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Purification and characterization of three novel enzymes of mitochondrial fatty acid beta-oxidation

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City University of New York, 1995

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PURIFICATION AND CHARACTERIZATION OF THREE NOVEL ENZYMES OF MITOCHONDRIAL FATTY ACID BETA-OXIDATION

by

Ming Jiang Luo

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fullfillment of the requirements of the degree of Doctor of Philosophy, The City University of New York.

1995

This manuscript has been read and accepted for the Graduate Faculty in Biochemisty in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chair of Examining Committee

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Executive Officer

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THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

PURIFICATON AND CHARACTERIZATION OF THREE NOVEL ENZYMES OF MITOCHONDRIAL FATTY ACID β-OXIDATION

by

Ming Jiang Luo

Adviser: Professor Horst Schulz

The presence of a trifunctional β-oxidation complex in pig heart and its relationship to the known long-chain enoyl-CoA hydratase (EC 4.2.1.74) from pig heart mitochondria were investigated. For this study, the complex was partially purified by chromatography on DEAE-cellulose in the absence of detergents and was purified to near homogeneity in the presence of Triton X-100. Both enzyme preparations contained long-chain specific activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase but were virtually inactive toward short-chain (C4) substrates. The molecular weights of the two subunits of the pig heart complex were estimated to be 81,000 and 45,000, respectively. The partially purified preparation, obtained in the absence of detergent, was identified as a membranous fraction enriched with respect to the inner mitochondrial membrane. It is concluded that long-chain enoyl-CoA hydratase (EC 4.2.1.74) is a component enzyme of the trifunctional β-oxidation complex which is associated with the inner membrane of pig heart mitochondria.

Mitochondrial $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, which catalyzes the conversion of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA, was purified from rat liver 370-fold at almost 30% yield by a six-step purification procedure. The final preparation appeared to be homogeneous as judged by gel electrophoresis. The molecular weights of the native enzyme and its subunit(s) were estimated to be 126,000 and 32,000, respectively. The purification of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase completes the characterization of the enzymes functioning in the NADPH-dependent pathway for the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms. This novel pathway may not be operative in peroxisomes because $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase was only detected in mitochondria.

Short-chain L-3-hydroxy-2-methylacyl-CoA dehydrogenase (SC-HMAD), a soluble mitochondrial enzyme, was purified 6000-fold from rat liver in 6% yield by a six-step purification procedure. The purified enzyme was homogeneous as judged by SDS-PAGE. The subunit molecular weight of this enzyme was estimated to be 28,000. The enzyme seems to be a dimer composed of two identical subunits. Immunoblotting with antibodies to pig heart L-3-hydroxyacyl-CoA dehydrogenase (HAD)(EC 1.1.1.35) revealed that SC-HMAD and HAD are immunologically unrelated proteins. SC-HMAD, but not HAD, catalyzes the NAD^+-dependent dehydrogenation of L-3-hydroxy-2-methybutyryl-CoA, a metabolite of isoleucine, to 2-methylacetoacetyl-CoA. Unbranched 3-hydroxyacyl-CoA thioesters are also substrates of SC-HMAD. The apparent Km values for L-3-hydroxy-2-methybutyryl-

CoA and L-3-hydroxybutyryl-CoA are 5 μM and 19 μM, respectively. The maximal velocities observed with these two substrates were similar. It is concluded that SC-HMAD catalyzes the second dehydrogenation step during the β-oxidation of the isoleucine metabolite 2-methylbutyryl-CoA. This enzyme may also be involved in the β-oxidation of natural and xenobiotic methyl-branched carboxylic acids.

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ABBREVIATIONS

CoA	coenzyme A
CoASH	coenzyme A
CPT	carnitine palmitoyltransferase
DEAE	diethylaminoethyl
DEHP	di(2-ethylhexyl)phthalate
E.coli	*Escherichia coli*
EDTA	ethylenediaminotetraacetic acid
EGTA	ethylene glycolbis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid
HAD	L-3-hydroxyacyl-CoA dehydrogenase
Hepes	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
KPi	Potassium phosphate
NAD+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine denucleotide
NADP+	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PAGE	polyacylamide gel electrophoresis
PEG	polyethylene glycol
PMSF	phenylmethysulfonyl fluoride
SC-HMAD	short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase
SDS	sodium dodecyl sulfate
UV	ultraviolet

INTRODUCTION

β-Oxidation of fatty acids is one of the major metabolic pathways in animals and in other living organisms (1). In the heart, fatty acids are the preferred fuel (2). In skeletal muscle, β-oxidation of fatty acids provides a major part of the energy (2) and even tissues, like brain (3), that do not derive energy from fatty acid oxidation, contain enzymes of β-oxidation. Two β-oxidation systems coexist in mammalian tissues: one in mitochonodria and the other in peroxisomes (1). Recent research has identified several inherited human diseases due to deficiencies of β-oxidation enzymes both in mitochondria and in peroxisomes. Depending on the deficient enzyme, these inherited defects may only cause mild muscle weakness (4-6) or may result in life-threatening coma or death (7). Given the importance of β-oxidation in mammalian energy metabolism it is important to understand all aspects of fatty acid oxidation. A major part of the effort addressed in this study is the purification and characterization of unknown enzymes functioning in the β-oxidation of fatty acids.

The mechanism of fatty acid uptake by animal cells is not fully understood even though significant progress has been made in the characterization of a 40-kDa fatty acid binding protein present in the plasma membrane of liver and other tissues (8). Some investigators maintain that the cellular uptake of fatty acids is a facilitated process, whereas others argue that fatty acids enter cells by simple diffusion (1). Medium-chain and short-chain fatty acids, in contrast to long-chain fatty acids, can directly enter the mitochondial matrix where

they are activated by medium-chain and short-chain acyl-CoA synthetase (1). The identification of fatty acid binding proteins in the cytosols of animal cells has prompted speculation that they may play a role in fatty acid transport, uptake and delivery (9). The β-oxidation of fatty acids requires their prior conversion to coenzyme A thioesters. The reaction is catalyzed by a group of acyl-CoA synthetases, which differ in their chain length specificities and subcellular locations. They are referred to as short-chain acyl-CoA synthetase (EC 6.2.1.1.), medium-chain acyl-CoA synthetase (EC 6.2.1.2.), and long-chain acyl-CoA synthetase (EC 6.2.1.3.)(10). Recently, a very-long-chain acyl-CoA synthetase was shown to exist in most animal tissues (11,12). The mitochondial membrane is impermeable toward acyl-CoAs formed by acyl-CoA synthetase. It was proposed that acyl residues are transferred by carnitine acyltransferase I (CPT I) from CoA to carnitine at the outer mitochondrial membrane to yield acylcarnitines (13,14), which pass through the inner mitochondrial membrane via carnitine-acylcarnitine translocase. In the matrix, acyl groups are transferred back form carnitine to coenzyme A by carnitine acyltransferase II (CPT II) (13). The active site of CPT I, which is thought to face the intermembrane space (14), is subject ot regulation by malonyl-CoA, whereas CPT II, facing the matrix space, is not affected by malonyl-CoA (15). Figure 1 shows the overall scheme for fatty acid activation, translocation and the four reaction involved in the β-oxidation cycle.

The pathway of saturated fatty acids oxidation in mitochondria, which consists of four reactions, is well established. The first reaction

is the dehydrogenation of acyl-CoA to 2-*trans*-enoyl-CoA catalyzed by a group of acyl-CoA dehydrogenases with different chain length specificities (EC 1.3.99.2 and EC 1.3.99.3.). They are short-chain, medium-chain, long-chain and very-long-chain acyl-CoA dehydrogenases. The purification and characterization of this group of acyl-CoA dehydrogenzses has been completed (16-20).

The second step in the β-oxidation is the hydration of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by enoyl-CoA hydratase or crotonase (EC 4.2.1.17) and long-chain enoyl-CoA hydratase (EC 4.2.1.74). The soluble matrix enzyme has been purified from bovine liver, pig heart and rat liver (21, 22). The reaction rates catalyzed by the soluble hydratase decrease as the length of the enoyl group increases form crotonyl-CoA to 2-hexadecenoyl-CoA (21, 22). It has been suggested that the long-chain enoyl-CoA hydratase complements crotonase to assure a high rate of hydration over the entire spectrum of enoyl-CoA intermediates (23).

The third reaction is the dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenases. The soluble matirx enzyme (EC 1.1.1.35) has been purified from pig heart (24) and rat liver (25). This enzyme is active toward the long-chain substrate 3-hydroxyhexadecanoyl-CoA but at a rate that is only 6% of the rate obtained with the medium-chain substrate 3-hydroxydecanoyl-CoA (26). A long-chain 3-hydroxyacyl-CoA dehydrogenase has recently been purified from rat liver (27), human liver (28) and subsequently form pig heart(29).

The last reaction is the thiolytic cleavage of 3-ketoacyl-CoA thioesters, which produces acyl-CoA chain-shortened by two carbon atoms and acetyl-CoA. There are three types of thiolases in mitochondria, which differ in their chain length specificities: acetoacetyl-CoA thiolase (EC 2.3.1.9) which only acts on acetoacetyl-CoA, 3-ketoacyl-CoA thiolase (EC 2.3.1.16) which is active with all substrates (30-32) and long chain 3-ketoacyl-CoA thiolase which is active only with medium-chain and long chain substrates (27-29).

β-Oxidation in peroxisomes is preceded by the conversion of fatty acids to their CoA thioesters which are degraded by a sequence of four reactions. The intermediates of peroxisomal β-oxidation are identical with the mitochondrial intermediates. However, the first step of dehydrogenation is catalyzed by acyl-CoA oxidase which transfers electrons from acyl-CoA to O_2 to form H_2O_2. Catalase, which is present in peroxisomes, cleaves H_2O_2 to O_2 and H_2O (33). The hydration of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA and its NAD^+-dependent dehydrogenation are catalyzed by the peroxisomal trifunctional enzyme having enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activities (33,34). The final step of β-oxidation in peroxisomes is the thiolytic cleavage of 3-ketoacyl-CoA catalyzed by 3-ketoacyl-CoA thiolase. All peroxisomal β-oxidation enzymes, except for acyl-CoA oxidase, act on the whole range of substrates. Acyl-CoA oxidase in mammalian peroxisomes is almost inactive toward octanoyl-CoA and shorter chain acyl-CoAs (33). The main function of β-oxidation in peroxisomes seems to be the partial metabolism of unusual fatty

acids, such as very-long-chain fatty acids, dicarboxylic acids, prostaglandins and xenobiotic carboxylic acids(35-39).

Most mitochondrial enzymes of β-oxidation are soluble and present in the matrix. Included in this group of enzymes are enoyl-CoA hydratase or crotonase (EC 4.2.1.17), L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) in addition to short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases (EC 1.3.99.2) and (EC 1.3.99.3)(1). Some of these soluble mitochondrial matrix enzymes exhibit low activities with long-chain substrates. In 1974, Schulz (23) isolated and partially purified a long-chain enoyl-CoA hydratase from pig heart muscle. The results suggested that the long-chain enoyl-CoA is associated with the mitochondrial membrane (23). The long-chain hydratase is active toward the medium and long-chain enoyl-CoA substrates, but is nearly inactive toward crotonyl-CoA (23). It was proposed that the complementary action of the two enoyl-CoA hydratase assures a high rate of hydration of enoyl-CoA intermediates of all chain lengths in fatty acid β-oxidation (40). In 1982, El-Fakhri and Middleton (41) reported that an inner-membrane-bound, long-chain specific 3-hydroxyacyl-CoA dehydrogenase was present in mammalian mitochondria. The long-chain 3-hydroxyacyl-CoA dehydrogenase was separated from the matrix 3-hydroxyacyl-CoA dehydrogenase by gel filtration. It has recently been reported that rat and human liver mitochondria contain a membrane-bound trifunctional β-oxidation complex which exhibits medium-chain and long-chain, but not short-chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA

dehydrogenase, and 3-ketoacyl-CoA thiolase (27,28). The purification of this β-oxidation complex, which is composed of two non-identical subunits, was prompted by an interest in long-chain L-3-hydroxyacyl-CoA dehydrogenase (41), which is deficient in some patients with inherited diseases of mitochondrial fatty acid oxidation (42,43). The study reported here was initiated with the aim of elucidating the relationship between long-chain enoyl-CoA hydratase from pig heart mitochondria (23) and the hydratase activity of the trifunctional β–oxidation complex by isolating and purifying the complex from pig heart.

Mitochondrial and peroxisomal β-oxidation of unsaturated and polyunsaturated fatty acids with even-numbered double bonds proceeds overwhelmingly by the reductase-dependent pathway (44). A small percentage of polyunsaturated fatty acids may be degraded in peroxisomes via an epimerase-dependent pathway (44). The auxiliary enzymes required for the β-oxidation of unsaturated fatty acids are 2,4-dienoyl-CoA reductase, Δ^3,Δ^2-enoyl-CoA isomerase and the peroxisomal D-3-hydroxyacyl-CoA dehydratase. D-3-Hydroxyacyl-CoA dehydratase, together with enoyl-CoA hydratase, catalyzes the epimerization of D-3-hydroxyacyl-CoA to L-3-hydroxyacyl-CoA. Unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms are assumed to be chain shortened to 3-enoyl-CoAs, which after isomerization to 2-*trans*-enoyl-CoAs by Δ^3,Δ^2-enoyl-CoA isomerase, are further degraded via the β-oxidation spiral (45). Figure 2 shows the β-oxidation of lineoleoyl-CoA. However, Tserng and Jin (46) reported that the chain shortening of 5-

enoyl-CoAs, which are the presumed precursors of 3-enoyl-CoAs, required NADPH. A detailed study of the mitochondrial β-oxidation of 5-octenoyl-CoA revealed a novel pathway (47) according to which 5-octenoyl-CoA is dehydrogenated by medium-chain acyl-CoA dehydrogenase to 2,5-octadienoyl-CoA. Isomerization of the latter compound by Δ^3,Δ^2-enoyl-CoA isomerase yields 3,5-octadienoyl-CoA which is isomerized to 2,4-octadienoyl-CoA by soluble extracts of either rat liver mitochondria or rat heart mitochondria. The further metabolism of 2,4-octadienoyl-CoA involves the NADPH-dependent reduction of one double bond catalyzed by 2,4-dienoyl-CoA reductase followed by isomerization of *3-trans*-octenoyl-CoA to *2-trans*-octenoyl-CoA and the complete degradation of the latter compound via the β-oxidation spiral. The isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA was attributed to a novel $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (47). Here I report the purification and characterization of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) from rat liver mitochondria which catalyzes the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA.

Isoleucine is metabolized to 2-methylbutyryl-CoA by deamination followed by oxidative decarboxylation. The latter compound undergoes one cycle of β-oxidation to yield acetyl-CoA and propionyl-CoA (48-50). Figure 3 shows the metabolic fate of isoleucine. The first step of the β-oxidation of 2-methylbutyryl-CoA is catalyzed by 2-methyl-branched chain acyl-CoA dehydrogenase, which is an enzyme distinct from the acyl-CoA dehydrogenases involved in fatty acid β-oxidation (51). The second reaction, the hydration of tiglyl-CoA, was

shown to be catalyzed by enoyl-CoA hydratase (crotonase)(EC 4.2.1.17) (50). The fourth and last step of the β-oxidation is catalyzed by mitochondrial acetoacetyl-CoA thiolase (EC 2.3.1.9) which is thought to function only in isoleucine and ketone body metabolism (52,53). The mammalian enzyme that catalyzes the third reaction, the dehydrogenation of 3-hydroxy-2-methylbutyryl-CoA to 2-methylacetoacetyl-CoA, had not been identified. However, an NAD^+-dependent dehydrogenase, referred to as 3-hydroxy-2-methylbutyryl-CoA dehydrogenase (EC 1.1.1.178). which is active with 3-hydroxy-2-methylbutyryl-CoA and 3-hydroxybutyryl-CoA was isolated and purified from *Pseudomonas putida* grown on isoleucine (54). In the same study it was observed that L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) from pig heart is inactive with 3-hydroxy-2-methylbutyryl-CoA as a substrate. This study was undertaken with the aim of characterizing all forms of 3-hydroxyacyl-CoA dehydrogenase present in mammalian mitochondria. As part of this investigation I purified and characterized short-chain 3-hydroxy-2-methyacyl-CoA dehydrogenase from rat liver.

EXPERIMENTAL PROCEDURES

Materials. Benzamidine hydrochloride, pepstatin A, leupeptin, PEG (average molecular weight 8000), Q-Sepharose, S-Sepharose, Sepharose CL-6B, Sephacryl S-200, phosphocellulose, DEAE cellulose, Blue Dextran Agarose, tiglyl-CoA, Polybuffer Exchanger 94, Polybuffer 96, Nycodenz, molecular weight markers for gel filtration, Triton X-100, CoASH, pig heart L-3-hydroxyacyl-CoA dehydrogenase, catalase, and most standard biochemicals were purchased from Sigma. Centricon 10 microconcentrator was purchased from Amicon. Molecular weight standards for SDS-PAGE, and 10%, 5-15% and 5-20% polyacrylamide mini-gels, hydroxylapatite, and the dye reagent for protein assays were bought from Bio-Rad. Di(2-ethylhexyl)-phthaltate was obtained from Aldrich. The following enzymes were purified or partially purified by established procedures: enoyl-CoA hydratase or crotonase from bovine liver (55), L-3-hydroxyacyl-CoA dehydrogenase from pig heart (24), and acetoacetyl-CoA thiolase from pig heart (30). Male Sprague-Dawley rats (250 g) were obtained from Taconic Farms (Germantown, NY) and kept on a diet of either normal rodent chow or rodent chow containing 2% (w/w) DEHP. Rabbit antiserum to HAD from pig heart was produced by Pocono Rabbit Farm and Laboratories (Canadensis, PA).

Synthesis of Substrates. The CoA thioesters of 2-hexadecynoic acid and 2-octynoic acid were synthesized by the mixed anhydride procedure as detailed by Goldman and Vagelos (56). 2-Octynoyl-CoA and 2-hexadecynoyl-CoA, the latter after purification by hydrophobic chromatography on octyl-Sepharose, were converted by crotonase to

3-ketooctanoyl-CoA and 3-ketohexadecanoyl-CoA, respectively, as described in principle by Thorpe (57). The concentrations of 3-ketooctanoyl-CoA and 3-ketohexadecanoyl-CoA were determined by measuring their complete reductions by NADH at pH 7 in the presence of L-3-hydroxyacyl-CoA dehydrogenase. The purity of 3-ketohexadecanoyl-CoA was determined to be 93% by high performance liquid chromatography (HPLC) on a Waters μBondpak C_{18} reverse-phase column (30 cm x 3.9 mm) attached to a Waters HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by increasing the acetonitrile content of the 10 mM ammonium phosphate elution buffer (pH 5.5) from 10% to 70%. The following substrates were synthesized according to published procedures: acetoacetyl-CoA (58), crotonyl-CoA (59), 3-*cis*-octenoyl-CoA (60). Concentrations of acyl-CoAs were determined by quantifying CoASH according to the method of Ellman (61) after cleaving the thioester bond with NH_2OH at pH 7.

Enzyme and Protein Assays. All enzyme activities were measured spectrophotometrically on a Gilford, Model 250 or 260, recording spectrophotometer at 25 ^{0}C. Enoyl-CoA hydratase was assayed at 280 nm as described by Fong and Schulz (21). An extinction coefficient of 5,100 M^{-1} cm^{-1} was used to calculate rates. Dienoyl-CoA isomerase was assayed by measuring the increase in absorbance at 300nm. A typical assay mixture contained 8 μM 3,5-octadienoyl-CoA in 0.2 M KP_i (pH 8) and enzyme to obtain an absorbance change of approxiamtely 0.04 per min. The assay was started by the addition of enzyme. An extinction coefficient of 27,800 M^{-1} cm^{-1} (62) was used to calculate rates. The assay

of L-3-hydroxyacyl-CoA dehydrogenase was based on the 3-ketoacyl-CoA-dependent dehydrogenation of NADH (60). An extinction coefficient of 6,220 M^{-1} CM^{-1} was used to calculate rates. 3-Ketoacyl-CoA thiolase was assayed at 303 nm by an established procedure (63). Long-chain 3-ketoacyl-CoA thiolase was assayed at 303 nm by the same procedure except that 0.025% Triton X-100 was included in the reaction mixture. Short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase (SC-HMAD) was assayed spectrophotometrically by measuring the increasing absorbance at 340 nm. A typical assay mixture contained 0.2 M KP_i (pH 9), 1 mM NAD^+, 30 μM tiglyl-CoA, crotonase (0.1 unit/ml), and SC-HMAD activity to give an absorbance change of 0.01 to 0.02 per min. The assays were initiated by the addition of enzyme. When kinetic parameters (Km, Vmax) of SC-HMAD were determined, the concentrations of tiglyl-CoA and crotonyl-CoA were varied betwen 4 μM and 80 μM at a fixed concentration of 1 mM NAD^+. Effective concentrations of L-3-hydroxy-2-methylbutyryl-CoA and L-3-hydroxybutyryl-CoA were calculated based on equilibrium ratios of 0.33 determined for [3-hydroxy-2-methylbutyryl-CoA]/[tiglyl-CoA] and 3.2 reported for [3-hydroxybutyrul-CoA]/[crotonyl-CoA] (64). The apparent Km value for NAD^+ was determined at a fixed concentration of 25 μM L-3-hydroxy-2-methylbutyryl-CoA. Kinetic parameters were obtained by nonlinear curve fitting using the Sigma plot program. The following enzymes were assayed by established procedures: Rotenone-insensitive NADH-cytochrome C reductase (65), succinate-cytochrome C reductase (65), malate dehydrogenase (66), and catalase (67). One unit (U) of enzyme activity is defined as the amount of enzyme that

catalyzes the conversion of 1 μmol of substrate to product per minute. Protein concentrations were determined by a dye binding assay with bovine serum albumin as a standard (68).

Effects of Detergents on the Activity of the Trifunctional β-Oxidation Complex. The effects of Triton X-100 and octyl glucoside on long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase and long-chain 3-ketoacyl-CoA thiolase activities were studied at different concentrations of detergent in the assay mixture. These three long chain enzyme activities were measured as described above. When assaying long-chain 3-hydroxyacyl-CoA dehydrogenase and long-chain 3-ketoacyl-CoA thiolase, 25 μM 3-ketohexadecanoyl-CoA was used as the substrate. Long-chain enoyl-CoA hydratase was assayed with 20 μM of 2-hexadecenoyl-CoA. A homogenate of rat liver mitochondria served as an enzyme source. When the effect of detergents on proteolysis was studied, subtilisin was added to a rat liver mitochondrial homogenate in 0.1 M KPi (pH 7.0) at the concentration of 0.0177 mg of subtilisin/mg homogenate protein in the presence of 0.4% octyl glucoside, and at the concentration of 0.177 mg of subtilisin /mg homogenate in the absence of octyl glucoside. The effect of subtilisin on the enzyme was monitored by assaying the 3-hydroxyacyl-CoA dehydrogenase activities with acetoacetyl-CoA and 3-ketohexadecanoyl-CoA at various time intervals.

Subcellular Distribution of Dienoyl-CoA Isomerase. A 30% (w/v) solution of Nycodenz containing 1 mM EDTA, 5 mM Hepes (pH 7.4), and 0.1 % ethanol was prepared at 4°C, and 7.6 ml of this solution was placed in a 10 ml centrifuge tube on top of 0.5 ml of a 60% sucrose

cushion. The gradient was generated by centrifugation at 64,400 x g in a T865.1 small angle rotor on a Du Pont RC70 ultracentrifuge at 4°C for 24 hours. Light mitochondria (0.5 mg of protein in 0.5 ml), prepared according to Appelmans *et al.* (69) were layered on top of the gradient. A 3-fold diluted isolation buffer (250 mM sucrose, 1 mM EDTA, 0.1% ethanol, 10 mM Tris) was applied as a cover solution. The sample was centrifuged for 60 min at 76,200 x g at 4°C. Fifteen fractions were collected from the bottom of the tube. Peroxisomes and mitochondria were localized by assaying the marker enzyme catalase and malate dehydrogenase, respectively. Dienoyl-CoA isomerase was assayed as described above. Dilutions of the Nycodenz fractions were made into 1 mM $NaHCO_3$ (pH 7.6) containing 1mM EDTA, 0.01 % Triton X-100, 0.1% ethanol, and bovine serum albumin (1 mg/ml).

Partial Purification of the Trifunctional β-Oxidation Complex from Pig Heart in the Absence of Detergent. Frozen pig heart (120 g) was thawed, trimmed of fat, cut into small pieces with a pair of scissors, and homogenized in a Waring blender at high speed for 3 min at 4°C in the presence of 500 ml of 50 mM KP_i (pH 7) containing 5 mM mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine, and 1 mM PMSF (buffer A). The resulting suspension was centrifuged at 10,000 x g for 10 min. PEG was added to the supernatant to a final concentration of 10% to precipitate membrane fragments. After two hours, the suspension was centrifuged at 6,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended with 200 ml of buffer A. After stirring for 30 min, the suspension was centrifuged at 8,000 x g for 20 min. The supernatant

was applied to a DEAE-cellulose column (2.5 cm x 25 cm) which previously had been equilibrated with 10 mM KP_i (pH 7) containing 5 mM mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine and 1 mM PMSF (buffer B). The column was washed with buffer B containing 100 mM KCl and was then developed with a gradient made up of 500 ml each of buffer B containing 0.1 M KCl and buffer B containing 1 M KCl. Fractions of 15 ml were collected and assayed for enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase. When succinate-cytochrome C reductase, rotenone-insensitive NADH-cytochrome C reductase and 3-ketohexadecanoyl-CoA reductase activities were determined, mercaptoethanol, EDTA, EGTA, benzamidine and PMSF were omitted from the buffer.

Purification of the Trifunctional β-Oxidation Complex from Pig Heart. Four frozen pig hearts weighing approximately 1 kg were thawed, trimmed of fat, cut into small pieces with a pair of scissors, and homogenized in a Waring blender at high speed for 3 min at 4°C with 4 l of 20 mM KP_i (pH 7.5) containing 5 mM mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 mM benzamidine, pepstatin A (1 mg/ml), and leupeptin (1 mg/ml) (buffer C). The resulting suspension was centrifuged at 1500 x g for 10 min. To the resultant supernatant PEG was added to reach a final concentration of 10% to precipitate membrane fragments. After two hours, the suspension was centrifuged at 8,000 x g for 10 min. The supernatant was discarded and the pellet, which contained mitochondrial and other membranes, was dissolved in buffer C containing 2% Triton

X-100 and stirred for 30 min. The suspension was centrifuged at 8,000 x g for 20 min. The supernatant was applied to a phosphocellulose column (5 cm x 20 cm), which was equilibrated with buffer C containing 0.5% Triton X-100 and washed with 500 ml buffer C containing 0.2 M KCl and 0.5% Triton X-100. The trifunctional β-oxidation complex, as reflected by the long-chain L-3-hydroxyacyl-CoA dehydrogenase activity, was eluted with buffer C containing 1 M KCl and 0.5% Triton X-100. The sample eluted from the phosphocellulose column was concentrated in an Amicon concentrator with a PX 10 membrane and diluted with buffer C containing 0.5 % Triton X-100 to lower the concentration of KCl. The resultant sample containing approximately 0.1 M KCl was applied to a DEAE-cellulose column (2.5 cm x 25 cm) previously equilibrated with buffer C containing 0.5% Triton X-100. The column was washed with buffer C containing 0.1 M KCl and 0.5% Triton X-100. The trifunctional β-oxidation complex did not absorb to the column and was detected by its long-chain 3-hydroxyacyl-CoA dehydrogenase activity. Fractions containing the trifunctional β-oxidation complex were combined and applied to a second phosphocellulose column (25 cm x 35 cm) equilibrated with buffer C containing 0.5% Triton X-100. The column was extensively washed with buffer C containing 0.2 M KCl and 0.5% Triton X-100 and thereafter developed with a gradient made up of 600 ml of buffer C containing 0.2 M KCl and 0.5% Triton X-100, and 600 ml of buffer C containing 1 M KCl and 0.5% Triton X-100. Fractions were assayed for protein, enoyl-CoA hydratase, L-3-hdyroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. Active fractions were combined and concentrated in an Amicon

concentrator with a PX 10 membrane. The concentrated sample was twice diluted with buffer C containing 0.5% Triton X-100 and concentrated to lower the KCl concentration. Glycerol was added to a final concentration of 40% (v/v) before storing the enzyme at -80°C.

Purification of Dienoyl-CoA Isomerase. All operations were carried out at temperatures between 4°C and 0°C. The liver from an adult Sprague-Dawley rat (250-350g), which had been fed rodent chow containing 2% (w/w) DEHP, was minced and homogenized with 200 ml of 10 mM KP_i (pH 8.3) containing 5 mM mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 1 mM PMSF, 1 μM pepstatin A, 1μM leupeptin (buffer D) in a loose-fitting Potter-Elvehjim type glass-Telfon homogenizer. The resulting suspension was sonicated for 6 x 10 sec with a Heat Systems sonifier (Model 385) and centrifuged at 8,000 x g for 20 min. The pellet was resuspended in buffer D, sonicated and centrifuged as described above. PEG was added to the combined supernatants, referred to as soluble extract, to give a final concentration of 8%. After keeping the suspension overnight, it was centrifuged at 6,000 x g for 20 min. The supernatant was discarded and the pellet was suspended in 200 ml of buffer D containing 0.2 M KCl. After stirring for 30 min, the suspension was centrifuged at 100,000 x g for one hour. The pellet was resuspended in 200 ml of buffer D containing 0.2 M KCl, stirred for 30 min, and centrifuged at 100,000 x g for one hour. The combined supernatants were diluted with 4 volumes of buffer D to lower the concentration of KCl to 40 mM and were applied to a Q-Sepharose column (2.5 x 40 cm) equilibrated with buffer D. After extensively washing the column

with buffer D, dienoyl-CoA isomerase was eluted with 400 ml of buffer D containing 0.2 M KCl. Fractions of 20 ml each were collected and those containing dienoyl-CoA isomerase were combined and, after adjusting the pH to 6.3 with 1 M KH_2PO_4, were applied to a hydroxylapatite column (1.5 x 40 cm) equilibrated with 5 mM KP_i (pH 6.3) containing 5 mM mercaptoethanol, 1 mM benzamidine, 1 mM PMSF (buffer E). The column was washed with buffer E containing 0.5 M KCl and then developed with a gradient made up of 300 ml of buffer E and 300 ml of buffer E containing 0.8 M KP_i (pH 6.3). Fractions of 10 ml were collected and assayed for dienoyl-CoA isomerase. Active fractions were combined and concentrated in an Amicon concentrator with a PX-10 membrane. The enzyme concentrate was applied to a Sepharose CL-6B column (2 x 70 cm) equilibrated with 50 mM KP_i (pH 7.4) containing 0.1 M KCl, 5 mM mercaptoethanol, 1 mM benzamidine, 1 mM PMSF and 20% glycerol (buffer F). The column was developed with buffer F and fractions of 1.5 ml were collected. Active fractions were combined and dialyzed overnight against 25 mM ethanolamine-acetic acid (pH 9.4) containing 5 mM mercaptoethanol, 1 mM benzamidine, 1 mM PMSF and 30% glycerol (buffer G). The dialyzed sample was applied to a chromatofocusing column (1 x 20 cm) containing Polybuffer Exchanger 94 and equilibrated with buffer G. The column was washed with buffer G and then developed with 200 ml of Polybuffer 96 adjusted to pH 6. Fractions of 5 ml were collected and assayed for dienoyl-CoA isomerase. Active fractions were combined and, after adjusting the pH to 6.3 with 1 M KH_2PO_4, were applied to a second hydroxylapatite column (1.5 x 40 cm) equilibrated with buffer E

containing 30% glycerol. After extensively washing the column with buffer E containing 30% glycerol and 0.5 M KCl, the column was developed with a gradient made up of 300 ml of buffer E containing 30% glycerol and 300 ml of buffer E containing 30 % glycerol and 0.8 M KP_i (pH 6.3). Fractions of 10 ml were collected and assayed for dienoyl-CoA isomerase and crotonase. Fractions with a high activity ratio of isomerase to crotonase were combined , dialyzed overnight against buffer D containing 40 % glycerol, and stored at -80 °C.

Purification of SC-HMAD. All operations were carried out at a temperature between 4^0C and 0^0C. Eight rat livers from adult Sprague-Dawley rats, which had been fed rodent chow containing 2% (w/w) DEHP for 6 weeks, were minced and homogenized with 500 ml of 20 mM KP_i (pH 8.0) containing 5 mM mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine in a loose-fitting Potter-Elvehjem type glass-Teflon homogenizer. The resulting suspension was sonicated for 4 X 15 seconds with a Heat System Sonifier (model 385) and centrifuged at 10,000 X g for 10 min. The pellets were resuspended in the above buffer, sonicated, and centrifuged as described above. PEG was added to the combined supernatants, referred to as soluble extract, to give a final concentration of 3%. After keeping the suspension for three hours, it was centrifuged at 10,000 X g for 10 min. The pellets were discarded and PEG was added to the supernatant to a final concentration of 15%. The suspension was kept overnight and then was centrifuged at 10,000 X g for 10 min. The supernatant was discarded and the pellet was resuspended in 500 ml of 10 mM KP_i (pH 6.8) containing 5 mM mercaptoethanol, 1 mM

benzamidine and 25% glycerol (buffer H), plus 1 mM PMSF and 1 μM leupeptin. The suspension was centrifuged at 100,000 X g for one hour and the pellet was discarded. KCl was added to the supernatant to a final concentration of 50 mM and this solution was applied to phosphocellulose column (2 X 40 cm) which had been equilibrated with buffer H containing 50 mM KCl. The column was washed with 500 ml buffer H containing 100 mM KCl and then developed with a gradient made up of 300 ml of buffer H containing 100 mM KCl and 300 ml of buffer H containing 500 mM KCl. Fractions of 20 ml were collected and assayed for SC-HMAD and HAD activities. Fractions with high SC-HMAD activity were combined and applied to a hydroxylapatite column (2 X 40 cm), which had been equilibrated with buffer H containing 200 mM KCl. The column was washed with 200 ml of buffer H containing 200 mM KCl, and then was developed with a gradient made up of 300 ml of buffer H and 300 ml of buffer H containing 300 mM KP_i (pH 6.8). Fractions with high SC-HMAD activity were combined and dialyzed overnight against 20 mM ethanolamine-acetic acid buffer (pH 8.5), containing 5 mM mercaptoethanol, 1 mM benzamidine, and 25% glycerol. After dialysis, the sample was applied to a chromatofocusing column (1.5 X 40 cm) containing Polybuffer Exchange 94 equilibrated with 20 mM ethanolamine-acetic acid (pH 9.0) containing 5 mM mercaptoethanol, 1 mM benzamidine, and 25% glycerol. The column was washed with the dialysis buffer and fractions were assayed for SC-HMAD activity. Fractions containing SC-HMAD, which did not bind to the column, were combined and adjusted to pH 7.0 with 1 M KP_i buffer (pH 6.0). The sample was applied to an S Sepharose column (1.5 X 40 cm)

equilibrated with buffer H. The enzyme was eluted by applying a gradient made up of 200 ml of buffer H and 200 ml of buffer H containing 500 mM KCl. Fractions with SC-HMAD activity were collected, combined, and dialyzed overnight against buffer H. The sample was applied to a Blue Dextran Agarose column (1 X 15 cm) which had been equilibrated with buffer H. The column was extensively washed with buffer H and then with 20 ml of buffer H containing 2 mM NADH. Active fractions, identified by measuring HAD activity with acetoacetyl-CoA as a substrate, were concentrated in Centricon 10 microconcentrators and applied to a Sepharose CL-6B column (2 X 70 cm), which had been equilibrated with buffer H containing 50 mM KCl. The column was developed with the same buffer and fractions with HAD activity were concentrated by centrifugation in Centricon 10 microconcentrators. The resultant sample of SC-HMAD was stored at -80^{0}C.

Molecular Weight Determinations and Electrophoresis of Protein. The subunit molecular weights of purified enzymes were determined by SDS-PAGE on a 10% polyacryamide mini gel for trifunctional β-oxidation complex, on a 4-15% polyacryamide mini gel for dienoyl-CoA isomerase, and on a 4-20% polyacylamide mini gel for SC-HMAD according to the general procedure of Laemmli (70). The gels were stained for protein with Coomassie blue. The molecular weight standards used for estimating the subunit molecular weights of purified enzymes were phosphorylase b (97,400), serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). The molecular weight of

native dienoyl-CoA isomerase was determined by gel filtration on a Sepharose CL-6B column (2 x 70 cm) equilibrated with 50 mM KP_i (pH 7.4) containing 0.1 M KCl. Molecular weight standards used for estimating the native molecular weight of dienoyl-CoA isomerase were carbonic anhydrase (29,000), albumin (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), apoferritin (443,000), and thyroglobulin (669,000). The molecular weight of native SC-HMAD was determined by gel filtration on a Sephacryl S-200 column (1 X 40 cm) equilibrated with 100 mM KPi (pH 7). The molecular standard used for estimating the native molecular mass of SC-HMAD were cytochrome C (12,400), carbonic anhydrase (29,000), serum albumin (66,000), alcohol dehydrogenase (150,000), and β-amylase (200,000). Gel electrophoresis of dienoyl-CoA on polyacrylamide gel under nondenaturing conditions was performed as described (71) with some modifications. A 5%-15% polyacrylamide gel was used and run with 25 mM Tris, 200 mM glycine buffer adjusted to pH 8.6 with KOH at 10°C and a constant current of 10 mA. Upon completion, half of the gel was stained with Coomassie blue while the other half was sliced into segments which were extracted with 0.1 M KP_i buffer (pH 8.0) containing 5 mM mercaptoethanol, bovine serum albumin (1 mg/ml) and assayed for dienoyl-CoA isomerase.

Western Blotting Analysis of L-3-Hydroxy-2-methylacyl-CoA Dehydrogenase. After SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane and then probed with rabbit antiserum to normal HAD. The primary antibody was detected by allowing it to react with goat-rabbit IgG alkaline

phosphase conjugate, and it was visualized by incubating the nitrocellulose membrane in the presence of the alkaline phosphase substrate (72).

PART I: Purification and Characterization of the Trifunctional β-Oxidation Complex from Pig Heart Mitochondria

RESULTS

Effects of Detergents on the Activities of the Trifunctional β-Oxidation Complex. The adddition of Triton X-100 to the long-chain hydratase assay mixture caused the absorbance at 280 nm to change in the absence of enzyme. Thus when long-chain enoyl-CoA hydratase was assayed, no Triton X-100 was added to the assay mixture except for the Triton X-100 already present in the enzyme sample. When long-chain 3-hydroxyacyl-CoA dehydrogenase was assayed, the activity decreased with an increasing concentration of Triton X-100 in the assay mixture (Fig. 4). Therefore no additional Triton X-100 was added to the long-chain dehydrogenase assay mixture. For assaying long-chain 3-ketoacyl-CoA thiolase, however, the addition of 0.025% of Triton X-100 in the assay mixture yielded optimal thiolase activity (Fig. 5). Thus 0.025% of Triton X-100 was routinely included in the long-chain thiolase assay mixture. The presence of detergent, like octyl glucoside, increased the proteolysis more than 10-fold, as judged by the decline of the dehydrogenase activities (Fig. 6). A protease inhibitor cocktail was included in the buffer to reduce proteolysis. Benzamidine and PMSF were added to inhibit serine proteases. Leupeptin was included to inhibit thiol proteases, whereas pepstatin A was used to inhibit acid proteases. EDTA and EGTA were also included in the buffer to remove divalent metal ions, which might activate metalloproteases. In the presence of the above protease

inhibitors, the trifunctional β-oxidation complex was purified in more than 30% yield.

Purification of Trifunctional β-Oxidation Complex from Pig Heart. The purification of the trifunctional β-oxidation complex from pig heart was based on the published purification procedure of the complex from rat liver (27). However, some modifications were introduced to facilitate the purification of the complex without having first to isolate mitochondria from a tough tissue like heart. As a result, the specific activity of long-chain L-3-hydroxyacyl-CoA dehydrogenase, which was measured to monitor the purification of the complex, was lower in the extract from pig heart membranes (0.22 units/mg; see Table I) than in the extract from the mitochondrial membrane of rat liver (2.84 units/mg; see Ref. 27). However, the specific activities of the final preparations from pig heart with 3-ketohexadecanoyl-CoA as substrate (26 units/mg; see Table I) and rat liver (26.3 units/mg; see Ref. 27) were virtually identical. A cocktail of protease inhibitors was present during the isolation and solubilization of membranes to reduce loss of enzyme activity due to proteolysis. The trifunctional complex, based on the long-chain L-3-hydroxyacyl-CoA dehydrogenase activity, was purified more than 100-fold at better than 30% yield. The degree and yield of this purification were somewhat higher than indicated in Table I because a fraction, estimated to be 15% of the dehydrogenase activity measured with 3-ketohexadecanoyl-CoA (C_{16}) as a substrate, was due to the general L-3-hydroxyacyl-CoA dehydrogenase which is reflected by the short-chain (C_4) activity.

Proof that the long-chain L-3-hydroxyacyl-CoA dehydrogenase activity reflected the trifunctional enzyme, was provided by assaying the fractions from the second phosphocellulose column for long-chain activities of 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and 3-ketoacyl-CoA thiolase. As shown in Fig. 7, all three enzyme activities were eluted coincidentally and in parallel to the second protein peak.

The final specific activities of the three long-chain enzymes were 26 units/mg for L-3-hydroxyacyl-CoA dehydrogenase with 3-ketohexadecanoyl-CoA as substrate, 25.5 units/mg for enoyl-CoA hydratase with 2-hexadecenoyl-CoA, and 19.2 units/mg for 3-ketoacyl-CoA with 3-ketohexadecanoyl-CoA. Activities with medium-chain (C_8) substrates were 87%, 71% and 73% of the long-chain (C_{16}) activities determined for L-3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase, respectively. The activities of the complex with corresponding short-chain (C_4) substrates were less than 2% of the long-chain (C_{16}) activities.

The purity of the final preparation was assessed by SDS-PAGE (see Fig. 8) which proved the presence of two types of subunits with estimated molecular weights of 81,000 and 45,000. Small amounts of impurities and/or degradation products related to the complex with molecular weights slightly higher than that of the 45-kDa subunit were detected by scanning the gel (see Fig. 8).

Partial Purification of the Trifunctional β-Oxidation Complex from Pig Heart in the Absence of Detergent. Since the mitochondrial long-

chain enoyl-CoA hydratase from pig heart had been separated from crotonase and partially purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose (23), a fraction of a pig heart homogenate was subjected to DEAE-cellulose chromatography and assayed for the long-chain activities L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in addition to enoyl-CoA hydratase. Shown in Fig. 9A are the results of such experiment which documents the coelution of the three long-chain-specific enzymes virtually devoid of the corresponding short-chain activities. Hence, long-chain enoyl-CoA hydratase (EC 4.2.1.74) is a component enzyme of the trifunctional β-oxidation complex. Since no detergents were used in this experiment and the trifunctional β-oxidation complex is membrane-bound (27), the fractions containing the complex should contain membranous material. In agreement with this prediction was the observed cloudiness of the fractions containing the complex. Further evidence for the presence of membranous material in fractions containing the complex was provided by assaying for marker enzymes of the inner and outer mitochondrial membrane. As shown in Fig. 9B, long-chain L-3-hydroxyacyl-CoA dehydrogenase of the complex and succinate-cytochrome C reductase, a marker enzyme for the inner mitochondria membrane (73), were coeluted. The same fractions also contained rotenone-insensitive NADH-cytochrome C reductase which is a marker enzyme of the outer mitochondrial membrane (73). However, the elution patterns of the two marker enzymes differed significantly thereby permitting the conclusion that the

trifunctional β-oxidation complex is associated with the inner mitochondrial membrane.

Since the reported chain length specificities of the partially purified long-chain enoyl-CoA hydratase from pig heart (23) and of the long-chain enoyl-CoA hydratase associated with the trifunctional β-oxidation complex from rat liver (27) differ significantly, the chain length specificity of enoyl-CoA hydratase associated with the purified pig heart complex was determined. At a fixed substrate concentration of 50 μM, the highest specific activity was observed with 2-tetradecenoyl-CoA (C_{14}) which was twice the activity determined with 2-hexadecenoyl-CoA (C_{16}). The activities with 2-hexenoyl-CoA (C_6), 2-octenoyl-CoA (C_8), and 2-decenoyl-CoA (C_{10}) were 57%, 71% and 86%, respectively, of the activity observed with 2-tetradecenoyl-CoA.

DISCUSSION

Studies of the enzymes of β-oxidation in the fifties and sixties led to the identification of three acyl-CoA dehydrogenases with different but overlapping chain length specificities and one enzyme each for the three additional reactions of the β-oxidation spiral (74). The latter three enzymes have broad chain length specificities and are active with substrates having acyl chains from four to sixteen carbon atoms. All of the β-oxidation enzymes are soluble in aqueous buffers and are located in the mitochondrial matrix. The low activity of enoyl-CoA hydratase (crotonase) toward long-chain substrates prompted a search for a long-chain-specific enoyl-CoA hydratase. Such enzyme, which

hydrates medium-chain and long-chain substrates but is inactive toward crotonyl-CoA, was detected in pig heart mitochondria (23). Surprisingly, it is associated with the mitochondrial membrane and not with the matrix fraction (23). Subsequently, evidence for the presence of a long-chain L-3-hydroxyacyl-CoA dehydrogenase in rat liver mitochondria was published (41). This enzyme is also membrane-bound and inactive with the short-chain substrate acetoacetyl-CoA (41). The purification and characterization of long-chain L-3-hydroxyacyl-CoA dehydrogenase led to the isolation of trifunctional β-oxidation complexes from rat liver (27) and human liver (28). The rat liver complex, which is composed of two types of subunits with molecular weights of 49-51 kDa and 79 kDa, respectively, exhibits medium-chain and long-chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA thiolase (27). The human liver complex is very similar (28). However, it remained unclear whether or not the previously described long-chain enoyl-CoA hydratase is part of the complex. The purified trifunctional complex from pig heart is composed of two subunits with molecular masses of 81 kDa and 45 kDa, analyzed by SDS-PAGE. Based on the amino acid sequence, however, the calculated molecular mass of the large subunit is 79 KDa (75). This report demonstrates that long-chain activities of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase are associated with long-chain enoyl-CoA hydratase which was partially purified from pig heart by the previously published procedure (23). Since purified pig heart complex (see Fig. 7) and partially purified pig heart long-chain enoyl-CoA hydratase (see Fig. 9A) contained similar relative long-

chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, it is concluded that long-chain enoyl-CoA hydratase (EC 4.2.1.74) is a component enzyme of the trifunctional β-oxidation complex. Additionally, evidence is provided for the association of the β-oxidation complex with the inner mitochondrial membrane.

The chain length specificities of long-chain enoyl-CoA hydratase associated with purified mitochondrial membranes from pig heart and the specificity of enoyl-CoA hydratase associated with the trifunctional β-oxidation complex from rat liver differ significantly. Although both hydratase activities are virtually inactive toward crotonyl-CoA, the hydratase of the purified complex is most active with 2-hexadecenoyl-CoA (27), whereas the hydratase associated with membranes is most active toward 2-octenoyl-CoA (23). However, when purified, the pig heart hydratase was found to be most active with the long-chain substrate 2-tetradecenoyl-CoA while simultaneously exhibiting high activities with medium-chain substrates from 2-hexenoyl-CoA to 2-decenoyl-CoA. The remaining difference between the specificities of the rat liver and pig heart hydratases may reflect species differences or, more likely, be due to differences in assay conditions which greatly affect the activity of this enzyme.

The association of long-chain-specific forms of the four β-oxidation enzymes with the inner mitochondrial membrane (20, 27-29), prompts the idea that the initial phase of β-oxidation might take place at the inner mitochondrial membrane. If so, fatty acyl groups after

passing through the inner mitochondrial membrane as acyl carnitines, would be transferred from carnitine to CoA by carnitine palmitoyl–transferase II and then chain shortened by the long-chain-specific β-oxidation system. This hypothesis raises the question as to whether long-chain-specific forms of 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase, which are required for the β-oxidation of unsaturated fatty acids, are also located at the inner mitochondrial membrane. The cooperative work done by XueYing He (29) in the same lab indicates that neither a long-chain 2,4-dienoyl-CoA reductase nor a long-chain Δ^3,Δ^2-enoyl-CoA isomerase is associated with the inner mitochondrial membrane.

The existence of a long-chain-specific β-oxidation system in the inner mitochondrial membrane in addition to the well-known matrix system active with short-chain, medium-chain and long-chain fatty acids raised many questions about the metabolic function(s) of these two systems. For example, is the trifunctional β-oxidation complex specifically involved in the acetyl-CoA-dependent elongation of fatty acids in mitochondria (74)? Moreover, conclusions that have been reached about regulatory sites and mechanisms of mitochondrial β-oxidation must be re-evaluated in view of the increased complexity of the mitochondrial β-oxidation system.

PART II: Purification and Characterization of $\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA Isomerase from Rat Liver Mitochondria

RESULTS

Subcellular Localization of Dienoyl-CoA Isomerase. Since mammalian cells contain two β-oxidation systems, one located in mitochondria and the other associated with peroxisomes, dienoyl-CoA isomerase may be present in either organelle or both. For the purpose of determining the subcellular location of dienoyl-CoA isomerase, a light mitochondrial fraction (L-fraction), containing both mitochondria and peroxisomes, was prepared from the liver of a rat fed chow containing 2% (w/w) DEHP to cause the proliferation of peroxisomes and the induction of the peroxisomal β-oxidation system (33). The light mitochondrial fraction was further fractionated by centrifugation on a Nycodenz density gradient. Fractions were assayed for dienoyl-CoA isomerase in addition to catalase and malate dehydrogenase which served as marker enzymes for peroxisomes and mitochondria, respectively. The results shown in Fig.10 demonstrate that dienoyl-CoA isomerase is a mitochondrial enzyme. The low level of dienoyl-CoA isomerase detected in fractions 1 through 3, which contain mostly peroxisomes, may be due to contaminating mitochondria as evidenced by residual malate dehydrogenase activity detected in the same fractions. Thus, dienoyl-CoA isomerase is absent from peroxisomes or is present only at a very low level.

Purification of Dienoyl-CoA Isomerase from Rat Liver. Since dienoyl-CoA isomerase is a soluble mitochondrial enzyme, a time consuming

preparation of subcellular organelles was unnecessary and hence a soluble extract prepared from whole rat liver was used as starting material for the purification of this enzyme. A liver from a rat fed chow containing 2% DEHP was used because the specific activity of dienoyl-CoA isomerase was increased compared to normal livers. The purification of dienoyl-CoA isomerase is summarized in Table 2. Precipitation by PEG and chromatography on Q-Sepharose were chosen as the initial purification steps because these methods can be easily upscaled to accommodate a large amount of proteins. The initial two steps resulted in the removal of 95% of the protein and an almost 16-fold purification. Further purification was achieved by column chromatographies on hydroxylapatite and Sepharose CL-6B followed by chromatofocusing. Finally, a second hydroxylapatite chromatography step resulted in the separation of the remaining enoyl-CoA hydratase (crotonase) from dienoyl-CoA isomerase (see Fig. 11) which was purified by this procedure 370-fold at almost 30% yield. The coincidence of the main protein peak with the peak of dienoyl-CoA isomerase activity is indicative of a highly purified enzyme preparation.

The purity of dienoyl-CoA isomerase was assessed by PAGE in the absence (Fig.12) and presence of SDS (Fig. 13). Under non-denaturing conditions, purified dienoyl-CoA isomerase yielded a single band of protein that exhibited isomerase activity (see Fig. 12). In the presence of SDS the isomerase gave rise to one protein band which upon careful inspection seemed to consist of two closely-spaced bands of near equal intensity (see Fig.13) Since the use of protease inhibitors

during its purification was important for obtaining good preparations of this enzyme, the impression is that the enzyme is sensitive to proteases. This property could be the cause for the presence of two types of subunits of almost equal size which may have been formed by proteolysis during the purification of dienoyl-CoA isomerase. However, it is also possible that the enzyme consists of two distinct types of subunits or that two unrelated but very similar proteins were co-purified. Further studies will have to resolve this problem.

The purified preparation of dienoyl-CoA isomerase was assayed for other enzymes of β-oxidation. Traces of enoyl-CoA hydratase (crotonase) and 3-hydroxyacyl-CoA dehydrogenase activities of less than 0.1 U/mg were detected, whereas no activities were observed when the preparation was assayed for Δ^3,Δ^2-enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, or thiolase.

Properties of Dienoyl-CoA Isomerase. The molecular weight of native dienoyl-CoA isomerase was estimated by chromatography on Sepharose CL-6B to be 126,000 (data not shown). Since SDS-PAGE yielded a subunit molecular weight of 32,000 (see Fig. 13), dienoyl-CoA isomerase seems to be composed of four subunits. The pH-activity profile of dienoyl-CoA isomerase has the appearance of a bell-shaped curve with an optimum close to pH 8 and half maximal activities at pH 5 and 10 (Fig. 14).

DISCUSSION

The reaction catalyzed by dienoyl-CoA isomerase and the mitochondrial location of this enzyme agree with the proposed

function of this isomerase in β-oxidation of unsaturated fatty acids (47). However, it was not clear whether this isomerase activity was due to a unique enzyme or was the additional activity of an otherwise known β-oxidation enzyme. In fact, it had been claimed that the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA is catalyzed by Δ^3,Δ^2-enoyl-CoA isomerase (76). This claim is disproved by the purification of dienoyl-CoA isomerase which is devoid of Δ^3, Δ^2-enoyl-CoA isomerase activity. Moreover, it was previously demonstrated that Δ^3, Δ^2-enoyl-CoA isomerase activities present in mitochondria and peroxisomes catalyze the isomerization of 2,5-octadienoyl-CoA to its 3,5-isomer, but not to the 2,4-isomer (47). It is likely that the dienoyl-CoA isomerase activity attributed to Δ^3, Δ^2-enoyl-CoA isomerase was due to the presence of dienoyl-CoA isomerase in the partially purified preparation of Δ^3, Δ^2-enoyl-CoA isomerase used in the previous study (76). The separation of enoyl-CoA hydratase (crotonase) from dienoyl-CoA isomerase and the absence of significant activities of other β-oxidation enzyme from the purified preparation support the conclusion that dienoyl-CoA isomerase is an unique and until now unidentified enzyme of β-oxidation.

The purification of dienoyl-CoA isomerase completes the characterization of the enzymes functioning in the novel NADPH-dependent pathway of β-oxidation by which unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms are degraded. Shown in Fig. 15 are the currently accepted, NADPH-independent pathway characterized by metabolites 2 through 6 and

the novel NADPH-dependent pathway involving metabolites 2 and 7 through 10. To which extent these two pathways contribute to the β-oxidation of unsaturated fatty acids depends on the fraction of 2,5-dienoyl-CoA (Fig. 15, compound 2) completing one cycle of β-oxidation (NADPH-independent pathway) as compared to the fraction of this compound undergoing isomerization to 3,5-dienoyl-CoA (Fig. 15, compound 7). It seems that once the two carbon-carbon double bonds are in conjugation, as in the $\Delta^{3,5}$-isomer, the metabolite is destined for the NADPH-dependent pathway. If so, Δ^3, Δ^2-enoyl-CoA isomerase plays a key role in determining the flux through the NADPH-dependent pathway, and hence knowledge about its chain length specificity is crucial for predicting the extent to which 2,5-dienoyl-CoA metabolites of different chain lengths are degraded via the NADPH-dependent pathway.

The absence of dienoyl-CoA isomerase from peroxisomes suggests that the NADPH-dependent pathway is not operative in peroxisomal β-oxidation. This conclusion is surprising because Δ^3, Δ^2-enoyl-CoA isomerase associated with the trifunctional peroxisomal enzyme catalyzes the isomerization of 2,5-octadienoyl-CoA to 3,5-octadienoyl-CoA (47). However, it is possible that 2,5-octadienoyl-CoA is not a peroxisomal metabolite because its formation requires linolenoyl-CoA to pass five times through the β-oxidation spiral, whereas peroxisomal β-oxidation in mammals was reported to chain shorten fatty acids only by two or three cycles (77).

Although this report definitely establishes that an NADPH-dependent pathway capable of oxidizing unsaturated fatty acids with

odd-numbered double bonds exists in mitochondria, the contribution of this pathway to the β-oxidation of unsaturated fatty acids remains to be established.

PART III: Purification and Characterization of 3-Hydroxy-2-methyacyl-CoA Dehydrogenase (SC-HMAD)

RESULTS

Purification of SC-HMAD from Rat Liver. Homogenization of rat liver followed by sonification resulted in the release of a soluble enzyme activity, referred to as short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase (SC-HMAD), which catalyzed the reduction of NAD^+ upon incubation with tiglyl-CoA in the presence of crotonase. Since crotonase is abundantly present in soluble liver extracts, it was only added to the assay mixture after it had been removed during the purification of SC-HMAD. The soluble extract from eight rat livers, after fractionation with PEG, was subjected to chromatography on phosphocellulose. Fractions were assayed for SC-HMAD with tiglyl-CoA and for HAD with acetoacetyl-CoA. The results of this purification step, shown in Fig. 16, clearly demonstrate that the SC-HMAD activity is separable from the main HAD activity. The SC-HMAD activity, after chromatography on phosphocellulose, was further purified by chromatography on hydroxylapatite, chromatofocusing and chromatographies on S-Sepharose, Blue Dextran Agarose followed by gel filtration on Sepharose CL-6B. As summarized in Table III, SC-HMAD was purified by this procedure 6000-fold and obtained in 6% yield.

Characterization of Purified SC-HMAD. When the purity of the SC-HMAD preparation was evaluated by SDS-PAGE, only a single band was observed (Fig. 17A, lane 2) which migrated ahead of the band

corresponding to HAD from pig heart (Fig. 17A, lane 3). Immunoblotting with antiserum to pig heart HAD resulted in the appearance of one strong band in the lane to which 0.4 μg of pig heart HAD had been applied (Fig. 17B, lane 6). A weaker band was detected at the position of HAD, in the lane to which a sample of soluble rat liver proteins containing 0.1-0.2 μg of rat liver HAD had been applied (Fig. 17B, lane 5). Thus, antibodies to pig heart HAD cross-react with rat liver HAD. However, as no band was detected in the lane with 0.5 μg of rat liver SC-HMAD, it is concluded that this enzyme is immunologically unrelated to HAD.

The subunit molecular weight of SC-HMAD was estimated to be 28,000 and hence its molecular weight is smaller than that of pig heart HAD for which a molecular weight of 34,000 was obtained close to the calculated value of 33,300 based on the published amino acid sequence for this protein (78). The molecular weight of native SC-HMAD was estimated by gel filtration on a Sephacryl S-200 column (1 X 50 cm) equilibrated with 0.1 M KP_i (pH 7). Pig heart HAD, and rat liver SC-HMAD were eluted in the same fractions in which serum albumin with molecular weight of 66,000 emerged (data not shown). Hence, SC-HMAD, like HAD, seems to be a dimer composed of two identical subunits.

The products of the reaction catalyzed by SC-HMAD were characterized spectrophotometrically and by HPLC. Incubation of tiglyl-CoA with NAD^+ and SC-HMAD at pH 9 yielded NADH, as reflected by an increase in absorbance at 340 nm, only when crotonase was added to catalyze the conversion of tiglyl-CoA to L-3-hydroxy-2-

methylbutyryl-CoA (see Fig. 18A). When pig heart HAD was substituted for SC-HMAD, no reaction was observed unless crotonyl-CoA was added to the incubation mixture (see Fig. 18A). This observation confirms that mitochondrial HAD does not act on 2-methyl-branched substrates. The degradation of tiglyl-CoA by a reconstituted β-oxidation system was evaluated. For this purpose, tiglyl-CoA was incubated with crotonase, SC-HMAD, and mitochondrial acetoacetyl-CoA thiolase in the presence of NAD^+ and CoASH. HPLC-analysis of acyl-CoA thioesters by Li Feng Mao (79) revealed the formation of propionyl-CoA and acetyl-CoA (see Fig. 18B). Together these experiments demonstrate that SC-HMAD catalyzes the NAD^+-dependent dehydrogenation of L-3-hydroxy-2-methylbutyryl-CoA to 2-methyl-acetoacetyl-CoA which is cleaved by acetoacetyl-CoA thiolase to acetyl-CoA and propionyl-CoA.

The apparent kinetic constants (V_{max}, K_m) were determined with L-3-hydroxy-2-methylbutyryl-CoA and L-3-hydroxybutyryl-CoA as substrates. Similar maximal velocities of 22 units/mg and 17 units/mg were obtained with the methyl branched and straight chain substrates, respectively. However, the apparent K_m value for L-3-hydroxy-2-methylbutyryl-CoA was only 5 μM as compared to 19 μM for L-3-hydroxybutyryl-CoA. Thus, the lower activity observed with the straight chain substrate as compared to the branched chain substrate is more a reflection of different K_m values than of different maximal velocities. If the K_m values are measures of the affinities of the substrates for the enzyme, it seems that the 2-methyl group makes a positive contribution to the binding of the substrate to the

enzyme. The apparent K_m value for NAD^+ with L-3-hydroxy-2-methylbutyryl-CoA as the substrate was determined to be 63 μM.

DISCUSSION

A study of the hepatic metabolism of 2-methyl fatty acids revealed that medium-chain and long-chain 2-methyl fatty acids can be degraded in rat liver mitochondria by β-oxidation (80). The enzymes acting on medium-chain and long-chain 2-methylacyl-CoA thioesters are long-chain acyl-CoA dehydrogenase (EC 1.3.99.3) and the trifunctional β-oxidation complex (80) which harbors medium-chain and long-chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (27-29). However, not all of the β-oxidation enzymes acting on short-chain 2-methylacyl-CoA thioesters, as for example 2-methylbutyryl-CoA, a metabolite of isoleucine, had been characterized. It was known that 2-methylbutyryl-CoA is dehydrogenated by 2-methyl-branched chain acyl-CoA dehydrogenase to tiglyl-CoA (51) which can be hydrated by enoyl-CoA hydratase (crotonase) to L-3-hydroxy-2-methylbutyryl-CoA (50). The mammalian dehydrogenase acting on L-3-hydroxy-2-methylbutyryl-CoA had not been identified, wherease the thiolase cleaving the dehydrogenation product, 2-methylacetoacetyl-CoA, was known to be mitochondrial acetoacetyl-CoA thiolase which is defective in patients with β-ketothiolase deficiency (53). This study reports the purification of SC-HMAD from rat liver and thereby completes the characterization of the enzymes which together catalyze the mitochondrial β-oxidation of short-chain 2-methylacyl-CoA thioesters, including 2-methylbutyryl-CoA, a metabolite of

isoleucine. Of the four β-oxidation enzymes involved in the degradation of short-chain 2-methylacyl-CoA only enoyl-CoA hydratase (crotonase) is required for the β-oxidation of fatty acids. The other three enzymes, 2-methyl-branched chain acyl-CoA dehydrogenase, SC-HMAD, and acetoacetyl-CoA thiolase apparently do not function in the β-oxidation of common fatty acids. However, mitochondrial acetoacetyl-CoA thiolase also functions in ketone body metabolism (52). This conclusion is supported by the observation that a deficiency of this enzyme (β-ketothiolase deficiency) is a cause for severe ketoacidosis (81). Thus, of the four β-oxidation enzymes necessary for the degradation of the isoleucine metabolite 3-hydroxy-2-methylbutyryl-CoA, only two, namely 2-methyl-branched chain acyl-CoA dehydrogenase and SC-HMAD, seem to be specifically involved in the degradation of short-chain 2-methylacyl-CoA thioesters. Known substrates of 2-methyl-branched chain acyl-CoA dehydrogenase are 2-methylbutyryl-CoA and isobutyryl-CoA which are metabolites of isoleucine and valine, respectively (51). Assuming that SC-HMAD does not function in fatty acid β-oxidation, the only known substrate of SC-HMAD is L-3-hydroxy-2-methylbutyryl-CoA, a metabolite of isoleucine. However, it is possible that SC-HMAD functions in the metabolism of other branched chain carboxylic acids that may be degraded by mitochondrial β-oxidation. For example, pristanic acid, which is formed from phytanic acid by β-oxidation, is believed to be chain-shorted by peroxisomal β-oxidation (77). The resultant short-chain and/or medium-chain 2-methylacyl-CoA thioesters may be further degraded by the mitochondrial β-oxidation system that includes SC-HMAD. Xenobiotic 2-branched chain

carboxylic acids may undergo mitochondrial β-oxidation involving SC-HMAD. An example is valproic acid (2-propylpentanoic acid) which is metabolized in liver to 3-ketovalproyl-CoA (3-keto-2-propylpentanoyl-CoA) (82).

This report describes a third mitochondrial type of L-3-hydroxyacyl-CoA dehydrogenase. These three enzymes, SC-HMAD, HAD, and long-chain HAD associated with the trifunctional β-oxidation complex, are clearly distinguishable by their different substrate specificities and molecular structures. Whereas SC-HMAD acts on short-chain and to a lesser extent on medium-chain 2-methyl-branched and straight chain substrates, HAD only acts on straight chain substrates with acyl chains having 4-16 carbon atoms. The latter enzyme is most active with medium-chain substrates, slightly less active with short-chain substrates, but exhibits little activity toward a long-chain substrate like L-3-hydroxyhexadecanoyl-CoA (26). The long-chain HAD activity of the trifunctional β-oxidation complex is active with straight-chain as well as 2-methyl-branched long-chain and medium-chain substrates (80) It is likely that HAD and long-chain HAD cooperate in the β-oxidation of fatty acids to assure high rates of dehydrogenation over the full spectrum of 3-hydroxyacyl-CoA intermediates. SC-HMAD functions in isoleucine metabolism but additionally may complement long-chain HAD to facilitate the complete β-oxidation of branched chain carboxylic acids. Direct evidence in support of the proposed functions of these three dehydrogenases has so far only been obtained for long-chain HAD. A deficiency of this enzyme in humans impairs fatty acid β-oxidation

and can cause life-threatening hypoketotic hypoglycemia (43). Together the three L-3-hydroxyacyl-CoA dehydrogenases can account for the degradation of all known subtrates of mitochondrial β-oxidation.

TABLE I

Purification of the Trifunctional β-Oxidation Complex from Pig Heart

Step	Total activity[a]		Total protein	Specific activity	Purification	Recovery
	C4	C16		C16		
	units		*mg*	*units/mg*	*fold*	%
Membrane extract	5,631	7,084	32,200	0.22	1	100
Phosphocellulose	644	5,036	2,675	1.88	8.5	71
DEAE-Cellulose	455	3,834	1,361	2.82	12.8	54
Phosphocellose	17	2,270	87.2	26	118	32

[a] The activity of L-3-hydroxyacyl-CoA dehydrogenase was measured with either acetoacetyl-CoA (C4) or 3-ketohexadecanoyl-CoA (C16) as substrate.

TABLE II

Purification of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase from rat liver

Step	Total activity [a] *units*	Total protein *mg*	Specific activity *units/mg*	Purification *fold*	Recovery %
Soluble Extract	713	4080	0.17	1	100
PEG precipitation	601	1400	0.43	2.5	84
Q-Sepharose	503	183	2.75	16	71
Hydroxylapatite	455	43.7	10.4	61	64
Sepharose CL-6B	336	24.7	13.6	80	47
Chromatofocusing	266	5.4	49	288	37
Hydroxylapatite	203	3.2	63	370	28

a. Dienoyl-CoA isomerase activity was determined with 8 μM 3,5-*trans*-octadienoyl-CoA as described under Experimental Procedures.

TABLE III
Purification of SC-HMAD from Rat Liver

Step	Total activity *units*	Total protein *mg*	Specific activity *units/mg*	Purification *fold*	Recovery %
Soluble Extract	42	13,650	0.003	1	100
PEG precipitation	37	5,580	0.0066	2.2	88
Phosphocellulose	31	1,670	0.019	6.2	74
Hydroxylapatite	26	715	0.036	12	62
Chromatofocusing	22	176	0.125	42	52
S-Sepharose	18	75	0.24	80	43
Blue Dextran Agarose and Sepharose CL-6B	2.5	0.14	18	6000	6

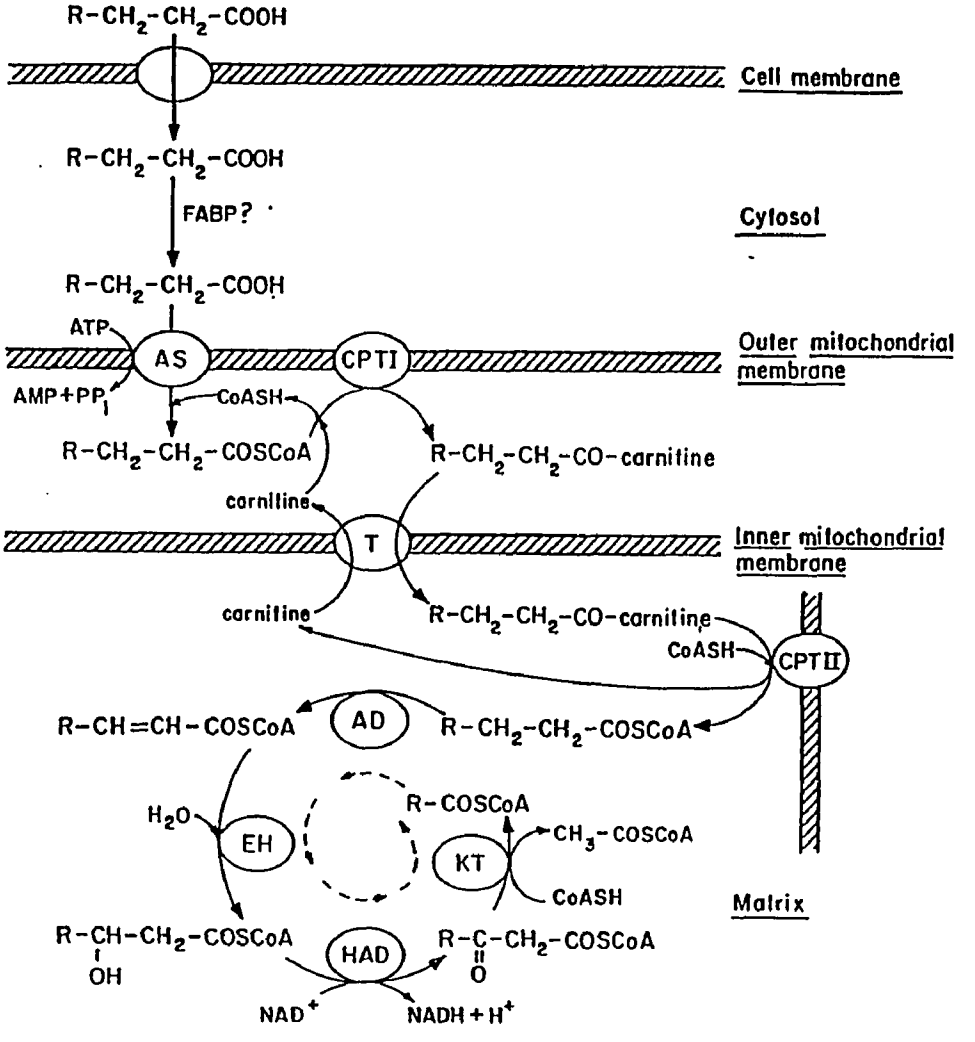


Figure 1. The overall scheme for fatty acid activation, transportation and the four reactions involved in the β-oxidation cycle. Enzymes of the pathway are: AS, acyl-CoA synthetase; CPT I, carnitine palmitoyltransferase I; T, carnitine:acylcarnitine transferase; CPT II, carnitine palmitoytransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HAD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase. Other abbreviation; FABP, fatty acid binding protein.

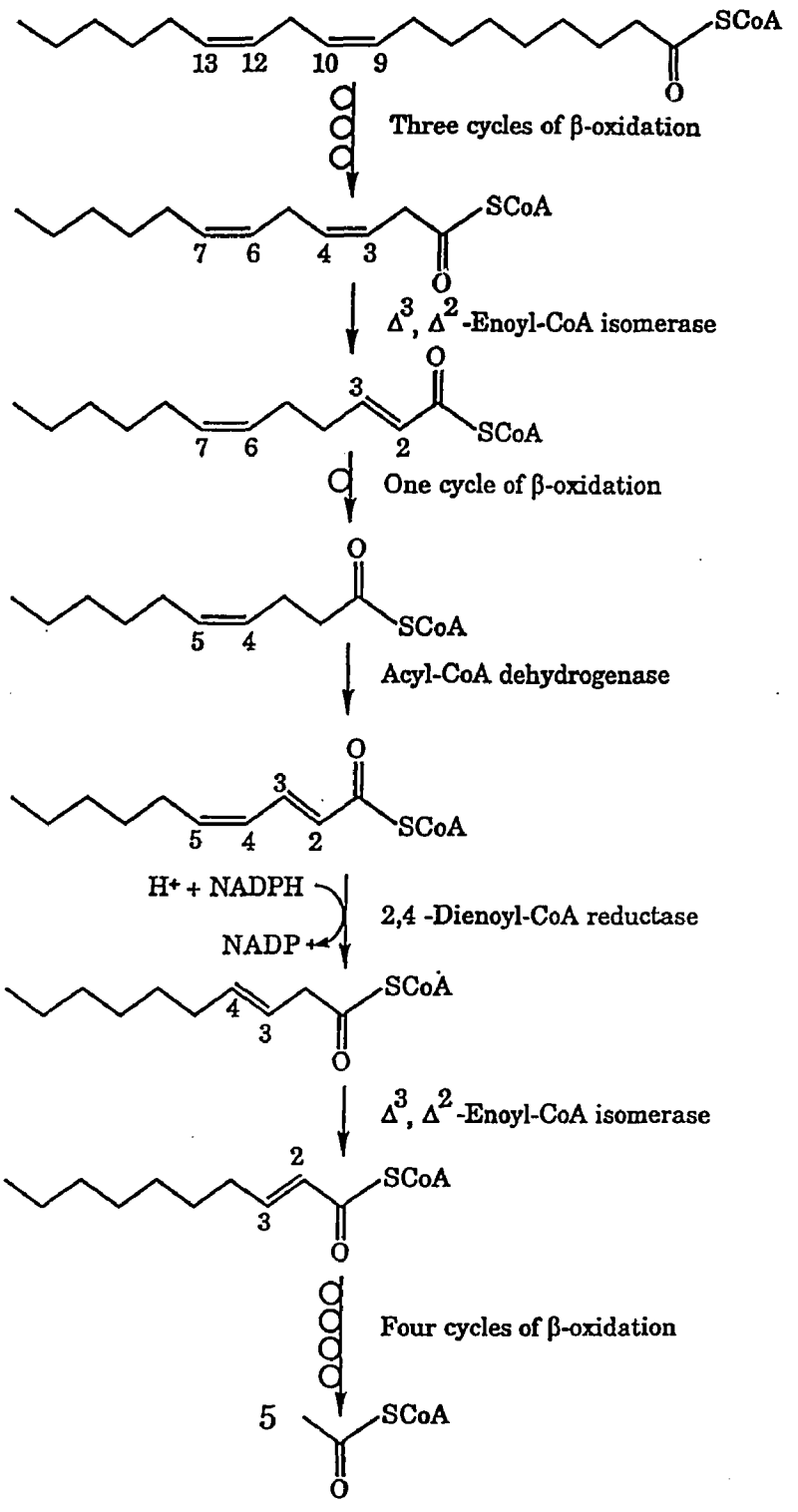


Figure 2. β-Oxidation of linoleoyl-CoA.

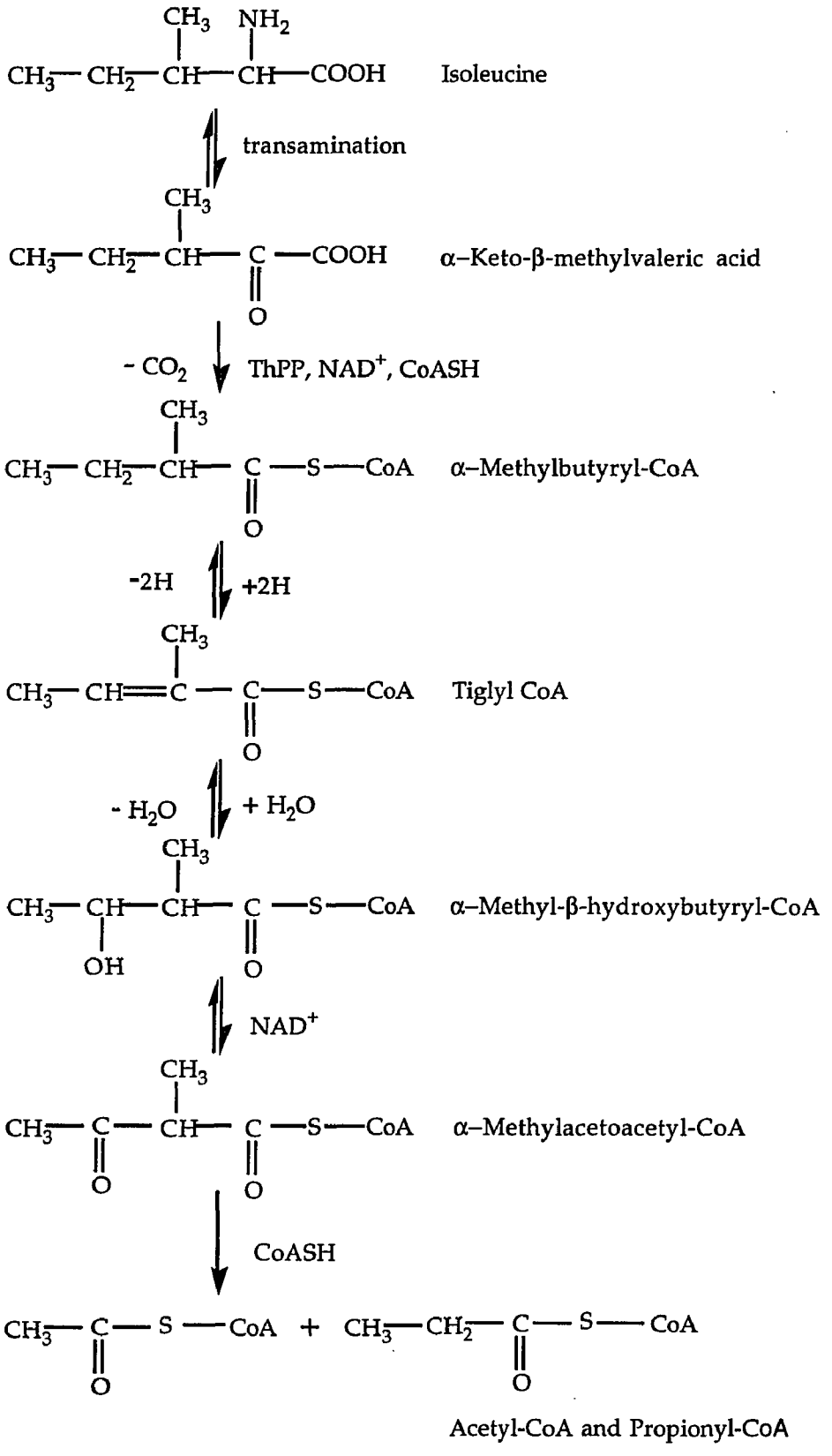


Figure 3. Metabolism of Isoleucine

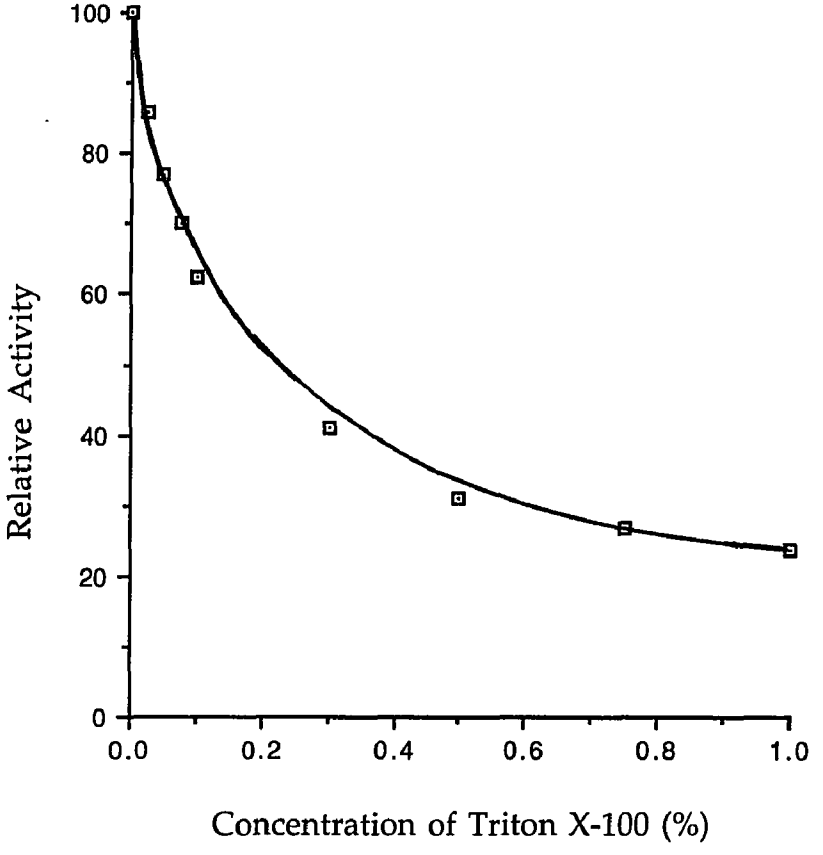


Figure 4. Effect of Triton X-100 on long-chain 3-hydroxyacyl-CoA dehydrogenase activity.

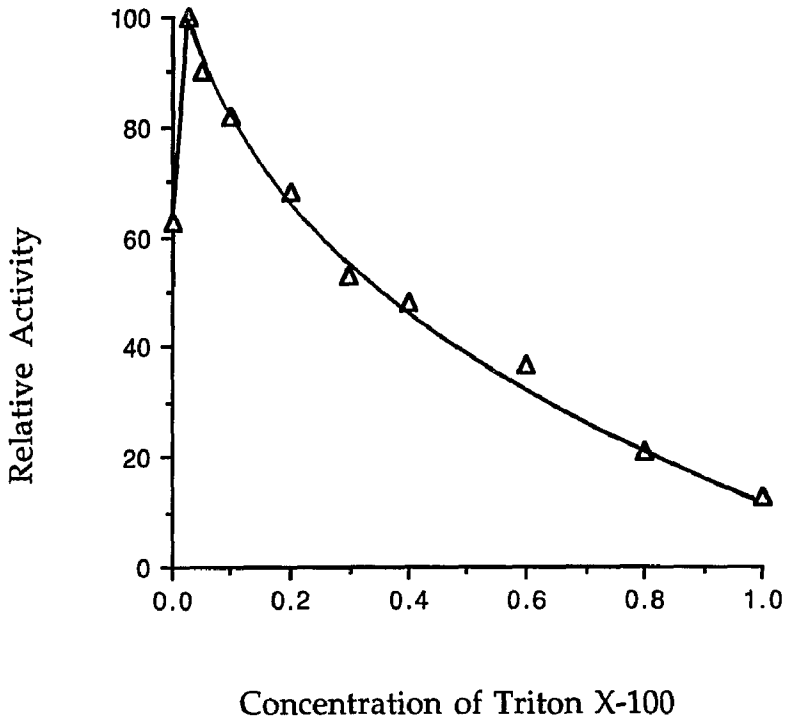


Figure 5. Effect of Triton X-100 on long-chain 3-ketoacyl-CoA thiolase activity.

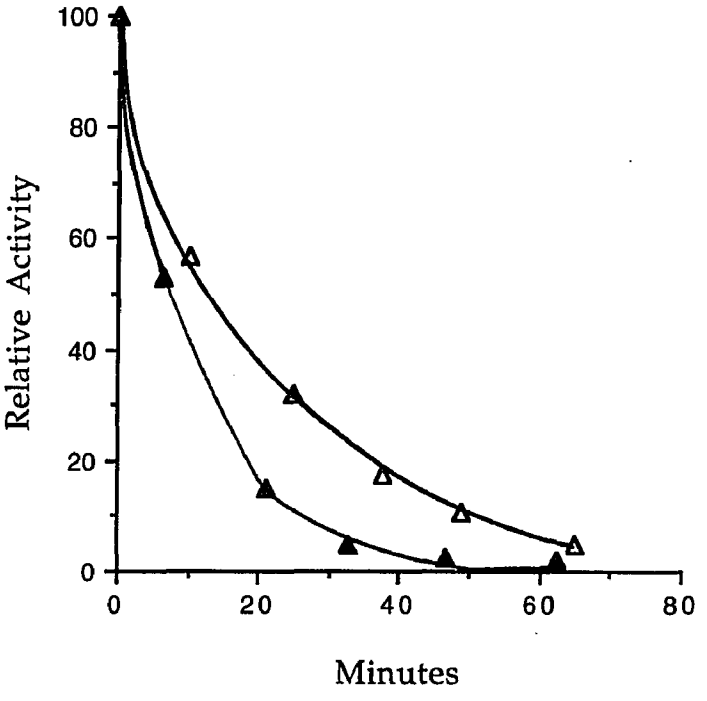


Figure 6. Effect of octyl glucoside on the subtilisin-dependent inactivation of HAD present in an extract obtained by sonication and centrifugation of rat liver mitochondria, suspended in 0.5 M KCl, 50 mM KPi (pH 7.5). HAD activities were measured with 3-ketohexadecanoyl-CoA as a substrate. The results are expressed as activities relative to activities measured in the absence of subtilisin.

a. The extract contained subtilisin (0.177 mg/mg of protein) and no octyl glucoside.

b. The extract contained subtilisin (0.0177 mg/mg of protein) and 0.5% octyl glucoside.

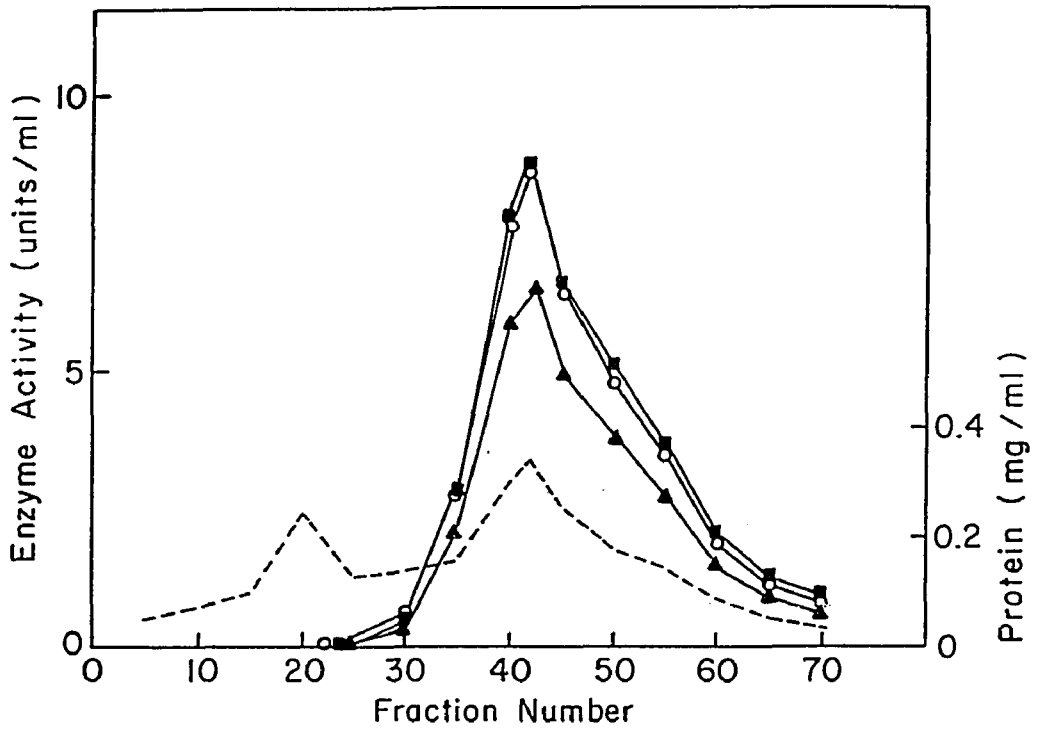


Figure 7. Purification of the trifunctional β-oxidation complex from pig heart by chromatography on phosphocellulose. Dashed line, protein concentration; solid line, enzymatic activities; (■) long-chain L-3-hydroxyacyl-CoA dehydrogenase with 3-ketohexadecanoyl-CoA as substrate; (o) long-chain enoyl-CoA hydratase with 2-hexadecenoyl-CoA as substrate; (▲) long-chain 3-ketoacyl-CoA thiolase with 3-ketohexadecanoyl-CoA as substrate.

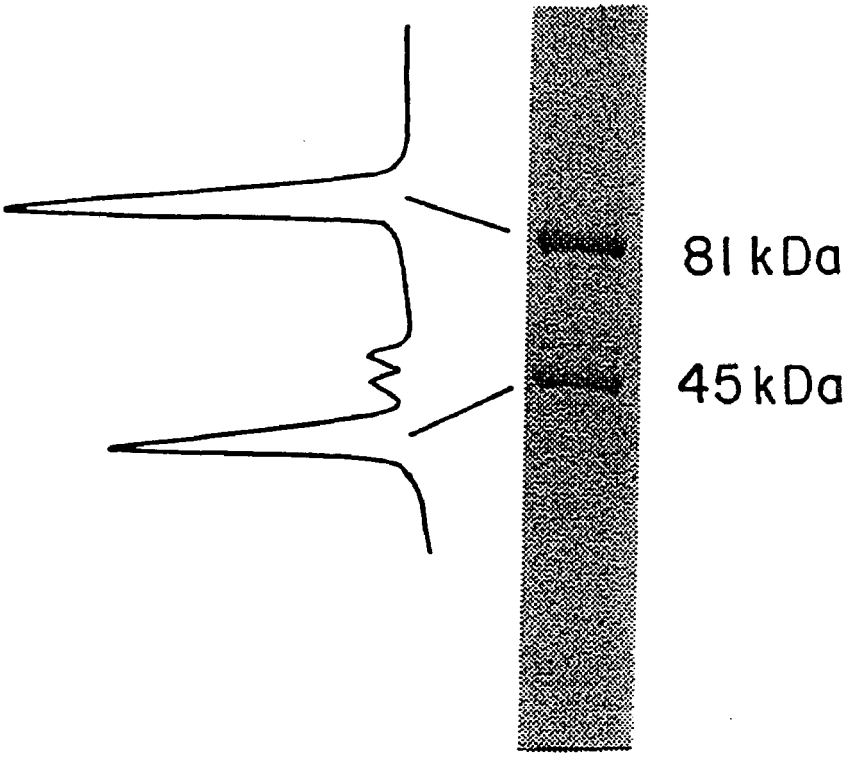


Figure 8. SDS-PAGE of the trifunctional β-oxidation complex from pig heart. The two subunits of the complex are marked 81 and 45 kDa. An absorbance scan of a gel slice stained with Coomassie blue for protein is displayed to the left of the gel.

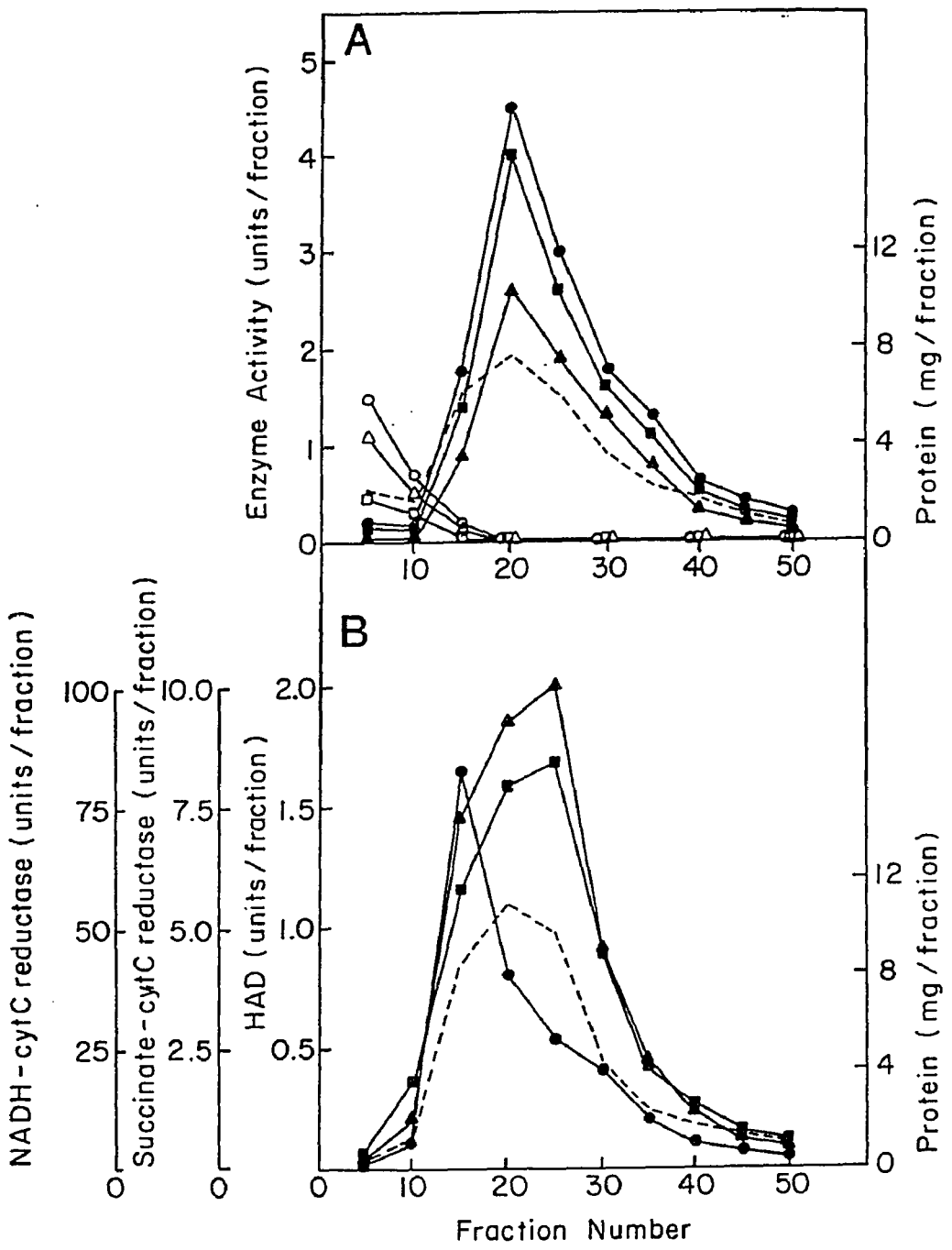

A
B
Enzyme Activity (units / fraction)
Protein (mg / fraction)
NADH - cytC reductase (units / fraction)
Succinate - cytC reductase (units / fraction)
HAD (units / fraction)
Fraction Number

Figure 9.

Figure 9. Chromatography of a pig heart homogenate fraction on DEAE-cellulose. A. Fractions were assayed for protein (dashed line) and for the following enzymes (solid lines): enoyl-CoA hydratase with crotonyl-CoA (O) and 2-hexadecenoyl-CoA (●), L-3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA (□) and 3-ketohexadecanoyl-CoA (■), and 3-ketoacyl-CoA thiolase with acetoacetyl-CoA (Δ) and 3-ketohexadecanoyl-CoA (▲). B. Fractions were assayed for protein (dashed line) and for the following enzymes (solid line): L-3-hydroxyacyl-CoA dehydrogenase with 3-ketohexadecanoyl-CoA (■), succinate-cytochrome c reductase (▲), and rotenone-insensitive NADH-cytochrome c reductase (●).

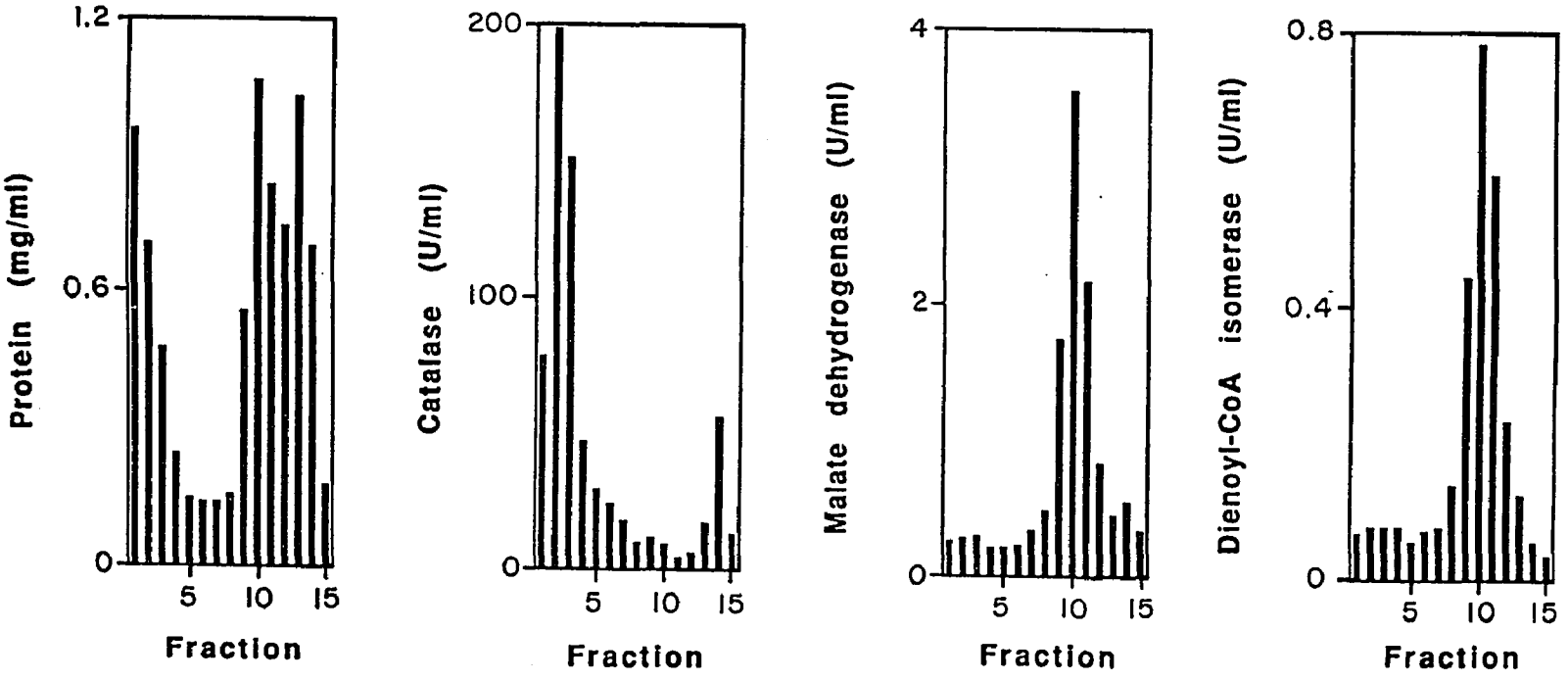


Figure 10. Separation of a light mitochondrial fraction from rat liver by centrifugation on a Nycodenz density gradient.

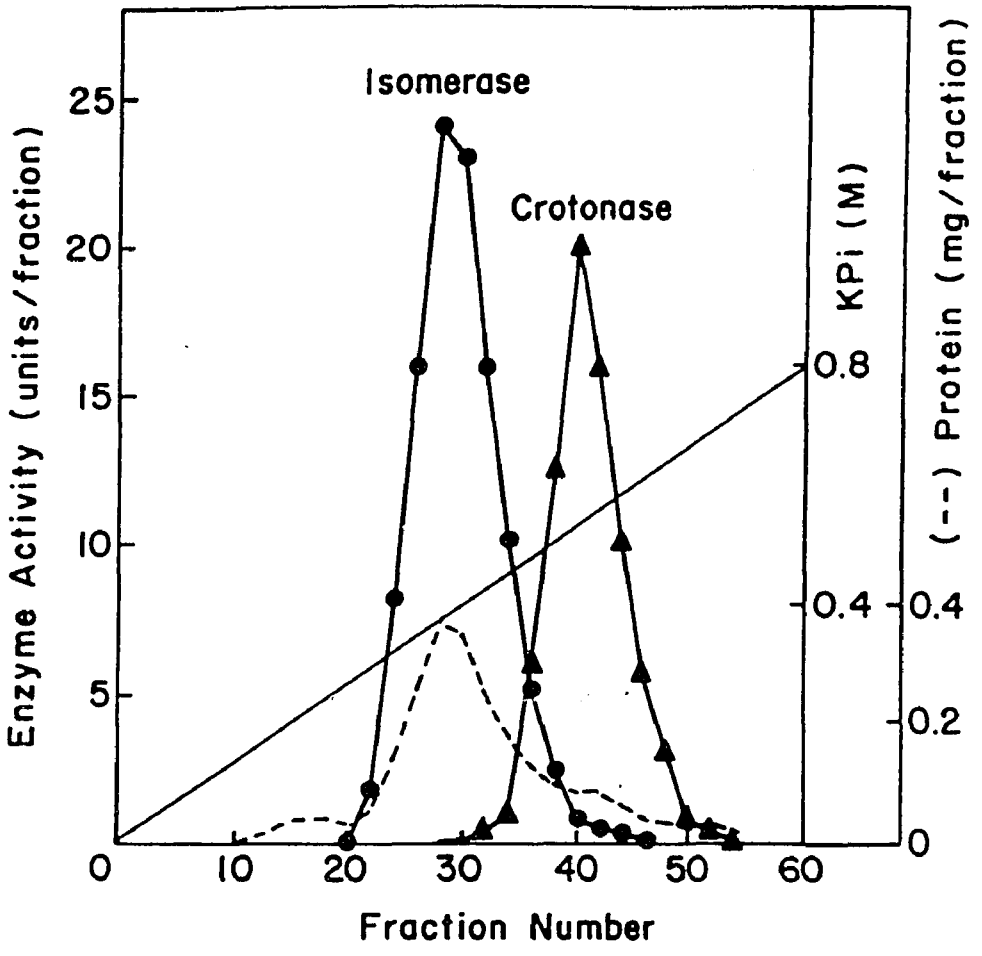


Figure 11. Final step in the purification of dienoyl-CoA isomerase by chromatography on hydroxylapatite. Fractions were assayed for dienoyl-CoA isomerase and enoyl-CoA hydratase (crotonase).

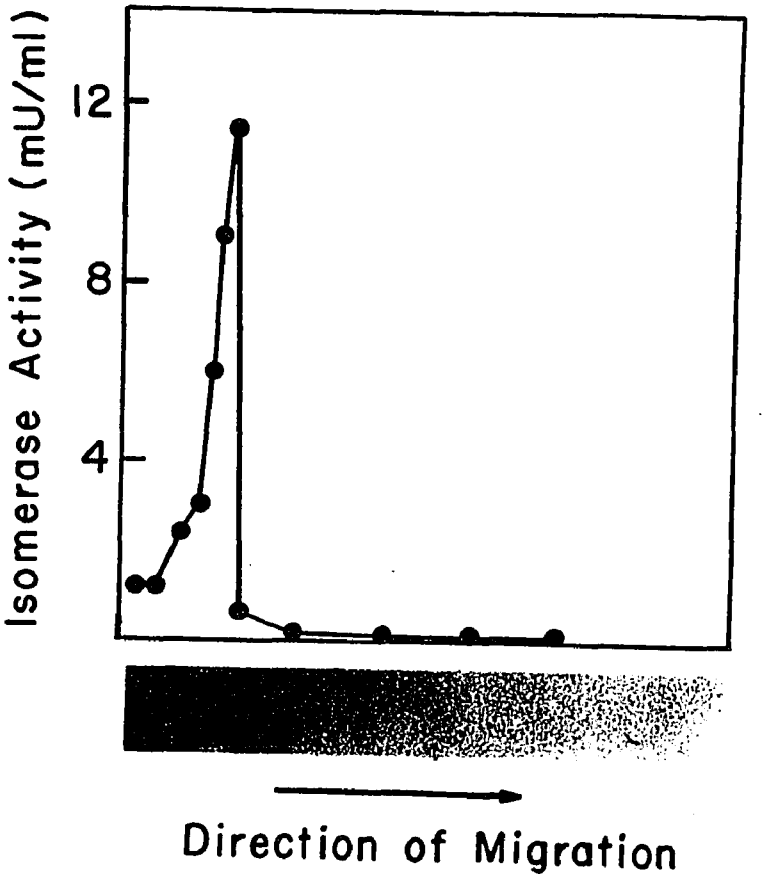


Figure 12. PAGE of native dienoyl-CoA isomerase. Half of the gel was stained for protein with Coomassic Blue, wheras the other half of the gel was sliced, extracted, and assayed for dienoyl-CoA isomerase.

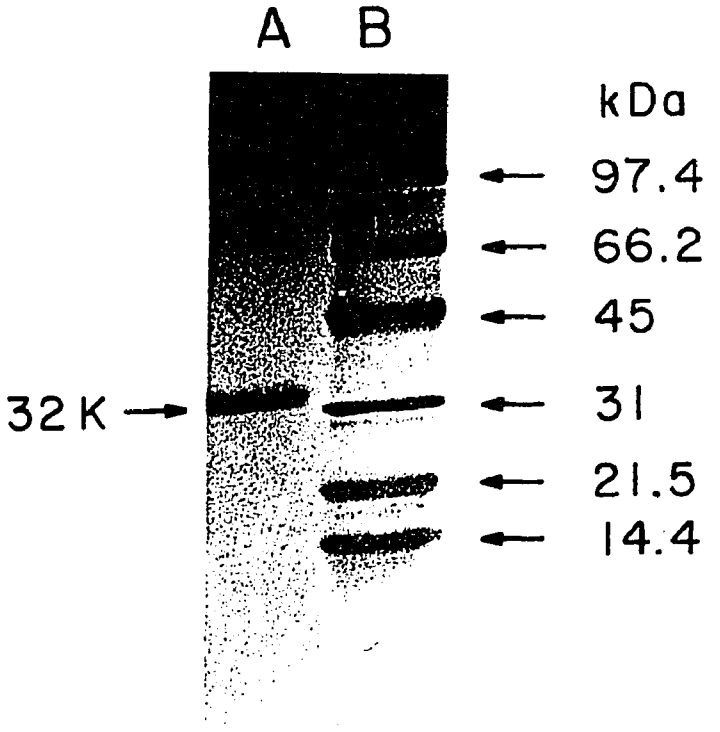


Figure 13. SDS-PAGE of dienoyl-CoA isomerase. *Lane A*, purified dienoyl-CoA isomerase; *lane B*, molecular weight standard: phosphorylase *b* (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

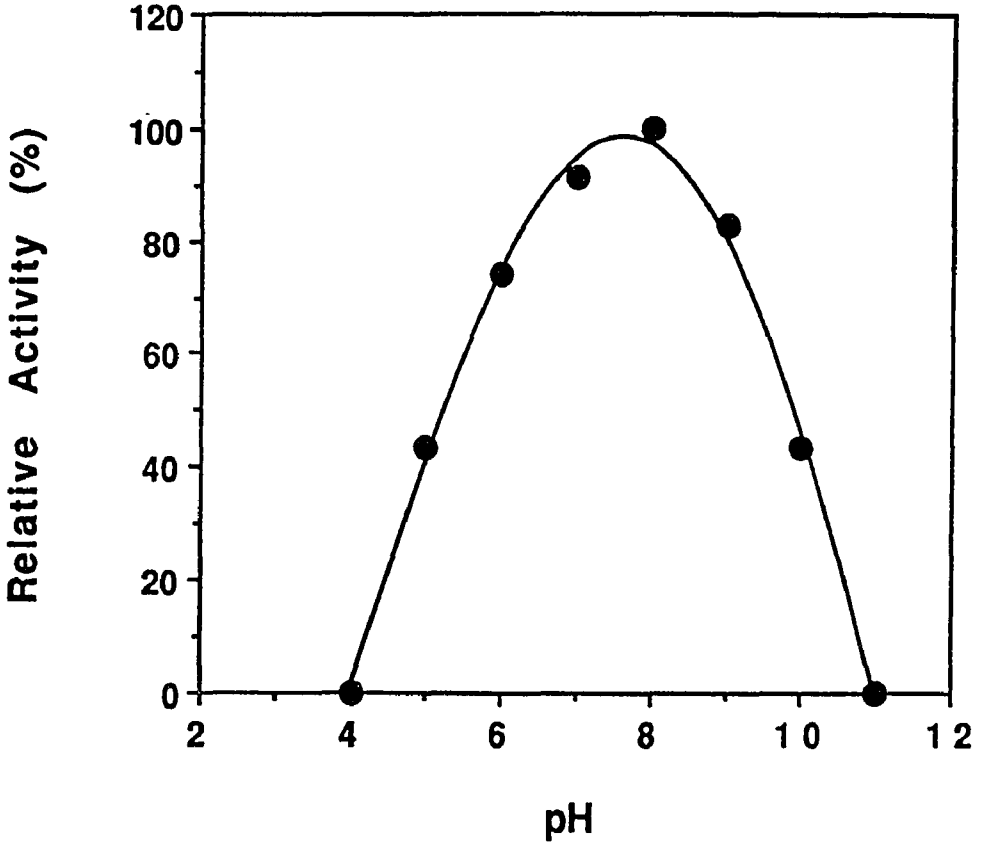


Figure 14. Activity of dienoyl-CoA isomerase as a function of pH. The potassium phosphate buffer of the same ionic strength as 50 mM KPi, pH 8.0 was used.

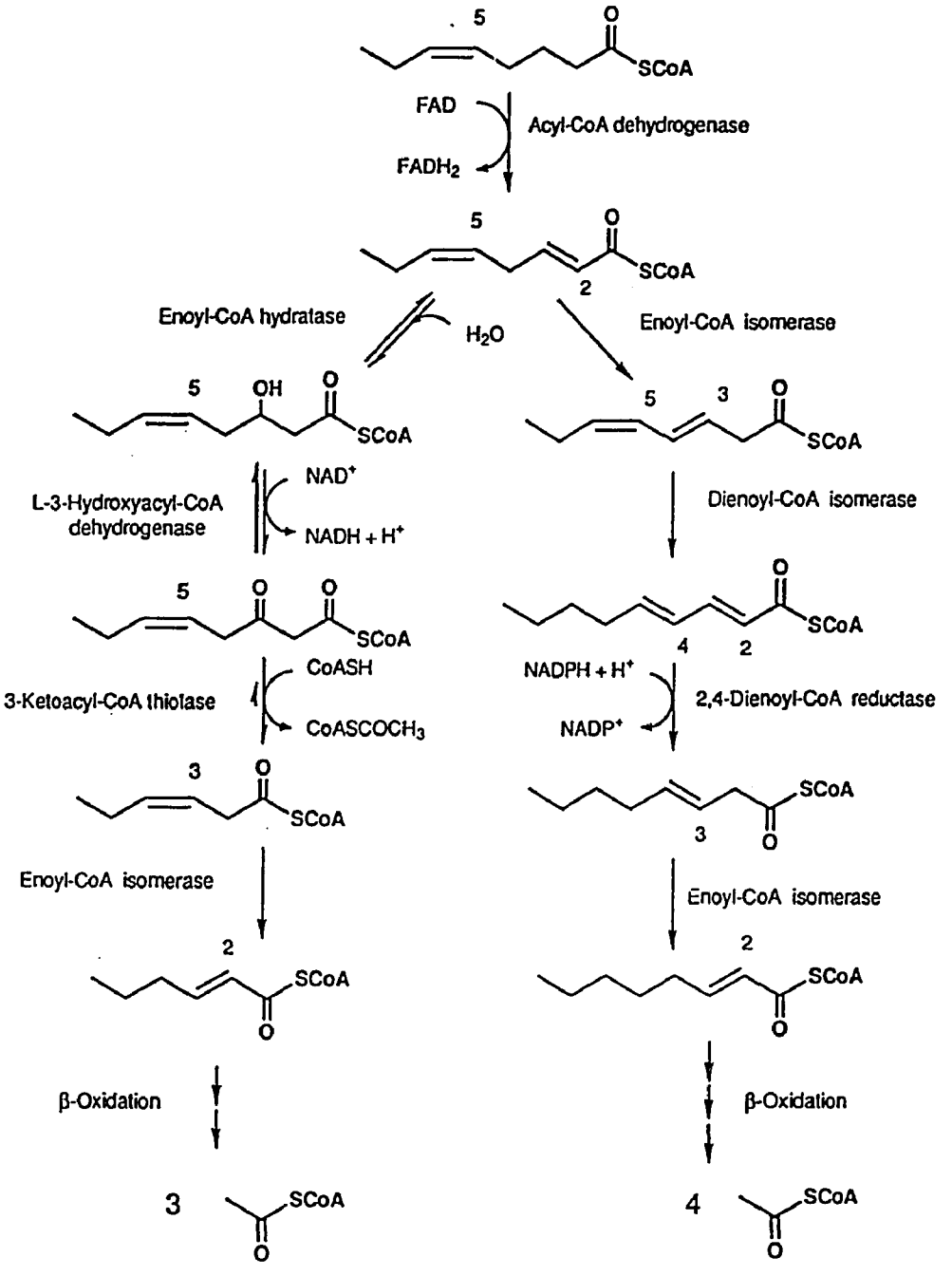


Figure 15. Pathway of the NADPH-independent and NADPH-dependent β–oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms.

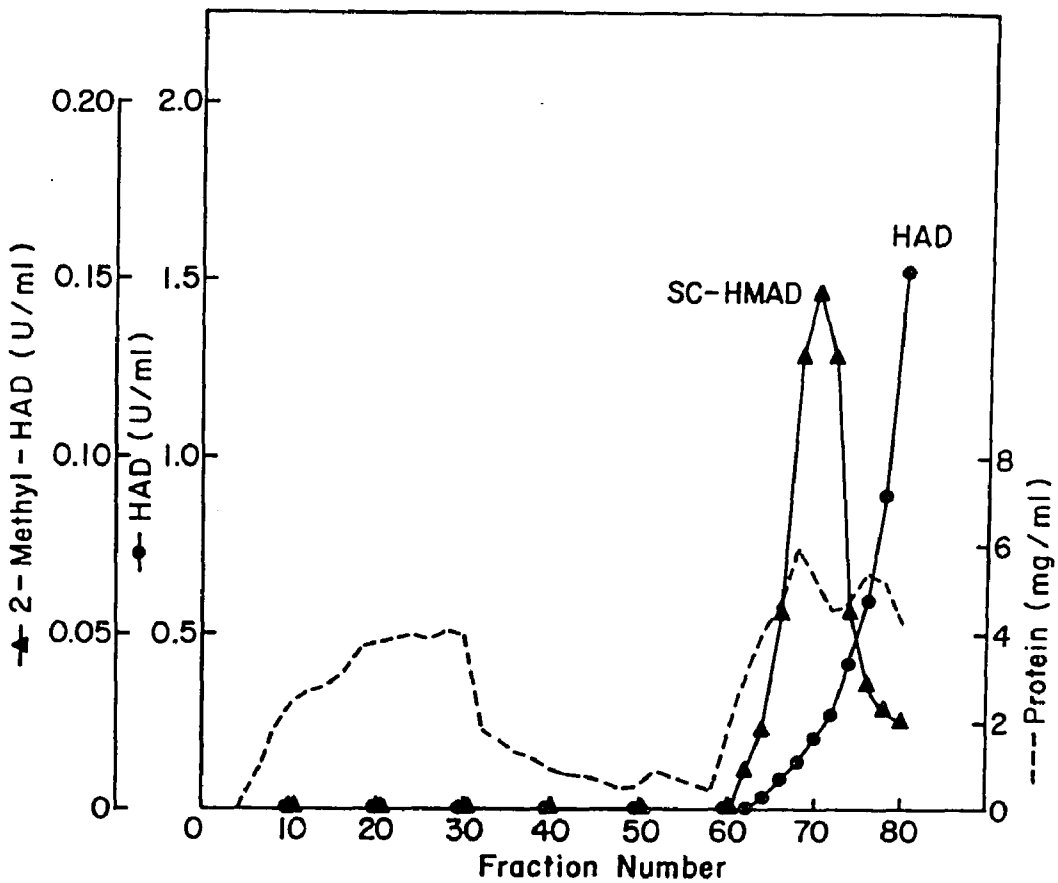


Figure 16. Chromatography of a rat liver extract after PEG fractionation on phosphocellulose. Fractions were assayed for SC-HMAD (2-Methyl-HAD) and HAD activities with tiglyl-CoA plus crotonase and acetoacetyl-CoA, respectively.

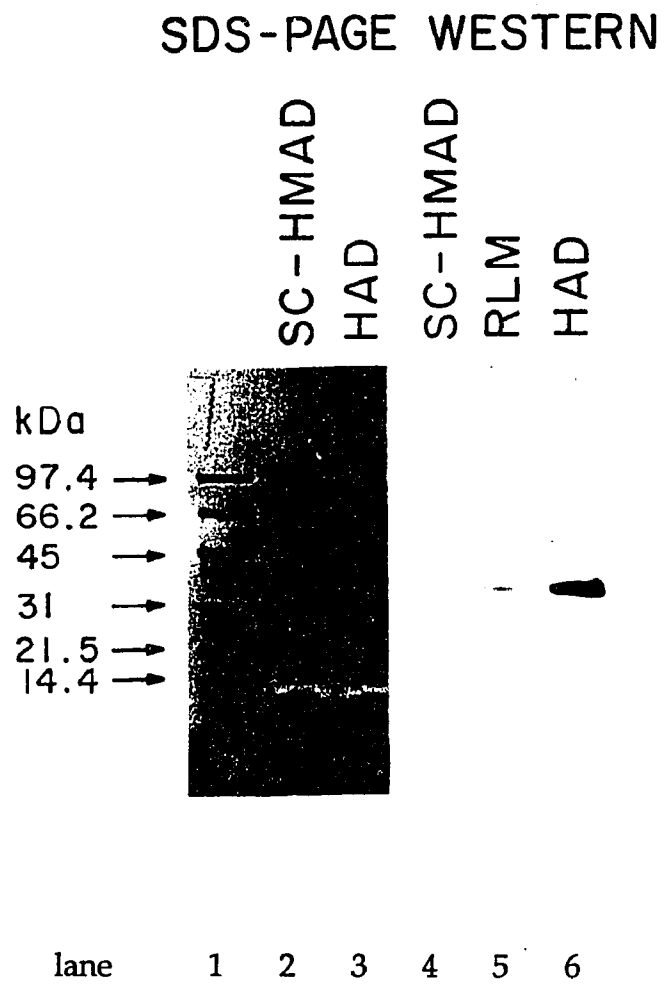
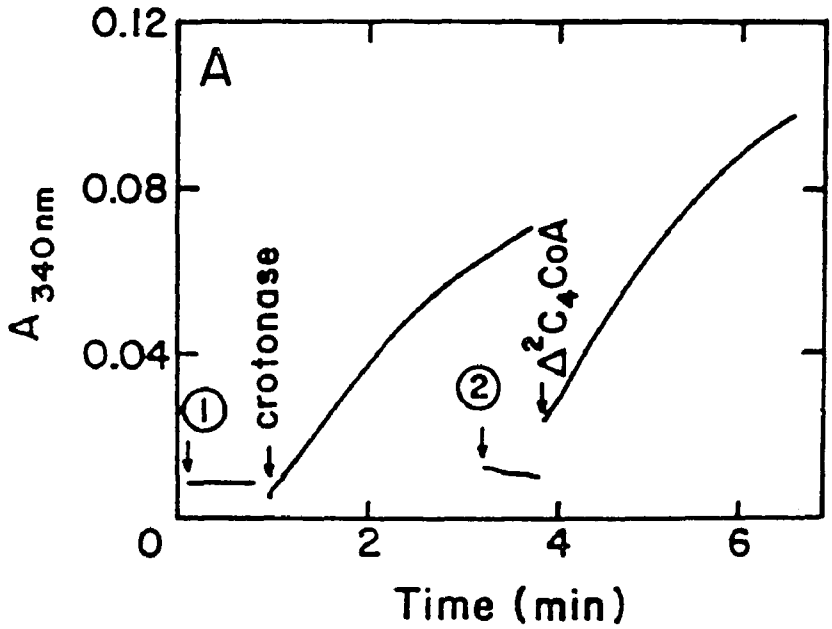


Figure 17. SDS-PAGE and Western blotting of purified SC-HMAD from rat liver and purified HAD from pig heart. A. Gel after staining with Coomassie Blue. B. Immunoblot after probing with antibodies to pig heart HAD. Lane 1, molecular weight stansards; lane 2 and 4, SC-HMAD (0.5 ug); lanes 3 and 6, HAD (0.4 ug); lane 5, rat liver extract (20 ug) after PEG fractionation.


A
0.12
0.08
0.04
0
A_{340nm}
crotonase
Δ^2C_4CoA
①
②
2
4
6
Time (min)

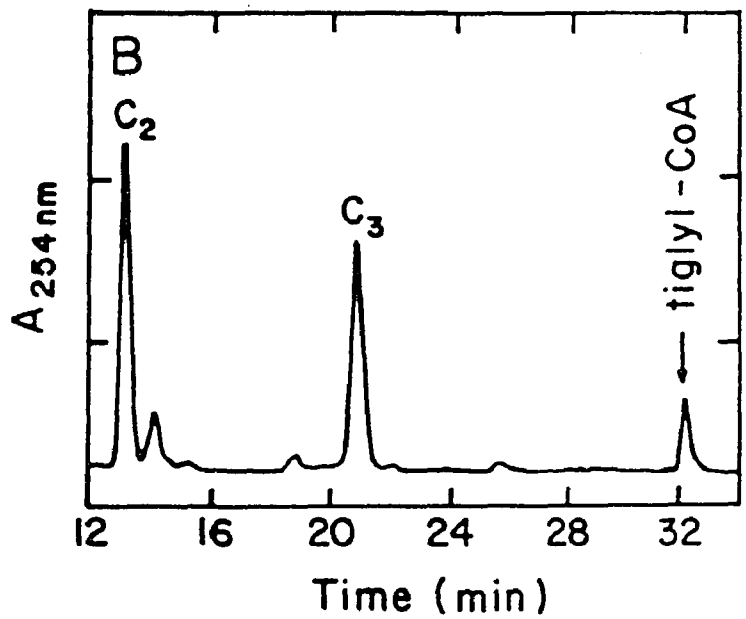

B
C_2
C_3
tiglyl-CoA
A_{254nm}
12
16
20
24
28
32
Time (min)

Figure 18. Analysis of products formed in reactions catalyzed by purified SC-HMAD from rat liver and purified HAD from pig heart. A. Spectrophotometric determination of NADH formation when (**1**) 40 μM tiglyl-CoA and 1 mM NAD^+ in 50 mM Tris-H_3PO_4 (pH 9) containing 150 mM KCl were incubated with SC-HMAD (0.8 μg) followed by the addition of crotonase (0.7 unit); (**2**) 40 μM tiglyl-CoA, 1 mM NAD^+, and crotonase (0.7 unit) in 50 mM Tris-H_3PO_4 (pH9) containing 150 mM KCl were incubated with HAD (4 μg) followed by the addition of 30 μM crotonyl-CoA (Δ^2 C_4), B. HPLC analysis of products formed when 40 μM tiglyl-CoA, 1 mM NAD^+, 0.3 mM CoASH in Tris-H_3PO_4 (pH 8) were incubated with crotonase (0.7 unit), SC-HMAD (0.8 μg) and acetoacetyl-CoA thiolase (8 μg) for 6 min. The sample was processed and analyzed by HPLC as described under Experimental Procedures. Peaks identified by use of authentic sample were C_2, acetyl-CoA; C_3 , propionyl-CoA. All other significant peaks were due to impurities present in the thiolase preparation.

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