely releases peroxisomal COT (34,39), while freeze/thaw treatment of microsomes releases < 10% of microsomal COT activity (39). While peroxisomal COT is a soluble, matrix enzyme, microsomal COT is firmly membrane-bound, requiring detergents for solubilization.

The mechanism of etomoxiryl-CoA and malonyl-CoA inhibition of microsomal COT seems to be different. Etomoxiryl-CoA inhibition is time dependent while malonyl-CoA inhibition does not require preincubation. Membrane bound microsomal COT is inhibited by malonyl-CoA and etomoxiryl-CoA (see Figure 2) while detergent (CHAPS) solubilized microsomal COT retains inhibition by etomoxiryl-CoA but is not inhibited by up to 200 μM malonyl-CoA (see Figure 3). It has been proposed that malonyl-CoA is a reversible inhibitor of CPT and etomoxiryl-CoA is an irreversible, active site directed inhibitor of CPT (22,23) , but the etomoxiryl-CoA inhibition of microsomal COT is reversed with detergent solubilization and desalting the solubilized supernatant. It has also been reported that the etomoxiryl-CoA inhibition of rat liver mitochondrial CPT can be reversed with dialysis (90). The reversibility of etomoxiryl-CoA inhibition of microsomal COT by detergent solubilization could indicate that a regulatory protein is separated from a catalytic protein.

There is no direct evidence that the [^{3}H]-etomoxir labeled proteins are microsomal COT or that they are involved in the etomoxiryl-CoA inhibition of microsomal COT. The reversal of etomoxiryl-CoA inhibition of microsomal COT with detergent solubilization suggests that etomoxiryl-CoA is not forming a covalent adduct with microsomal COT and that the [^{3}H]-etomoxir labeled proteins are not microsomal COT. The incorporation of label into

the approximately 87,000 dalton molecular weight microsomal protein is decreased by preincubation with malonyl-CoA suggesting that this protein could be involved in the malonyl-CoA inhibition of microsomal COT. The labeling in the major microsomal protein with a molecular weight of approximately 51-57,000 daltons is not decreased by preincubation and is decreased by a chase incubation with unlabeled etomoxiryl-CoA indicating it may have other functions unrelated to microsomal COT activity..

Chapter 3. Kinetic Characterization of Membrane Bound Microsomal COT

Rat liver endoplasmic reticulum contains malonyl-CoA sensitive medium/long-chain carnitine acyltransferase activity (microsomal COT). The kinetic constants for microsomal COT determined using a radiochemical assay show the $K_{0.5}$ for L-carnitine is 0.42 ± 0.04 mM and the $K_{0.5}$ for decanoyl-CoA is 1.9 ± 0.1 μM. Microsomal COT exhibits Michaelis-Menten kinetics; Hill coefficients are 0.91 ± 0.03 for decanoyl-CoA as varied substrate and 0.96 ± 0.12 for L-carnitine as varied substrate. Microsomal COT is inhibited by DL-aminocarnitine. The concentration of DL-aminocarnitine required for 50% inhibition of malonyl-CoA sensitive COT is 0.5 ± 0.12 mM. DL-Aminocarnitine inhibits microsomal COT competitively with respect to L-carnitine with a K_i of 40 μM. Decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine also inhibit microsomal COT. The concentrations of decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine required for 50% inhibition of malonyl-CoA sensitive microsomal COT are 6.8 ± 1.1 μM and 4.3 ± 1.0 μM, respectively. The results show that the DL-aminocarnitine inhibition of malonyl-CoA sensitive medium/long-chain carnitine acyltransferase activity of rat liver endoplasmic reticulum is similar to the L-aminocarnitine inhibition of the malonyl-CoA sensitive medium/long-chain carnitine acyltransferase activity of outer mitochondrial membrane enriched vesicles of rat liver. The kinetic characteristics of microsomal COT can be used to distinguish it from mitochondrial CPT and peroxisomal COT.

Introduction

Medium/long-chain carnitine acyltransferase activity in rat liver is associated with mitochondria, peroxisomes, and endoplasmic reticulum. The medium/long-chain carnitine acyltransferase activity associated with rat liver endoplasmic reticulum (microsomal COT) is strongly inhibited by malonyl-CoA (see Chapter 2), like the well characterized inhibition of the outer mitochondrial medium/long-chain carnitine acyltransferase (mitochondrial CPT_o). (30,71,72,85). Medium/long-chain carnitine acyltransferase activity is also located on the inner side of the inner mitochondrial membrane (mitochondrial CPT_i); mitochondrial CPT_i is not sensitive to inhibition by malonyl-CoA (71,72). It is not known if CPT_o and CPT_i are the same protein with different locations and regulatory properties or if they are distinct proteins (19). Mitochondrial CPT_o has been reported to be located both on the outer side of the inner mitochondrial membrane (25-28) and on the inner side of the outer mitochondrial membrane (11,29-32). Peroxisomal medium/long-chain carnitine acyltransferase activity is associated with the matrix (peroxisomal COT) (14,33-35).

The kinetic characteristics of membrane bound microsomal COT were determined for comparison with the known kinetic properties of mitochondrial CPT_o and CPT_i and with peroxisomal COT. Mitochondrial enriched fractions of rat liver homogenates prepared using differential centrifugation can be contaminated by significant quantities of peroxisomes and endoplasmic reticulum (35,58,59,61). The kinetic properties of membrane bound microsomal COT could be useful in determining the fraction of medium/long-chain carnitine acyltransferase

activity associated with rat liver mitochondria that is due to microsomal contamination.

Aminocarnitine (3-amino-4-trimethylaminobutyric acid) is a noncovalent inhibitor of carnitine acyltransferases (67-70). Aminocarnitine has been proposed as a antiketogenic agent which could be useful in the treatment of ketoacidosis associated with diabetes mellitus (67-69). The aminocarnitine inhibition of both mitochondrial and peroxisomal medium/long-chain carnitine acyltransferase activities in rat liver has been studied (67-70) but the aminocarnitine inhibition of rat liver microsomal medium/long-chain carnitine acyltransferase activity has not been characterized. Herein the DL-aminocarnitine inhibition of microsomal COT and the inhibition of microsomal COT by decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine were determined. The DL-aminocarnitine inhibition of microsomal COT is similar to the L-aminocarnitine inhibition reported for the CPT_o activity of outer mitochondrial membrane enriched vesicles.

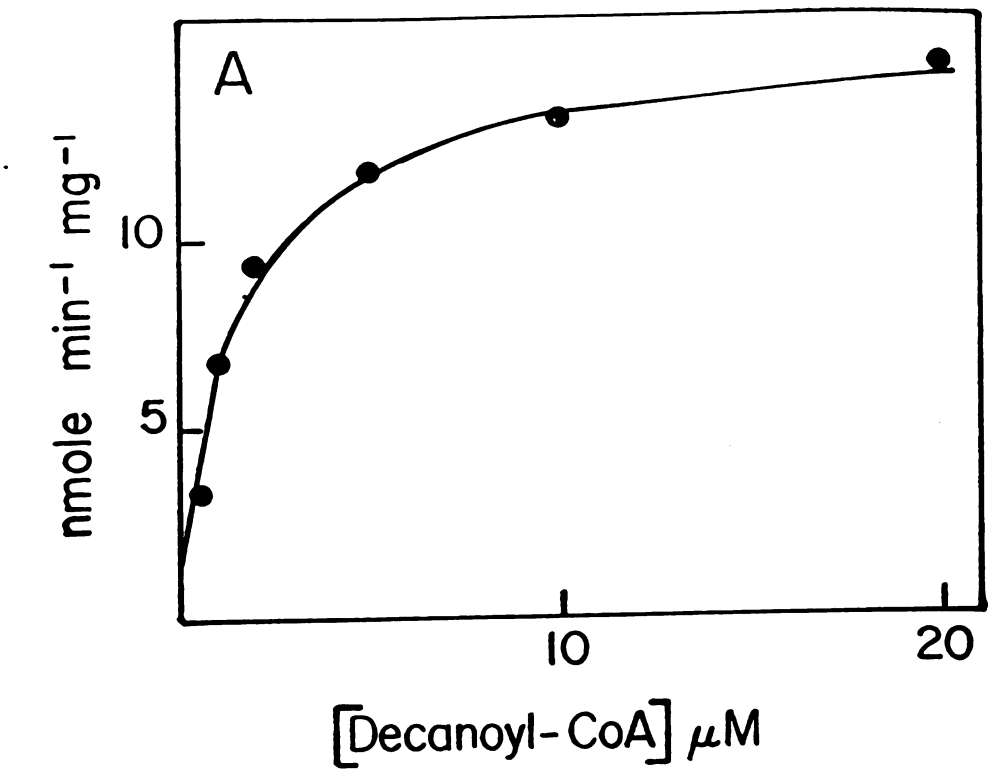
Results

Determination of Kinetic Constants

Mitochondrial CPT, microsomal COT, and peroxisomal COT contribute to the cytosolic production of medium-chain and long-chain acylcarnitines in liver. The kinetic properties of purified mitochondrial CPT and purified peroxisomal COT have been studied. The kinetic properties of membrane-bound microsomal COT were determined for comparison. A velocity *versus* decanoyl-CoA concentration plot for membrane bound microsomal COT is shown in Figure 1A and a velocity *versus* L-carnitine concentration plot is shown in Figure 2A. The velocity *versus* substrate concentration plots for microsomal COT are hyperbolic; Hill coefficients determined from the slope of a Hill plot are 0.91 ± 0.03 for decanoyl-CoA as varied substrate and 0.96 ± 0.12 for L-carnitine as varied substrate. The double reciprocal plot with decanoyl-CoA as the varied substrate is shown in Figure 1B and for L-carnitine as the varied substrate in Figure 2B. The double-reciprocal plots were linear indicating microsomal COT follows Michaelis-Menten kinetics. Kinetic constants calculated from the equation for the line show $K_{0.5}$ for decanoyl-CoA is 1.9 ± 0.1 μM and V_{max} is 16.3 ± 1.1 nmole min^{-1} mg^{-1} protein while the $K_{0.5}$ for L-carnitine is 0.42 ± 0.04 mM and the V_{max} is 10.7 ± 0.8 nmole min^{-1} mg^{-1}.

Figure 1. Velocity *versus* Decanoyl-CoA Concentration Curve and Double-Reciprocal Plot for Microsomal COT. Microsomal COT activity was determined in the presence of increasing decanoyl-CoA concentrations. Velocity *versus* decanoyl-CoA concentration plot is shown in Panel A. Double-reciprocal plot is shown in Panel B; the line was fitted by least-squares regression with correlation coefficient (r) = 0.989. Kinetic constants calculated from the equation for the line show $K_{0.5}$ = 1.9 ± 0.1 μM and V_{max} = 16.3 ± 1.1 nmole min^{-1} mg^{-1} protein. Data plotted as mean of three experiments.

Figure 1.


A
nmole min⁻¹ mg⁻¹
10
5
10
20
[Decanoyl-CoA] μM

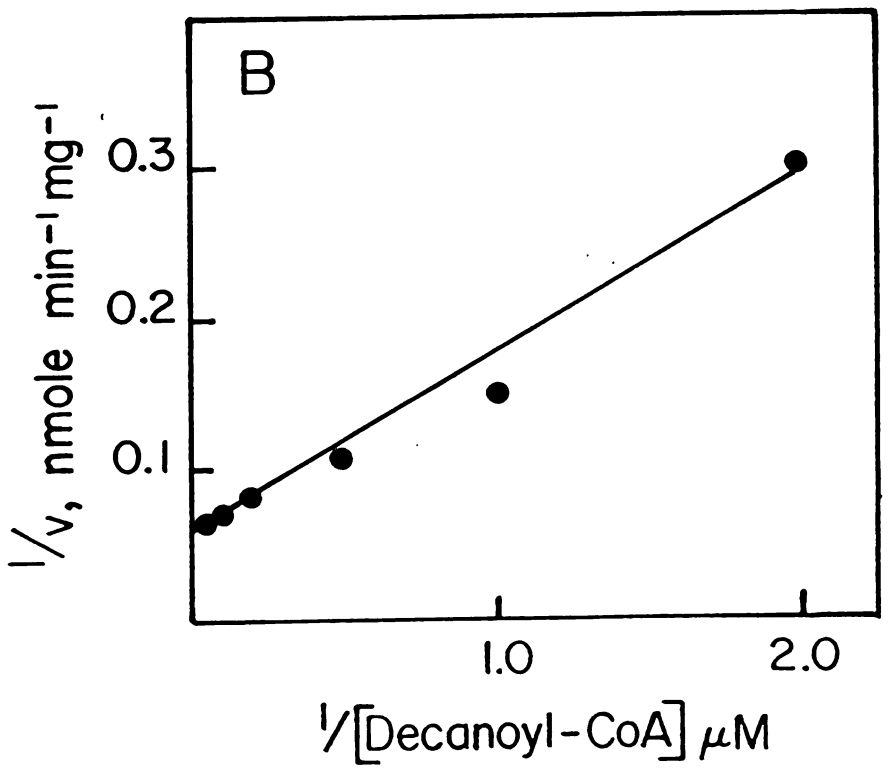
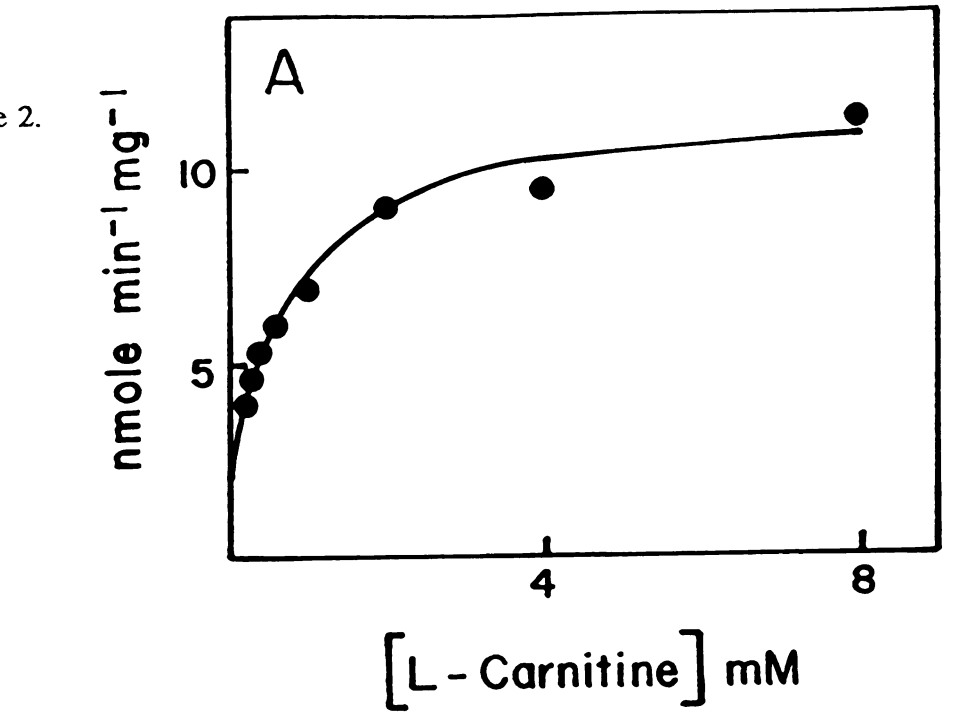
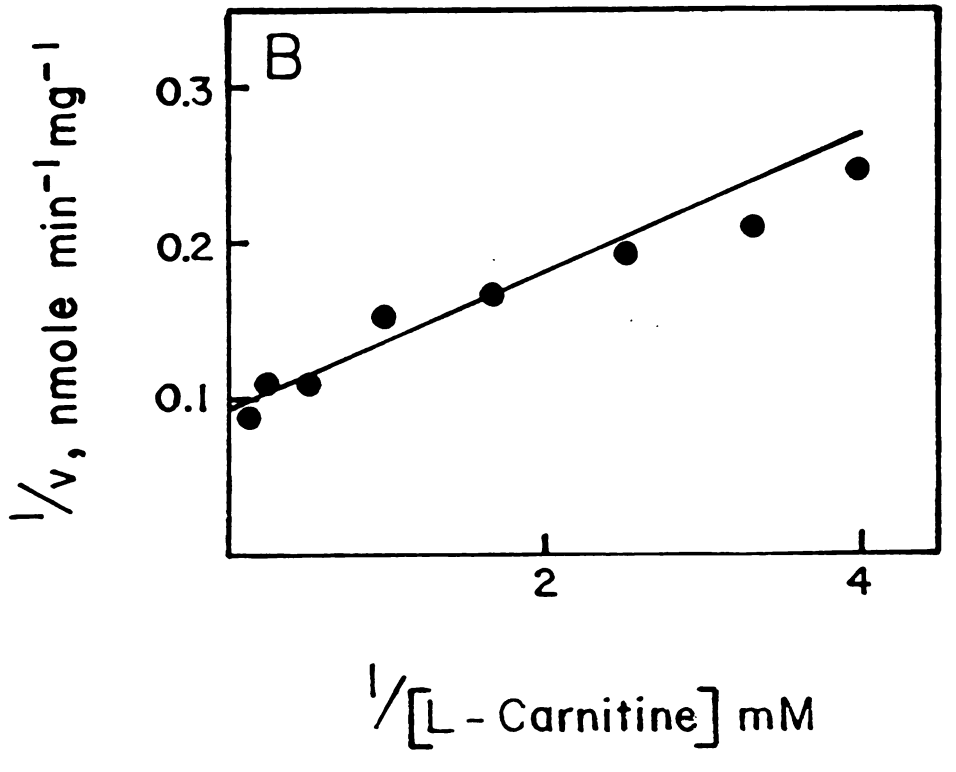

B
1/v, nmole min⁻¹ mg⁻¹
0.3
0.2
0.1
1.0
2.0
1/[Decanoyl-CoA] μM

Figure 2. Velocity *versus* L-Carnitine Concentration Curve and Double-Reciprocal Plot for Microsomal COT. Microsomal COT activity was determined in the presence of increasing L-carnitine concentrations. Velocity *versus* decanoyl-CoA concentration plot is shown in Panel A. Double-reciprocal plot is shown in Panel B; the line was fitted by least-squares regression with correlation coefficient (r) = 0.985. Kinetic constants calculated from the equation for the line show $K_{0.5} = 0.42 \pm 0.04$ mM and $V_{max} = 10.8 \pm 0.8$ nmole min^{-1} mg^{-1} protein. Data plotted as mean of two experiments.

Figure 2.


A
nmole min⁻¹ mg⁻¹
10
5
4
8
[L - Carnitine] mM


B
1/v, nmole min⁻¹ mg⁻¹
0.3
0.2
0.1
2
4
1/[L - Carnitine] mM

Effect of pH on Microsomal COT Activity

The effect of pH on microsomal COT activity is shown in Figure 3. The pH optimum for membrane bound microsomal COT assayed in the forward direction is 8.5.

DL-Aminocarnitine Inhibition of Microsomal COT

The effect of DL-aminocarnitine concentration on microsomal COT activity is shown in Figure 4. Membrane bound microsomal COT is completely inhibited by DL-aminocarnitine. The concentration of DL-aminocarnitine required to inhibit 50% of microsomal COT is 0.5 ± 0.12 mM.

The effect of DL-aminocarnitine on the kinetic parameters of microsomal COT with L-carnitine as variable substrate is shown in Figure 5. DL-Aminocarnitine is a competitive inhibitor with respect to L-carnitine. The K_i for DL-aminocarnitine is 40 μM calculated from a replot of $K_{m\,app}$ versus [DL-aminocarnitine]. DL-aminocarnitine is a substrate of microsomal COT with decanoyl-CoA as cosubstrate. Microsomal COT activity with 10 mM DL-aminocarnitine is 0.12 nmole min^{-1} mg^{-1} protein.

Decanoyl-DL-Aminocarnitine and Palmitoyl-DL-Aminocarnitine Inhibition of Microsomal COT

The effect of decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine concentration of microsomal COT activity is shown in Figure 6. Decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine inhibit microsomal COT. The concentrations of decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine required to inhibit 50% of microsomal COT activity are 6.8 ± 1.1 μM and 4.3 ± 1.0 μM, respectively.

Figure 3. The pH Optimum of Microsomal COT. Microsomal COT was assayed with 6 mM L-carnitine and 16 μM [1-^{14}C]-decanoyl-CoA in 50 mM potassium phosphate at the pH values indicated. The pH optimum for membrane bound microsomal COT assayed in the forward direction is 8.5. Data plotted as the mean of two experiments ± SEM.

Figure 3.

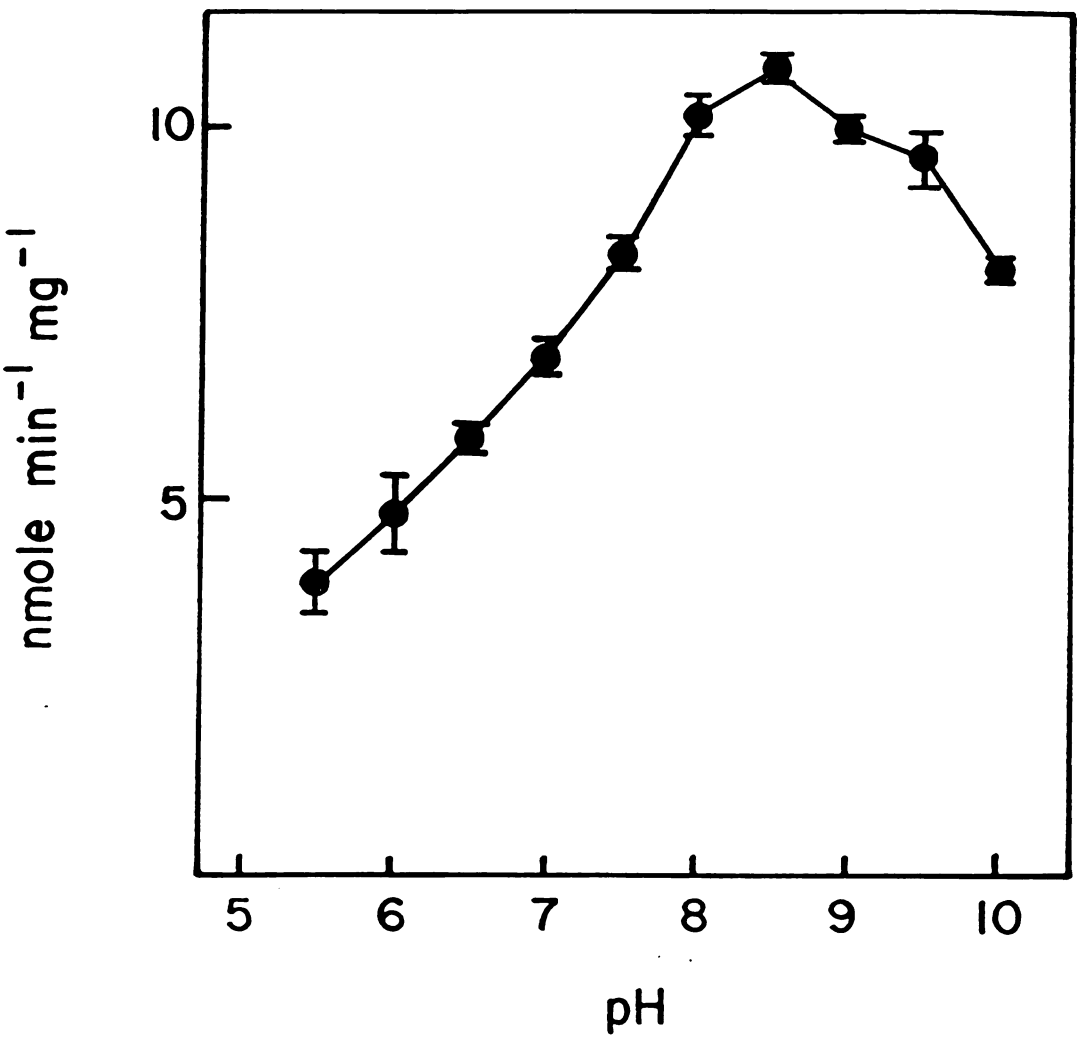
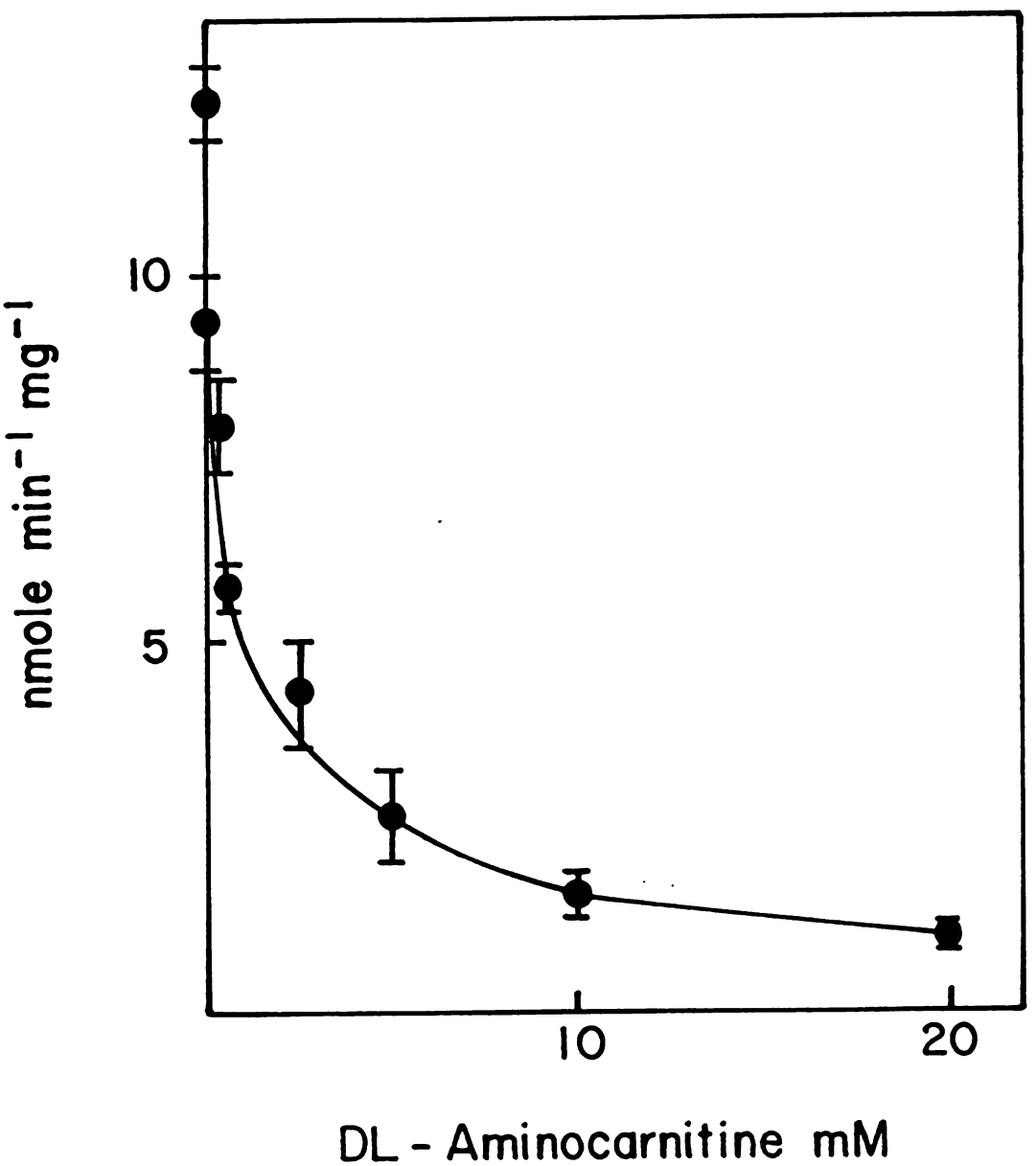


Figure 4. DL-Aminocarnitine Inhibition of Microsomal COT. Microsomal COT activity was determined in the presence of increasing concentrations of DL-aminocarnitine as indicated. The concentration of DL-aminocarnitine required to inhibit 50% of microsomal COT is 0.5 ± 0.12 mM. Data plotted as the mean of two experiments ± SEM.

Figure 4.



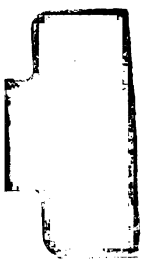


Figure 5. Effect of DL-Aminocarnitine on the Kinetic Parameters of Microsomal COT. Microsomal COT was assayed with increasing concentrations of L-carnitine in the presence of the indicated concentrations of DL-aminocarnitine. A double-reciprocal plot shows DL-aminocarnitine is a competitive inhibitor of microsomal COT with respect to L-carnitine. A replot of $K_{0.5app}$ *versus* [DL-aminocarnitine] gives a K_i = 40 μM. Data plotted as the mean of two experiments.

Figure 5.

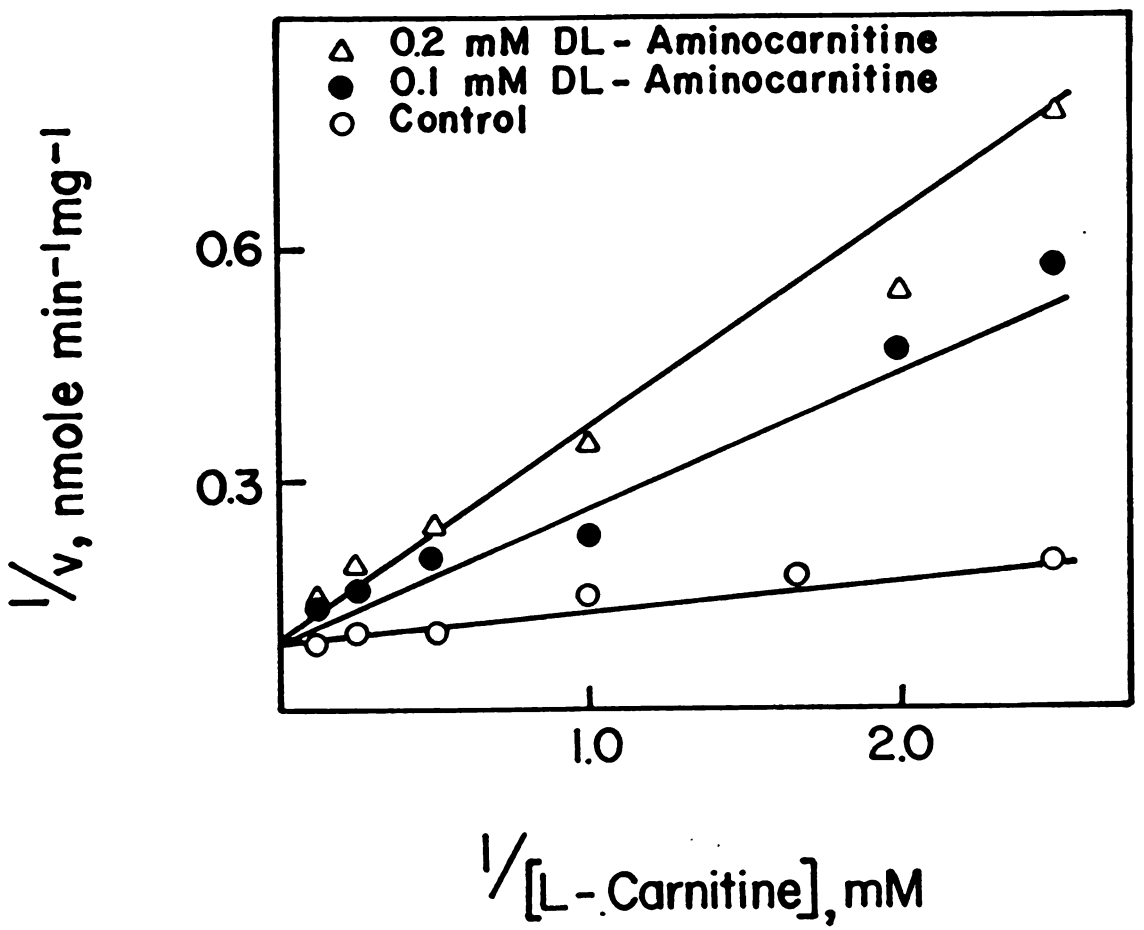
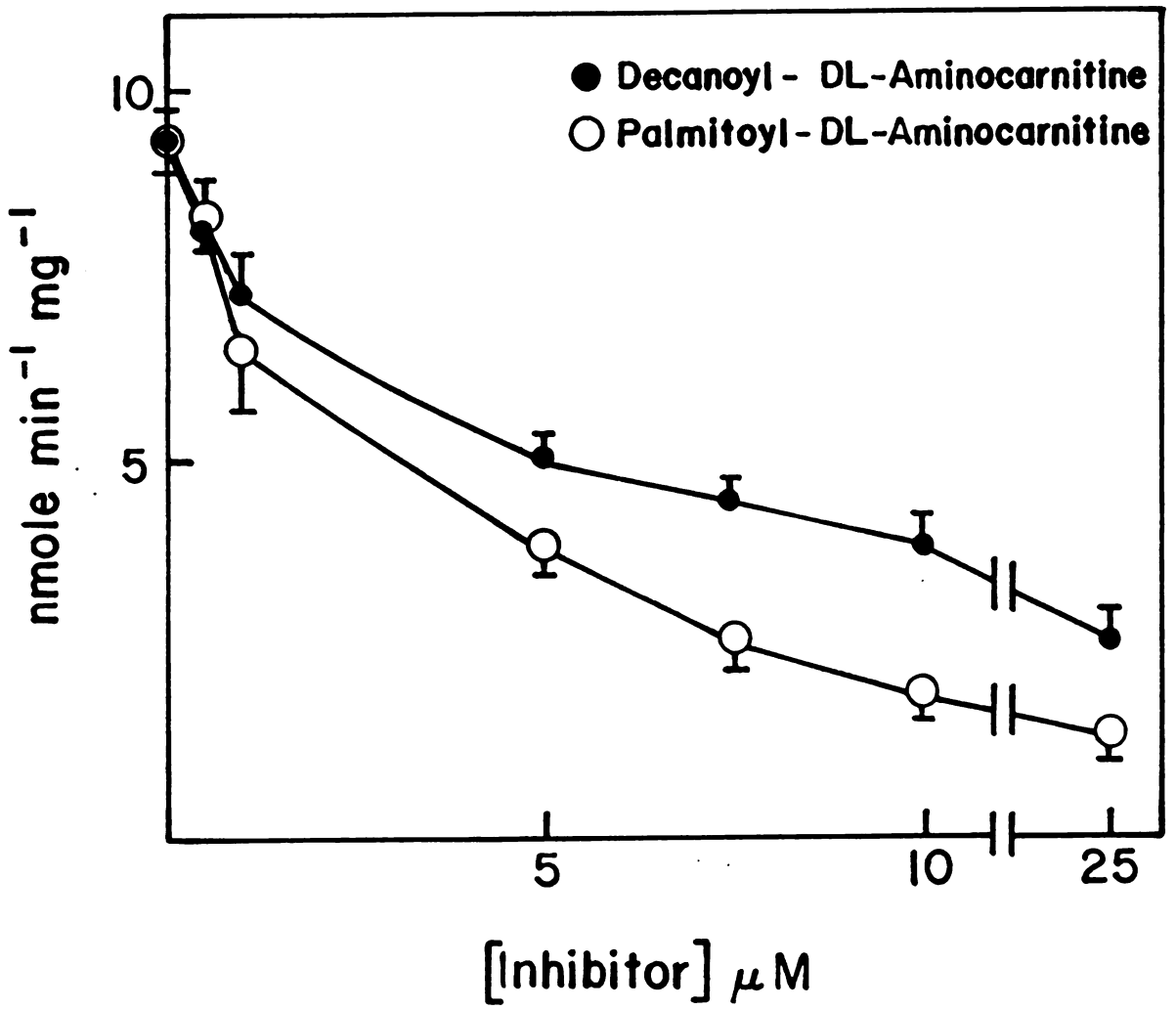

0.2 mM DL - Aminocarnitine
0.1 mM DL - Aminocarnitine
Control
0.6
0.3
1.0
2.0
1/v, nmole min⁻¹mg⁻¹
1/[L-Carnitine], mM

Figure 6. Decanoyl-DL-Aminocarnitine and Palmitoyl-DL-Aminocarnitine Inhibition of Microsomal COT. Microsomal COT activity was determined in the presence of increasing concentrations of decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine as indicated. The concentrations of decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine required to inhibit 50% of microsomal COT are 6.8 ± 1.1 μM and 4.3 ± 1.0 μM, respectively. Data plotted as the mean of two experiments ± SEM.

Figure 6.


● Decanoyl - DL-Aminocarnitine
○ Palmitoyl - DL-Aminocarnitine
10
5
nmole min⁻¹ mg⁻¹
5
10
25
[Inhibitor] μM

Discussion

Rat liver contains medium/long-chain carnitine acyltransferase activity located in microsomes, peroxisomes, and mitochondria (19,41). Microsomal COT, like purified peroxisomal COT, exhibits Michaelis-Menten kinetics. Velocity *versus* substrate plots for microsomal COT are hyperbolic with a hill coefficients of 1 (see Figures 1 and 2). Hill coefficients for purified mouse liver peroxisomal COT for acyl-CoA as variable substrate are 1-1.2 (14). Purified rat liver peroxisomal COT also exhibits Michaelis-Menten kinetics (34). In contrast, purified beef heart mitochondrial CPT and membrane bound CPT of intact rat heart mitochondria show allosteric kinetics with sigmoid velocity *versus* substrate plots indicating cooperative binding of acyl-CoA and L-carnitine (5,20). Purified rat liver mitochondrial CPT also shows complex, biphasic kinetics (34). Microsomal COT can be distinguished from mitochondrial CPT since microsomal COT does not show cooperative substrate binding.

Although it is difficult to compare kinetic constants determined using different assay conditions, the $K_{0.5}$ for decanoyl-CoA of microsomal COT is similar to values reported for purified mouse liver peroxisomal COT and membrane bound rat heart and liver mitochondrial CPT. The $K_{0.5}$ of purified peroxisomal COT for L-carnitine with decanoyl-CoA as cosubstrate, however is ~7-8 fold less than the $K_{0.5}$ of membrane bound microsomal COT and membrane bound mitochondrial CPT with decanoyl-CoA as cosubstrate. The $K_{0.5}$ of microsomal COT for decanoyl-CoA is 1.9 μM and 0.42 mM for L-carnitine (see Figures 1 and 2) determined using a rate forward, spectral assay at pH 7.5 using DTBP as a thiol trapping agent. The $K_{0.5}$

of purified mouse liver peroxisomal COT for decanoyl-CoA is 2.2 μM and 55 μM for L-carnitine determined with a rate forward, spectral assay at pH 8.0 using DTNB (14). The $K_{0.5}$ of membrane bound rat heart mitochondrial CPT for decanoyl-CoA is 3 μM and 0.2-0.7 mM for L-carnitine determined with a rate forward, spectral assay at pH 8.0 using DTNB (5). The $K_{0.5}$ of membrane bound rat liver mitochondrial CPT_o for palmitoyl-CoA is 27 μM and 0.16 mM for L-carnitine determined with an isotope forward assay using [^{14}C]-carnitine (91). The $K_{0.5}$ of mitochondrial CPT and peroxisomal COT for L-carnitine varies with acyl-CoA chain length (14,20). The $K_{0.5}$ of microsomal COT for L-carnitine was only determined with decanoyl-CoA as cosubstrate. Microsomal COT can be distinguished from peroxisomal COT by its higher $K_{0.5}$ for L-carnitine with decanoyl-CoA as cosubstrate.

The pH optimum for membrane bound microsomal COT is different than the reported pH optimum values for purified mitochondrial CPT and peroxisomal COT. The pH optimum for microsomal COT assayed in the forward direction is 8.5 (see Figure 3). The pH optimum for purified mouse liver peroxisomal COT assayed in the reverse direction is 8.0 (14). Purified beef heart mitochondrial CPT in octylglucoside assayed in the forward direction has a pH optimum at pH 7.0 (92). Membrane bound microsomal COT can be distinguished from peroxisomal COT and mitochondrial CPT by its higher pH optimum.

Aminocarnitine Inhibition

The data presented herein shown that while microsomal COT is less sensitive to inhibition by DL-aminocarnitine than mitochondrial CPT activity, the DL-aminocarnitine inhibition of microsomal COT is similar to the L-aminocarnitine inhibition reported for CPT activity of outer mitochondrial membrane enriched vesicles (OMV CPT). Microsomal COT

is less sensitive to DL-aminocarnitine inhibition than the CPT activity of Triton X-100 treated mitochondria and to the L-aminocarnitine inhibition reported for mitochondrial CPT. The kinetic properties of microsomal COT are also different than the kinetic properties reported for mitochondrial CPT and peroxisomal COT. Microsomal COT is inhibited by DL-Aminocarnitine with an I_{50} value of 0.5 mM (see Figure 4) and OMV CPT is inhibited by L-aminocarnitine with an I_{50} value of 0.25 mM (70). Microsomal COT uses DL-aminocarnitine as substrate with decanoyl-CoA as cosubstrate although presumably only L-aminocarnitine is acylated. OMV CPT uses L-aminocarnitine as substrate with octanoyl-CoA as cosubstrate but not with palmitoyl-CoA as cosubstrate (70). Microsomal COT, though, is inhibited by high concentrations of palmitoyl-CoA (see Figure 4 of Chapter 2). The DL-aminocarnitine inhibition of microsomal COT is also similar to the L-aminocarnitine inhibition of the medium/long-chain carnitine acyltransferase activity associated with intact, gradient purified peroxisomes (70).

The DL-aminocarnitine inhibition of microsomal COT differs from the aminocarnitine inhibition of CPT activity of detergent treated mitochondria, intact mitochondria, inner mitochondrial membrane enriched vesicles (IMV CPT) and purified mitochondrial CPT. DL-Aminocarnitine at a concentration of 5 μM inhibits 64% of the CPT activity of rat liver mitochondria treated with Triton X-100 (67). Decanoyl-DL-Aminocarnitine and palmitoyl-DL-aminocarnitine at 5 μM inhibit 75% and 99%, respectively, of the CPT activity of Triton X-100 treated mitochondria (68). The I_{50} for inhibition of CPT_o of intact rat liver mitochondria is 62.5 μM by L-aminocarnitine and 2.2 μM by palmitoyl-L-aminocarnitine (69). The I_{50} for inhibition of IMV CPT activity and for purified mitochondrial CPT is 25 μM (70). In contrast, microsomal COT is less sensitive to aminocarnitine inhibition. The I_{50} for DL-aminocarnitine

inhibition of microsomal COT is 0.5 mM (see Figure 4) and the I_{50} for decanoyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine inhibition of microsomal are 7 and 4 μM, respectively (see Figure 6).

Microsomal COT also differs from mitochondrial CPT in the ability to use aminocarnitine as substrate. Microsomal COT like the OMV CPT activity uses aminocarnitine as substrate with decanoyl-CoA as cosubstrate for microsomal COT and octanoyl-CoA as cosubstrate for OMV CPT (70). Triton X-100 treated mitochondria do not use aminocarnitine as substrate with palmitoyl-CoA as cosubstrate (67). It has been reported that intact rat liver mitochondria use aminocarnitine as substrate with palmitoyl-CoA as cosubstrate (69). Rat liver mitochondria, though, can be significantly contaminated by microsomes (61), so the enzyme responsible for the formation of the palmitoyl-L-aminocarnitine could be microsomal. Purified mitochondrial CPT, IMV CPT and purified peroxisomal COT do not use aminocarnitine as substrate with either octanoyl-CoA or palmitoyl-CoA as cosubstrate (70).

The sensitivity of liver medium/long-chain carnitine acyltransferase activity to aminocarnitine inhibition has been proposed as a method for distinguishing mitochondrial CPT activity from peroxisomal COT activity (70). Aminocarnitine inhibition of microsomal COT activity can also be used to distinguish medium/long-chain carnitine acyltransferase activity of microsomal origin from mitochondrial CPT and peroxisomal COT. The DL-aminocarnitine inhibition of microsomal COT is similar, though, to the L-aminocarnitine inhibition reported for the CPT activity of outer mitochondrial membrane enriched vesicles and the COT activity associated with intact peroxisomes (70); further research is needed to demonstrate if the medium/long-chain carnitine acyltransferase activity located in the endoplasmic reticulum, outer mitochondrial membrane enriched vesicles, and intact peroxisomes is due to one enzyme or

if it is due to multiple enzymes.

Chapter 4. Attempted Purification of Microsomal COT

Rat liver endoplasmic reticulum contains a medium/long-chain carnitine acyltransferase referred to herein as microsomal carnitine octanoyltransferase (COT). Microsomal COT activity is tightly associated with the microsomal membrane and requires detergents or high pH conditions for solubilization. Greater than 90% of microsomal COT can be solubilized with the zwitterionic detergent, CHAPS. Microsomal COT can also be solubilized at pH>10.5. Purification of CHAPS solubilized COT using column chromatography was not successful. Gel filtration, anion exchange, hydrophobic interaction, and dye affinity chromatography were used. CHAPS solubilized COT eluted from an anion exchange column with a lower specific activity than the initial sample. Reconstitution of the low activity COT eluted from the anion exchange column with phospholipids did not lead to recovery of activity.

Introduction

Rat liver contains at least three carnitine acyltransferases with medium/long-chain acyl-group specificity: mitochondrial carnitine palmitoyltransferase (CPT), peroxisomal carnitine octanoyltransferase (COT), and microsomal carnitine octanoyltransferase (COT) (19,41). The role of mitochondrial CPT in the transport of long-chain acyl-CoA through the inner membrane of mitochondria for subsequent ß-oxidation is well established (19,85). It has been proposed that peroxisomal COT is involved in the transport of peroxisomal ß-oxidation chain-shortened acyl-CoA from the peroxisome to the mitochondria (36). The function of microsomal COT is not known. Mitochondrial CPT and peroxisomal COT in rat liver have been purified to homogeneity (34). Microsomal COT is tightly associated with the outer surface of the microsomal membrane (42). Characterization of microsomal COT has been hindered by the instability of the detergent solubilized enzyme (37,39), with the result that the enzyme has not yet been purified.

Mitochondrial CPT is also membrane bound and is located both outside (CPT_o) and inside (CPT_i) the inner mitochondrial membrane (19). It is not known if CPT_o and CPT_i are different proteins or if they are the same protein with different locations and regulatory properties. Only one protein with medium/long-chain carnitine acyltransferase activity from liver mitochondria has been purified to homogeneity (19). The purified liver mitochondrial CPT is oligomeric with a native molecular weight of 280,000 - 320,000 estimated by gel filtration chromatography and a subunit molecular weight of ~70,000 estimated by SDS-PAGE

(34). Purified CPT exhibits complex allosteric kinetics with cooperative binding of acyl-CoA and carnitine (5,34). Although it is thought that purified CPT and CPT_i are the same protein, it is not known if CPT_o and purified CPT are the same protein. Unlike purified CPT, CPT_o is inhibitable by malonyl-CoA (19,30). It has been reported that CPT_o is a different protein than either purified CPT or CPT_i and that it is destroyed by detergent solubilization (22-24).

Peroxisomal COT is soluble and is located in the matrix of peroxisomes (39). Peroxisomal COT has been purified to homogeneity from rat liver with a subunit molecular weight of 66,000 daltons estimated by SDS-PAGE (34); peroxisomal COT purified from mouse liver has a native and subunit molecular weight of 60,000 daltons estimated by gel filtration chromatography and SDS-PAGE (14). Peroxisomal COT is not inhibited by malonyl-CoA (34).

The data presented herein describe an attempted purification of microsomal COT. The characteristics of purified microsomal COT could be compared to the known properties of purified mitochondrial CPT or purified peroxisomal COT. Although microsomal COT was not purified to homogeneity, the attempted purification steps can be used to gain insight into the solubility characteristics and stability of the solubilized enzyme. Column chromatography and ammonium sulfate fractionation purification attempts of solubilized microsomal COT are reported.

Results

Preparation of Microsomes

Microsomes were prepared by differential centrifugation of a rat liver homogenate in the presence of the protease inhibitor PMSF. The average yield of microsomes was 10.1 ± 1.5 mg / g wet weight of liver (n=5). The specific activity of COT was ~6 nmole min^{-1} mg^{-1} protein (see Table 2 of Chapter 2) giving a yield of microsomal COT activity of ~60 munit / g wet weight of liver.

An attempt was made to scale-up the preparation of microsomes by using $CaCl_2$ precipitation of rat livers which were stored frozen at -70°C. The rat livers were collected and stored in 0.25 M Mannitol, 1 mM EDTA, pH 7.4, and 25 µg/ml PMSF. A large scale preparation of microsomes was done using $CaCl_2$ precipitation of the post-mitochondrial supernatant obtained from the frozen livers. An unusually high yield of COT activity (383 munits COT / g wet weight of liver) was obtained. The specific activity of COT was 20.7 nmole min^{-1} mg^{-1} protein which was also high compared to a typical specific activity of ~5 nmole min^{-1} mg^{-1} protein obtained from fresh rat livers (see Table 2 of Chapter 2). Marker enzymes were assayed and the specific activity of the microsomal marker enzyme, NADPH cytochrome *c* reductase was 17.6 nmole min^{-1} mg^{-1} protein which was low compared to a typical specific activity of ~50 nmole min^{-1} mg^{-1} protein from fresh rat liver (see Table 1 of Chapter 2). The specific activity of the marker for the outer mitochondrial membrane, monoamine oxidase, was 6.3 nmole min^{-1} mg^{-1} protein which is higher than the typical value

of ~0.7 nmole min^{-1} mg^{-1} protein from fresh rat livers (see Table 1 of Chapter 2). $CaCl_2$ precipitated microsomes obtained from frozen rat livers contain high amounts of monoamine oxidase indicating they are significantly contaminated by mitochondria.

Solubilization

Microsomal COT is firmly membrane bound and is not solubilized from the membrane by freeze/thawing or sonication (37,39). Microsomal COT can be solubilized from the microsomal membrane using either detergent or high pH conditions. The effect of different detergents on COT activity is shown in Table 3 of Chapter 2. The optimum conditions for detergent solubilization were to make the microsomal membranes 8 mM in CHAPS and incubating on ice for one hour followed by freezing at -70°C for at least 12 hours. The microsomes containing CHAPS were thawed at room temperature and centrifuged at 100,000 x g for 30-60 minutes at 5°C. For a representative experiment, the specific activity of the membrane bound microsomal COT was 7.9 ± 1.1 nmole min^{-1} mg^{-1} protein while the specific activity of the CHAPS-solubilized supernatant was 4.7 ± 0.6 nmole min^{-1} mg^{-1} protein (n=2). CHAPS-solubilized COT is stable both at 4°C and to freezing at -20°C.

Microsomal COT can also be solubilized from the membrane using high pH conditions. The optimum conditions for high pH solubilization were to make the microsomes pH 10.5 with the addition of NH_4OH followed by three cycles of freezing in a dry ice/acetone bath and thawing at room temperature. The frozen-thawed microsomes were made 1 mM in dithiothreitol, flushed with N_2, and sonicated 4 times at power setting 4 for 30 seconds using a Model W-220F Heat Systems - Ultrasonics, Inc. sonicator. The sonicated microsomes were centrifuged at 100,000 x g for 30-60 minutes at 5°C. For a representative experiment the

extent of solubilization was 78%; of an initial 141.6 munits of membrane bound microsomal COT there were 110.2 munits of COT in the 100,000 x g supernatant fluids and there were 25.3 munits of COT in the 100,000 x g pellet. For this experiment the specific activity of the high pH solubilized COT was 14.1 nmole min^{-1} mg^{-1} protein.

The high pH solubilized microsomal proteins re-aggregated when the pH was lowered and were pelleted when the sample was centrifuged at 100,000 x g. The effect of pH on the solubility of the high pH solubilized microsomes is shown in Table 1. The solubilized microsomes were adjusted to the pH values indicated and incubated at 4°C for 12 hours. The samples were again centrifuged at 100,000 x g and the COT activity in the supernatant fluid and pellet was determined. COT activity remained soluble after the 12 hour incubation at pH 9.7, and pH 8.5, while 81% of the COT activity had re-aggregated at pH 6.6.

Microsomal COT can also be solubilized from the microsomal membrane by a combination of high pH and detergent solubilization techniques. CHAPS was included during the high pH solubilization to optimize the solubility of the high pH solubilized enzyme. Optimum conditions were to make the microsomes 2 mM in CHAPS and pH 10.5 with the addition of NH_4OH and to incubate on ice for at least 1 hour. The high pH - CHAPS microsomes were subjected to three cycles of freezing in a dry ice/ acetone bath and thawing at room temperature followed by centrifugation at 100,000 x g for 30-60 minutes. Sonication was not used. For a representative experiment the specific activity of the membrane bound microsomal COT was 11.4 nmole min^{-1} mg^{-1} protein while the specific activity of the high pH - CHAPS solubilized supernatant was 8.4 nmole min^{-1} mg^{-1} protein.

Table 1. Effect of pH on the Solubility of Microsomal COT

pH of extracted COT	9.7	8.5	6.6
Activity of extracted COT nmole min^{-1}	10.72	15.32	16.84
The extracted COT was incubated at 4°C for 12 hours then centrifuged at 100,000 x g.			
Activity of Soluble COT (100,000 x g Supernatant) nmole min^{-1}	11.48	14.56	2.28
Activity of Re-aggregated COT (100,000 x g Pellet) nmole min^{-1}	0.76	2.3	9.94

Ammonium Sulfate Fractionation

Ammonium sulfate fractionation of microsomal COT solubilized in 1% Tween 20 is shown in Figure 1. The optimum concentration for ammonium sulfate fractionation was 40%. The 40% ammonium sulfate precipitated protein does not pellet with centrifugation at 10,000 x g. The 40% ammonium sulfate supernatant and pellet were separated by filtering through a column of glass wool and the precipitated protein was eluted with 5 mM potassium phosphate, pH 7.5, 1 mM EDTA containing 0.5% Tween 20. The COT activity of the resuspended 40% ammonium sulfate precipitate was not soluble and pelleted with centrifugation at 100,000 x g. Conditions were not found to solubilize stable COT activity from the resuspended 40% ammonium sulfate precipitate.

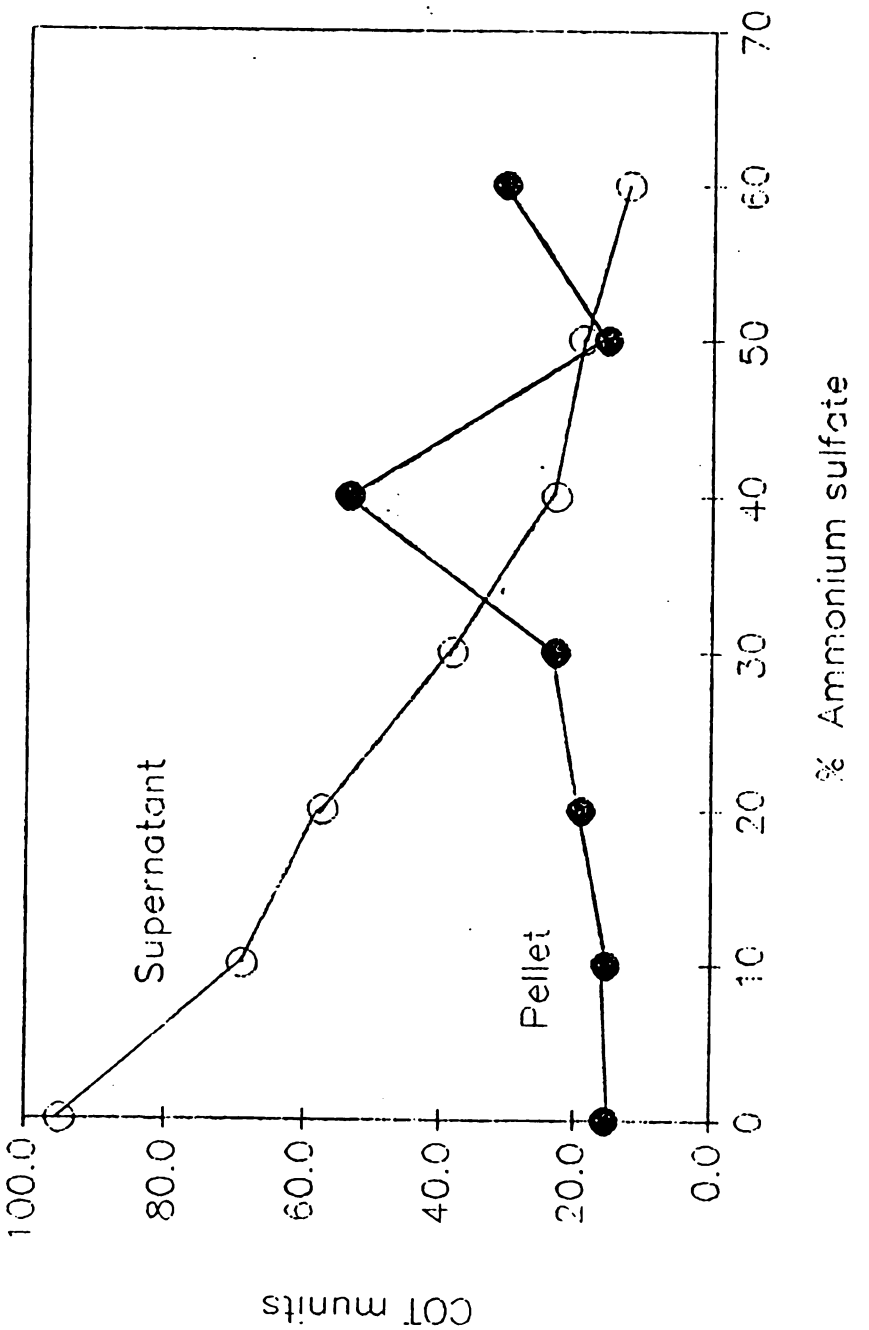
Column Chromatography

A. Purification of high pH-CHAPS solubilized microsomal COT was attempted using gel filtration chromatography. Analytical scale HPLC gel filtration chromatography using a Dupont G-250 sieving column showed that solubilized microsomal COT eluted with a molecular weight ~60,000. The column was equilibrated in 200 mM ammonium acetate pH 8.0 containing 20% glycerol. The solubilized microsomal COT eluted with a retention time of 11.1 min. while the retention time of bovine serum albumin which has a molecular weight of 66,000 daltons was 10.3 min. The recovery of COT eluted from the column was 3.8%; 6.9 munits were loaded and 0.26 munits were recovered.

B. Purification of microsomal COT using gel filtration was scaled up for use as a preparative chromatographic step. A 2.5 cm x 80 cm Bio gel P100 column was prepared and equilibrated in 50 mM potassium phosphate, pH 7.5 with 20% glycerol. The column was calibrated with the following molecular weight standards: Ribonuclease A, Chymotrypsinogen

Figure 1. Ammonium Sulfate Fractionation of Detergent Solubilized Microsomal COT. Aliquots of 1% Tween-20 solubilized COT containing 100 munits of COT activity were made the percentage of ammonium sulfate indicated and the supernatant and pellet were collected and assayed spectrally for COT as described in Materials and Methods.

Figure 1.

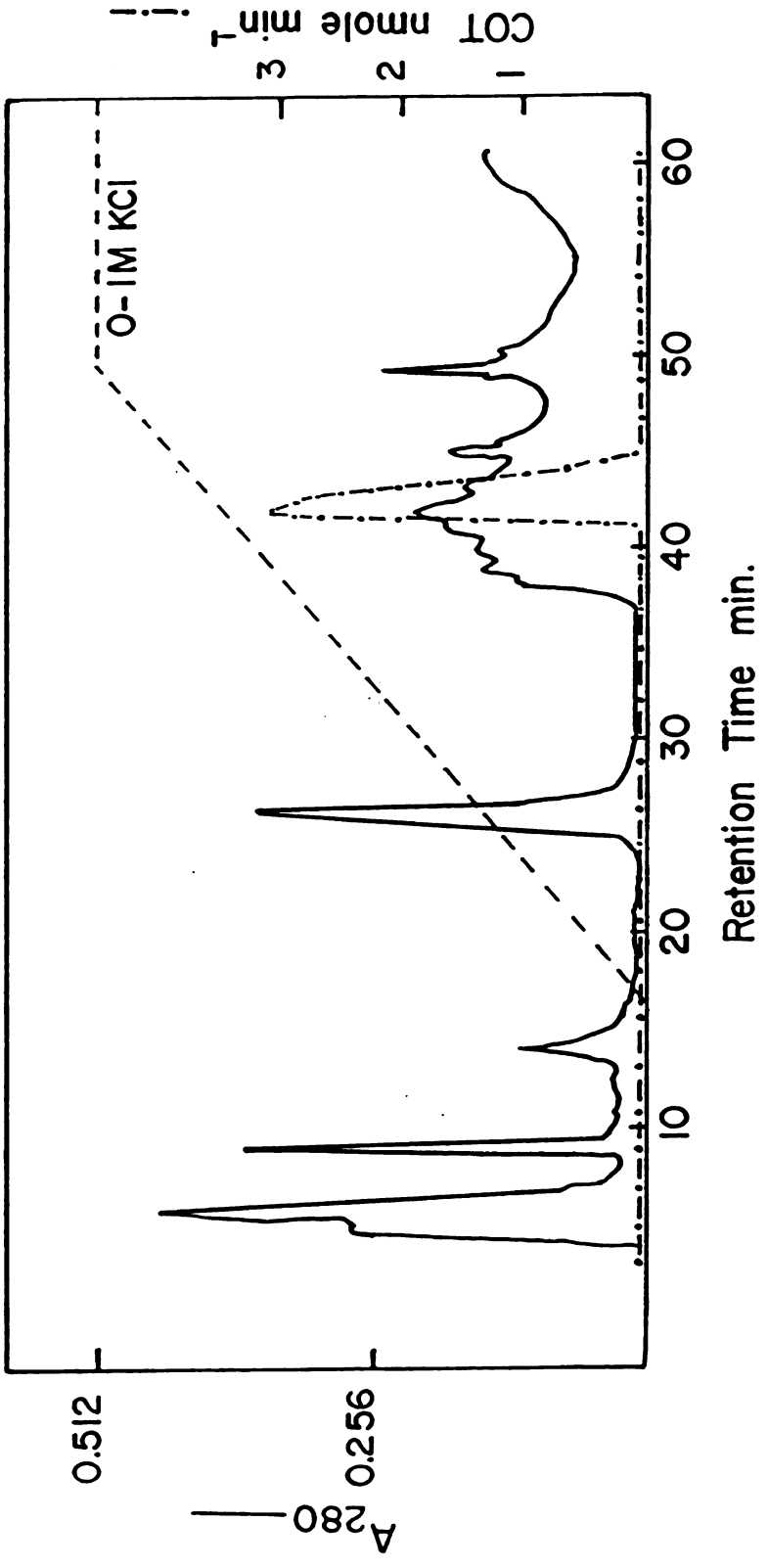

CAT munits
100.0
80.0
60.0
40.0
20.0
0.0
0
10
20
30
40
50
60
70
% Ammonium sulfate
Supernatant
Pellet

A, Ovalbumin and, Bovine Serum Albumin; Blue Dextran was used to determine the void volume. High pH-CHAPS solubilized microsomal COT eluted from the column in the void volume with a 10.4% recovery of initial activity indicating the solubilized COT had re-aggregated during the chromatography. The P100 column was equilibrated in 50 mM Tris-Cl pH 8.5, 1 mM EDTA, 4 mM CHAPS, and 1 mM DTT to attempt to optimize conditions for COT stability and solubility. The high pH-CHAPS solubilized COT again eluted with the void volume with a 37% recovery again indicating the COT had re-aggregated. Conditions were not found to allow preparative scale gel filtration chromatography of the solubilized enzyme.

C. An HPLC weak anion exchange separation of the high pH - CHAPS solubilized microsomal COT was attempted. A Synchropak AX 300 column was equilibrated in 10 mM potassium phosphate pH 7.5, 2 mM CHAPS containing 20% glycerol (buffer A). The solubilized COT was loaded and the column washed with buffer A until the A_{280} was ~0. The COT was eluted with a linear gradient of buffer A containing 1 M KCl. A representative column profile is shown in Figure 2. For this experiment 17.2 munits of COT with a specific activity of 10.8 nmole min^{-1} mg^{-1} protein were loaded onto the column. The recovery of COT eluted from the column was 2.7 munits (15.7%); the specific activity of the COT eluted from the column was 7.05 nmole min^{-1} mg^{-1} protein. COT eluted from the column was soluble and did not pellet with centrifugation at 100,000 x g. Fractions containing COT activity were separated on a 10% SDS polyacrylamide gel and silver stained. SDS-PAGE showed the presence of at least 27 polypeptides with a molecular weight range of ~24,000 - 100,000 daltons. No conditions were found using anion exchange chromatography which resulted in an increase in the specific activity of microsomal COT.

Figure 2. HPLC Weak Anion Exchange Separation of High pH - CHAPS Solubilized Microsomal COT. A Synchropak AX 300 column was equilibrated in 10 mM potassium phosphate pH, 7.5, 2 mM CHAPS, and 20% glycerol (buffer A) at a flow rate of 0.5 ml min^{-1}. COT (17.2 munits) was loaded isocratically. The column washed with buffer A until the A_{280} was ~0 and proteins eluted with a linear gradient of buffer A containing 1 M potassium chloride beginning at 18 minutes as indicated. One ml fractions were collected and assayed spectrally for COT activity as indicated.

Figure 2.


COT nmole min⁻¹
1
2
3
0-1M KCl
A280
0.512
0.256
10
20
30
40
50
60
Retention Time min.

D. Additional column chromatography techniques which were attempted included: dye affinity, hydroxylapatite, and hydrophobic interaction column separations. Cibacron blue sepharose and orange sepharose columns were used but the yield of COT activity was low. Hydroxylapatite chromatography in one experiment gave a 17.6% recovery of COT activity. For this experiment a Biogel-HTP column was used and of the 930 munits of COT loaded on the column, 164 munits of COT were eluted with a linear potassium phosphate gradient. COT activity was not stable during hydrophobic interaction chromatography; phenyl-sepharose and octyl-sepharose columns were used.

Restoration of COT Eluted from an Anion Exchange Column

Restoration of microsomal COT eluted from the anion exchange column was attempted to increase specific activity. The specific activity of the microsomal COT eluted from the column was 7.05 nmole min^{-1} mg^{-1} protein. Addition of 0.2% Tween-20 to the assay mix did not change the specific activity of the COT. Chloroform/methanol extracted microsomal phospholipids added to the assay also did not change the specific activity. Addition of 0.001% asolectin to the assay gave a specific activity of 7.7 nmole min^{-1} mg^{-1} protein. A neutralized acid extract of microsomes was also added to the assay and the specific activity of the COT was decreased to 2.8 nmole min^{-1} mg^{-1} protein. Addition of 1 mM ATP and 1 mM $MgCl_2$ to the assay also decreased the specific activity of COT to 5.6 nmole min^{-1} mg^{-1} protein. No conditions were found that increased the specific activity of the COT containing fractions eluted from the anion exchange column.

Discussion

The purification of microsomal COT was attempted from a microsomal membrane pellet prepared by differential centrifugation of a post mitochondrial supernatant obtained from fresh rat livers. The preparation of microsomes using $CaCl_2$ precipitation of a post mitochondrial supernatant obtained from livers which were stored frozen at -70°C resulted in a microsomal fraction that contained high amounts of monoamine oxidase activity. Monoamine oxidase is a marker enzyme for the outer mitochondrial membrane indicating the fraction was significantly contaminated by the outer membrane of mitochondria. Since there have been reports that in liver mitochondrial medium/long-chain carnitine acyltransferase activity is located in the outer membrane (11,29-32), these microsomal preparations were not used for further purification.

Microsomal COT is tightly associated with the microsomal membrane and is not solubilized by freeze-thaw treatment of microsomes (39). Microsomal COT can be solubilized by treatment of the microsomes with 1% Triton X-100 containing 0.4 M potassium chloride but the solubilized COT activity was not stable (39). The data reported herein show that microsomal COT can be solubilized using detergent or high pH conditions; 20% glycerol was present during solubilization. Osmolytes such as glycerol have been shown to act as protein stabilizers during detergent solubilization (93). Optimum detergent solubilization is achieved using the zwitterionic detergent, CHAPS. CHAPS - solubilized microsomal COT activity is stable but purification attempts of detergent solubilized COT activity were not successful.

Microsomal COT is also solubilized from the membrane using high - pH conditions (pH 10.5) although the enzyme re-aggregates when the pH is lowered (see Table 1). A recent report in abstract form describes solubilization of the three most hydrophobic polypeptides of the *b6f* complex of *b* cytochromes using pH > 10.5 and proposed that electrostatic forces could be important is stabilizing this complex in the membrane (94). This suggests that microsomal COT is solubilized from the membrane at pH 10.5 due to disruption of electrostatic forces.

A combination of high pH and detergent conditions also solubilized microsomal COT from the membrane although the enzyme still re-aggregated when the pH was lowered for gel filtration chromatography. In contrast, the high pH - CHAPS solubilized microsomal COT eluted from an anion exchange column was soluble (see Figure 2). Microsomal COT eluted from the anion exchange column with a lower specific activity than the initial high - pH solubilized sample (compare 7.05 nmole min^{-1} mg^{-1} protein with 10.8 nmole min^{-1} mg^{-1} protein). Restoration of COT activity eluted from the anion exchange column using asolectin or microsomal phospholipids was not successful.

The purification attempts of microsomal COT reported herein were not successful in purifying the enzyme to homogeneity. One explanation of the difficulty of solubilizing microsomal COT from the membrane and loss of COT activity with anion exchange chromatography is that microsomal COT may require association with another protein for maximum activity and stability. Future purification of microsomal COT could include attempts to restore the low specific activity COT fractions eluted from the anion exchange column with other protein containing fraction separated by the column to yield a high activity, stable enzyme complex.

Another purification attempt could focus on restoring the malonyl-CoA sensitivity of

solubilized microsomal COT. Membrane bound microsomal COT is inhibited by malonyl-CoA while detergent solubilized microsomal COT is not inhibited by up to 200 μM malonyl-CoA (see Figure 3 of Chapter 2). Membrane bound microsomal COT may be sensitive to inhibition by malonyl-CoA through association with a putative malonyl-CoA binding regulatory protein. Such a putative regulatory protein has been proposed to be involved in the malonyl-CoA inhibition of mitochondrial CPT (74) although direct evidence is lacking (19). If the putative malonyl-CoA binding regulatory protein is purified from mitochondria it could be used to try and reconstitute the fractions with low COT activity eluted from the anion exchange column to yield a high activity, malonyl-CoA sensitive enzyme.

Chapter 5. Summary and Conclusions

Rat liver contains at least three carnitine acyltransferases with medium-chain to long-chain acyl-group specificity located in mitochondria, peroxisomes, and endoplasmic reticulum. Medium/long-chain carnitine acyltransferase activity located in mitochondria with access to cytosolic acyl-CoA's (mitochondrial CPT_o) and medium/long-chain carnitine acyltransferase activity located in the endoplasmic reticulum are regulated through inhibition by malonyl-CoA. Medium/long-chain carnitine acyltransferase activity associated with the matrix of peroxisomes is not inhibitable by malonyl-CoA. Malonyl-CoA, an intermediate in fatty acid synthesis, acts to inhibit fatty acid oxidation via inhibition of cytosolic medium/long-chain acylcarnitine production preventing a futile cycle of synthesis and oxidation. Table 1 summarizes a comparison of previously reported characteristics of medium/long-chain carnitine acyltransferase activity located in mitochondria and peroxisomes with data given in this thesis characterizing medium/long-chain carnitine acyltransferase activity located in the endoplasmic reticulum.

The data show that microsomal COT has different characteristics than either mitochondrial CPT or peroxisomal COT although there are also similarities between these enzymes. Microsomal COT, peroxisomal COT and mitochondrial CPT all transfer medium-chain and long-chain acyl-groups between acyl-CoA and L-carnitine. Microsomal COT is antigenically different than either mitochondrial CPT or peroxisomal COT. Microsomal COT

Table 1. Summary of the Properties of Rat liver Mitochondrial CPT, Peroxisomal COT, and Microsomal COT

	Mitochondrial CPT_o[1], CPT_i[2]	Peroxisomal COT	Microsomal COT[3]
Membrane bound	CPT_o + CPT_i yes	No Located in matrix (37)	Yes (37,39) Located on outer surface of ER (42)
Kinetic Characteristics	Complex, allosteric Cooperative substrate binding with acyl-CoA and L-carnitine (34)	Michaelis-Menten (34) Hill n = 1-1.2 from mouse liver with acyl-CoA (14)	Michaelis Menten Hill n ~ 1 with acyl-CoA and L-carnitine
Antigenic Characteristics			
Immunoprecipitated by Antimitochondrial-CPT	CPT_o and CPT_i yes (16)	No (16)	No
Immunoprecipitated by Antiperoxisomal-COT	No (14)	Yes (14)	No
Palmitoyl-CoA Inhibition	OMV CPT[4] inhibited by > 18 μM IMV CPT[5] not inhibited by 100 μM (29)	Not inhibited by 300 μM (15)	Membrane bound I_{50} = 11 μM
Malonyl-CoA Inhibition	CPT_o K_i 1-2 μM (71) CPT_i not inhibited (71) Solubilized CPT not inhibited (19)	Not inhibited[6] (34)	Membrane bound I_{50} = 5 μM Solubilized not inhibited

Table 1. Continued

	Mitochondrial CPT_o[1], CPT_i[2]	Peroxisomal COT	Microsomal COT[3]
Etomoxiryl-CoA Inhibition	CPT_o I_{50} = 3 nM (22) Purified (beef heart) CPT decrease Hill n (96)	K_i = 1 μM, mixed type inhibitor (95)	Membrane bound I_{50} = 0.7 μM Solubilized I_{50} = 1.5 μM
L-Aminocarnitine Inhibition	OMV CPT[4] I_{50} = 250 μM (70) IMV CPT[5] I_{50} = 25 μM (70) Purified CPT I_{50} = 25μM (70)	Not inhibited by 2 mM (70)	Membrane bound I_{50} = 500 μM (DL-aminocarnitine)

[1] Malonyl-CoA sensitive form of CPT in contact with the cytosol
[2] Form of CPT in contact with the matrix of mitochondria
[3] Unless otherwise indicated data given in Chapters 2, 3, and 4
[4] CPT activity of outer membrane enriched vesicles
[5] CPT activity of inner membrane enriched vesicles
[6] Membrane bound CPT activity associated with intact peroxisomes is inhibitable by malonyl-CoA with I_{50} = 2.2 μM (40)

like mitochondrial CPT is membrane bound and unlike peroxisomal COT which is soluble. Microsomal COT like peroxisomal COT shows Michaelis-Menten kinetics while mitochondrial CPT shows complex allosteric kinetics. Membrane bound microsomal COT like membrane bound mitochondrial CPT is inhibited by malonyl-CoA while peroxisomal COT is not inhibited by malonyl-CoA. Microsomal COT, both membrane bound and soluble, and peroxisomal COT are inhibited by micromolar concentrations of etomoxiryl-CoA while membrane bound mitochondrial CPT is inhibited by nanamolar concentrations of etomoxiryl-CoA. Microsomal COT is less sensitive to aminocarnitine inhibition than mitochondrial CPT while peroxisomal COT is not inhibited by aminocarnitine.

Microsomal COT has similar properties to the CPT activity of outer mitochondrial membrane enriched vesicles (OMV CPT) and the CPT activity associated with intact peroxisomes. These properties include: inhibition by malonyl-CoA and aminocarnitine. Rat liver mitochondria prepared by differential centrifugation can be significantly contaminated by both peroxisomes and mitochondria (35,58,59,61). Further studies are needed to clarify the relationship between microsomal COT, OMV CPT and the CPT associated with intact peroxisomes to determine if they are due to a single enzyme activity or to separate enzymes with similar properties.

Possible Functions of Microsomal COT

Although the function of the malonyl-CoA sensitive COT of microsomes is not established, the strong inhibition by low amounts of malonyl-CoA indicates it is subject to short-term metabolite regulation in a manner that would reduce or prevent long-chain and medium-chain acylcarnitine formation in the fed state. The condensing enzyme(s) of rat liver

involved in microsomal fatty acid elongation are located on the cytosolic surface of the endoplasmic reticulum (97). This enzyme can catalyze the condensation of palmitoyl-CoA with malonyl-CoA, the initial step in the microsomal chain elongation system. The strong inhibition of microsomal COT by palmitoyl-CoA and malonyl-CoA suggests that microsomal acylcarnitine formation is inhibited under metabolic conditions that promote fatty acid elongation.

CPT, purified from beef heart mitochondria exhibits a log relationship between the acyl-CoA chain length and the $K_{0.5}$ for carnitine, indicating that at physiological, non-saturating concentrations of L-carnitine, it has the capacity to kinetically select for long-chain acyl-CoA derivatives (20). Although it is well-established that medium-chain fatty acids can be activated in the mitochondrial matrix, the fraction of medium-chain fatty acids activated in the cytosolic compartment compared to the matrix compartment *in vivo* is not known. Due to the acyl-CoA impermeable barrier, carnitine is required for the mitochondrial β-oxidation of cytosolic medium-chain acyl-CoAs; thus, regulation of their conversion to acylcarnitines in the cytosolic compartment should be expected. Microsomal COT can convert cytosolic medium-chain acyl-CoAs to acylcarnitines which subsequently enter mitochondria for β-oxidation. This could permit β-oxidation of both cytosolic long-chain acyl-CoAs and medium-chain acyl-CoAs when mixtures of cytosolic acyl-CoAs are present, especially in the fasted state.

Alternatively, medium-chain carnitine acyltransferase activity in the microsomes may function in the detoxification of acyl residues as carnitine conjugates that can be eliminated in the urine of humans. Valproic acid therapy and pivampicillin therapy cause the excretion of both valproylcarnitine and pivaloylcarnitine, respectively (98,99). Similarly, several human

disease states promote urinary excretion of specific acylcarnitines (100,101). The carnitine acyltransferase(s) responsible for the formation of these acylcarnitines has not been determined. Since many detoxification systems are located in the endoplasmic reticulum of liver, this may prove to be a function of the microsomal COT.

Future Research

It is important to establish if microsomal COT is a separate enzyme than either mitochondrial CPT or peroxisomal COT. The purification of microsomal COT to homogeneity could allow studies to be done to determine the relationship between these enzymes. Mitochondrial CPT (24) and peroxisomal COT (102) have been cloned and the cDNA sequenced to determine the deduced amino acid sequence. N-Terminal amino acid sequence obtained from the purified enzyme or deduced amino acid sequence obtained from a cDNA for microsomal COT could be used to unequivocally establish is microsomal COT is a separate enzyme.

The mechanism of malonyl-CoA inhibition of medium/long-chain carnitine acyltransferase activity is not known (19). It is important to determine if the carnitine acyltransferase itself binds malonyl-CoA or if the carnitine acyltransferase associates with a regulatory protein which binds malonyl-CoA. The purification of microsomal COT could allow studies to be done to determine the mechanism of malonyl-CoA inhibition of microsomal COT. For example it could be possible to purify malonyl-CoA sensitive microsomal COT and determine if it is a single protein or a complex of proteins. Reconstitution of the purified microsomal COT protein(s) into phospholipid vesicles may be required since microsomal COT is malonyl-CoA sensitive when it is membrane bound. It has been shown that the sensitivity

of solubilized mitochondrial CPT_o to malonyl-CoA inhibition is enhanced by reconstitution into asolectin liposomes (29).

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