Beta-Oxidation of Elaidic Acid and Delta 3, Delta 2-Enoyl-CoA Isomerases in the Rat

by

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ABSTRACT

BETA-OXIDATION OF ELAIDIC ACID AND DELTA 3, DELTA 2-ENOYL-COA ISOMERASE IN THE RAT

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The degradation of elaidic acid, oleic acid, and stearic acid by rat mitochondria was studied to determine if the presence of a *trans* double bond in place of a *cis* double bond or no double bond affects β-oxidation. Rat mitochondria from liver or heart effectively degraded the coenzyme A derivatives of all three fatty acids. However, with elaidoyl-CoA as substrate, a major metabolite accumulated in the mitochondrial matrix. This metabolite was isolated and identified as 5-*trans*-tetradecenoyl-CoA. In contrast, little or none of the corresponding metabolites were detected with oleoyl-CoA or stearoyl-CoA as substrates. A kinetic study of long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase (VLCAD) revealed that 5-*trans*-tetradecenoyl-CoA is a poorer substrate of LCAD than is 5-*cis*-tetradecenoyl-CoA, while both unsaturated acyl-CoAs are poor substrates of VLCAD when compared to myristoyl-CoA. Tetradecenoic acid and tetradecenoylcarnitine were detected by gas chromatography/mass spectrometry and tandem mass spectrometry, respectively,

when rat liver mitochondria were incubated with elaidoyl-CoA but not when oleoyl-CoA was the substrate. These observations support the conclusion that 5-*trans*-tetradecenoyl-CoA accumulates in the mitochondrial matrix because it is less efficiently dehydrogenated by LCAD than its *cis* isomer and that the accumulation of this β-oxidation intermediate facilitates its hydrolysis and conversion to 5-*trans*-tetradecenoylcarnitine thereby permitting a partially degraded fatty acid to escape from mitochondria.

The degradation of unsaturated fatty acids by β-oxidation involves Δ^3, Δ^2-enoyl-CoA isomerases (enoyl-CoA isomerases) that catalyze 3-*cis* → 2-*trans* and 3-*trans* →2-*trans* isomerizations of enoyl-CoAs and the 2,5 →3,5 isomerization of dienoyl-CoAs. An analysis of rat liver enoyl-CoA isomerases revealed the presence of a monofunctional enoyl-CoA isomerase (ECI) in addition to mitochondrial short-chain enoyl-CoA isomerase (mECI) in mitochondria, whereas peroxisomes contain ECI and multifunctional enzyme 1 (MFE1). Thus ECI, which previously had been described as mono-functional peroxisomal enoyl-CoA isomerase, was found to be present in both peroxisomes and mitochondria. This enzyme seems to be identical with mitochondrial long-chain enoyl-CoA isomerase. The mature form of rat liver mECI was produced by molecular cloning and heterologous expression. The crystal structure of this mECI has been solved and clearly shows that rat liver recombinant mECI is a trimer and not a dimmer as previously reported. Its general features are similar to those of other members of the hydratase/isomerase super-family.

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ABBREVIATIONS

ECI	Δ^3, Δ^2-enoyl-CoA isomerase
mECI	mitochondrial Δ^3, Δ^2-enoyl-CoA isomerase
pECI	peroxisomal Δ^3, Δ^2-enoyl-CoA isomerase
MFE1	peroxisomal multifunctional enzyme 1
MECH	mitochondrial enoyl-CoA hydratase or crotonase
DECI	dienoyl-CoA isomerase
DCPIP	2,6-dichlorophenoindophenol
LCAD	long chain acyl-CoA dehydrogenase
VLCAD	very long chain acyl-CoA dehydrogenase
PMS	phenazine methosulfate
C18:0	octadecanoic acid (stearic acid)
cΔ^9C18:1	9-cis-octadecenoic acid (oleic acid)
tΔ^9C18:1	9-trans-octadecenoic acid (elaidic acid)
cΔ^5C14:1	5-cis-tetradecenoic acid
tΔ^5C14:1	5-trans-tetradecenoic acid
SDS	sodium dodecylsulfate
PAGE	polyacylamine gel electrophoresis
HPLC	high-performance liguid chromatograph
GC-MS	gas chromatograph-mass spectrometry

INTRODUCTION

Fatty acids are very important to nearly all free-living organisms. They not only serve as a source of concentrated metabolic energy, but also are components of membranes, and precursors of hormones and intracellular messengers. The consequences of fatty acid oxidation dysfunctions can be severe and contribute to complications of many diseases, such as ischemia (1,2), cancer (3), diabetes (4), and obesity (5).

β-Oxidation of fatty acids in animals can occur in both mitochondria and peroxisomes, which have similar and distinctive features (6). Generally, the degradation of fatty acids is accomplished by the sequential removal of two-carbon units, a process called β-oxidation. Substrates of this process are fatty acyl-CoAs that are formed by ligation of fatty acids to CoA in an ATP-dependent reaction catalyzed by acyl-CoA synthetases. The main pathway of β-oxidation proceeds sequentially through four steps: the oxidation of a fatty acyl-CoA to a 2-enoyl-CoA; the hydration of the 2-enoyl-CoA to 3-hydroxyacyl-CoA; the dehydrogenation of the 3-hydroxyacyl-CoA to a 3-ketoacyl-CoA; the thiolysis of the 3-ketoacyl-CoA to acetyl-CoA and a fatty acyl-CoA shortened by a two carbon unit. In mitochondria, the enzymes involved in this process are acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. The corresponding enzymes of β-oxidation in peroxisomes are acyl-CoA oxidase,

multifunctional enzyme 1 (MFE 1) / multifunctional enzyme 2 (MFE 2) and 3-keto-acyl-CoA thiolase. Both MFE 1 and MFE 2 contain enoyl-CoA hydratase and 3-hydroxyacyl-CoA degydrogenase activities. Additionally, MFE 1 contains Δ^3, Δ^2-enoyl-CoA isomerase. 3-Hydroxyacyl-CoAs formed by MFE 1 have the L-configuration, while the hydroxyacyl-CoAs produced by MFE 2 have the D-configuration (7). Obviously, the differences between the enzymes involved in the β-oxidation spiral in mitochondria and peroxisomes result in different metabolic outcomes. β-Oxidation of fatty acids in mitochondria may go to completion without formation of significant amount of intermediates. In contrast, the substrates for peroxisomes, such as very long-chain fatty acids, eicosanoids, dicarboxylic acids, pristanic acid and hydroxylated cholestanic acids (8), which are poor substrates of mitochondrial β-oxidation, are partially degraded, excreted and in some instances further degraded in mitochondria.

The complete metabolism of unsaturated and polyunsaturated fatty acids, such as oleate, linoleate, linoleneate, requires the assistance of additional enzymes besides the enzymes necessary for β-oxidation of saturated fatty acids (6). The auxiliary enzymes are Δ^3, Δ^2-enoyl-CoA isomerase, 2, 4-dienoyl-CoA reductase and $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase. The known pathways for the degradation of unsaturated fatty acids are shown in Fig.1. Unsaturated fatty acids with odd-numbered and even-numbered double bonds yield 5-*cis*-enoyl-CoA and 4-*cis*-enoyl-CoA intermediates, respectively, as the result of chain shortening. 5-Enoyl-

CoAs intermediates either are chain shortened to 3-enoyl-CoAs, isomerized to 2-enoyl-CoAs by Δ^3, Δ^2-enoyl-CoA isomerase, and completely oxidized, or are converted to 3,5-dienoyl-CoAs by Δ^3, Δ^2-enoyl-CoA isomerase after their dehydrogenation to 2,5-dienoyl-CoAs during the first step of β-oxidation. 3,5-Dienoyl-CoAs are converted by $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (DECI) to 2,4-dienoyl-CoAs which are reduced by 2,4-dienoyl-CoA reductase to 3-*trans*-enoyl-CoAs. 4-Enoyl-CoAs are dehydrogenated to 2,4-dienoyl-CoAs, which are reduced by 2,4-dienoyl-CoA reductase to 3-*trans*-enoyl-CoAs. Both 3-*cis* and 3-*trans*-enoyl-CoAs are converted by enoyl-CoA isomerase to 2-*trans*-enoyl-CoAs, which can enter the main pathway of β-oxidation. In addition, Δ^3, Δ^2-enoyl-CoA isomerase can catalyze the isomerization of 2,5-dienoyl-CoA intermediates to 3,5-dienoyl-CoAs. Thus, enoyl-CoA isomerase plays a very important role during the β-oxidation of unsaturated fatty acids.

At least five mammalian enzymes are known that have Δ^3, Δ^2-enoyl-CoA isomerase (ECI) activity (see Table 1). Present in mitochondria are short chain Δ^3, Δ^2-enoyl-CoA isomerase (mECI) (9,10), long-chain Δ^3, Δ^2-enoyl-CoA isomerase (11), and 2-enoyl-CoA hydratase (mECH) or crotonase (12), while peroxisomes contain multifunctional enzyme 1 (MFE 1) (13), and peroxisomal Δ^3, Δ^2-enoyl-CoA isomerase (pECI) (14). Δ^3, Δ^2-Enoyl-CoA isomerase (mECI) has been purified from rat heart (15), hog liver (16), bovine liver (17) and rat liver (15, 18, 19). This enzyme was reported to be a homodimer with a subunit molecular weight of close

to 30,000. The major function of crotonase is the hydration of the 2-enoyl-CoAs, while its enoyl-CoA isomerase activity is insignificant at 1/5000 of its hydratase activity (12). Although peroxisomes contain MFE 1 that harbors enoyl-CoA isomerase activity (13), additionally a monofunctional enoyl-CoA isomerase was identified in peroxisomes by a genomic search based on its sequence similarity to the yeast monofunctional Δ^3, Δ^2-enoyl-CoA isomerase (20). Human pECI contains a type-1 peroxisomal targeting signal (21,22) and was localized to peroxisomes by both subcellular fractionation and immunofluorescence microscopy (14). The specific activities of recombinant enoyl-CoA isomerases indicate that mECI and pECI may be the major enoyl-CoA isomerases in mitochondia and peroxisomes (14,23), respectively. Little is known about the "mitochondrial long-chain Δ^3, Δ^2-enoyl-CoA isomerase", which was described by Kilponen, JM et al. in 1990 (11) based on two lines of evidences: (I) A new ECI activity was detected in rat liver mitochondria when the tissue extract was subjected to chromatography on hydroxylapatite (HAP). The ratio of activities with 3-*trans*-enoyl-CoAs having 6, 10, 12, and 16 carbon acyl chains was 1 : 2 : 3 : 1.7, which prompted the designation "long-chain enoyl-CoA isomerase". (II) Antibodies to mECI and MFE1 did not recognize this novel mitochondrial enzyme. However, since this enzyme has never been purified and characterized, the evidence in support of a long-chain enoyl-CoA isomerase is less than compelling. It is possible that this ECI activity is the component enzyme of a complex or, like crotonase, is just another enzyme with a minor enoyl-CoA activity, or even is identical with crotonase or pECI. Even

though immunoblotting revealed that long-chain Δ^3, Δ^2-enoyl-CoA isomerase differs from mECI (9,10) and MFE1 (13), its relationship to crotonase (12) and pECI (14) was unknown. Crotonase can be identified by its hydratase and isomerase activities as well as by immunoblotting; as for pECI, a new method has been developed in this lab for the identification of this enoyl-CoA isomerase besides immunoblotting. Kinetic results indicate that mECI prefers shorter chain substrates with a 3-*cis* double bond, whereas human pECI prefers longer chain substrates with a 3-*trans* double bond (24). It was observed that mECI has a 2-3-times higher activity with 3-*cis*-octenoyl-CoA than with 3-*trans*-octenoyl-CoA, whereas pECI prefers 3-*trans*-octenoyl-CoA over 3-*cis*-octenoyl-CoA as substrate. This significant difference between activities with 3-*cis*, and 3- *trans*-enoyl-CoAs can be used as a tool to distinguish pECI from mECI and other isoenzymes. This situation has aided efforts to identify and characterize mitochondrial long-chain Δ^3, Δ^2-enoyl-CoA isomerase.

It is known that pECI, mECI and crotonase (mECH) belong to the hydratase/isomerase enzyme super-family, which is characterized by low sequence identity (20-30%), while having the same overall 3-D folding (25). So far the crystal structures of four enzymes in this family have been solved, which show that they have very similar 3-D structures. These four enzymes are: 2-enoyl-CoA hydratase (crotonase) (26), 4-chlorobenzoyl-CoA dehalogenase (27), $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (28), and yeast monofunctional peroxisomal enoyl-CoA

isomerase (29). The crystal structures of these enzymes, except for that of 4-chlorobenzoyl-CoA dehalogenase, revealed the presence of a tight homotrimer with extensive interactions at the subunit-subunit interface. Residues, which contribute to these interactions, are conserved in other members of this super-family. Furthermore two trimers form a hexamer even though few inter-trimer contacts are involved. Active-site pockets of these enzymes are formed by residues of one subunit together with residues of the adjacent subunit in the same trimer (26, 28, 29). This situation raises the questions of how the activities of pECI, mECI and crotonase are related to their quaternary structures (see Fig.2). To answer this question, it would be important to also know the quaternary structure of mECI. Even though mECI belongs to the same enzyme super-family (25), previous studies with mECI are suggestive of a dimeric structure for this enzyme (15-19).

It is well known that β-oxidation in mitochondria is a tightly controlled metabolic process. Normally, only very small amounts of intermediates or no intermediates are present in mitochondria possibly due to the intermediate channeling (30), except when inhibitors are used or enzymes of β-oxidation are deficient. The formation of an intermediate by the beating rat heart perfused with elaidic acid, a *trans* fatty acid, prompted the investigation of elaidate β-oxidation in rat mitochondria (31). *Trans* fatty acids, such as *trans* monoenoic acids (elaidic acid), *trans* dienoic acids (conjugated linoleic acid) are geometrical isomers of the more common *cis* unsaturated fatty acids. There are two main sources of *trans* fatty

acids in the human diet: hydrogenated vegetable oils (e.g. margarines, shortenings, and baked goods) and meats and dairy products of ruminant (32). Concerns about the increasing consumption of *trans* fatty acids have been fueled by a number of clinical studies, which have shown that *trans* fatty acids, in contrast to the *cis* isomers, increase plasma levels of total cholesterol, triacylglycerols, LDL cholesterol, and atherogenic lipoprotein (33-35). Thus, intake of *trans* fatty acids may have an adverse effect on the plasma lipid profile and increase the risk for cardiovascular diseases (36). I have extended my study of the β-oxidation of unsaturated fatty acids to include unsaturated fatty acids with *trans* double bonds because of an interest in understanding the consequences of *trans* fatty acid consumption and to fully explore the molecular mechanisms of double bond metabolism during β-oxidation. Elaidic acid, 9-*trans*-octadecenoic acid, was chose as a substrate because it contains only one double bond and because it is the geometric isomer of oleic acid whose degradation by β-oxidation has been studied in detail (37). Moreover, it has been reported that elaidic acid is partially converted to 5-*trans*-tetradecenoic acid when it serves as a substrate in the perfused rat heart (31). That observation is surprising as mitochondrial β-oxidation is thought to go to completion without the accumulation of significant amounts of intermediates (30). The unusual leakage of an intermediate during the β-oxidation of a *trans* fatty acid and the potential for gaining a better understanding of how β-oxidation is coordinated in intact mitochondria have prompted this investigation

EXPERIMENTAL PROCEDURES

Materials— CoASH, FAD, NADH, ADP, steraroyl-CoA, oleoyl-CoA, elaidoyl-CoA, palmitoyl-CoA, pentadecanoyl-CoA, tetradecanoyl-CoA, dodecanoyl-CoA, decanoyl-CoA, octanoyl-CoA, hexanoyl-CoA, butyryl-CoA were purchased from Life Science Resources. Acyl-CoA oxidase from *Arthrobacter* species, CHAPS, fatty acid-free bovine serum albumin (BSA), 2,2-Dimethoxypropane, N-ethylmaleimide, phenazine methosulfate, dichlorophenolindophenol, Sephacryl S-200HR, Q Sepharose, Blue Sepharose CL-6B, DEAE-cellulose, Octyl-Sepharose CL-4B, CM-52, and all standard biochemicals were obtained from Sigma. Matrix Gel-Red A was bought from Amicon. A PCR Advantage kit was purchased from Clontech. PGEM-T Easy was obtained from Promega. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction endonucleases, T4 polynucleotide kinase, T4 ligase and T4 DNA polymerase were supplied by Biolabs. Hydroxylapatite, the dye reagent for protein assays, Sequi-Blot PVDF membrane, 4-20% polyacrylamide ready gels, the low M_r standard kit for SDS/PAGE, and materials for immunoblotting including the goat anti-rabbit IgG conjugated with alkaline phosphatase were all bought from Bio-Rad. Rabbit anti-serum against human pECI, mECI, and crotonase were raised by Pocono Rabbit Farms and Laboratory, Canadensis, PA. Boron trichloride in methanol was obtained from SUPELCO. 5-

trans-Tetradecenoic acid was kindly provided by Dr. Howard Sprecher, Ohio State University. 5-*cis*-Tetradecenoic acid was synthesized by Cayman Chemical (Ann Arbor, MI). Iodixanol (Optiprep) was from Nycomed Pharma AS, Oslo, Norway. Male Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. Burdick & Jackson (Muskegon, MI) was the source of ethyl ether. Hexane was bought from Fisher Scientific. Sep-Pak C_{18} cartridges and μBondapak C_{18} columns (30 cm × 3.9 mm) were purchased from Waters Associates. Rat liver very long chain acyl-CoA dehydrogenase (VLCAD) (38), bovine liver enoyl-CoA hydratase (crotonase) (39), rat liver enoyl-CoA isomerase (15), recombinant rat liver dienoyl-CoA isomerase (40), pig heart 3-ketoacyl-CoA thiolase (41) were purified by published procedures.

Synthesis of Substrates — 3-*cis*-Octenoic acid (42), acetoacetyl-CoA (43), and crotonyl-CoA (44) were synthesized according to published procedures. 2-*trans*-Tetradecenoic acid was synthesized by reacting malonic acid with *n*-dodecanal as described in principle by Linestead *et al* (45). 3-*trans*-Octenoyl-CoA, 3-*cis*-octenoyl-CoA, 5-*cis*-tetradecenoyl-CoA, 5-*trans*-tetradecenoyl-CoA, and 2-*trans*-tetradecenoyl-CoA were synthesized from their acidic forms by the mixed-anhydride method as described by Fong and Schulz (46). 2-*trans*-Tetradecenoyl-CoA was partially converted to L-3-hydroxytetradecanoyl-CoA by hydration in the presence of crotonase in 0.1 M KP_i (pH 8.0). 3-Keto-hexadecanoyl-CoA was synthesized as described (47). All products were purified by high-performance

liquid chromatography (HPLC). The pH values of the acyl-CoA derivatives were adjusted approximately to 3–4, and the concentration of thioesters was determined by the method of Ellman (48) after cleaving the thioester bond with hydroxylamine at pH 7.

Chromatography on a Hydroxylapatite (HAP) Column — Tissue segments (4g portions) from rat liver, and/or heart were homogenized (20%, w/v) with a POLYTRON tissue homogenizer in 10 mM K_3PO_4 buffer, pH7.4, containing 0.2M KCl, 0.5 mM EDTA, 1mM benzamidine (BA) and 0.5 mM dithiothreitol (DTT) (buffer A). Extracts were sonicated and centrifuged at 100,000 *g* for 1hr. The supernatants were dialyzed against buffer B (20 mM K_3PO_4, pH7.0, 0.5mM BA and 0.5 mM DTT). After dialysis, samples were applied to hydroxylapatite columns (1.5 cm x 15 cm) equilibrated with buffer B at a flow rate of 10ml/h. Proteins bound to the column were eluted with a linear gradient of KPi (20 to 500 mM, pH 7.0) in 200ml.

Immunoblotting — Twenty-five μl of protein samples from fractions eluted from the HAP column were combined with 25 μl of SDS sample buffer and boiled for 5 min before being loaded onto an SDS-PAGE ready gel. After electrophoresis at 90 V for 90 min, proteins in the gel were transferred onto a nitrocellulose membrane by semi-dry blotting (49). The membrane was soaked in 20 mM Tris-HCl (pH 7.4), 0.9% NaCl (TBS) containing 5% nonfat milk at 37 degree for 1h,

then washed with TBS buffer containing 0.2% nonfat milk, incubated with a 1000 fold diluted rabbit antiserum raised against pECI, or mECI, or MFE1 under gentle shaking. After washing with TBS buffer, the membrane was incubated for 90 min with 3000 fold diluted goat anti-rabbit IgG cross-linked with alkaline phosphatase, then developed with the buffer containing the enzyme substrates: nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine until bands appeared on the membrane.

Partial Purification of pECI from Rat Heart — Frozen rat hearts were homogenized in 5-fold (w/v) excess of 10 mM K_3PO_4, pH7.4, containing 0.2M KCl, 0.5 mM EDTA, 1mM BA and 0.5 mM DTT (buffer A). Rat heart extracts were sonicated and centrifuged at 100,000g for 1hr. Then the supernatants were dialyzed against buffer B (20 mM K_3PO_4, pH7.0, 0.5mM BA and 0.5 mM DTT). After dialysis, the samples were applied to hydroxylapatite columns (1.5 cm x 15 cm) equilibrated with buffer B at a flow rate of 10ml/h. The proteins bound on the column were eluted with a linear gradient of KPi (20 to 500 mM, pH7.0) in 200ml. After assaying the fractions for enoyl-CoA isomerase with 3-*cis*- and 3-*trans*-octenoyl-CoA and after immunoblotting, fractions containing pECI were pooled, concentrated, and dialyzed against 20 mM KPi, pH 6, containing 1 mM BA, 1mM DTT, and 10% glycerol (buffer C). The protein sample was then loaded onto a CM-52 column equilibrated with buffer C. After washing the column with buffer C, a gradient consisting of 100 ml of buffer C and 100 ml of buffer C plus 0.75 M

sodium chloride was applied for elution. Active fractions were collected and concentrated. After overnight dialysis against 10 mM KPi (pH 8.0), 0.3 mM EDTA, 15% glycerol (buffer D), the sample was applied to a Blue Sepharose CL-6B column (1.5 × 10 cm) equilibrated with buffer D. The column was eluted with a linear gradient from 0 to 0.6 M KCl in buffer D. The active fractions were combined, concentrated, and further purified by gel filtration on a Sephacryl S-200 HR column (1.5 cm x 30 cm) with a buffer containing 20 mM KPi, pH 7.0, 0.5 mM BA, 0.5 mM DTT, and 10% glycerol. The most active fractions were pooled and concentrated prior to storage at –80 °C. The purity of ECI peak was checked by SDS-PAGE with at least 25μg of protein.

N-Terminal Sequencing of Proteins — N-terminal amino acid sequencing of partially purified rat pECIs, and purified mECI was performed by Stephen Bobin at Dartmouth College Molecular Biology Core Facility after SDS-PAGE, and transferring the desired material to Sequi-Blot™PVDF membranes.

Cloning, Expression and Purification of Recombinant Rat Liver mECI — Rat mitochondrial enoyl-CoA isomerase was cloned, expressed, and purified to homogeneity for crystallization. Briefly, rat liver Marathon-Ready cDNA (CLONTECH) was used as the template for cloning the cDNA of the putative mature form of mECI (corresponding to amino acids Ala26 or Phe29 through Gly289) by touch-down PCR according to the protocol of CLONTECH. The

primers used were 5'-CAGGATCCCATATGGCGCGTCGCTTCTCTAACAAGC-3' or 5'-CAGGATCCCAT ATG TTC TCT AAC AAG CGG GTG TTGG-3' and 5'-CAGTAAGCTTATCAGCCCTTCTTTTGCTTGAGCTT-3'. The PCR product was inserted into vector pGEM-T Easy and amplified. Thereafter, it was subcloned into the *Bam*HI-*Hin*dIII site of vector pND-1 (a gift from Dr. Didier Negre) to obtain an expression plasmid. This plasmid was used to transform *E. coli* BL21 (DE3) pLysS cells. The transformants were grown in LB media to an absorbance of about 1.0 at 600 nm and the expression of mECI was induced by the addition of 0.6 mM IPTG for 4 h. Cells were harvested by centrifugation at 3000×*g* for 5 min and stored at –80 °C. The supernatant of the cell pellet after sonication and centrifugation was loaded onto a hydroxylapatite column that had been equilibrated overnight with 20 mM KPi, pH 7, containing 1mM BA, 1mM DTT, and 10% glycerol (buffer A). The column was washed with buffer A for 2 hrs at 60 ml/hr. Thereafter the column was developed with a gradient consisting of 300 ml of buffer A and 300 ml of 0.5 M KPi, pH 7, containing 1 mM BA, 1mM DTT, and 10% glycerol at a rate of 30 ml/hr. Active fractions were pooled, concentrated to about 15 ml, and dialyzed against 50 mM KPi, pH 6, containing 1 mM BA, 1mM DTT, and 10% glycerol (buffer B) for CM-52. The recombinant mECI was then loaded onto a CM-52 column equilibrated with buffer B. After washing the column with buffer B, a gradient consisting of 100 ml of buffer B and 100 ml of buffer B plus 0.75 M sodium chloride was applied to elute mECI. Active fractions were collected and concentrated to about 0.2 mg/ml. After chromatography on the CM-52 column,

the sample was diluted 4-fold with buffer C (35 mM KPi, pH 7, containing 5 mM 2-mercaptoethanol, 0.5 mM BA, and 10% glycerol), then loaded onto a Matrix Gel-Red A column (1.5 cm x 3.5 cm) which had been equilibrated with buffer C overnight. After washing the column with buffer C, a gradient consisting of 150 ml buffer C and 150 ml of buffer C plus 2 M potassium chloride was applied to elute mECI. Active fractions were collected and concentrated to 2.8 ml. The mECI sample was further purified by gel filtration on a Sephacryl S-200 HR column (2.5 cm x 44 cm) with a buffer containing 20 mM KPi, pH 7.25, and 1mM EDTA. The most active fractions were pooled and concentrated to about 6 mg/ml prior to storage at –80 °C.

Purification of Rat Liver Crotonase and Recombinant Pig Liver L-3-Hydroxyacyl-CoA Dehydrogenase (HAD) — Rat liver crotonase (39) and 3-hydroxylacyl-CoA dehydrogenase were purified according to the methods described previously (50).

Examination of the Interactions among Rat Liver mECI, Crotonase, and 3-Hydroxyacyl-CoA Dehydrogenase by Chromatography on Hydroxylapatite — Approximately 3ml of recombinant mECI (7.25 U/ml, 8u/mg) was mixed with 1ml of rat liver crotonase (1885 U/ml, 321 U/mg) and 2ml pig liver 3-hydroxyacyl-CoA dehydrogenase (390.5 U/ml, 164.1 U/mg). Then the enzymes mixture was diluted

to 50 ml with 20 mM KPi (pH 7), containing 1mM BA, 1mM DTT, and kept at 4^0 C for 3 hrs. The KPi concentration was adjusted to 0.15 M with 0.55M KPi (pH 7) and glycerol was added to a final concentration of 10% (v/v). The mixture was concentrated to about 5 ml with an Amicon concentrator. Then sample was applied to a hydroxylapatite column (1.5 cm X 6cm) equilibrated overnight with 20 mM KPi (pH7) buffer containing 1mM BA, 1mM DTT, 10% glycerol (buffer A). The column was washed with buffer A for 2 hrs at 30 ml/hr, and eluted with a gradient consisting of 120 ml buffer A and 120 ml of buffer A containing 0.5 M KPi (pH7) at a rate of 10 ml/hr. Fractions were assayed for the activities of mECI, crotonase and 3-hydroxyacyl-CoA dehydrogenase.

Rat Liver Mitochondrial Respiration Measurements - Rat liver mitochondria were isolated as described by Nedergaard and Cannon (51) from male Sprague-Dawley rats (240-260 g) kept on a standard chow and then fasted for 24 h before the isolation of mitochondria. For respiration measurements, 1.5 mg of rat liver mitochondria were incubated in 1.9 ml of incubation buffer containing 20 mM Tris-HCl (pH7.4), 4 mM KP_i, 0.1 M KCl, 4 mM $MgCl_2$, 0.1 mM EGTA, BSA (0.5mg/ml), and 0.5 mM L-malate. L-Carnitine (1 mM) was added to the mixture and after preincubation for 2 min. Respiration was initiated by the addition of 15 μM of the indicated fatty acyl-CoA and 1 mM ADP to achieve state 3 respiration.

Rates of respiration were measured polarographically with a Clark oxygen electrode attached to a YS-oxygraph.

Analysis of Acyl-CoAs Present in the Mitochodrial Matrix - When fatty acyl-CoAs were analyzed that are present in the mitochondrial matrix, the incubation mixture used for respiration measurements was scaled up to 20 ml. The reaction was terminated by the addition of 25 ml of methanol at the indicated time. After addition of 15 nmoles of pentadecanoyl-CoA as internal standard, the suspension was centrifuged at 17,500 × *g* for 15 min. The supernatant was diluted five-fold before it was passed slowly through a C_{18} Sep-Pak column. The bound CoA derivatives were eluted with 2.5 ml methanol. The extraction process was repeated, and 1 ml of 50 mM ammonium phosphate (pH 5.5) was added to the combined methanolic extracts. After removal of methanol under a stream of N_2 or by reduced pressure, samples were applied to a μBondapak C_{18} reverse-phase column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H_2O (9:1, v/v) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 20% to 80% in 30 min at a flow rate of 2 ml/min. Metabolites were quantified by integrating areas under the peaks by use of the Millennium software from Waters Corporation and by using the determined recovery (~35%) of the internal standard to calculate concentrations of acyl-CoAs. To identify the major metabolite of elaidoyl-CoA, the appropriate HPLC fractions

were collected and freed of acetonitrile by evaporation under reduced pressure. The resultant aqueous solution was mixed with an equal volume of 4 N KOH and kept at room temperature for 1 hr to hydrolyze the thioester bond. The mixture was adjusted pH to 1 with concentrated H_2SO_4 and extracted three times with ether. The combined extracts were dried over anhydrous Na_2SO_4 and filtered. After removal of ether by evaporation under a stream of N_2, the residue was dissolved in 1 ml boron trichloride in methanol (12% w/w) and 2,2-dimethoxypropane (100 µl). After this mixture was heated at 60^0C for 10 min, 1 ml of H_2O was added to terminate the reaction. The mixture was extracted three times with 1 ml of hexane each. The combined hexane extracts were dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under N_2 and the residue was subjected to GC-MS analysis. For the spectrophotometric identification of the major metabolite of elaidoyl-CoA, the HPLC-purified compound, presumably 5-*trans*-tetradecenoyl-CoA, was sequentially reacted in phosphate buffer (pH 8) with 0.1 unit of acyl-CoA oxidase, 8 mU of enoyl-CoA isomerase, and 4 mU of dienoyl-CoA isomerase. Absorbance spectra were recorded between 200nm and 400nm to follow the progress of the reactions and to document the final UV absorbances.

Identification of 5-trans-Tetradecenoic Acid and 5-trans-Tetradecenoyl-L-carnitine after Incubation of Rat Liver Mitochondria with Elaidoyl-CoA – For identification of 5-*trans*-tetradecenoic acid, the incubation mixture used for respiration measurements with rat liver mitochondria was scaled up to 20 ml. After

incubation for 5 min with elaidoyl-CoA, oleoyl-CoA, or stearoyl-CoA and ADP, the reaction was terminated by the addition of 4 ml of 1.2 N HCl. Twenty μg of pentadecanoic acid were added as an internal standard. Ether of the highest purity was used to extract free fatty acids. The ether extract was dried over anhydrous sodium sulfate and evaporated under a stream of nitrogen. The sample was dissolved in CH_2Cl_2 and directly analyzed by GC-MS, or was analyzed after conversion of acids to methyl esters by treatment with boron trichloride in methanol. Aliquots of 1 μl of the fatty acid methyl esters were injected at 250 °C into a GC/MS instrument (Shimadzu Scientific Instruments) consisting of a gas chromatograph (model GC-17A) interphased with a mass spectrometer (QP-5000) and equipped with a capillary column (30 m, ID: 0.25 mm, film thickness: 0.25 μm; EC-5, Alltech Associates Inc., Deerfield, IL). The oven temperature was raised from 100 °C to 230 °C at 5 °C/min, to 300 °C at 20 °C/min and then held constant for 6 min. The mass spectrometer served as a detector and was operated at 280 °C. For the identification of 5-*trans*-tetradecenoyl-L-carnitine, the incubation mixture used to measure respiration was scaled up to 10 ml. After incubation for 5 min with ADP and 15 μM elaidoyl-CoA, oleoyl-CoA, or stearoyl-CoA, the reaction was terminated by the addition of 2 ml of 1.2 N HCl and 2.5 μl of 2 mM myristoyl-L-carnitine in CH_3OH/H_2O (3:1,v/v) was added as internal standard. After keeping the mixture for 5 min at 4°C, the pH was adjusted to ~5 and the incubation was continued for another 5 min. The resulting suspension was centrifuged at 17,500 x g for 15 min and the supernatant was collected for acylcarnitine measurements.

Acylcarnitines were analyzed as butyl esters by stable isotope dilution electrospray ionization tandem mass spectrometry (ESI MS/MS) using a modification of the method of Rashed et al. (52). Deuterium labeled acylcarnitines (0.2 pmol of C4-C14 and 0.4 pmol of C16-C18) were added as internal standards to 15 µl of neutralized extract and centrifuged. The supernatant was dried under a stream of nitrogen, then butylated by reacting it with 0.1 ml of 3 N HCl in butanol at 55°C for 15 min. The sample was dried under a stream of nitrogen and dissolved in 0.15 ml of acetonitrile/water (4:1, v/v). Twenty µl were used for flow injection analysis by ESI MS/MS. Data was acquired by precursor ion scanning of the common product ion at *m/z* 85 for labeled and unlabeled acylcarnitines. The concentrations of acylcarnitines were calculated from the peak intensities of unlabeled acylcarnitines relative to labeled internal standards.

Expression and Purification of Rat Long Chain Acyl-CoA Dehydrogenase (LCAD) - A cDNA insert corresponding to the mature form of rat LCAD and its expression vector were prepared by PCR as described (53). *E.coli* strain XL1-Bla transformed with this plasmid was grown in LB media at 30^0C in the presence of ampicillin to an absorbance of about 1.0 at 600 nm, and then induced in the presence of 0.6 mM isopropyl-β-D-thiogalactoside for 6 h. Cells were harvested by centrifugation for 20 min at 8000 × *g*, and pellets were suspended in 10 mM Tris-HCI (pH7.5) buffer containing 10% glycerol, 0.3 mM EDTA, and 1 mM FAD (buffer A). The suspension was sonicated 10 times for 20 s each at 4^0C, and then

centrifuged at 100,000 × *g* for 1 h. The supernatant was loaded onto a DEAE-Sepharose column (2.5 × 40 cm) that had been equilibrated overnight with buffer A. After washing the column with buffer A, the column was eluted with a linear gradient from 0 to 300 mM NaCl in buffer A. Fractions were assayed for long-chain acyl-CoA dehydrogenase. Active fractions were pooled, concentrated in an Amicon concentrator with a YM-10 membrane, and dialyzed against 50 mM KP_i (pH 7.6) containing 10% glycerol, 0.3 mM EDTA and 1μM FAD (buffer B). The sample was applied to a hydroxylapatite column (2.5 × 25 cm) equilibrated with buffer B and eluted with a linear gradient from 50 to 400 mM KP_i (pH 7.6) in buffer B. Active fractions were identified, collected and concentrated. After overnight dialysis against 10 mM KP_i (pH 8.0), 0.3 mM EDTA, 15% glycerol (buffer C), the sample was applied to a Blue Sepharose CL-6B column (1.5 × 10 cm) equilibrated with buffer C. The column was developed with a linear gradient from 0 to 0.6 M KCl in buffer C. Active fractions were combined, concentrated, and dialyzed against 10 mM KP_i (pH 7.5), 0.3 mM EDTA, and 20% glycerol (buffer D). The sample was loaded onto a Q Sepharose column (0.5 × 5 cm) previously equilibrated with buffer D. The column was developed with a linear gradient from 0 to 0.5 M KP_i in buffer D. Active fractions were pooled, concentrated to 0.6 ml, diluted with glycerol to ~ 1 ml, and stored at -80^0C.

Partial Purification of Mitochondrial Thioesterase and CPT II from Rat Liver Mitochondria – For the partial purification of thioesterase, rat liver mitochondria

were purified by gradient density centrifugation in a self-generated gradient of iodixanol (Optiprep). Equal volumes of iodixanol (50%, w/v) containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Mops-NaOH (pH 7.4) and a suspension of heavy mitochondria were mixed (final iodixanol concentration = 25%; ρ = 1.150g/ml) and then transferred to 10 ml tubes and centrifuged at 180,000 g_{av} for 3 h in a T865-1 fixed angle rotor at 4 °C using the slow acceleration and braking modes. Fractions were collected from the bottom after slowly inserting a thin glass tube through the bottom of the tube. Catalase and malate dehydrogenase were assayed as marker enzymes for peroxisomes and mitochondria, respectively. Fractions containing most mitochondria were combined and diluted 2-fold with MST isolation buffer (210mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA) before they were harvested by centrifugation at 17,500 × g for 20 min. The mitochondrial pellet was suspended, dialyzed against MST isolation buffer for at least 4-6 hours, and then sonicated 6-times for 20 s each at intervals of 30 s to keep the temperature of the suspension at 4 °C. The suspension of sonicated mitochondria was centrifuged for 1 h at 100,000 × g and the resultant supernatant was brought to 70% saturation with ammonium sulfate. Precipitated proteins were collected by centrifugation at 15,000 × g for 10 min, dissolved in 3 ml of 20 mM KP_i (pH 7.0) containing 0.5 mM BA, 0.5 mM DTT, 10% glycerol, 30 mM NaCl, and applied to a Sephacryl S-200HR column (2.5 × 40 cm). After elution with the same buffer, active fractions were identified, pooled, concentrated to 0.6 ml, and stored at –80 °C. For the purification of CPT II, mitochondria (1.3 mg/ml) in 20

mM KP_i (pH 7.4) containing 1 M KCl (buffer A) were treated with 0.2% CHAPS for 10 min at 4°C. The resultant suspension was centrifuged at 1,500 x g for 10 min and the supernatant was applied to an Octyl Sepharose CL-4B column (1x4 cm) equilibrated with buffer A containing 0.2% CHAPS. CPT II was eluted with buffer A containing 1% CHAPS and active fractions were pooled, concentrated, and stored at –80°C.

Enzyme and Protein Assays— Spectrophotometric assays were performed on a Gilford, model 260, recording spectrophotometer at 25°C. Enoyl-CoA isomerase, crotonase, and L-3-hydroxyacyl-CoA dehydrogenase (HAD) activities were measured as described by Binstock and Schulz (54). For enoyl-CoA isomerase, a standard assay mixture contained 0.2 M KPi, pH 8.0, 325uM NAD^+, 100 μM CoASH, 35 μM 3-*cis*, or *trans*-octenoyl-CoA, coupling enzymes, and an aliquot of the sample. A molar extinction coefficient of 6220 $M^{-1}cm^{-1}$ at 340 nm was used to calculated rates. The crotonase activity was measured at 263 nm by the direct assay method. A standard assay mixture contained 0.2 M KPi, pH8.0, 50μM crotonyl-CoA, and an aliquot of the sample. A molar extinction coefficient of 6700 $M^{-1}cm^{-1}$ was used to calculate rates. The HAD activity was measured at 340 nm with acetoacetyl-CoA as substrate. A standard assay mixture contained 0.15M KPi, pH7.0, 100 μM NADH, 30μM substrate, and an aliquot of the sample. Both rat liver mitochondrial thioesterase and CPT II activities were determined by measuring the release of CoASH from acyl-CoA with Ellman's reagent (48) at 412

nm on a Hitachi, model U-3010, spectrophotometer. A typical assay mixture for thioesterase contained 0.175 M KPi, 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DNTB, Ellman's regent), and 20 μM acyl-CoA. For CPT II, the assay mixture contained 115 mM Tris-HCl, 0.1% Triton X-100, 0.11 mM DNTB, 1.1 mM L-carnitine and 35μM acyl-CoA at pH 8.0. The extinction coefficient used to calculated rates for both enzymes was 13,600 $M^{-1}cm^{-1}$. The need to correct rates was most pronounced when partially purified enzymes were used, which generally contain proteins that have sulfhydryl groups. Acyl-CoA dehydrogenase was assayed spectrophotometrically at 600 nm with phenazine methosulfate as the primary electron acceptor and dichorophenolindophenol as the final electron acceptor (55). Using tetradecanoyl-CoA, 5-*cis*-tetradecenoyl-CoA and 5-*trans*-tetradecenoyl-CoA as substrates, kinetic parameters of rat LCAD and VLCAD were determined by the fluorometric assay with 1mM electron transferring flavoprotein (ETF) as electron acceptor as detailed by Mohsen and Vockley (56). Data were analyzed by nonlinear curve fitting using the Sigma plot program. One unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate to product per min. Protein concentrations were determined by the dye binding assay as described by Bradford (57) with bovine serum albumin (BSA) as standard.

RESULTS

Part I: Enoyl-CoA Isomerases in the Rat

Characterization of Mitochondrial Long-chain Δ^3, Δ^2-Enoyl-CoA Isomerase - Mitochondrial long-chain enoyl-CoA isomerase had been detected by Kilponen *et al.* (11) who separated it from mECI and peroxisomal MFE1 by chromatography on hydroxylapatite (Fig. 3A). However, they did not purify it any further. Because this enoyl-CoA isomerase was more active with 3-*trans*-dodecenoyl-CoA than with 3-*trans*-hexenoyl-CoA as substrate, they named it long-chain enoyl-CoA isomerase. They also concluded that it was a mitochondrial enzyme. I repeated their experiment by separating a soluble extract from rat liver by chromatography on hydroxylapatite. However, I assayed each fraction with 3-*trans*-octenoyl-CoA and 3-*cis*- octenoyl-CoA because the ratio of these two activities aids in distinguishing between mECI and pECI. The former isomerase is more active with 3-*cis*-enoyl-CoAs as substrates, whereas the later enzyme prefers substrates with a *trans* double bonds. Shown in Fig. 3B is the result of this experiment. The activity pattern obtained with 3-*trans*-octenoyl-CoA as substrate was similar to that observed by Kilponen *et al.* (11). But the activity pattern revealed with 3-*cis*-octenoyl-CoA as substrate was quite different. The isomerase activity present in fractions 13-18 in Fig. 3B is easily missed, whereas the existence of two isomerase activities,

presumably corresponding to MFE1 and mECI, in fractions 20-40 was more clearly revealed than with the 3-*trans* substrate. A *trans/cis* activity ratio of close to 2 determined for fractions 13-16 was similar to that of pECI, which has a *trans/cis* activity ratio of 2 in contrast to mECI and MFE1 with *trans/cis* activity ratios below 1. The presence of pECI in fractions 13-18 was confirmed by immunoblotting (data not shown). Thus, it seems that mitochondrial long-chain enoyl-CoA isomerase is identical with pECI.

Characterization of pECI in the Rat --- pECI was reported to be a monofunctional peroxisomal enoyl-CoA isomerase (14) while long-chain Δ^3, Δ^2-enoyl-CoA isomerase was identified as a mitochondrial enzyme (11). These reports do not agree with my hypothesis that pECI and long-chain enoyl-CoA isomerase are the same enzyme. In order to resolve this contradiction, the subcellular localization of rat pECI was again determined. Rat pECI was detected in a rat liver homogenate by immunoblotting with the antiserum raised against human pECI. Rat pECI was also detected in a heart homogenate after chromatography on hydroxylapatite by enzyme assay and immunoblotting (Fig.4). Because rat heart contains few peroxisomes, the enzyme is most likely present in heart mitochondria. Hence, the subcellular localization of rat pECI seems to be different from that of pECI in human fibroblast (14). My further experiments proved that rat pECI could be detected in both purified rat liver peroxisomes and mitochondria (Fig.4B). Careful examination of the N-terminal sequence of human pECI revealed a number

of basic and hydrophobic residues that may form a positively charged helix, which is the characteristic feature of a putative mitochondrial targeting signal (58). Overall, these data agree with the notion that mitochondrial long-chain enoyl-CoA isomerase and rat pECI are the same enzyme which is present in both rat liver mitochondria and peroxisomes. Therefore, this isomerase will be referred to as Δ^3,Δ^2-enoyl-CoA isomerase (ECI).

Since this type of mammalian ECI has never been purified from natural sources, and possibly more than one mature form of rat ECI exists in different organelles of rat heart and liver, purification of these isoforms from their organelles was necessary. Purification of ECI from a rat heart mitochondria extract was attempted by chromatography on hydroxylapatite to first separate ECI from mECI, crotonase, MFE1 and other proteins. Then the fractions containing ECI were pooled, concentrated, and purified by other chromatographic methods, such as chromatography on CM52, Sephacryl S-200, and Blue Sepharose CL-6B. The purity of the sample after those purification steps was checked by SDS-PAGE. Unfortunately this enzyme was only partially purified. However, it was sufficiently pure to separate it from other impurities by SDS-PAGE and transfer the desired material to a Sequi-Blot^{TM} PVDF membrane for N-terminal sequencing. The ten N-terminal amino acids of rat heart ECI were found to be ATQQDFGNAG. This sequence is highly homologous to that of the mouse pECI (ASQQDFENAL) (14).

Based on this information, the rat ECI cDNA could be identified and amplified for further cloning of this protein.

Quaternary Structure Study of Recombinant Rat Liver mECI - Mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase (mECI, EC5.3.3.8) is an important enzyme in mitochondrial β-oxidation of unsaturated fatty acids. Its unprocessed precursor is comprised of 289 amino acid residues with a molecular mass of 32,254 Da (19). Its N-terminal amino acid residue is protected and therefore has not been determined. An attempt to sequence mECI purified from rat liver confirmed the previous conclusion that the N-terminal residue is protected but also provided slight evidence that the mature enzyme has an N-terminal Ala or Phe. Based on this evidence and the suggestion by Stoffel (19) that the mature mECI begins with Ala 26, the cDNA sequences coding for Ala 26 to Gly 289 and for Phe 29 to Gly 289 were generated by molecular cloning, and the corresponding proteins were expressed in *E. coli*. Since the recombinant protein Phe 29 → Gly 289 did not bind to CM-52, whereas recombinant protein Ala 26 → Gly 289 did bind as did the mature enzyme isolated from mitochondria, it was concluded that the mature protein extends from Ala 26 to Gly 289. The level of expression of mature mECI was low. Efforts were made to purify this protein by chromatography on hydroxylapatite, CM-52, Matrix Gel-Red A and Sephacryl S-200 HR. The final product was homogenous as judged by SDS-PAGE (Fig.5). A total amount of 4.1 mg of purified mECI was obtained after 78-fold purification (Table 2). The yield

was 15% and the specific activity of this preparation was 14.1 U/mg, which was slightly higher than the activity of the enzyme isolated from rat liver and assayed by the same method (62).

To date, all crystal structures of enzymes belonging to the hydratase/isomerase super-family are trimers or dimers of trimers (26-29). However, rat liver mECI was reported to be a dimer based on gel filtration and ultracentrifugation measurements (15-19) even though it belongs to the same super-family. Human mECI was also proposed to be a homodimer (59). Analysis of recombinant rat liver mECI by chromatography on Sephacryl S-200 HR (Fig.6) was indicative of a native molecular weight close to 65 kDa, a value that suggests the presence of two subunits with a subunit molecular weight close to 30 kDa. Rat liver mECI was crystalized in 1992 by Zeelen et al. (60), but no detailed structural information was obtained due to the low resolution. Recently, Dr. Hubbard and Dr. Kim in Medical College of Wisconsin have been successful to grow crystals of the recombinant rat liver mECI produced by me. Its crystal structure has been solved and clearly shows that rat liver recombinant mECI is a trimer, in which the general features of this superfamily of enzymes are conserved, such as the N-terminal domain fold, oxyanion hole, and CoA binding site (Fig.7).

Do Interactions between mECI, Crotonase and/or 3-Hydroxyacyl-CoA Dehydrogenase (HAD) Affect Their Activities? – When enoyl-CoA isomerase

isoenzymes from rat heart were separated by chromatography on hydroxylapatite, an unusual distribution of mECI activity was observed (Fig.8). The second peak of ECI activity, from fraction 30 to fraction 50 in Fig.8A, corresponds to mECI because of its higher activity with 3-*cis*-octenoyl-CoA than with 3-*trans*-octenoyl-CoA. This conclusion is also confirmed by immunoblotting using antiserum raised against mECI (Fig.8B). However, the ratio of mECI activities measured with *cis*- and *trans*-substrates changed dramatically across these fractions. For example, fraction 38, which contains the highest quantity of mECI according to immunoblotting, has the lowest ratio of ECI activities with 3-*cis*-octenoyl-CoA versus 3-*trans*-octenoyl-CoA but has the highest crotonase activity. The changing ratio of enoyl-CoA activities may be due to an unknown enoyl-CoA isomerase isoenzyme, or perhaps may reflect an intermolecular interaction between mECI and crotonase or HAD which may affect their activities. Efforts have been made to find a reasonable explanation. For this purpose, the elution of mECI from a hydroxylapatite column was studied with purified mECI in the presence of crotonase or/and HAD. First, I analyzed separately the elutions of recombinant rat liver mECI, crotonase, and pig liver HAD by hydroxylapatite chromatography. Then mixtures of two or three enzymes were examined by chromatography on hydroxylapatite to determine if the changing activity ratios of mECI with 3-*trans*, and *cis*-enoyl-CoAs (Fig.8) are due to intermolecular interactions. Fig.9 demonstrates that the activity profile of mECI was not affected by either crotonase

or HAD when probed by elution from hydroxylapatite. Thus, the activity pattern shown in Fig.8 most likely is not due to mECI interacting with crotonase or HAD.

Part II: β-Oxidation of Elaidic Acid

Respiration Rates of Rat Mitochondria with cis and trans Monounsaturated Fatty Acyl-CoAs as Substrates – The perfused rat heart was observed to convert part of elaidic acid to 5-*trans*-tetradecenoic acid, which appears to be a sufficient energy source for sustaining the heart beat (31). In an effort to compare the effectiveness of elaidic acid with oleic acid and stearic acid as substrates of mitochondrial β-oxidation, I measured respiration rates of coupled rat liver and heart mitochondria with several fatty acyl-CoAs as substrates. As shown in Fig.10, stearoyl-CoA and elaidoyl-CoA supported equal rates of respiration in rat liver mitochondria while oleoyl-CoA sustained a rate that was approximately 50% higher than the rates observed with the other two substrates. Rates obtained with myristoyl-CoA, 5-*cis*-tetradecenoyl-CoA, and 5-*trans*-tetradecenoyl-CoA were higher than the rates supported by any of the three longer chain acyl-CoAs. In agreement with a previous report (61), elaidoyl-CoA was degraded in rat heart mitrochondria at a rate that was 50% and 33% lower than rates observed with oleoyl-CoA and stearoyl-CoA, respectively (results not shown). This data indicates that elaidic acid is an adequate substrate of β-oxidation in both rat liver and heart mitochondria.

Detection and Identification of 5-trans-Tetradecenoyl-CoA in Rat Mitochondria Incubated with Elaidoyl-CoA – In an effort to detect possible differences between the β-oxidation of elaidoyl-CoA and oleoyl-CoA, coupled rat liver mitochondria were incubated with either elaidoyl-CoA, oleoyl-CoA, or stearoyl-CoA at state 3 respiration and fatty acyl-CoAs were analyzed by HPLC. Since no CoA was added to the incubation mixture, fatty acyl-CoAs other than the substrate were overwhelmingly, if not completely, present in the mitochondrial matrix. Shown in Fig.11 are HPLC chromatograms of fatty acyl-CoAs that were isolated from rat liver mitochondria after incubation with stearoyl-CoA (Fig.11A), oleoyl-CoA (Fig.11B), or elaidoyl-CoA (Fig.11C) for 2 min. One major peak in each chromatogram corresponds to the fatty acyl-CoA that served as substrate. A second peak marked "IS" is due to pentadecanoyl-CoA that was added as an internal standard after the incubation was completed to facilitate the quantification of metabolites. However in Fig.11C, a third prominent peak is visible, which, based on its elution time, was tentatively identified as 5-*trans*-tetradecenoyl-CoA ($t\Delta^5C14$). After further purification by HPLC, this material was hydrolyzed and converted to its methyl ester. Analysis of the methyl ester by gas chromatography/mass spectrometry proved this material to be a homogeneous compound with a molecular ion at m/z of 258 as expected of methyl tetradecenoate upon chemical ionization in the presence of ammonia (data not shown). The material presumed to be 5-*trans*-tetradecenoyl-CoA had a UV spectrum with an absorbance maximum around 260 nm that is typical of an acyl-CoA (see spectrum

#1 in Fig.12). Upon treatment with acyl-CoA oxidase, spectrum #1 changed to spectrum #2. The observed increase in the absorbance around 260 nm agrees with the expected conversion of 5-*trans*-tetradecenoyl-CoA to 2,5-*trans*-tetradecadienoyl-CoA. Treatment of the latter compound with enoyl-CoA isomerase yielded spectrum #3 that is characteristic of 3,5-dienoyl-CoAs. Finally, when the compound, presumed to be 3,5-tetradecadienoyl-CoA, was incubated with dienoyl-CoA isomerase, spectrum #3 changed to spectrum #4 with absorbance maxima at 260 nm and 300 nm, which is characteristic of 2,4-dienoyl-CoA. Taken together, the data demonstrate that 5-tetradecenoyl-CoA accumulated in the matrix of mitochondria that oxidized elaidoyl-CoA. Because the intermediate most likely retained the original *trans* double bond, it was assumed to be 5-*trans*-tetradecenoyl-CoA. The amount of this metabolite after 1 to 2 min of incubation was 0.88 ± 0.22 nmol/mg of mitochondrial protein. When oleoyl-CoA served as a substrate, a metabolite with chromatographic behavior identical to that of authentic 5-*cis*-tetradecenoyl-CoA was observed (see Fig.11B). However, the putative *cis* metabolite was present at a level 10-times lower than the *trans* compound (compare Figs.11B and 11C) and therefore no attempt was made to further identify it. Altogether, it is very likely that the metabolite of oleoyl-CoA was 5-*cis*-tetradecenoyl-CoA. Tetradecanoyl-CoA (myristoyl-CoA), the corresponding metabolite of stearoyl-CoA, was not detected and therefore did not accumulate at a level that was higher than the background noise.

Kinetics of the Dehydrogenation of 5-trans- and 5-cis-Tetradecenoyl-CoA and Myristoyl-CoA by Long-chain (LCAD) and Very Long-chain Acyl-CoA Dehydrogenases (VLCAD) – The observed accumulation of 5-*trans*-tetradecenoyl-CoA in the matrix of rat liver mitochondria raised the question as to the cause for the built-up of this metabolite. I investigated the possible role of long chain acyl-CoA dehydrogenase (LCAD) and very long chain acyl-CoA dehydrogenase (VLCAD) in the accumulation of Δ^5-*trans*-tetradecenoyl-CoA. Elaidoyl-CoA is an 18-carbon fatty acyl-CoA with one *trans*-double bond at the 9-position. After two cycles of β-oxidation, it is converted to Δ^5-*trans*-tetradecenoyl-CoA. The built-up of Δ^5-*trans*-tetradecenoyl-CoA indicates that its dehydrogenation catalyzed by acyl-CoA dehydrogenases may be the rate-limiting step (62) of the β-oxidation reactions of elaidoyl-CoA. Therefore the 5-*trans* double bond of 5-*trans*-tetradecenoyl-CoA may interfere with the rapid dehydrogenation of this compound. Four acyl-CoA dehydrogenases, named short-chain, medium-chain, long-chain, and very long-chain acyl-CoA dehydrogenase according to their chain-length specificities, cooperate to catalyze the dehydrogenation of β-oxidation. Only two of the four acyl-CoA dehydrogenases may act on 5-*trans*-tetradecenoyl-CoA. One is long-chain acyl-CoA dehydrogenase (LCAD), which is active with acyl-CoAs having acyl chains with 6 to 20 carbon atoms. The other is very long-chain acyl-CoA dehydrogenase (VLCAD), which acts on acyl-CoAs with acyl chains having 10 to 24 carbon atoms (63). To study the kinetics of these two enzymes, a recombinant rat LCAD was prepared and purified to avoid interferences by other acyl-CoA

dehydrogenases. Rat liver VLCAD was partially purified from rat liver mitochondria. It is a mitochondrial membrane protein that can be easily separated from mitochondrial matrix enzymes. The mature form of rat LCAD was obtained by molecular cloning and expression in *E.coli*. A cellular extract containing LCAD was purified by chromatographies on DEAE-cellulose, hydroxylapatite, Blue-Sepharose CL-6B, and Q-Sepharose. Even though the level of expression was low, LCAD was purified to near homogeneity and approximately 1.1mg of LCAD was obtained (0.81units/mg) (Table 3). Fig.13 shows the purity of recombinant LCAD at different stages of purification. Using tetradecanoyl-CoA, 5-*cis*-tetradecenoyl-CoA and 5-*trans*-tetradecenoyl-CoA as substrates, kinetic parameters of rat LCAD and VLCAD were determined by the fluorometric assay with 1mM electron transferring flavoprotein as the electron accepter (56). The results of this kinetic study, presented in Table 4, indicate that there is no significant difference between the V_{max} values of rat LCAD with the three substrates. But the catalytic efficiency (k_{cat}/K_m) of LCAD was 4-times lower with 5-*trans*-tetradecenoyl-CoA as substrate than with 5-*cis*-tetradecenoyl-CoA or tetradecanoyl-CoA (myristoyl-CoA). This lower catalytic efficiency is due to a 4-fold higher K_m for 5-*trans*-tertatedecenoyl-CoA as compared to the K_m values for the other two substrates. In contrast VLCAD acted equally well on the *cis* and *trans* isomers of 5-tetradecenoyl-CoA, which, however, were poorer substrates of this enzyme than was tetradecanoyl-CoA. The latter observation agrees with a previous report showing that saturated

acyl-CoAs are better substrates of VLCAD than the corresponding unsaturated substrates with 4,5- or 5,6-double bonds (64).

Identification of 5-trans-Tetradecenoic Acid as a Product of Elaidate β-Oxidation in Rat Liver Mitochondria – The reported formation of 5-*trans*-tetradecenoic acid in rat hearts perfused with elaidic acid (31) suggested that 5-*trans*-tetradecenoyl-CoA, a metabolite of elaidate β-oxidation, may be hydrolyzed in mitochondria and the resultant free fatty acid may exit from cells. To test this idea, isolated rat liver mitochondria were incubated with elaidoyl-CoA, oleoyl-CoA, or stearoyl-CoA for 5 min and the resultant acidic products were extracted and analyzed by gas chromatography/mass spectrometry (GC/MS). Shown in Fig. 14 are the gas chromatograms of the acids extracted after incubating mitochondria with elaidoyl-CoA or oleoyl-CoA. Only the incubation of mitochondria with elaidoyl-CoA yielded a compound, marked C14:1 acid, with an elution time identical to that of 5-*trans*-tetradecenoic acid (see Fig.14A&B). The mass spectrum of this compound was virtually identical with that of 5-*trans*-tetradecenoic acid (compare panels C & D of Fig.14). Hence 5-*trans*-tetradecenoyl-CoA, which is formed by β-oxidation of elaidoyl-CoA in mitochondria and accumulates in the matrix, was hydrolyzed. It was estimated that 1-2% of the elaidoyl-CoA present in the incubation mixture was converted to 5-*trans*-tetradecenoic acid. The hydrolysis of 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix requires an acyl-CoA thioesterase that was detected in the soluble extract from rat liver mitochondria.

Fig.15 shows the substrate profile of a partially purified preparation of this thioesterase(s). The enzyme was most active with substrates having acyl chains with 12 and 14 carbon atoms and hence is best classified as a long-chain acyl-CoA thioesterase. The enzyme was highly active with 5-tetradececenoyl-CoA, although slightly more so with the *cis* than the *trans* isomer. Intermediates of β-oxidation, e.g. 2-*trans*-tetradecenoyl-CoA, 3-hydroxytetradecanoyl-CoA, and 3-ketohexadecanoyl-CoA, were poorer substrates than regular fatty acyl-CoAs of equal chain length. K_m and V_{max} values for the thioesterase-catalyzed hydrolysis of tetradecanoyl-CoA (myristoyl-CoA) were determined to be 12.6±0.8 μM and 22.8±0.6 milliunits/mg, respectively, and for the hydrolysis of 5-*trans*-tetradecenoyl-CoA 7±1 μM and 12.6±0.6 milliunits/mg, respectively. Thus, the catalytic effeciency of this enzyme is unaffected by the presence of the 5-*trans* double bond.

Formation of 5-trans-Tetradecenoylcarnitine During the β-Oxidation of Elaidic Acid in Rat Liver Mitochondria – The observed hydrolysis of 5-*trans*-tetradecenoyl-CoA in mitochondria raised the question as to whether the 5-*trans*-tetradecenoyl residue also is transferred to carnitine? To answer this question, rat liver mitochondria were incubated with elaidoyl-CoA or oleoyl-CoA for 5 min and the resultant aqueous phases after deproteination were analyzed by tandem mass spectrometry to identify acylcarnitines. The mass spectrum of acylcarnitines that were detected after incubating mitochondria with elaidoyl-CoA (see Fig.16A)

shows a peak labelled C14:1 that corresponds to tetradecenoylcarnitine with an m/z of 426.4. Also detected was a peak that corresponds to elaidoylcarnitine with an m/z of 482.5. All other acylcarnitines, including myristoylcarnitine (C14) with an m/z of 428.4 and a mixture of deuterated acylcarnitines marked by astericks were added as internal standards at the end of the incubation period or prior to derivatizing the acylcarnitines. Most important was the demonstration that tetradecenoylcarnitine (C14:1), presumably 5-*trans*-tetradecenoylcarnitine, was formed during the β-oxidation of elaidoyl-CoA (see Fig.16B), whereas β-oxidation of oleoyl-CoA did not yield such metabolite (see Fig.16C). It was estimated that 4-5% of elaidoyl-CoA present in the incubation mixture was converted to 5-*trans*-tetradecenoylcarnitine. The latter compound was most likely formed from 5-*trans*-tetradecenoyl-CoA by carnitine palmitoyltransferase II (CPT II) in the mitochondrial matrix. To confirm this idea, CPT II was partially purified from rat liver mitochondria under conditions that resulted in the inactivation of CPT I (65). This preparation of CPT II was used to determine kinetic properties of this enzyme at a fixed concentration of 1 mM carnitine. The apparent K_m values for tetradecanoyl-CoA and 5-*trans*-tetradecenoyl-CoA as substrates were found to be 21.6±2 μM and 10.6±1.3 μM, respectively, while the corresponding V_{max} values were 286±10 milliunits/mg and 387±17 milliunits/mg, respectively. Thus, the catalytic efficiency of CPT II is approximately 3-times greater with 5-*trans*-tetradecenoyl-CoA than with tetradecanoyl-CoA as substrate.

An attempt was made to estimate the relative rates at which 5-*trans*-tetradecenoyl-CoA was hydrolyzed and converted to the carnitine derivative in the mitochondrial matrix. For this purpose, activities of CPT II and thioesterase were measured in extracts of rat liver mitochondria at one concentration of 5-*trans*-tetradecenoyl-CoA. The values thus obtained together with the kinetic parameters determined for the partially purified enzymes with the same substrate were used to estimate activities of the two enzymes as a function of the concentration of 5-*trans*-tetradecenoyl-CoA (see Fig.17). The data show that the conversion of 5-*trans*-tetradecenoyl-CoA to 5-*trans*-tetradecenoylcarnitine is favored by a factor of 1.5 over the hydrolysis of 5-*trans*-tetradecenoyl-CoA to 5-*trans*-tetradecenoic acid (see Fig.17). This conclusion agrees with the observed greater accumulation of 5-*trans*-tetradecenoylcarnitine than 5-*trans*-tetradecenoic acid.

In conclusion, during the β-oxidation of elaidic acid (9-*trans*-octadecenoic acid) in rat mitochondria, 5-t*rans*-tetradecenoyl-CoA accumulates in the mitochondrial matrix. 5-*trans*-Tetradecenoyl-CoA accumulates because the 5-*trans* intermediate is dehydrogenated less efficiently than the 5-*cis* isomer by long-chain acyl-CoA dehydrogenase (LCAD). At the elevated concentration of 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix, this intermediate is either hydrolyzed to 5-*trans*-tetradecenoic acid by thioesterase or converted to 5-*trans*-tetradeceoylcarnitine by CPT II (see Fig.18).

DISCUSSION

Identification of the Mitochondrial Long-chain Δ^3, Δ^2-Enoyl-CoA - The results of this investigation prove that at least two enoyl-CoA isomerases each are present in mitochondria and peroxisomes of rat liver. The surprising conclusion reached during their characterization was that one of these isomerases, henceforth referred to as Δ^3,Δ^2 -enoyl-CoA isomerase or ECI, has a dual subcellular localization in mitochondria and peroxisomes. Moreover, this isomerase is identical with peroxisomal enoyl-CoA isomerase (14), pECI, and is indistinguishable from mitochondrial long-chain enoyl-CoA isomerase (11). When initially identified and characterized, pECI was shown to be located predominantly in peroxisomes of human fibroblasts (14). It remains to be determined whether the apparent absence of this enzyme from human mitochondria is characteristic of all human cells or is a unique feature of human fibroblasts. The identification of ECI as the mitochondrial long-chain enoyl-CoA isomerase is based on the limited amount of information published about the latter enzyme (11). Specifically, the immunological characterization of mitochondrial long-chain enoyl-CoA isomerase after its separation from other isomerases by chromatography on hydroxylapatite and its preference for long-chain substrates support the conclusion that this isomerase and ECI are the same enzyme. It is unclear why this enzyme was not detected in peroxisomes during the previous investigation (11). It could be the consequence of

its low activity compared with the isomerase activity of MFE1 in peroxisomes isolated from livers of clofibrate-treated rats. ECI was expected to be present in peroxisomes because human isomerase has a C-terminal serine-lysine-leucine peroxisomal targeting sequence, whereas the mouse enzyme has a proline-lysine-leucine signal (14). The signal responsible for directing this enzyme to the mitochondrial matrix has not yet been identified. However, the N-terminal 16 residues of ECI are predicted to form an α-helix with amphiphatic properties, which may facilitate the entry of this protein into mitochondria.

Quaternary Structure of Rat Liver mECI - The unprocessed precursor of rat liver mECI was comprised of 289 AA (19). But the primary structure of the mature form of mECI had not been determined because its N-terminal residue is protected. N-terminal sequencing of mature mECI from rat liver confirmed that mECI seems to be protected because only a weak signal was obtained when the protein was subjected to sequencing by Edman degradation. Ninety percent of mECI amenable to sequencing was composed of 264 amino acid residues starting with Ala 26. This sequence agrees with the sequence of mature mECI as suggested by Stoffel (19). Ten percent of mECI started with Phe 29, an observation consistent with the result of Palosaari, who reported the mature mECI contained 261 amino acids (66). Based on this information, two mature mECIs were cloned. Both clones, referred to as mECI-1 and mECI-2 were expressed, even though the levels of expression were low. But unlike mECI-1, recombinant mECI-2 behaved differently than natural

mECI upon chromatography on CM-52. Thus mECI-2 might have been truncated during its purification, thereby loosing its three N-terminal residues with two arginine residues. An analysis of the crystal structure of recombinant rat liver mECI demonstrates that it exists as a trimer as other members of hydratase/isomerase super-family. However, results of gel filtration and ultracentrifugation experiments suggested that this enzyme might be dimeric (15). Actually, it is difficult to distinguish between a dimmer and trimer by these techniques.

Possible Interactions of mECI with Crotonase and/or 3-Hydroxyacyl-CoA Dehydrogense (HAD) – It was speculated that the changing activity ratios of enoyl-CoA activities might be due to intermolecular interactions between mECI and crotonase and/or HAD. After simulating the isolation of mECI on hydroxylapatite chromatography by mixing purified mECI with crotonase or/and HAD and subjecting this mixture to chromatography on hydroxylapatite, no evidence was obtained for the existence of mECI forms that were distinguishable by different activity ratios with *trans* and *cis* substrates. Thus, it is unlikely that the activity pattern shown in Fig.8 is the result of mECI interacting with either crotonase or/and HAD. I also prepared an affinity column with antibodies to mECI as ligand to investigate the interaction of mECI with crotonase and/or HAD. The outcome was negative. No crotonase or HAD activities were retained when mECI was bound by the antibody column. Should such interations occur in intact cells, they are too

weak to be detected under the conditions used in these experiments. Thus, for the time being the cause for the variable ratio of ECI activities of mECI with *cis* and *trans* substrates remains unexplained.

Investigation of Intermediate Accumulation During the β-Oxidation of Elaidoyl-CoA in Rat Liver Mitochondria - This study was initiated with the aim of analyzing the mitochondrial β-oxidation of *trans* fatty acids at the molecular level. The reported formation of 5-*trans*-tetradecenoic acid from elaidic acid (9-*trans*-octadecenoic acid) in perfused rat hearts (31) prompted the idea that β-oxidation of *trans* fatty acids may be an atypical process because the substrate or part of the substrate was incompletely degraded. In contrast, oleic acid did not give rise to a partially degraded substrate. In spite of its incomplete degradation, elaidic acid seemed to be a sufficient energy source for the beating heart (31). Moreover, elaidoyl-CoA was observed to support high rates of respiration in isolated rat heart and liver mitochondria (see Ref. 61 and this study). Hence, the energy production in rat mitochondria does not seem to be compromised when elaidic acid serves as substrate even though part of it is not completely degraded. The premature termination of elaidate β-oxidation is most likely related to the accumulation of 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix. Apparently 5-*trans*-tetradecenoyl-CoA is more rapidly formed than degraded by β-oxidation, whereas the corresponding intermediates of common dietary fatty acids, including 5-*cis*-tetradecenoyl-CoA derived from oleic acid, are degraded without accumulating to a

significant extent. The most obvious reason for the accumulation of 5-*trans*-tetradecenoyl-CoA would be its slower dehydrogenation by LCAD or/and VLCAD as compared to its formation from elaidoyl-CoA by two cycles of β-oxidation (see Fig.18). A kinetic analysis revealed that 5-*trans*-tetradecenoyl-CoA is a poorer sustrate of LCAD than is 5-*cis*-tetradecenoyl-CoA or myristoyl-CoA. Moreover, both unsaturated fatty acyl-CoAs are poor substrates of VLCAD when compared with myristoyl-CoA. This data supports the conclusion that the presence of a 5-*trans* double bond in place of a 5-*cis* or no double bond in the substrate reduces the catalytic efficiency of LCAD. However, since the lower catalytic efficiency of LCAD with 5-*trans*-tetradecenoyl-CoA compared to its efficiency with the 5-*cis* isomer is due only to a higher K_m for the former substrate, an increase in the concentration of 5-*trans*-tetradecenoyl-CoA should eliminate or minimize the difference between the rates at which the two isomeric substrates are dehydrogenated. Thus, the accumulation of 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix is expected to cause the rate of its dehydrogenation to increase to a level that is achieved with a lower concentration of 5-*cis*-tetradecenoyl-CoA. This conclusion is supported by the observation that 5-*trans*-tetradecenoyl-CoA supports a rate of respiration that is only 20% lower than rates obtained with 5-*cis*-tetradecenoyl-CoA or myristoyl-CoA as substrates. Any remaining difference between the rates of elaidate and oleate β-oxidation may be due to the inhibitory effects of accumulated 5-*trans*-tetradecenoyl-CoA or to the adverse effect of the *trans* double bond on other reactions of β-oxidation.

Another consequence of the accumulation of 5-*trans*-tetradecenoyl-CoA is the effective competition of other enzymes for this intermediate, which thereby will be diverted from β-oxidation. As summarized in Fig.18, 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix is not only a substrate of LCAD and VLCAD but also of thioesterase and carnitine palmitoyltransferase II (CPT II). The hydrolysis by thioesterase yields 5-*trans*-tetradecenoic acid that is assumed to pass through the mitochondrial and cellular membranes to enter the circulation where it has been observed previously (31). Alternatively, it will move from the mitochondrial matrix into the cytosol where it can be activated by conversion to its CoA derivative and utilized for lipid synthesis, mitochondrial β-oxidation, or perhaps for protein acylation in place of myristoyl-CoA. The long-chain thioesterase activity present in rat liver mitochondria seems to be due to one enzyme that has been purified to apparent homogeneity and shown to be highly active with palmitoyl-CoA and myristoyl-CoA (67). The transfer of the 5-*trans*-tetradecenoyl residue from CoA to carnitine is catalyzed by CPT II that normally operates in the opposite direction to supply substrates for β-oxidation. The formation of 5-*trans*-tetradecenoylcarnitine is favored when the intramitochondrial concentration of medium-chain and long-chain acyl-CoAs is high (65). Both the hydrolysis of 5-*trans*-tetradecenoyl-CoA and its conversion to 5-*trans*-tetradecenoylcarnitine will only contribute significantly to the metabolism of elaidic acid at an elevated concentration of the intermediate because the K_m values of thioesterase and CPT II for 5-*trans*-tetradecenoyl-CoA are 4 to 10-times higher than the K_m values of LCAD and

VLCAD for the same substrate while the intramitochondrial activities of thioesterase and CPT II are lower than those of LCAD and VLCAD (see Ref. 64 and data from this study).

The partial degradation of elaidic acid to 5-*trans*-tetradecenoic acid and 5-*trans*-tetradecenoylcarnitine raises the question as to the fate of these metabolites and their possible impact on cellular metabolism and/or physiology. 5-*trans*-Tetradecenoylcarnitine can be converted back to 5-*trans*-tetradecenoyl-CoA by CPT II and degraded by β-oxidation either immediately or after leaving and reentering mitochondria in the same or other tissues. 5-*trans*-Tetradecenoic acid, however, must be reactivated by conversion to its CoA derivative in the cytosol before it can be utilized as substrate of β-oxidation, lipid synthesis, and possibly protein myristoylation. Its possible substitution for myristoyl-CoA in the modification of proteins, especially of G proteins, could affect the functions of such proteins in cell signaling because the presence of double bonds in the acyl chain has been shown to reduce the affinity of myristoylated proteins for lipid rafts (68, 69). In the retina, 5-*cis*-tetradecenoyl and 5-*cis*, 8-*cis*-tetradecadienoyl residues partially replace the myristoyl group in transducin and some other G proteins (reviewed in Ref. 70). This heterogeneous acylation of transducin and other retinal proteins is assumed to be physiologically significant although this idea remains to be proven. The incomplete degradation of elaidic acid raises the spector of other monounsaturated and polyunsaturated *trans* fatty acids yielding novel fatty acids by

partial β-oxidation. Because of continuing health concerns about the consumption of *trans* fatty acids, it seems prudent to study the β-oxidation of major *trans* fatty acids and to assess the biological effects of products that are formed by partial β-oxidation. Such evaluation should include conjugated linoleic acids (CLA) because they contain *trans* double bonds and are important constituents of the human diet due to their presence in dairy products, meat of ruminants, and partially hydrogenated vegetable oils. The observed incomplete degradation of a fraction of elaidic acid contradicts the general conclusion that under normal conditions mitochondrial β-oxidation facilitates the complete breakdown of fatty acids and proceeds without the accumulation of extensive amounts of intermediates (reviewed in Ref. 30). The accumulation of substantial quantities of partially degraded fatty acids has only been observed in cases of enzyme deficiencies (71, 72) or when inhibitors of β-oxidation enzymes or respiration were added to mitochondria (73). However, under such conditions β-oxidation usually is severely or completely inhibited. The prevailing view of mitochondrial β-oxidation is that of a system operating in a highly integrated fashion, perhaps as the result of intermediate channeling due an intramitochondrial organization of the enzymes of β-oxidation. It was assumed that the accumulation of intermediates would interfere with a high flux of fatty acids through β-oxidation. This concern does not seem to apply to the built-up of 5-*trans*-tetradecenoyl-CoA and by extension may not be a problem when acyl-CoAs that are substrates of acyl-CoA dehydrogenases accumulate in the mitochondrial matrix. The presumed reason for avoiding an accumulation of

significant amounts of intermediates in mitochondria during β-oxidation was their potential for inhibiting the process by inhibiting individual enzymes and/or depleting free CoA. This argument may apply to 2-enoyl-CoA, 3-hydroxyacyl-CoAs, and 3-ketoacyl-CoAs that have been shown to strongly inhibit certain enzymes of β-oxidation at micromolar concentrations (reviewed in Ref. 74). However, saturated fatty acyl-CoAs seem to be less toxic. Total depletion of free CoA would compromise β-oxidation by preventing the formation of fatty acyl-CoAs from their carnitine derivatives by CPT II and the thiolase-catalyzed cleavage of 3-ketoacyl-CoAs in the mitochondrial matrix. However, the likelihood of such situation developing may be small due to the regeneration of free CoA via the tricarboxylic acid cycle and by hydrolysis of acyl-CoAs

.

In conclusion, this study demonstrates that the accumulation of 5-*trans*-tetradecenoyl-CoA and possibly of other saturated fatty acyl-CoA intermediates does not reduce the effectiveness of mitochondrial β-oxidation. This conclusion changes our understanding of how this pathway operates and is essential for interpreting observations made when β-oxidation is compromised by inhibitions or deficiencies of enzymes.

Table 1.

Mammalian Δ^3, Δ^2-enoyl-CoA isomerases

Isoenzyme	Abbre-viation	Location	Function	Specific activity[a] (U/mg)
Short chain enoyl-CoA isomerase	mECI	Mitochondria	Enoyl-CoA isomerase	64 (7, 8)
Long chain enoyl-CoA isomerase	None	Mitochondria	Enoyl-CoA isomerase	N.D.
Enoyl-CoA hydratase (crotonase)	mECH	Mitochondria	Enoyl-CoA hydratase	0.2 (10)
Enoyl-CoA isomerase	pECI	Peroxisomes	Enoyl-CoA isomerase	27 (12)
Multifunctional enzyme 1	MFE 1	Peroxisomes	3-Hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA isomerase	5 (11)

[a]Measured with 35 μM 3-*trans*-octenoyl-CoA. N.D., not determined

Table 2

Purification of recombinant mitochondrial enoyl-CoA isomerase (mECI)

	Total protein (mg)	Total activity (U)	Spec. Act. (U/mg)	Yield (%)
Cell Extract	2208	390	0.18	100
HAP	57.7	108.7	1.9	28
CM-52	11.1	81.0	7.3	21
Matrix Gel-Red-A	6.2	65.1	10.5	17
Sephacryl-S-200HR	4.2	59.2	14.1	15

Table 3

Purification of recombinant rat long-chain acyl-CoA dehydrogenase (LCAD)

	Total protein (mg)	Total activity (U)	Spec. Act. (U/mg)	Yield (%)
Extract	722	28.9	0.04	100
DEAE-Sepharose	21.1	5.7	0.27	19.7
HAP	3.08	3.3	1.1	11.4
Blue-Sepharose CL-6B	1.2	1.2	1.1	4.2
Q-Sepharose	1.1	0.9	0.8	3.1

Table 4

Kinetic parameters of long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase (VLCAD) with tetradecanoyl-CoA (C14:0-CoA), 5-*cis*-tetradecenoyl-CoA (5c-C14:1-CoA), and 5-*trans*-tetradecenoyl-CoA (5t-C14:1-CoA) as substrates

Kinetic Const.	LCAD*			VLCAD*		
Substrate	C14:0-CoA	5c-C14:1-CoA	5t-C14:1-CoA	C14:0CoA	5c-C14:1-CoA	5t-C14:1-CoA
V_{max} (U/mg)	3.3±0.27	3.0±0.13	2.9±0.20	1.4±0.13	0.32±0.018	0.88±0.073
Km (μM)	0.41±0.14	0.40±0.06	1.60±0.23	0.57±0.19	0.44±0.091	0.97±0.28
k_{cat} (s^{-1})	9.9 ±0.8	9.0±0.4	8.7±0.6	2.0±0.2	0.42±0.02	1.12±0.09
k_{cat}/Km	24	22	5	4	1	1

*Fluorescence-based assay with ETF as the second substrate. For details see "Experimental Procedures".

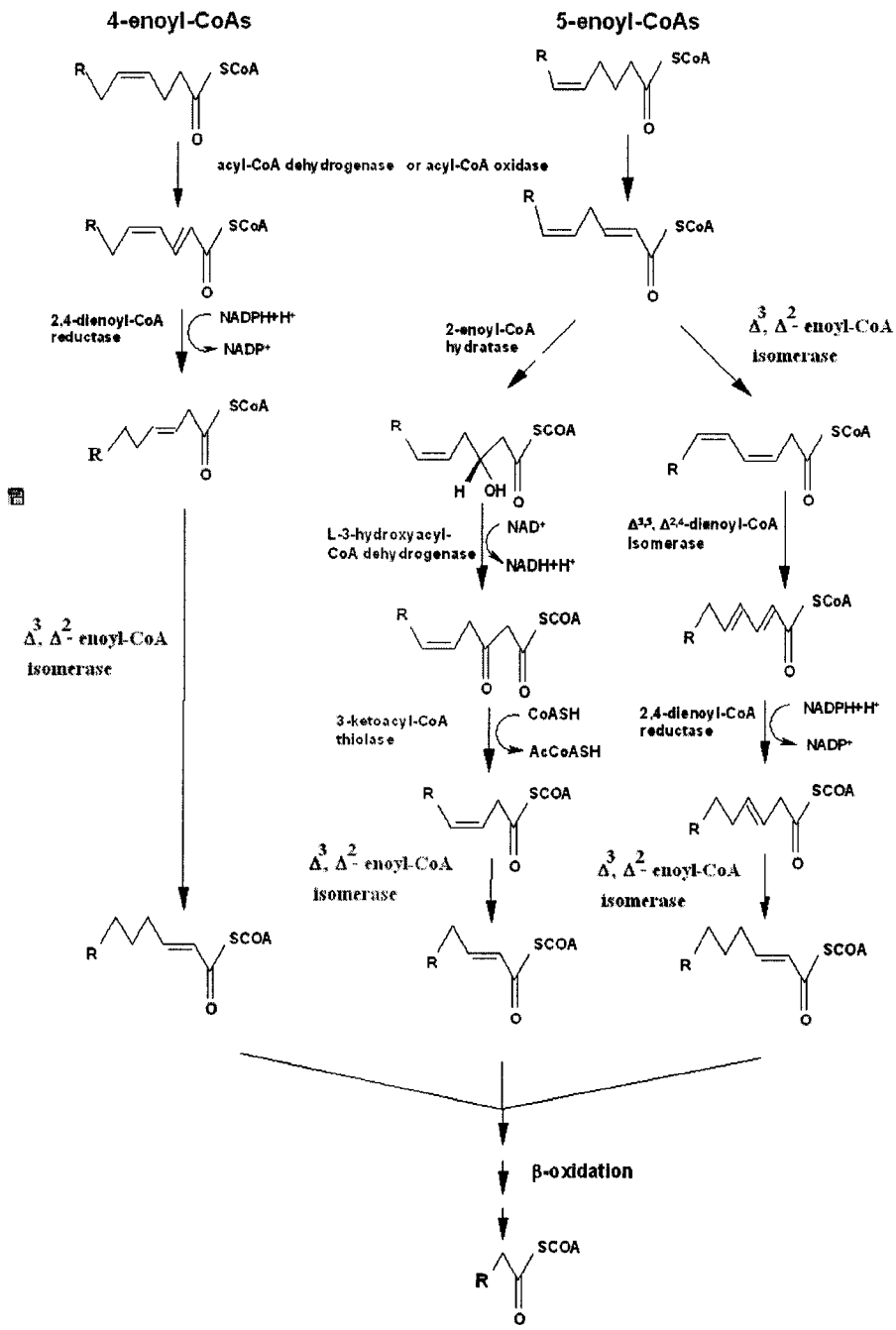


Fig.1. β-Oxidation of unsaturated fatty acids with even-numbered and odd-numbered double bonds.

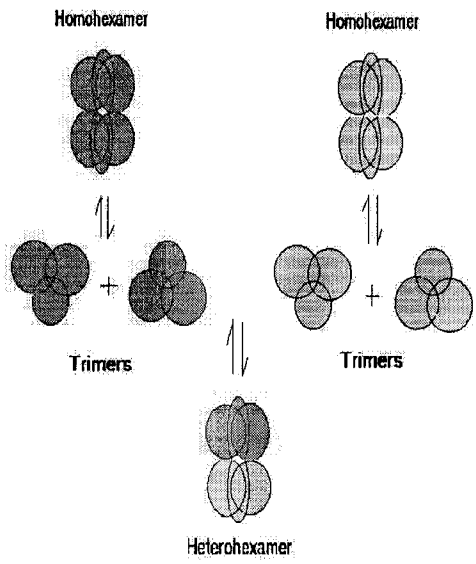


Fig.2. Diagram of the hypothetical generation of heterohexamers between members of the hydratase/isomerase super-family.

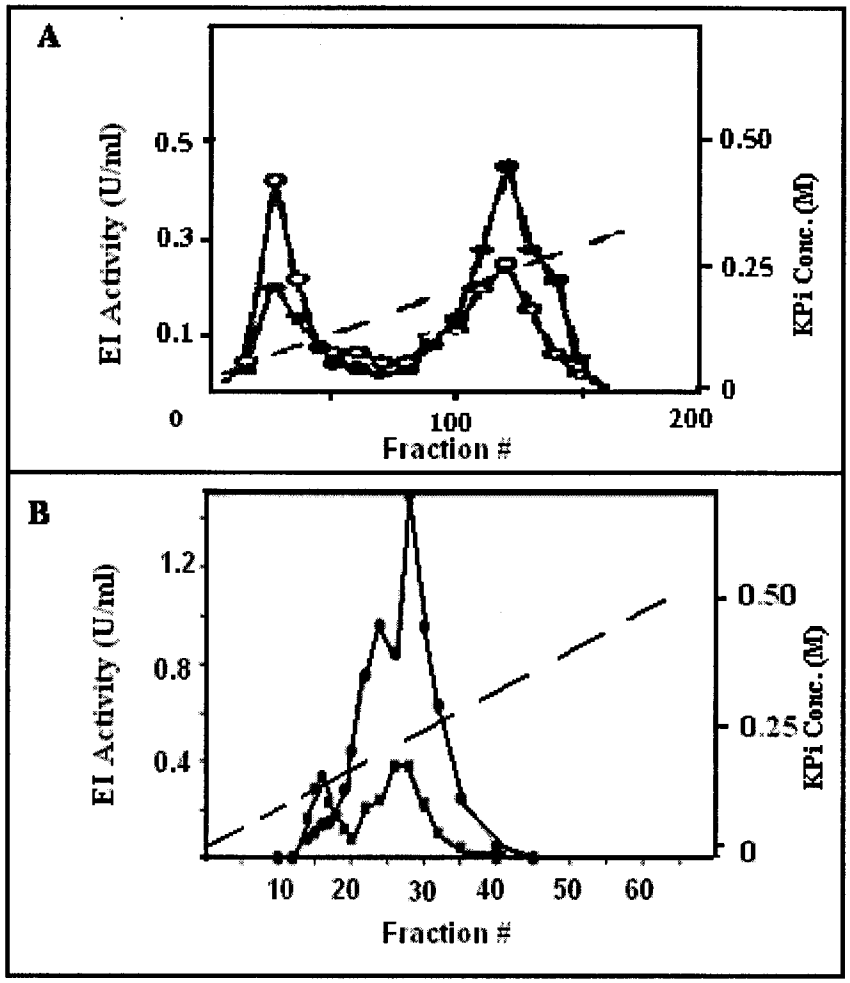


Fig.3. Elution profiles of ECI present in a rat liver homogenate after chromatography on hydroxylapatite.

A. Profile published in Ref. 11. Activitites were measured with either 3-*trans*-dodecenoyl-CoA (o) or 3-*trans*-hexenoyl-CoA (•) as substrate.

B. Profile obtained when fractions were assayed with 3-*trans*-octenoyl-CoA (■) and 3-*cis*- octenoyl-CoA as substrates (●).

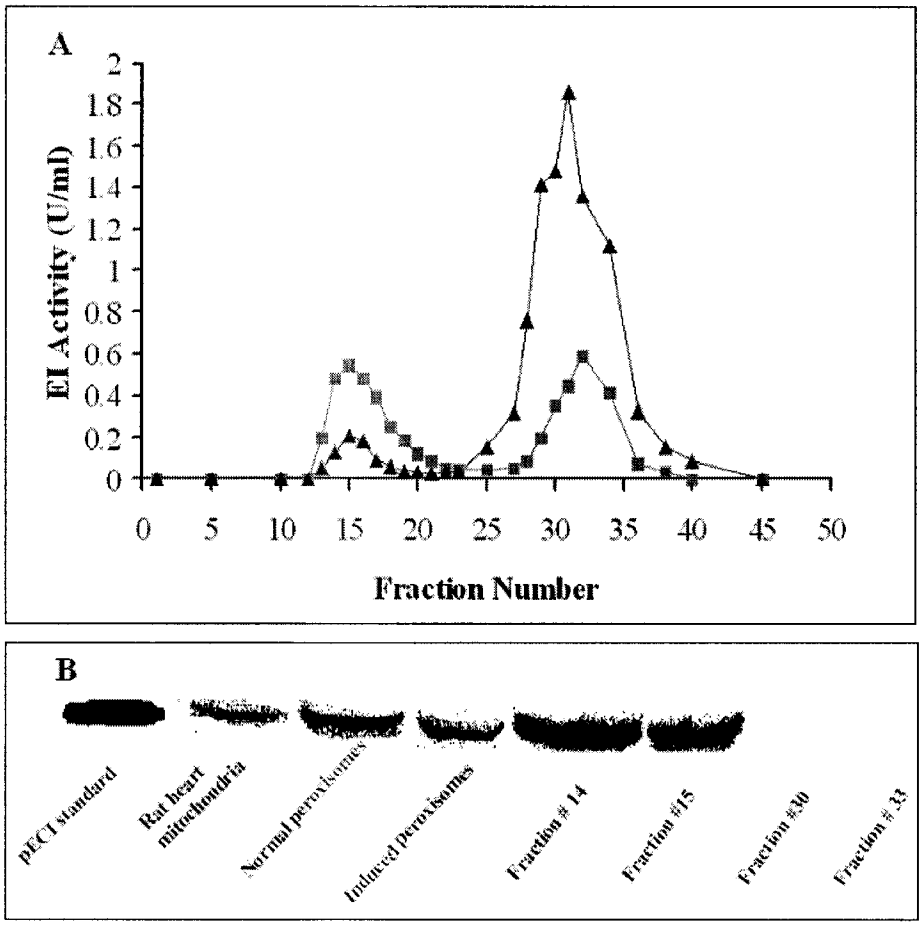


Fig.4. Detection of ECI in rat heart.

A. Elution profile of ECI present in a rat heart homogenate after chromatography on hydroxylapatite. Activities were measured with either 3-*trans*-octenoyl-CoA (■) or 3-*cis*-octenoyl-CoA (▲).

B. Immunoblotting of organelles and fractions with antiserum to human peroxisomal enoyl-CoA isomerase.

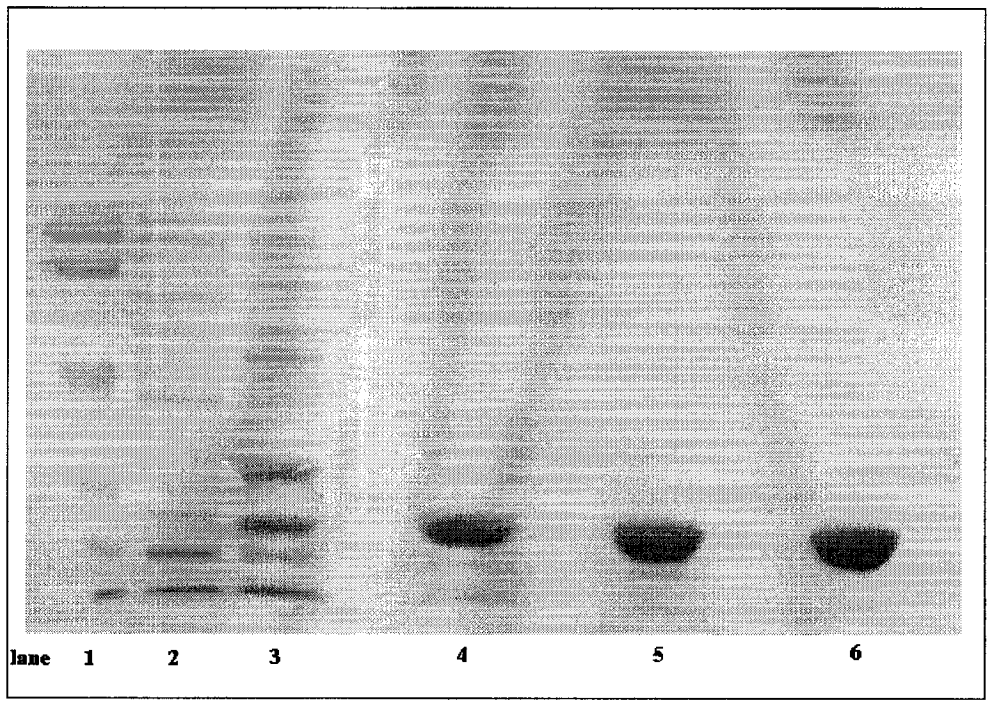


Fig.5. SDS-PAGE of recombinant rat liver mECI. Lane 1: prestained protein marker. Lane 2: mECI in cell extract. Lanes 3, 4, 5, 6: mECI purified after chromatographic steps on HAP, CM-52, Matrix Gel Red-A and Sephacryl S-200 HR, respectively.

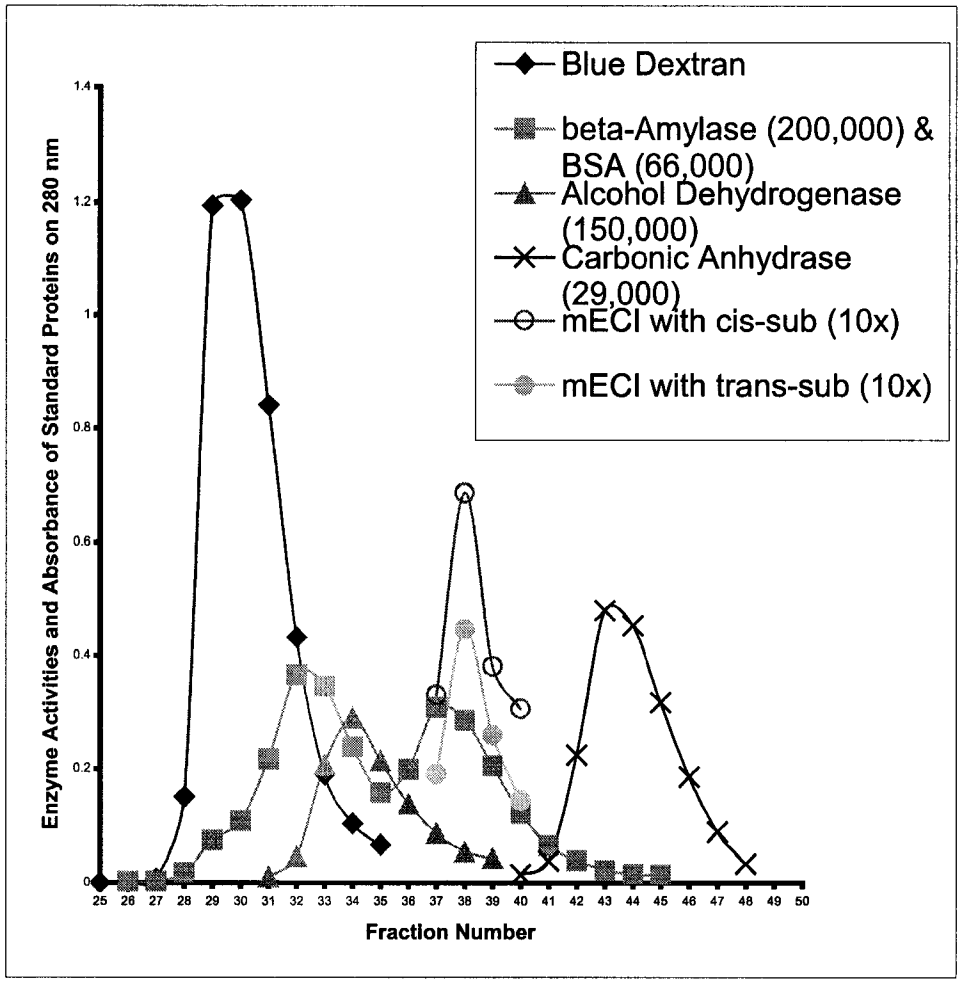
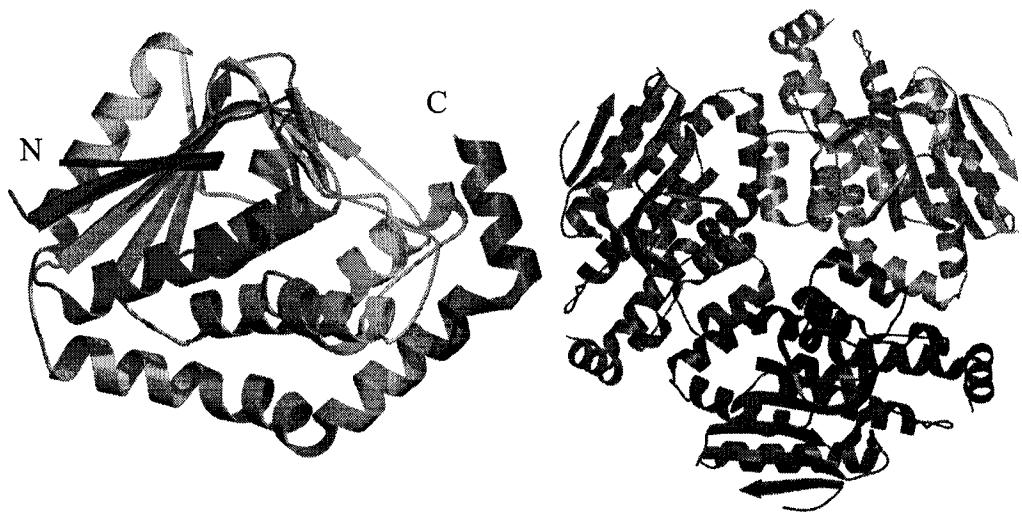


Fig.6. Estimation of the molecular weight of recombinant rat liver mECI by chromatography on Sephacryl S-200 HR



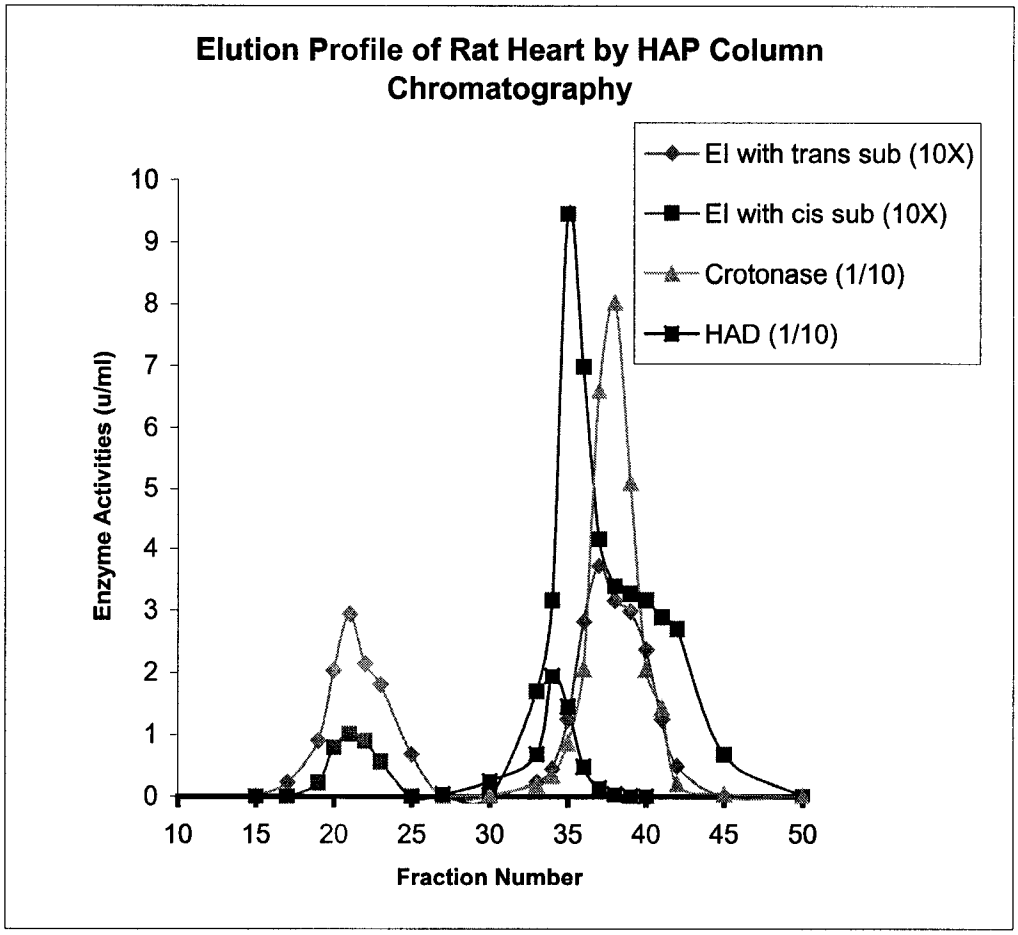
(A) **(B)**

Fig.7. Ribbon diagram showing the overall fold of recombinant rat liver mECI (*adopted from Hubbard, Yu, Schulz, and Kim, unpublished results*).

A, a single monomer, as found in the asymmetric unit. The color is ramped from blue, at the N-terminus, to red, at the C-terminus. Note the three α-helices of the C-terminal cat tail at the bottom and right of the figure.

B, the trimeric form of the enzyme, viewed down the 3-fold crystallographic axis. Each subunit is colored differently.

A



B

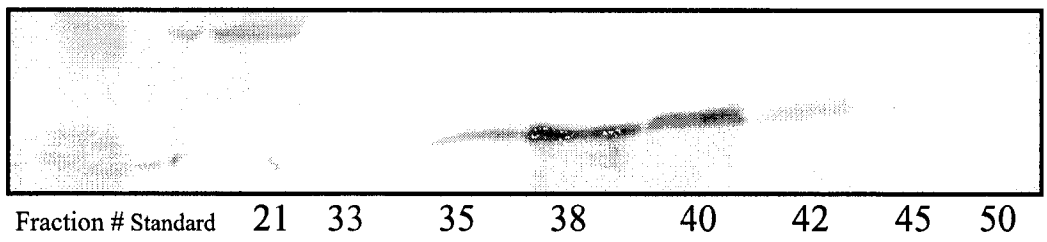


Fig.8

Fig.8. A. Separation of a rat heart homogenate by chromatography on hydroxyapatite. Fractions were collected and assayed for ECI, crotonase and 3-hydroxyacyl-CoA dehydrogenase activities. B. Immunoblotting of fractions (shown above) with antiserum to mECI

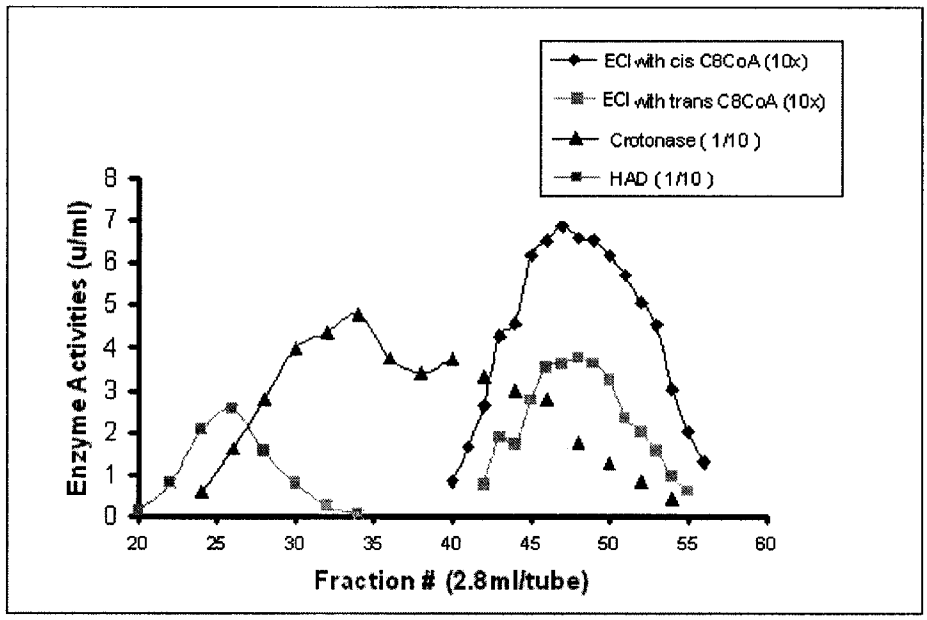


Fig.9. Separation of purified mECI, rat liver crotonase and pig liver HAD by chromatography on hydroxylapatite.

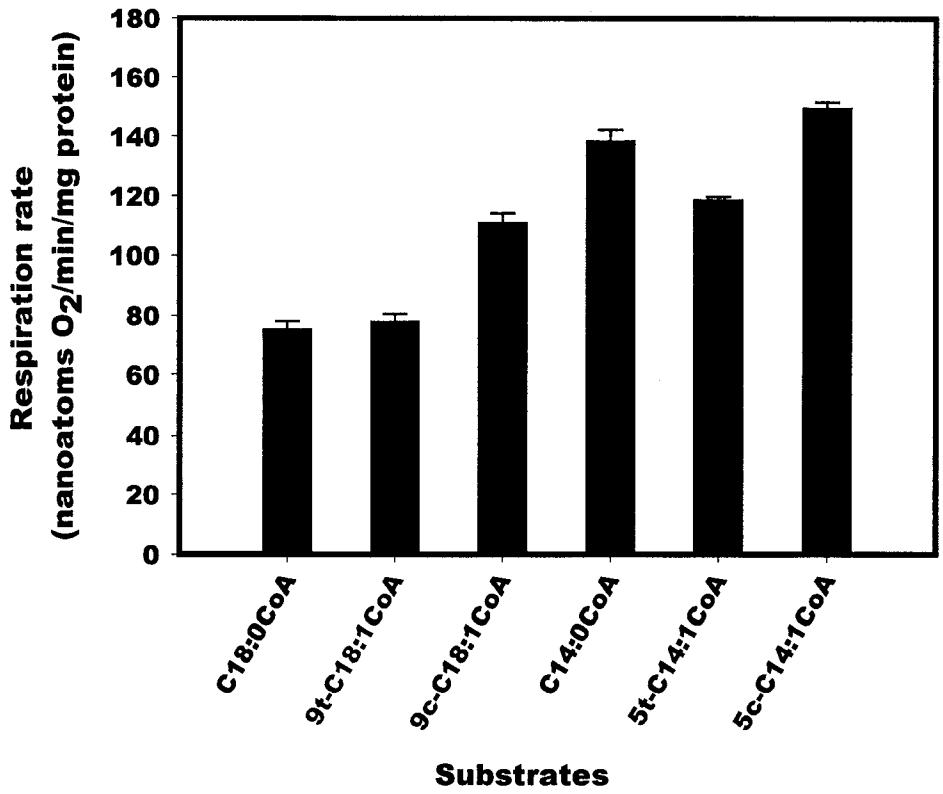


Fig.10. Respiration rates of rat liver mitochondria supported by β-oxidation. Substrates: C18:0CoA, stearoyl-CoA; 9t-C18:1CoA, elaidoyl-CoA; 9c-C18:1CoA, oleoyl-CoA; C14:0CoA, myristoyl-CoA; 5t-C14:1CoA, 5-*trans*-tetradecenoyl-CoA; 5c-C14:1CoA, 5-*cis*-tetradecenoyl;-CoA. For experimental details see "Experimental Procedures".

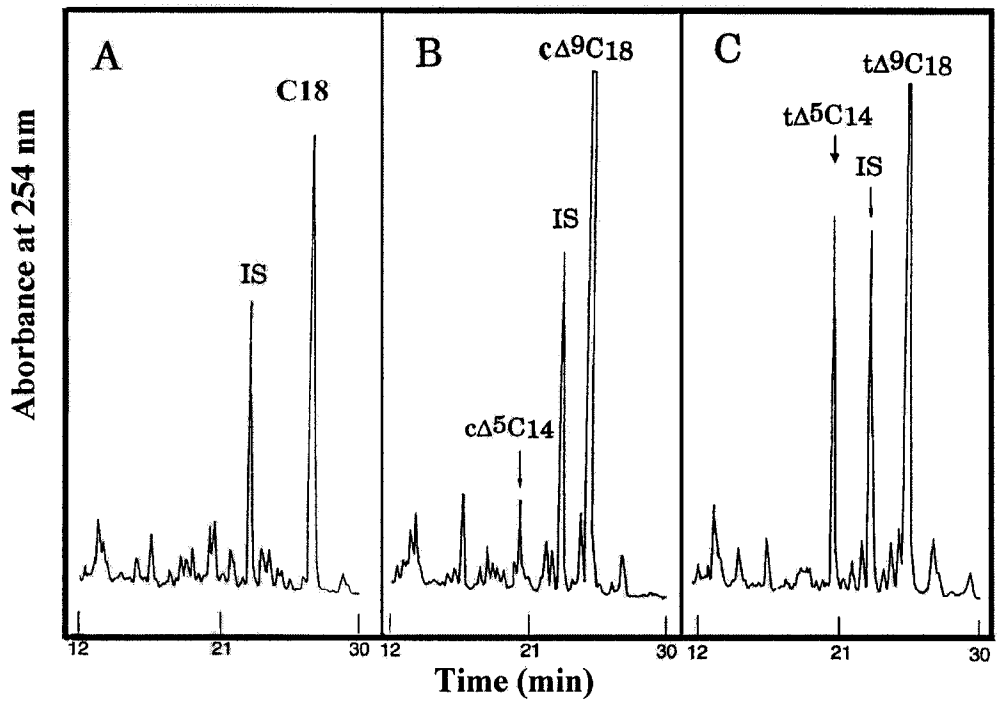


Fig.11. HPLC analysis of acyl-CoAs extracted from rat liver mitochondria after incubation with (A) stearoyl-CoA, (B) oleoyl-CoA, or (C) elaidoyl-CoA. Abbreviations: IS, internal standard (pentadecanoyl-CoA); C18, stearoyl-CoA; cΔ5C14, 5-*cis*-tetradecenoyl-CoA; cΔ9C18, oleoyl-CoA; tΔ5C14, 5-*trans*-tetradecenoyl-CoA; tΔ9C18, elaidoyl-CoA.

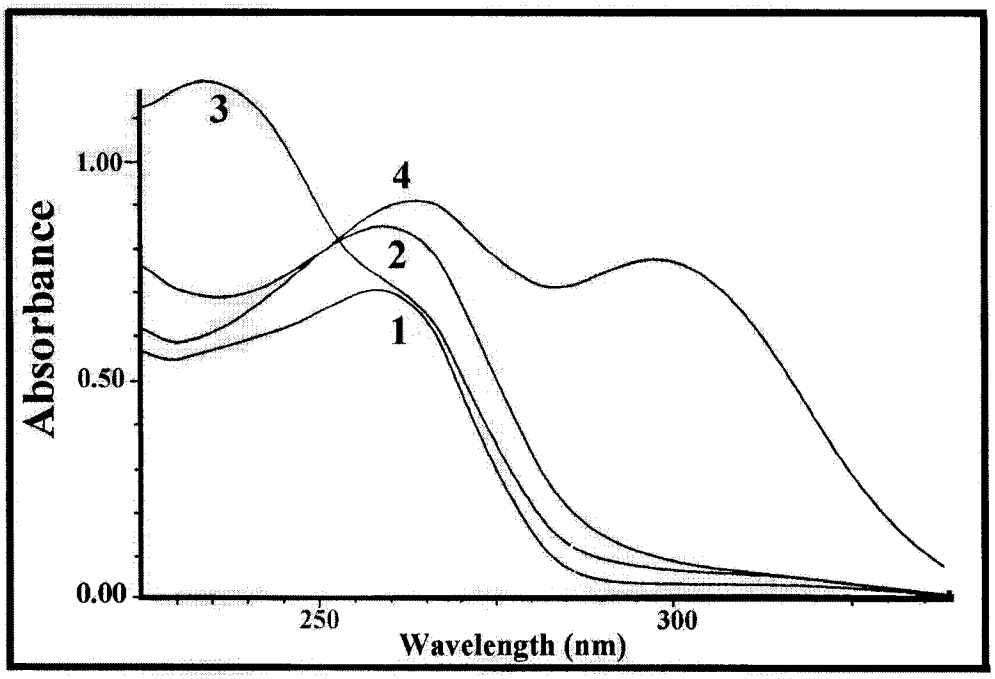
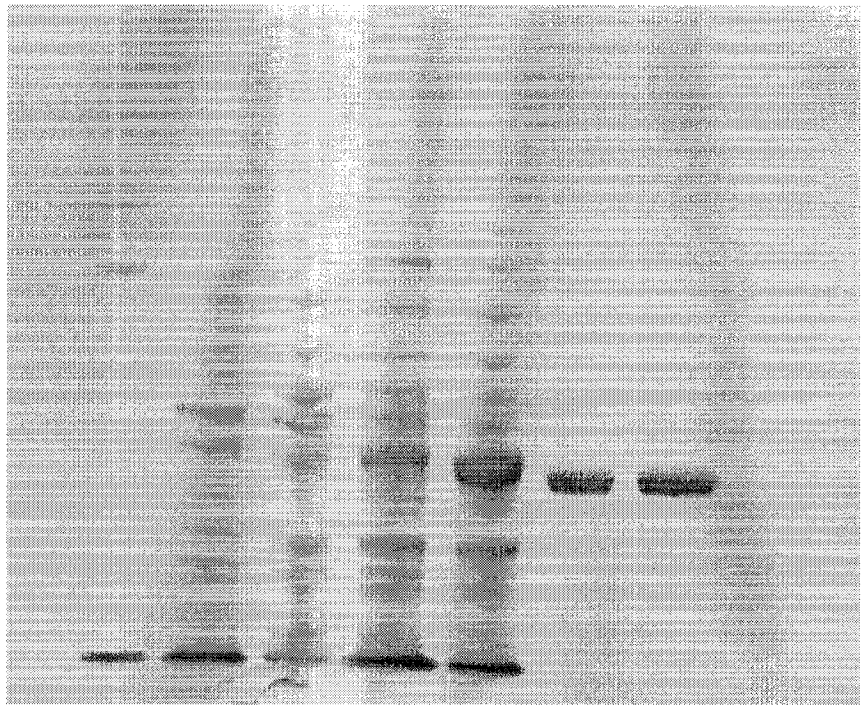


Fig.12. Structure proof of 5-tetradecenoyl-CoA by analyzing its enzymatic conversions spectrophotometrically. 5-Tetradecenoyl-CoA (spectrum 1) was dehydrogenated by acyl-CoA oxidase to 2,5-tetradecadienoyl-CoA (spectrum 2) that was isomerized by enoyl-CoA isomerase to 3,5-tetradecadienoyl-CoA (spectrum 3), which was converted to 2,4-tetradecadienoyl-CoA (spectrum 4) by dienoyl-CoA isomerase. For experimental details see "Experimental Procedures".



Lane 1 2 3 4 5 6 7

Fig.13. SDS-PAGE of recombinant LCAD. 5 μg of protein was loaded onto each lane. Lane 1, protein markers. Lane 2 & 3, *E.coli* extract expressing rat LCAD before and after IPTG induction. Lane 4 & 5, *E.coli* extract expressing mouse LCAD before and after IPTG induction. Lane 6 & 7, rat LCAD after purification on Blue-Sepharose CL-6B and Q-Sepharose.

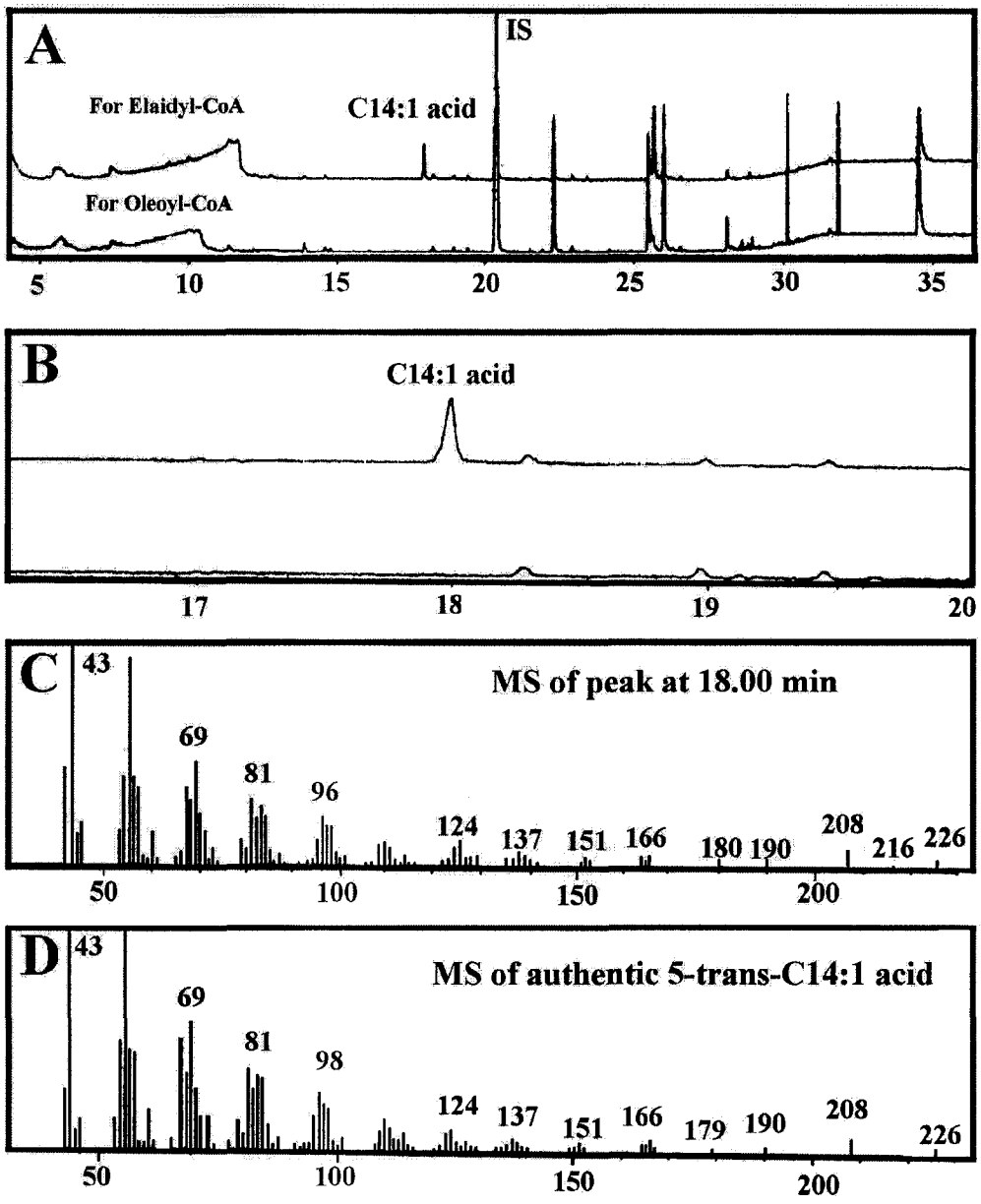


Fig.14

Fig.14. Identification of 5-*trans*-tetradecenoic acid as a product of elaidoate β-oxidation in rat liver mitochondria. (A) gas chromatogram of acidic products extracted from rat liver mitochondria after incubation with elaidoyl-CoA or oleoyl-CoA. (B) expanded region of the gas chromatogram where 5-*trans*-tetradecenoic acid was eluted. (C) mass spectrum of the material corresponding to the peak detected at 18 min in the gas chromatogram. (D) mass spectrum of authentic 5-*trans*-tetradecenoic acid. Abbreviations: C14:1 acid, 5-*trans*-tetradecenoic acid; IS, internal standard (pentadecanoic acid).

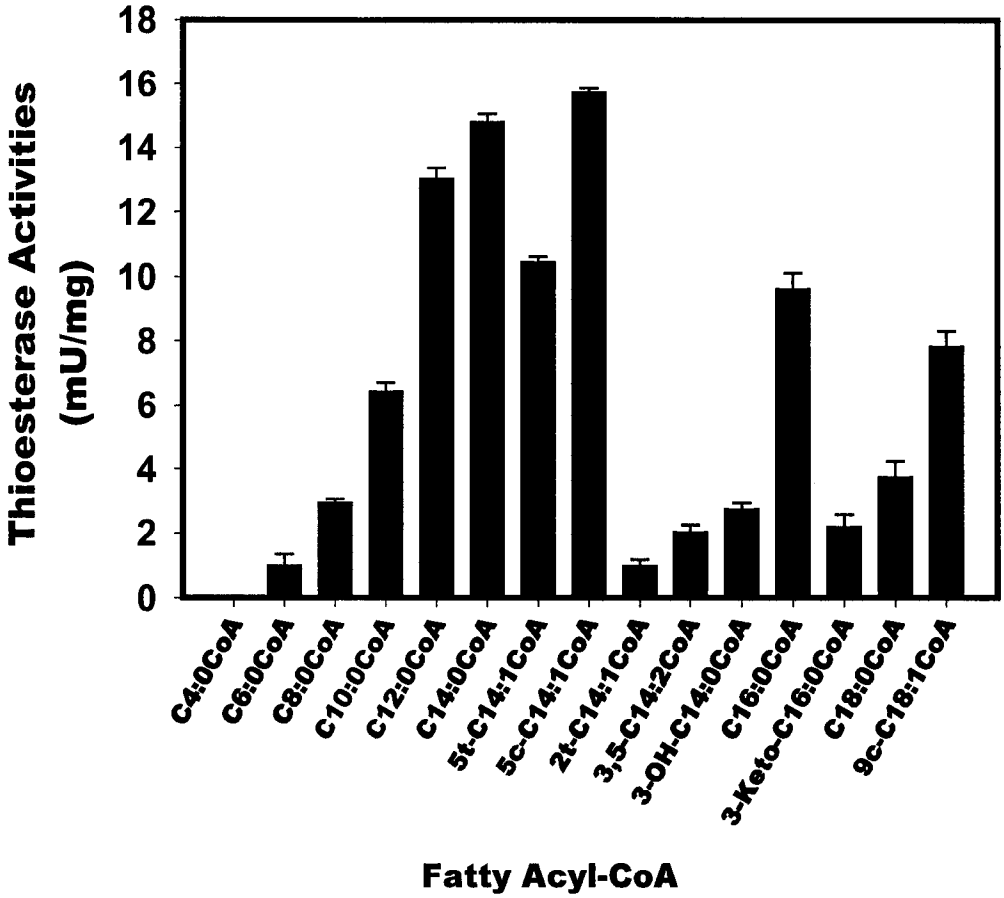


Fig.15

Fig.15. Substrate specificity of partially purified thioesterase from rat liver mitochondria. Specific activities in milliunits/mg of protein (mU/mg) of the partially purified thiesterase preparation with 20 μM of the following substrates: C4:0CoA, butyryl-CoA; C6:0CoA, hexanoyl-CoA; C8:0CoA, octanoyl-CoA; C10:0CoA, decanoyl-CoA; C12:0CoA, dodecanoyl-CoA; C14:0CoA, tetradecanoyl-CoA (myristoyl-CoA); 5t-C14:1CoA, 5-*trans*-tetradecenoyl-CoA; 5c-C14:1CoA, 5-*cis*-tetradecenoyl-CoA; 2t-C14:1CoA, 2-*trans*-tetradecenoyl-CoA; 3,5-C14:2CoA, 3,5-tetradecadienoyl-CoA; 3-OH-C14:0CoA, 3-hydroxytetradecanoyl-CoA; C16:0CoA, palmitoyl-CoA; 3-Keto-C:16CoA, 3-ketohexadecanoyl-CoA; C18:0CoA, stearoyl-CoA; 9c-C18:1CoA, oleoyl-CoA.

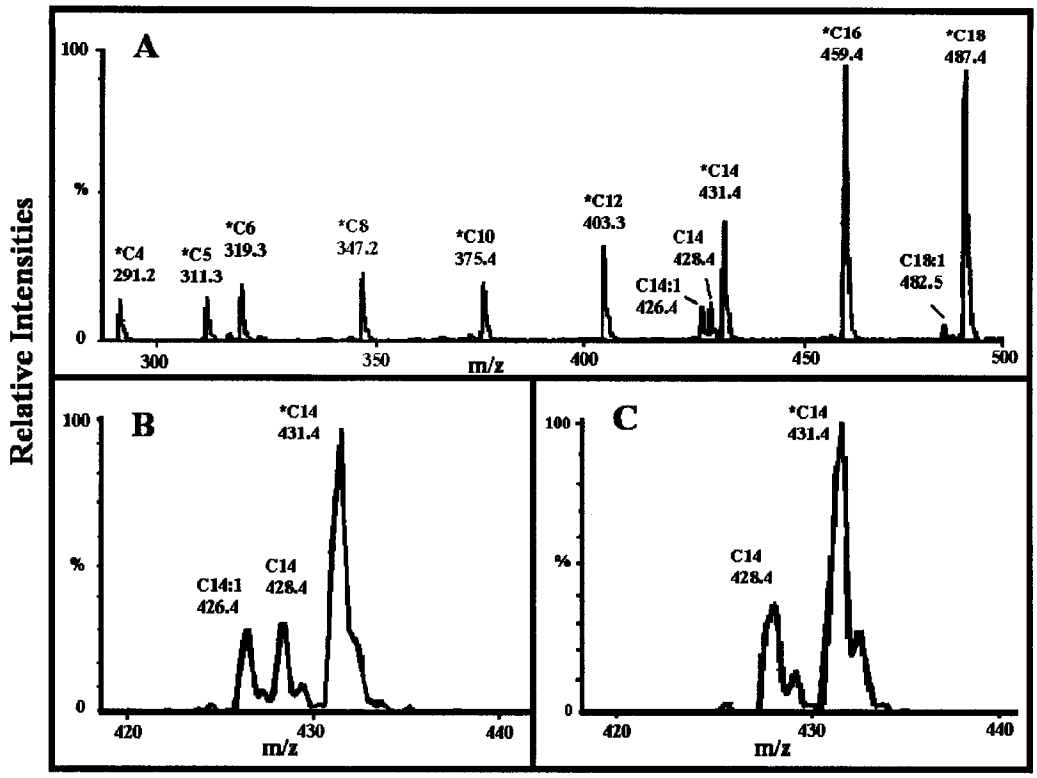


Fig.16

Fig.16. Acylcarnitine profiles. Acylcarnitines were identified and quantified after incubating rat liver mitochondria with (A,B) elaidoyl-CoA or (C) oleoyl-CoA plus carnitine. At the end of the incubation period, tetradecanoylcarnitine (myristoylcarnitine) labeled C14 (m/z = 428.4) was added as an internal standard. Carnitine derivatives (marked with asterisks) of trideuterated straight-chain, even-numbered carboxylic acids from acetate (C2) to stearate (C18) and nonadeuterated isovalerylcarnitine were added immediately before butylation and analysis of acylcarnitines present in the incubation mixture. For experimental details see "Experimental Procedures".

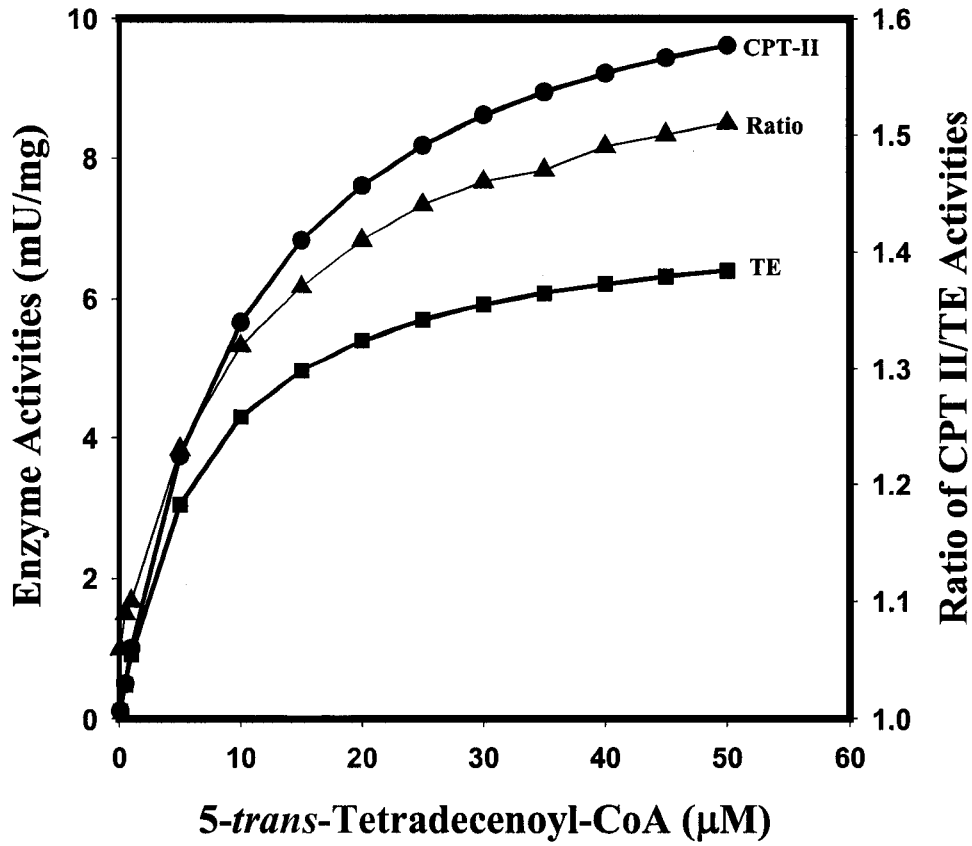


Fig.17

Fig.17. Activties of thioesterase and CPT II in rat liver mitochondria as a function of the concentration of 5-*trans*-tetradecanoyl-CoA. Specific activities of thioesterase (TE) and CPT II in rat liver mitochondria were calculated based on activities measured with 20 μM tetradecanoyl-CoA and the kinetic parameters determined with the partially purified enzyme preparations. Also plotted is the ratio of CPT II to thioesterase activities. For experimental details see "Experimental Procedures".

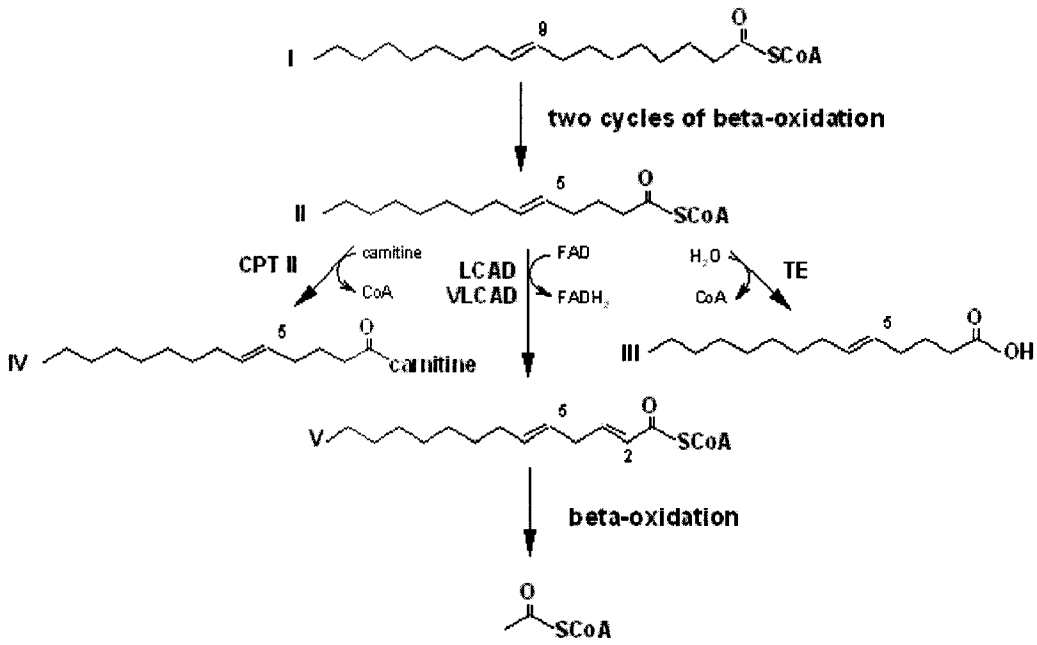


Fig.18 Degradation of elaidoyl-CoA in rat liver mitochondria. Compound I, elaidoyl-CoA; compound II, 5-*trans*-tetradecenoyl-CoA; compound III, 5-*trans*-tetradecenoic acid; compound IV, 5-*trans*-tetradecenoylcarnitine; compound V, 2-*trans*,5-*trans*-tetradecadienoyl-CoA. Abbreviations: TE, thioesterase; LCAD, long-chain acyl-CoA dehydrogenase; VLCAD, very long-chain acyl-CoA dehydrogenase; CPT II, carnitine palmitoyltransferase.

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