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Beta-Oxidation of Unsaturated Fatty Acids in Rat Heart Mitochondria and *Escherichia coli*

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ABSTRACT

BETA-OXIDATION OF UNSATURATED FATTY ACIDS IN RAT HEART MITOCHONDRIA AND *ESCHERICHIA COLI*

by

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Unsaturated fatty acids with odd-numbered double bonds, e.g. oleic acid, can be degraded by β-oxidation via the isomerase-dependent pathway or reductase-dependent pathway. The degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA, a metabolite of oleic acid, was studied with rat heart mitochondria. It is concluded that both pathways are essential for the degradation of unsaturated fatty acids with odd-numbered double bonds inasmuch as the isomerase-dependent pathway facilitates the major flux through β-oxidation and the reductase-dependent pathway prevents the accumulation of 3,5-*cis*-tetradecadienoyl-CoA, an otherwise undegradable metabolite.

This study relied on the use of a mitochondrial extract that permitted the analysis of intermediates but did not maintain the supramolecular organization of enzymes as they exist in intact mitochondria. The use of an organized β-oxidation system uncompromised by its isolation as present in *E. coli* would be advantageous. Data obtained with the *E. coli* fatty acid oxidation complex prompt the conclusion that oleate is mostly degraded via the isomerase-dependent pathway, but that a small amount of 2-*trans*,5-*cis*-tetradecadienoyl-CoA is diverted from that pathway via conversion to 3,5-*cis*-tetradecadienoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase. The 3,5-intermediate, which would strongly inhibit β-oxidation of oleate, is hydrolyzed and the resultant 3,5-tetradecadienoate is excreted into the growth medium. The substrate profile and kinetic data of the purified recombinant *E. coli* thioesterase II support the conclusion that this enzyme is responsible for hydrolyzing 3,5-*cis*-tetradecadienoyl-CoA.

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ABBREVIATIONS

CPT II	carnitine palmitoyltransferase II;
enoyl-CoA isomerase	Δ^3,Δ^2-enoyl-CoA isomerase;
dienoyl-CoA isomerase	$\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase;
MECI	mitochondrial enoyl-CoA isomerase;
MFE1	peroxisomal multifunctional enzyme 1;
ECI	monofunctional enoyl-CoA isomerase;
FAO complex	multienzyme complex of fatty acid oxidation;
kan	kanamycin;
cml	chloramphenicol;
cyclodextrin	methyl-β-cyclodextrin;
crotonase	enoyl-CoA hydratase;
EDTA	ethylenediaminetetraacetic acid;
ETF	electron transferring flavoprotein;
BSA	bovine serum albumin;
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis;
KPi	potassium phosphate;
HPLC	high-performance liquid chromatography;
PMSF	phenylmethylsulfonyl fluoride;

INTRODUCTION

Fatty acids are an important source of energy in many organisms but also are components of membranes and precursors of different signal molecules.

Abnormalities of fatty acids β-oxidation in humans are associated with many diseases, such as inborn errors of fat metabolism, diabetes (1), cancer (2) and cardiovascular diseases (3,4). The first fatty acid oxidation disorder was described in 1973 when carnitine and carnitine palmitoyltransferase II (CPT II) deficiency were reported to cause muscle weakness (5,6). Three clinical phenotypes of congenital CPT II deficiency, the mild adult muscular form (5,6), severe infantile (7,8), and neonatal form (9) have been reported. Both of the latter forms usually cause death during the first months of life. Years later, several other fatty acids disorders have been described, which are due to deficiencies of acyl-CoA dehydrogenases, 3-hydroxyacyl-CoA dehydrogenase, trifunctional β-oxidation complex, 2,4-dienoyl-CoA reductase, and other enzymes (10). The most common of these relatively rare disorders is medium-chain acyl-CoA dehydrogenase deficiency (6). It is characterized by nonketotic hypoglycemia and encephalopathy (10,11) during the first two years of life. Precipitated by a febrile illness, especially gastro-enteritis and fasting, patients look quite normal between episodes. Among diagnosed patients, 25% die and about 33% of rest will have irreversible neurological damage (12,13).

Most dietary fatty acids from plants and animals have an even number of carbon atoms, usually between 14 and 22. More than half of them are unsaturated or polyunsaturated. Therefore, an in-depth understanding of the β-oxidation of unsaturated and polyunsaturated fatty acids would be important for studying and interpreting abnormalities of fatty acid metabolism in humans.

Mitochondria and peroxisomes are two subcellular organelles that have the capacity to degrade fatty acids by β-oxidation. Only in animals, fatty acids are degraded in mitochondria, while peroxisomal β-oxidation occurs in all eukaryotes. In lower eukaryotes, such as yeast, fatty acids β-oxidation is confined to peroxisomes and results in the complete degradation of fatty acids. In animals, the main biological role of peroxisomes is to partially degrade very long chain fatty acids, dicarboxylic acids, methyl-branched carboxylic acids and hydroxylated cholestanoic acids (14) that are poor substrates of mitochondrial β-oxidation. The resultant chain-shortened acyl-CoAs are excreted or further degraded in mitochondria.

In animals, fatty acids entering mitochondria are completely degraded by β-oxidation that is tightly linked to oxidative phosphorylation. The well established β-oxidation of saturated fatty acids occurs by four sequential reactions that are catalyzed by distinct enzymes. The process begins with the oxidation of fatty acyl-CoAs to 2-*trans*-enoyl-CoAs catalyzed by acyl-CoA dehydrogenase. 2-Enoyl-CoA is hydrated by enoyl-CoA hydratase to L-3-hydroxyacyl-CoA which is dehydrogenated to 3-ketoacyl-CoAs by L-3-hydroxyacyl-CoA dehydrogenase. In the last reaction, 3-ketoacyl-CoA is cleaved by 3-ketoacyl-CoA thiolase to yield acetyl-CoA and a chain-shortened acyl-CoA. The observation that only very small amounts or no β-oxidation intermediates are present in mitochondria and the characterization of at least two isozymes for each of the four reactions of the β-oxidation spiral supports a model for the physical and functional organization of β-oxidation enzymes, which may result in the channeling of intermediates (see Fig. 1) (15). According to this model, long-chain fatty acids entering mitochondria are first partially degraded by the membrane bound, very long-chain acyl-CoA dehydrogenase and trifunctional β-oxidation complex that contains the long-chain and medium-chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. Chain-shortened fatty acyl-CoAs are then completely degraded by the matrix system of soluble β-oxidation enzymes that have a preference for medium- and short-chain fatty acyl-CoAs.

Unsaturated and polyunsaturated fatty acids also are degraded by β-oxidation. But when the double bonds move closer to the β-carbon of the fatty acyl-CoA residue due to chain shortening, enzymes in addition to the enzymes necessary for the β-oxidation of saturated fatty acids spiral are required to act on preexisting double bonds (16).

One of the most abundant unsaturated fatty acids in the human diet is oleic acid, which has one odd-numbered double bond that can be either isomerized or reduced during β-oxidation (for a review, see Ref. 16). As summarized in Scheme 1, after two cycles of β-oxidation, oleoyl-CoA (I) is chain-shortened to 5-*cis*-tetradecenoyl-CoA (II). Dehydrogenation of the latter compound by long-chain acyl-CoA dehydrogenase (17) produces 2-*trans*,5-*cis*-tetradecadienoyl-CoA (III), which can complete its pass through the β-oxidation cycle. After the resultant 3-*cis*-dodecenoyl-CoA (VI) is isomerized to 2-*trans*-dodecenoyl-CoA (VII) by Δ^3,Δ^2-enoyl-CoA isomerase (enoyl-CoA isomerase) (EC 5.3.3.8), the latter compound can be completely degraded via the β-oxidation spiral. This pathway is referred to as the isomerase-dependent pathway because it only requires enoyl-CoA isomerase as an auxiliary enzyme (for review, see Ref. 14). Most textbooks only mention this pathway when they discuss the β-oxidation of oleate or linoleate. However, the observation that a double bond at position 5 of an unsaturated fatty acid can be reduced by rat mitochondria in the presence of NADPH (18) led to an investigation that resulted in the characterization of an alternate pathway of β-oxidation for unsaturated fatty acids with odd-numbered double bonds (19). The reductive removal of the double bond of 5-enoyl-CoAs in mitochondria occurs by a four-step reaction sequence (19) that converts 2-*trans*,5-*cis*-tetradecadienoyl-CoA (III) to 2-*trans*-tetradecaenoyl-CoA (XIV) and thereby diverts it from the classical isomerase-dependent pathway as the result of its isomerization to the thermodynamically more stable 3,5-tetradecadienoyl-CoA (XI) catalyzed by enoyl-CoA isomerase. The 3,5-isomer is converted by the second auxiliary enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) (20), to 2,4-*trans*-tetradecadienoyl-CoA (XII) that is reduced in an NADPH-dependent reaction catalyzed by 2,4-dienoyl-CoA reductase (EC 1.3.1.34) to 3-*trans*-tetradecaenoyl-CoA (XIII). The latter intermediate is converted by enoyl-CoA isomerase to 2-*trans*-tetradecaenoyl-CoA (XIV), which is a substrate of the β-oxidation spiral and can be completely degraded by this process. The reduction of the double bond by dienoyl-CoA reductase was the reason for naming the alternate pathway reductase-dependent pathway.

Even-numbered double bonds, such as the 12-*cis* double bond of linoleic acid, are reductively removed via two reactions that are catalyzed by the auxiliary enzymes 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase. As shown in Scheme 2, linoleoyl-CoA (I) is chain-shortened to 4-*cis*-decenoyl-CoA (IV) that is dehydrogenated by acyl-CoA dehydrogenase. The resultant 2-*trans*,4-*cis*-decadienoyl-CoA (V) is reduced by the NADPH-dependent auxiliary enzyme 2,4-dienoyl-CoA reductase to yield 3-*trans*-decenoyl-CoA (VI). Finally, enoyl-CoA isomerase catalyzes the conversion of 3-*trans*-decenoyl-CoA to its 2-*trans* isomer (VII), which can be completed degraded by the traditional β-oxidation spiral. Among the auxiliary enzymes involved in the degradation of unsaturated or polyunsaturated fatty acids, both enoyl-CoA isomerase and dienoyl-CoA reductase are required for the degradation of unsaturated fatty acids with even-numbered and odd-numbered double bonds. Enoyl-CoA isomerase has been purified from rat liver (21,22), rat heart (22), and bovine liver (23). This enzyme has been reported to be a homodimer with a subunit molecular weight of around 30,000 (21-23) but recently was shown to be a trimer[1] (submitted for publication). So far, three enzymes have been identified in the rat that have enoyl-CoA isomerase activity. They are mitochondrial enoyl-CoA isomerase (MECI), peroxisomal multifunctional enzyme 1 (MFE1) and monofunctional enoyl-CoA isomerase (ECI) (24). Although all three enzymes have broad chain length specificities, they may preferentially act on different metabolites (24). ECI, which was located to both peroxisomes and mitochondria, is the major enzyme for the isomerization of 3-*trans* to 2-*trans* enoyl-CoAs. MECI has a preference for the conversion of 3-*cis* to 2-*trans* enoyl-CoAs isomer and 2,5 to 3,5-dienoyl-CoAs in mitochondria while MFE1 is most active in the isomerization of 2,5 to 3,5 isomers in peroxisomes (24).

Dienoyl-CoA reductase which requires NADPH as a cofactor catalyzes the reduction of 2-*trans*,4-*trans*-dienoyl-CoAs and 2-*trans*,4-*cis*-dienoyl-CoAs to 3-enoyl-CoAs. It also functions in the reduction of 2,4,6-octatrienoyl-CoA, which is assumed to be an intermediate formed during the β-oxidation of fatty acids with conjugated double bonds (25). It was purified from bovine liver and shown to be a homotetramer with a molecular weight of 124,000 (26).

Although the mitochondrial and peroxisomal enzymes of β-oxidation catalyze the same or similar reactions, most of them are structurally different, are encoded by different genes, and show different substrate specificities (14). Only few enzymes, e.g. dienoyl-CoA isomerase, are encoded by one gene but located in both

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[1] Hubbard, P., Yu, W., Schulz, H. and Kim, J.-J., personal communication.

peroxisomes and mitochondria because they have both peroxisomal and mitochondrial targeting signals at the C-terminus and N-terminus, respectively (27). Dienoyl-CoA isomerase has been purified from rat liver (20) and rat heart (28). It is a homohexamer with a subunit molecular weight of about 32,000 (28,29) and interestingly also exhibits $\Delta^{3,5,7}$,$\Delta^{2,4,6}$-trienoyl-CoA isomerase activity (30). Three acidic residues, Asp176, Glu196 and Asp204, are located at the catalytic site of dienoyl-CoA isomerase (29). Two of these residues Glu196 and Asp204, catalyze the simultaneous proton transfers at both ends of the diene or triene chromophore, which result in the isomerization of the conjugated double bonds.

The degradation of fatty acids has also been studied in prokaryotes. Most of the studies were carried out with *E. coli*. In *E. coli*, the activated fatty acid is dehydrogenated by acyl-CoA dehydrogenase to 2-*trans*-enoyl-CoA that is completely degraded by a multienzyme complex of fatty acid oxidation (FAO complex). *E. coli* FAO complex has two α- and β-subunits each and exists in the form of an $\alpha_2\beta_2$ complex. The large α-subunit contains activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and enoyl-CoA isomerase (31) while 3-ketoacyl-CoA thiolase is present in the small β-subunit (32). Only one additional auxiliary enzyme, 2,4-dienoyl-CoA reductase, is required for the metabolism of unsaturated fatty acids with even-numbered double bonds.

Thioesterases I and II are two enzymes in *E. coli* that cleave the thioester bond of acyl-CoA to yield CoA and fatty acids. Both enzymes were reported to be less active toward palmitoyl-ACP than toward palmitoyl-CoA (33). Thioesterase I, which was identified as a periplasmic enzyme (34), is encoded by the *tesA* gene and has a molecular weight at 22,000 (35). In contrast, thioesterase II, a 122 kDa cytosolic homotetramer, is encoded by the *tesB* gene (36). Thioesterase I only cleaves long-chain acyl-CoAs (C12-C18), whereas thioesterase II has a broader chain length specificity that extends from hexanoyl-CoA to stearoyl-CoA (36,37). Also, thioesterase II is active toward β-hydroxyacyl-CoAs that are not substrates of thioesterase I (36,38). The high activities of both thioesterases toward acyl-CoAs suggest that these enzymes may function in preventing an intracellular accumulation of acyl-CoAs that have not been observed in *E. coli* (39-41). But so far the physiological roles of these thioesterases haven't been clarified because the growth properties of *E. coli* cells seem to be unaffected by overexpressing both enzymes or silencing one or both thioesterase genes (34,37,42).

Attempts to determine the relative fluxes through the reductase-dependent and isomerase-dependent pathways have not yet produced completely satisfactory answers regarding the degradation of typical long-chain dietary fatty acids. A study of oleate β-oxidation with 5-*cis*-tetradecenoyl-CoA, an oleate metabolite, came to the conclusion that 86% and 65% (43) of this metabolite is degraded via the reductase-dependent pathway with liver and heart mitochondria, respectivcely. However, that study analyzed fatty acid metabolites in intact mitochondria. Though the intramitochondrial organization of enzymes was preserved, these metabolites are not true intermediates of β-oxidation but rather are products that have leaked from the pathway, especially when functionally compromised mitochondria are involved (44). Hence, it is very doubtful that these values are meaningful estimates of the flux through the reductase-dependent pathway. In fact, an earlier investigation in this lab using 2,5-octadienoyl-CoA, a medium-chain intermediate of α-linolenic acid β-oxidation, to study fluxes through the reductase-dependent and isomerase-dependent pathway in the presence of NAD^+, CoASH, and NADPH with a soluble extract of rat liver mitochondria, reached the conclusion that 80% of the metabolite was degraded via the isomerase-dependent pathway, while only 20% passed through the reductase-dependent pathway (45). The uncertainty about the contributions of the two pathways to the β-oxidation of long-chain dietary fatty acids prompted this study of the degradation of the oleate metabolite, 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) by solubilized rat heart mitochondria. This study relied on the use of a mitochondrial extract that permitted the analysis of intermediates but did not maintain the supramolecular organization of enzymes as they exist in intact mitochondria. Since the organization of these enzymes may affect the flux through one pathway relative to the other, the use of an organized β-oxidation system uncompromised by its isolation would be advantageous. Such system is present in *E. coli* where a FAO complex is highly expressed when cells are grown on long-chain fatty acids as the sole carbon source (46). The purified complex contains the cellular activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, and enoyl-CoA isomerase (32) that are necessary for fatty acid β-oxidation in *E. coli*. A study of fatty acid oxidation in *E. coli* would also reveal whether the alternative pathway of oleate β-oxidation, which requires dienoyl-CoA isomerase, exists in prokaryotes. If yes, it may be a ubiquitous process that is operative in all organisms capable of oxidizing fatty acids. All of the above considerations prompted the following study of oleate β-oxidation in both mitochondria and *E. coli*.

EXPERIMENTAL PROCEDURES

Materials - CoASH, NAD^+, NADH, NADPH, stearoyl-CoA, palmitoyl-CoA, tetradecanoyl-CoA, dodecanoyl-CoA, decanoyl-CoA, octanoyl-CoA, butyryl-CoA, acetyl-CoA and isopropyl-beta-D-thiogalactopyranoside were purchased from Life Science Resources (Milwaukee, WI). 5-*cis*-Tetradecenoic acid was synthesized by Cayman Chemical (Ann Arbor, MI). Oleic acid was obtained from Matreya, Inc. (Pleasant Gap, PA). BCl_3-methanol (12%, w/w) was purchased from Supelco (Bellefonte, PA). Burdick & Jackson (Muskegon, MI) was the source of ethyl ether, whereas hexane was from Fisher. Sep-Pak C_{18} cartridges used for concentrating acyl-CoAs and μBondapak C_{18} columns (30 cm x 3.9 mm) were purchased from Waters Associates. 10% Ready gels were purchased from Bio-Rad Laboratories (Hercules, CA). Acyl-CoA oxidase from *Arthrobacter* species, chloramphenicol (cml), kanamycin (kan), methyl-β-cyclodextrin (cyclodextrin) and most of the standard biochemicals were obtained from Sigma. Bovine liver enoyl-CoA hydratase (crotonase) (47), recombinant pig liver L-3-hydroxyacyl-CoA dehydrogenase (48), pig heart 3-ketoacyl-CoA thiolase (49), recombinant human peroxisomal enoyl-CoA isomerase (50), rat liver enoyl-CoA isomerase (22), recombinant rat liver dienoyl-CoA isomerase (28) and *E. coli* FAO complex (51) were purified by published procedures. *E. coli* strain HC74 (*tesA::kan*^{r} & *tesB::cml*^{r} thioesterase double mutant, *fadE*), and strain LE392 (*hasR, galK, trpR, metB, lacY*) (52)) were kindly provided by Dr. Stuart Smith (Children's Hospital

Oakland Research Institute) and the *E. coli* Genetic Stock Center (Yale University), respectively.

Syntheses of Substrates and Metabolites - 2-*trans*-Dodecenoic acid and 2-*trans*-tetradecenoic acid were synthesized by reacting malonic acid with n-decanal and n-dodecanal, respectively, as described in principle by Linestead et *al.* (53). Oleoyl-CoA, 5-*cis*-tetradecenoyl-CoA, 2-*trans*-tetradecenoyl-CoA, and 2-*trans*-dodecenoyl-CoA were synthesized from oleic acid, 5-*cis*-tetradecenoic acid, 2-*trans*-tetradecenoic acid, and 2-*trans*-dodecenoic acid, respectively, by the mixed anhydride method as described by Fong and Schulz (54). All products were purified by HPLC. 2-*trans*-Tetradecenoyl-CoA was partially converted to L-3-hydroxytetradecanoyl-CoA by hydration in the presence of crotonase in 0.1 M KPi (pH 8.0). The resultant L-3-hydroxytetradecanoyl-CoA was purified by HPLC. 3-*cis*-Tetradecenoyl-CoA (24) and 3-ketohexadecanoyl-CoA (55) were synthesized as described.

For the synthesis of 2-*trans*-5-*cis*-tetradecadienoyl-CoA, a solution of 5 μmol of 5-*cis*-tetradecenoyl-CoA in 30 ml of 0.1 M KPi (pH 9.0) was saturated with air for 30 min and dehydrogenated by acyl-CoA oxidase at room temperature. The near-complete conversion was achieved by the addition of 10-20 units of acyl-CoA oxidase in several aliquots over a period of 45 min. 3,5-*cis*-Tetradecadienoyl-CoA was synthesized by incubating 5 μmol of 5-*cis*-tetradecadienoyl-CoA in 15 ml of 0.1 M KPi (pH 8.0) with 12 units of acyl-CoA oxidase at room temperature. The progress of both reactions was monitored by HPLC. When a maximal conversion of 5-*cis*-tetradecenoyl-CoA to 2-*trans*,5-*cis*-tetradecadienoyl-CoA was achieved as indicated by the disappearance of 5-*cis*-tetradecaenoyl-CoA, 0.2 unit of purified rat liver dienoyl-CoA isomerase were added to the reaction mixture to remove traces of 3,5-tetradecadienoyl-CoA by conversion to 2,4-tetradecadienoyl-CoA, while 0.2 unit of crotonase was used to convert 2-*trans*-5-*cis*-tetradecadienoyl-CoA to L-3-hydroxy-5-*cis*-tetradecadineoyl-CoA when synthesizing 3,5-*cis*-tetradecadienoyl-CoA. The pH of the solution was adjusted to 1.5 with 6 N HCl to terminate the reaction. Precipitated protein was removed by filtering the solution through a 0.22-μm pore size membrane. After adjusting the pH to 4 with 4 N KOH, the solution was concentrated by passing it through a Sep-Pak C_{18} cartridge and eluting it with a small volume of methanol, which subsequently was evaporated under reduced pressure. The resultant 2-*trans*-5-*cis*-tetradecadienoyl-CoA or 3,5-*cis*-tetradecadienoyl-CoA was purified by HPLC. Fractions containing 2-*trans*-5-*cis*-tetradecadienoyl-CoA or 3,5-tetradecadienoyl-CoA were combined, concentrated as described above, and finally dissolved in deionized water. The pH of the final preparation was adjusted to ≈ 3-4, and the thioester concentration of this solution was determined spectrophotometrically by quantification of CoASH with Ellman's reagent (56) after cleaving the thioester bond with NH_2OH at pH 7.0 (54). The concentration of 2-*trans*,5-*cis*-tetradecadienoyl-CoA or 3,5-tetradecadienoyl-CoA was calculated by subtracting the concentration of 3,5-*cis*-tetradecadienoyl-CoA or 2-*trans*,5-*cis*-tetradecadienoyl-CoA from that of 2-*trans*,5-*cis*-tetradecadienoyl-CoA plus 3,5-*cis*-tetradecadienoyl-CoA. The concentrations of 2-*trans*,5-*cis*-tetradecadienoyl-CoA plus 3,5-*cis*-tetradecadienoyl-CoA and of 3,5-*cis*-

tetradecadienoyl-CoA were determined by measuring the absorbance changes at 300 nm caused by their conversions in 0.1 M KPi (pH 8.0) to 2,4-tetradecadienoyl-CoA on additions of 0.1 unit of dienoyl-CoA isomerase plus 0.05 unit of enoyl-CoA isomerase and of 0.1 unit of dienoyl-CoA isomerase, respectively. Concentrations of 2,4-dienoyl-CoA were calculated using an extinction coefficient of 28,000 $M^{-1}cm^{-1}$ (57).

L-3-Hydroxy-5-*cis*-tetradecenoyl-CoA was prepared by incubating 2 µmol of 2-*trans*,5-*cis*-tetradecadienoyl-CoA in 10 ml of 0.1 M KPi buffer (pH 8.0) with 9 units of enoyl-CoA hydratase at room temperature and separating the product from the substrate by HPLC. For the synthesis of 3-keto-5-*cis*-tetradecenoyl-CoA, 1.5 µmol of L-3-hydroxy-5-*cis*-tetradecenoyl-CoA in 10 ml of 20 mM KPi buffer (pH 8.0) was incubated with 0.5 mM pyruvate and 0.5 mM NAD^+ in the presence of 5 units of L-3-hydroxyacyl-CoA dehydrogenase and 7 units of lactate dehydrogenase at room temperature. The desired product was purified by HPLC. L-3-Hydroxydodecanoyl-CoA and 3-ketododecanoyl-CoA were synthesized from 2-*trans*-dodecenoyl-CoA and L-3-hydroxydodecanoyl-CoA, respectively, as described above for the corresponding longer chain acyl-CoAs. 2-*trans*,4-*trans*-Tetradecadienoyl-CoA was prepared by incubating 3,5-*cis*-tetradecadienoyl-CoA with dienoyl-CoA isomerase and purifying the product by HPLC.

Preparation of a Solubilized Extract from Rat Heart Mitochondria - Rat heart mitochondria were isolated as described by Chappell and Hansford (58) and stored at –80 ^{0}C. The thawed rat heart mitochondria were suspended in 0.2 M KPi containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 mM benzamidine, and 5 mM 2-mercaptoethanol and incubated for 30 min on ice. The mixture was centrifuged at 100,000 x g at 4 ^{0}C for 30 min, and the supernatant was used for metabolic assays.

Isolation of fadE Revertants from E. coli Strain HC74 - A *fadE* mutation had been introduced into *E. coli* strain LE392 by use of transposable element Tn*10* (34). Subsequently the Δ*tesA::kan*^{r} and then the Δ*tesB::cml*^{r} mutation had been introduced into this strain to create strain HC74 (34). Since *E. coli* electron transferring flavoprotein (ETF) is the product of *fadE*, strain HC74 would not grow on fatty acids as sole carbon source because of this mutation in β-oxidation. The aim of this research project was to study the β-oxidation of unsaturated fatty acid in *E. coli*, and hence it was important to obtain a revertant of the *fadE* mutation. Given that *E. coli* strain HC74 lacks ETF, it could not grow on a medium with palmitic acid as the sole carbon source. However, cells that would grow on such medium might be *fadE* revertants of *E. coli* strain HC74. To select such revertants, *E. coli* cells (strain HC74) were plated on LB agar containing kanamycin and chloramphenicol. After overnight incubation at 37 ^{0}C, these plates served as master plates for replica plates containing M9 mineral salts (52) with palmitic acid (0.1%, w/v) as the main carbon source, which additionally contained 1% trypton, 2 mM $MgSO_4$, 10 μM $CaCl_2$, 1 μM $FeCl_3$, 0.3 mM methionine, 0.02 mM tryptophan, 0.4% Triton X-100, kanamycin, and chloramphenicol. Single colonies that grew on these plates after 7 days of incubation at 37 ^{0}C were picked and grown in a liquid medium containing kanamycin and chloramphenicol to mid- logarithmic phase. Cells from the resultant culture were spread on agar plates containing M9 mineral salts and palmitic acid as described above. The same procedure was repeated once more and *fadE* revertants (strain YR1) were collected after growth in LB medium to an absorbance of 1 at 600 nm and stored at –80 ^{0}C.

Bacterial Growth Conditions - *E. coli* cells (strain B, YR1, and LE392) were grown on LB medium from single colonies. The initial culture was diluted 5-fold into M9 minimal medium containing 1% trypton, 2 mM $MgSO_4$, 10 μM $CaCl_2$, 1 μM $FeCl_3$ with or without 0.3 mM methionine and 0.02 mM tryptophan and additionally either glucose (0.5%, w/v), oleic acid (0.1%, v/v) or palmitic acid (0.1%, w/v) in the presence of 0.4% Triton X-100 or cyclodextrin depending on the experimental purposes. Long-chain fatty acids were solubilized with cyclodextrin at a 1:6 molar ratio in the growth medium. Kanamycin (20 μg/ml) and chloramphenicol (20 μg/ml) were added to all media used to grow *E. coli* strain YR1. The cultures were grown at 37 ^{0}C in a shaker incubator to an absorbance of 1 at 600 nm after which they were diluted 20-times into the same growth medium but without trypton. The cultures of strain YR1 and LE392 were repeatedly diluted 10-times with the same growth medium. Growth curves were obtained by measuring the absorbance change at 600 nm. *E. coli* cells were harvested by centrifugation at 3,500 x g for 30 min at 4 ^{0}C. Cell pellets were washed twice with M9 minimal medium and stored at -80 ^{0}C.

Preparation and Fractionation of Bacterial Extracts - Seven g of *E. coli* cell paste were suspended in 14 ml of 0.1 M KPi (pH 7.0) containing 1 mM phyenylmethylsulfonyl fluoride, 1 mM benzamidine and 10% glycerol, sonicated for a total of 2 min (10 sec x 12) at 0 ^{0}C and centrifuged at 100,000 x g for 1 hr at 4 ^{0}C. The resultant supernatant was collected for enzyme assays and protein purification. The soluble *E. coli* extract was dialyzed overnight against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol and 1 mM benzamidine. The dialyzed supernatant was applied to a DEAE-cellulose column (13.5 x 2.5 cm) equilibrated with dialysis buffer. The column was then developed with a linear gradient made up of 500 ml each of 0.02 M Tris-HCl (pH 7.8) containing either 50 mM NaCl or 500 mM NaCl. Fractions were assayed for thioesterase activity and those with high activities were pooled and stored at -80 ^{0}C.

Metabolic and Enzyme Assays - Rates of degradation of 2-*trans*-5-*cis*-tetradecadienoyl-CoA via the isomerase-dependent pathway were determined by incubating various amounts of the substrate in 0.2 M KPi (pH 8) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) in the presence of 1mM NAD$^+$ plus 0.3 mM CoASH and measuring the rate of NADH formation spectrophotometrically at 360 nm. An extinction coefficient of 4,140 M^{-1}cm^{-1} was used to calculate rates. A concentration of 1 mM was chosen for NAD$^+$ because it is saturating (59,60), whereas 0.3 mM is the estimated concentration of free CoASH in mitochondria at state 3 respiration with palmitoyl-L-carnitine as substrate (61). The conversion of 2-*trans*-5-*cis*-tetradecadienoyl-CoA to 2,4-

tetradecadienoyl-CoA was measured by incubating the substrate in 0.2 M KPi (pH 8) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) fortified with 0.1 unit of dienoyl-CoA isomerase. The absorbance change at 300 nm was recorded and an extinction coefficient of 28,000 $M^{-1}cm^{-1}$ was used to calculate rates. When the time-dependent formation of metabolites was studied, 20 μM 2-*trans*-5-*cis*-tetradecadienoyl-CoA or 20 μM 3,5-*cis*-tetradecadienoyl-CoA was incubated in 0.2 M KPi (pH 8.0) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) in the presence of 1 mM NAD^+, 0.3 mM CoASH, and 0.5 mM NADPH. Reactions were terminated by adjusting the pH to 1.5 with 6 N HCl. The pH was readjusted to 4.5 with 4 N KOH before the reaction mixtures were clarified by filtration through 0.22 μm pore size membranes and analyzed by HPLC.

Experimental conditions were quite similar in the concentration-dependent and time-dependent assays when studying the metabolism of 2-*trans*,5-*cis*-tetradecadienoyl-CoA in *E. coli*. Instead of using an extract of solubilized rat heart mitochondria, 0.6 μg of purified *E. coli* FAO complex plus bovine serum albumin (BSA) (0.1mg/ml) were used with or without 0.04 unit of dienoyl-CoA isomerase. Absorbance changes were recorded at 340 nm and used for calculating the rates.

Metabolites were quantified by the following procedure. Areas under the peaks were obtained by integration with Millennium software from Water Corporation. Peak areas were normalized by use of extinction coefficients of 15,000, 19,650, and 28,8000 $M^{-1}cm^{-1}$ that were determined for acyl-CoA thioesters with a saturated α-carbon, one double bond, and two double bonds in conjugation with the thioester function, respectively, at 254 nm. The sum of all normalized peak areas remained fairly constant throughout the experiment. Hence, the sum of all metabolites was 20 μM, the concentration of the substrate that was added to the incubation mixture. The normalized area of one peak relative to the sum of all normalized areas gives the percentage of substrate converted to the indicated metabolite. These values, referred to as *Metabolites* (%), are plotted as a function of time.

Enoyl-CoA isomerase activities were measured by a coupled assay (62). The isomerization of 30 μM 3-*cis*-octenoyl-CoA to 2-octenoyl-CoA was coupled to the hydration of the latter compound by crotonase, dehydrogenation of the 3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydrogenase in the presence of NAD^+. The resultant 3-ketoacyl-CoA was finally thiolytically cleaved by 3-ketoacyl-CoA thiolase. Formation of NADH was spectrophotometrically measured at 340 nm and used to calculate the rates of isomerization with an extinction coefficient of ε=6,220 $M^{-1}cm^{-1}$.

Dienoyl-CoA isomerase activities were assayed as described by Luo *et al.* (20). A reaction mixture contained 20 μM 3,5-octadienoyl-CoA in 0.2 M KPi (pH 8). The increase in absorbance at 300 nm (ε=28,000 $M^{-1}cm^{-1}$) due to the conversion of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA was the basis for calculating rates of isomerization.

When testing the activity of 2,4-dienoyl-CoA reductase, an assay mixture contained 30 μM 2,4-decadienoyl-CoA in 0.2 M KPi (pH=7.0) and the oxidation of

NADPH at 340 nm was measured spectrophotometrically as described by Kunau (63).

Thioesterase was assayed by measuring the release of CoASH from acyl-CoAs with Ellman's reagent (56). A standard assay mixture contained 0.175 M KPi (pH 8), 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent), and 20 μM acyl-CoA. The progress of the reaction was determined spectrophotometrically at 412 nm and rates were calculated using an extinction coefficient of 13,600 $M^{-1}cm^{-1}$. Kinetic analyses were performed with purified thioesterase II from *E. coli* and palmitoyl-CoA, myristoyl-CoA and 3,5-*cis*-tetradecadienoyl-CoA as substrates. Kinetic constants (K_m, V_{max}) were obtained by nonlinear curve fitting using the SigmaPlot 2000 program. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product in 1 min.

Isolation and Analysis of Fatty Acids Present in the Growth Medium - E. coli cells were grown to early exponential or to an absorbance of 1 in M9 medium containing oleate (0.1%, v/v) and Triton X-100 (0.4%) or were grown to an absorbance of 1 on M9 medium containing glucose (0.5%, w/v) plus Triton X-100 or palmitate (0.1%, w/v) plus Triton X-100. Cells were separated from the growth medium by centrifugation at 3,000 x g for 30 min at 4 ^{0}C. The supernatant was acidified (pH 1-2) with 2 N H_2SO_4 and then extracted four times with 100 ml ether each. The organic phase was extracted with aqueous sodium bicarbonate. After acidifying the aqueous phase with 2 N H_2SO_4, it was extracted 3-times with 8 ml ether each. The combined ether extracts were dried over anhydrous sodium sulfate and the residual material, after removal of drying agent by filtration and ether by evaporation under a stream of N_2, was methylated by reacting it with 2 ml of BCl_3-methanol (12%, w/w) for 10 min at 60 ^{0}C. After allowing the reaction mixture to cool down, 1 ml of H_2O and 1 ml of hexane were added. The organic layer was carefully removed and dried over anhydrous sodium sulfate. The residue after the removal of sodium sulfate and evaporation of ether was dissolved in a minimal volume of anhydrous ethanol. This fraction, which contained the methyl esters of fatty acids that were present in the growth medium, was analyzed by gas chromatography in combination with mass spectrometry (GC/MS). For the purpose of identifying methyl 3,5-tetradecadienoate, a sample containing 20 nmol of 3,5-tetradecadienoyl-CoA and 20 nmol of n-pentadecanoyl-CoA (internal standard) was hydrolyzed by reacting it with 4 N KOH at 25 ^{0}C for 1 hr. The reaction mixture was acidified (pH 1-2) with 2 N H_2SO_4 and extracted three times with 8 ml of ether each. The extracted fatty acids were converted to their methyl esters as described above. Aliquots of 1 μl of the fatty acid methyl esters were injected at 250 ^{0}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, inner diameter: 0.25 mm, film thickness: 0.25 μm, EC-5, Alltech Associates Inc., Deerfield, IL). The oven temperature was raised from 100 ^{0}C to 230 ^{0}C at 5 ^{0}C/min, to 300 ^{0}C at 20 ^{0}C/min and then held constant for 6 min. The mass spectrometer served as a detector and was operated at 280 ^{0}C.

Purification of E. coli thioesterase II - E. coli cells harboring pUC *tesB* were grown overnight at 37 ^{0}C in LB medium containing 50 μg/ml ampicillin. The initial culture was diluted 50-fold into the same medium. And this culture was grown to an absorbance of 1 at 600 nm. Isopropyl-beta-D-thiogalactopyranoside was added to a final concentration of 1mM and the culture was incubated at 37 ^{0}C for 4 hr. Cells were harvested by centrifugation at 3,000 g for 10 minutes. Cell pellets were washed with 10 volumes of 0.1 M KPi (pH 8) and stored at –80 ^{0}C. One g of cell paste was suspended in 2 ml 20 mM of KPi (pH 8) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, homogenized by sonication for a total of 2 min (20 sec x 6) at 0 ^{0}C and centrifuged at 100,000 x g for 1 hr at 4 ^{0}C. The supernatant was collected and brought to 50% saturation with $(NH4)_2SO_4$ after which it was centrifuged at 10,000 g for 10 min. The precipitated protein were dissolved in a minimal volume of 20 mM Tris-HCl (pH 7.8) containing 10% glycerol and loaded onto a DEAE-cellulose column (8 x 2.5 cm) equilibrated with the dissolving buffer. The column was developed with a linear gradient made up of 500 ml each of 0.02 M Tris-HCl (pH 7.8) containing either 50 mM NaCl or 500 mM NaCl. Fractions were assayed for thioesterase activity and those with high activities were pooled and stored at –80 ^{0}C.

Electrophoresis - Purified thioesterase was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% ready gel at 100 V for 1.5 hr (64).

Purification and Analyses of Acyl-CoA Thioesters by HPLC - Acyl-CoA thioesters were purified and metabolites were analyzed by reverse-phase HPLC on a Waters μBondapak C_{18} column (30cm x 3.9mm) attached to a Waters gradient HPLC system. The absorbance of the eluate was monitored at 254 nm. Separation of substrates and metabolites was achieved by washing the μBondapak C_{18} column with 50 mM ammonium phosphate (pH 5.5) containing 40% of acetonitrile/water (9:1, v/v) for 20 min and then eluting acyl-CoAs by linearly increasing the organic phase from 40 to 70% in 20 min at a flow rate of 2 ml/min. All samples were cleared of particulate matter by passing them through a 0.22-μm pore size membrane before they were injected into the HPLC system. Diluted samples were concentrated by passing them through Sep-Pak C_{18} cartridges and eluting them with small amounts of methanol, which subsequently were removed by evaporation under reduced pressure.

RESULTS

Studies with Rat Heart Mitochondria

Kinetics of 2-trans,5-cis-Tetradecadienoyl-CoA Degradation - 2-*trans*,5-*cis*-Tetradecadienoyl-CoA (Scheme 1, compound III) is an intermediate that is formed during the β-oxidation of oleate and that can be further metabolized by either the isomerase-dependent pathway or the reductase-dependent pathway (see Scheme 1). The kinetics of 2-*trans*,5-*cis*-tetradecadienoyl-CoA degradation via these two pathways were studied with rat heart mitochondria because of their minimal contamination with peroxisomes that are estimated to account for less than 3% of cardiac fatty acid β-oxidation (65). Treatment of rat heart mitochondria with 1% Triton X-100 yielded a soluble extract that contained all β-oxidation enzymes required for the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) to decanoyl-CoA (Scheme 1, compound X) and dodecanoyl-CoA (Scheme 1, compound XVII). Because it had previously been determined that Triton X-100 at the applied concentration did not affect the activities of the enzymes of the β-oxidation spiral (66,67), it was only necessary to assess how Triton X-100 affects the activities of enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and dienoyl-CoA isomerase. Such tests revealed that none of these three auxiliary enzymes was negatively affected by 1% Triton X-100 (data not shown). When 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) was incubated with an extract of rat heart mitochondria in the presence of 1 mM NAD$^+$

and 0.3 mM CoASH but in the absence of NADPH, it was possible to determine rates of β-oxidation via the isomerase-dependent pathway without interference from the reductase-dependent pathway by measuring spectrophotometrically the formation of NADH at 360 nm. The entry into the reductase-dependent pathway was determined separately by measuring at 300 nm the accumulation of 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII) in the absence of any cofactor. The results of these experiments are shown in Fig. 2. Specific activities for the isomerase-dependent pathway are based on initial velocity measurements that were linear during the first 2 min when, on the average, 1.5 mol of NADH were produced per mol of degraded 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III). The conversion of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) to 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII) was measured 30 s after initiation of the reaction, when rates were linear. When the flux of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) through the isomerase-dependent pathways is compared with its entry into the reductase-dependent pathway, it is obvious that the former pathway is dominant and that the ratio of rates for the two pathways does not vary significantly over a considerable range of substrate concentrations (see Fig. 2). Consequently, the results that were obtained by studying the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA at one concentration may reflect the situation in intact mitochondria, for which the concentrations of true intermediates are unknown. In subsequent experiments I analyzed the time-dependent formation of metabolites that accumulate when 20 μM 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) was incubated with an extract of solubilized rat heart mitochondria in the presence of 1 mM NAD^+, 0.3 mM CoASH, and 0.5 mM NADPH. Representative HPLC chromatograms are shown in Fig. 3. Product analysis 5 s after initiating the incubation revealed the rapid hydration of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 1, compound III) to 3-hydroxy-5-*cis*-tetradecenoyl-CoA (Scheme 1, compound IV) (see Fig. 3A). Because the hydration is freely reversible, both intermediates can enter either pathway. In addition, traces of 2-dodecenoyl-CoA (Scheme 1, compound VII) and 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII) were detected. These two metabolites are committed to proceed through the isomerase-dependent pathway and reductase-dependent pathway, respectively. After 1 min of incubation, all intermediates of the isomerase-dependent pathway with the exception of 3-ketododecanoyl-CoA (Scheme 1, compound IX) were present at detectable levels (see Fig. 3B). Decanoyl-CoA was the final product of this metabolic sequence due to the absence of cofactors that are necessary for its further degradation by β-oxidation. Entry into the reductase-dependent pathway had also continued as indicated by the formation of more 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII), whereas dodecanoyl-CoA (Scheme 1, compound XVII) remained undetectable (see Fig. 3B). Five minutes after initiating the incubation, dodecanoyl-CoA (Scheme 1, compound XVII), the end product of the reductase-dependent pathway under the prevailing experimental conditions, was present together with its precursor, 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII) (see Fig. 3C). 3,5-Tetradecadienoyl-CoA was difficult to detect because it was insufficiently separated from 2-*trans*,5-*cis*-tetradecadienoyl-CoA. However, it is unlikely to accumulate because of the high activity of dienoyl-CoA isomerase in the mitochondrial extract. After a total reaction time of 5 min, 2-*trans*,5-*cis*-tetradecadienoyl-CoA had been completely metabolized, and all intermediates of the isomerase-dependent pathway had been converted to decanoyl-CoA (Scheme 1, compound X). The small amount of material marked Δ^2-C_{12}-CoA was identified as a nonmetabolizable side product that seems to be formed from either 2,5-tetradecadienoyl-CoA (Scheme 1, compound III) or 2,4-tetradecadienoyl-CoA (Scheme 1, compound XII) in a time-dependent manner and that was eluted from the reverse-phase column together with Δ^2-C_{12}-CoA.

The kinetics of 2-*trans*,5-*cis*-tetradecadienoyl-CoA degradation and metabolite formation are shown in Fig. 4. Most dramatic was the rapid hydration of 2-*trans*,5-*cis*-tetradecadienoyl-CoA to 3-hydroxy-5-*cis*-tetradecenoyl-CoA. This reaction preceded the slower dehydrogenation of the 3-hydroxy intermediate and the even slower appearance of the final product, decanoyl-CoA (C_{10}-CoA). It was noted that the utilization of 2-*trans*,5-*cis*-tetradecadienoyl-CoA and 3-hydroxy-5-*cis*-tetradecenoyl-CoA followed a similar time course and that the concentrations of 2-dodecenoyl-CoA and 3-hydroxydodecanoyl-CoA changed almost in parallel (see Fig. 4). Thus, the two hydration reactions seem to be at or near equilibrium. The fact that the levels of 3-ketoacyl-CoAs and 3-*cis*-dodecenoyl-CoA were low or undetectable suggests that these intermediates are rapidly degraded. Taken together, the observed kinetics of intermediate formation and degradation point to the dehydrogenations of 3-hydroxyacyl-CoAs as the reactions that exert the greatest control over the flux through the isomerase-dependent pathway. The entry of 2-*trans*,5-*cis*-tetradecadienoyl-CoA into the reductase-dependent pathway was initially quite rapid as indicated by the formation of 2,4-tetradienoyl-CoA but declined as the concentration of 2-*trans*,5-*cis*-tetradecadienoyl-CoA decreased because of its hydration. However, dodecanoyl-CoA, the end product of this pathway, was formed very slowly with the result that only a fraction of its precursor, 2,4-tetradecadienoyl-CoA, was converted to the final product during the five-minute incubation period, which was sufficient for the complete conversion of all intermediates of the isomerase-dependent pathway to the final product decanoyl-CoA. Thus, it seems that the NADPH-dependent reduction of 2,4-tetradecadienoyl-CoA restricts the flux through the reductase-dependent pathway.

Degradation of 3,5-Tetradecadienoyl-CoA - 3,5-*cis*-Tetradecadienoyl-CoA is an assumed intermediate of oleate β-oxidation that we did not detect during the characterization of metabolites formed from 2-*trans*,5-*cis*-tetradecadienoyl-CoA because it was not separated from its precursor by HPLC. In addition we asked whether 3,5-*cis*-tetradecadienoyl-CoA could be metabolized via the isomerase-dependent pathway in addition to being degraded by the reductase-dependent pathway. To address these issues, 3,5-*cis*-tetradecadienoyl-CoA was incubated with an extract of rat heart mitochondria in the presence of NAD^+ and CoASH, and its metabolites were analyzed by HPLC. Because the absence of NADPH prevented its degradation via the reductase-dependent pathway, the flux through the isomerase-dependent pathway can be evaluated. As shown in Fig. 5A, 3,5-*cis*-tetradecadienoyl-CoA was rapidly converted to its 2,4 isomer, but did not enter the isomerase-dependent pathway to a significant degree. After 5 min of incubation, a trace of decanoyl-CoA was detected (data not shown), which could have been formed either via the isomerase-dependent pathway as outlined in Scheme 1 or more likely by direct β-oxidation of 2,4-tetradecadienoyl-CoA. The rapid degradation of 3,5-*cis*-tetradecadienoyl-CoA via the reductase-dependent pathway was demonstrated by incubating it with an extract of rat heart mitochondria in the presence of all required cofactors including NAD^+, CoASH, and NADPH. As shown in Fig. 5B, 3,5-cis-tetradecadienoyl-CoA was rapidly converted to its 2,4 isomer, which was slowly reduced as indicated by the delayed appearance of dodecanoyl-CoA (C_{12}-CoA) in the absence of significant amounts of downstream metabolites. This experiment demonstrates that 3,5-*cis*-tetradecadienoyl-CoA is only metabolized via the reductase-dependent pathway and additionally confirms the conclusion reached during the first part of this study that the reduction of 2,4-tetradecadienoyl-CoA is the rate-limiting reaction in the reductase-dependent pathway.

Effects of NADH and Acetyl-CoA on the β-Oxidation of 2-trans,5-cis-Tetradecadienoyl-CoA - The metabolic studies described above were carried out with NAD^+, CoASH, and NADPH as cofactors but in the absence of NADH and acetyl-CoA that are present in mitochondria. Because NADH and acetyl-CoA may inhibit β-oxidation enzymes and thereby the flux through the pathways, we assessed their effects on the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA. For this purpose, we determined the formation of decanoyl-CoA (C_{10}-CoA) and dodecanoyl-CoA (C_{12}-CoA) plus 2,4-tetradecadienoyl-CoA ($\Delta^{2,4}$-C_{14}-CoA) as a function of the incubation time to measure fluxes through the isomerase-dependent pathway and reductase-dependent pathway, respectively. Shown in Fig. 6 are the results that were obtained when no NADH (Fig. 6A), 0.17 mM NADH (Fig. 6B), or 0.5 mM NADH (Fig. 6C) was included in the incubation mixture in addition to the required cofactors NAD^+, CoASH, and NADPH. When the product formation during the first 3 min was evaluated, the presence of NADH at the lower level resulted in slightly lower rates of β-oxidation but did not affect the relative flux through the reductase-dependent pathway of ~10%. At the higher NADH concentration, the rate of product formation was further reduced, whereas the relative flux through the reductase-dependent pathway was only slightly increased to ~15% of the total. Thus, NADH inhibits β-oxidation without significantly affecting the relative contributions of the two pathways to the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA. The effect of acetyl-CoA on the operation of the two pathways was also investigated. An increasing substitution of up to 80% of CoASH in the incubation mixture by acetyl-CoA did not affect the rate of 2-*trans*,5-*cis*-tetradecadienoyl-CoA β-oxidation, nor did it change the contributions of the two pathways to this process (Table I).

Studies with *E. coli*

Degradation of 2-trans,5-cis-Tetradecadienoyl-CoA by the E. coli Fatty Acid Oxidation Complex - The degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (compound III in Scheme 3) via the classical isomerase-dependent pathway of β-oxidation (Scheme 3, pathway A) or via the alternate pathway that is initiated by isomerization to 3,5-tetradecadienoyl-CoA was also investigated in *E. coli* (Scheme 3, compound XI) (see Scheme 3). The enzymes necessary for these two divergent metabolic processes are enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, and enoyl-CoA isomerase, all of which are associated with the *E. coli* FAO complex that can be isolated and purified as an intact entity. The availability of this purified multienzyme complex provides the opportunity to study *in vitro* the entry of 2-*trans*,5-*cis*-tetradecadienoyl-CoA into the classical and alternate pathways of β-oxidation, because the key reactions of both pathways, the isomerization of the 2,5- to the 3,5-isomer (Scheme 3, III to compound XI) and completion of the β-oxidation cycle (Scheme 3, compound III to VI), are practically irreversible as shown in the experiments with rat heart mitochondria. Work done by Chinhung Chu & Julia Aguirre in this laboratory demonstrated that ~90% of 2-*trans*,5-*cis*-tetradecadienoyl-CoA was degraded via the classical pathway, whereas a small but significant amount (~10%) of this intermediate was converted to 3,5-tetradecadienoyl-CoA and thereby diverted from the main pathway. The fraction of 2-*trans*,5-*cis*-tetradecadienoyl-CoA entering either of the two pathways was relatively constant over a wide concentration range of this compound. Hence, subsequent studies were carried out at a fixed concentration of 20 μM 2-*trans*,5-*cis*-tetradecadienoyl-CoA.

The time course for the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA by the FAO complex in the presence of NAD^+ and CoASH was determined by analyzing and quantifying metabolites by HPLC. Since 3,5-*cis*-tetradecadienoyl-

CoA was coeluted with its 2,5-isomer, dienoyl-CoA isomerase was added to the incubation mixture to convert the 3,5-isomer to the 2,4-isomer, which is well separated from the starting material. Shown in Fig. 7 is the spectrum of metabolites that were formed during the first minute of the incubation period. All expected metabolites of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (see Scheme 3) were detected and identified by use of authentic compounds that were synthesized by chemical and enzymatic reactions. Changes of metabolite concentrations as a function of the incubation time are shown in Fig. 8A. The rapid hydration of the starting material, 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 3, compound III), led to a built-up of 3-hydroxy-5-cis-tetradecenoyl-CoA (Scheme 3, compound IV), because the latter compound seemed to be more slowly dehydrogenated than formed. Since the product of the dehydrogenation, 3-keto-5-*cis*-tetradecenoyl-CoA (Scheme 3, compound V), only accumulated to a limited extent (see Fig. 7), it seems that initially the dehydrogenation step limited the flux through the pathway. The subsequent build-up of 3-dodecenoyl-CoA (Scheme 3, compound VI) at 1 min followed by a more pronounced accumulation of 2-dodecenoyl-CoA (Scheme 3, compound VII) at 2 min suggests that a competition between these compounds for the hydratase/isomerase active site (68) may restrict the flux through the pathway during the later part of the incubation period. The time course for the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA (Scheme 3, compound III) by the FAO complex was quite different when dienoyl-CoA isomerase was omitted from the incubation mixture (see Fig. 8B). Most dramatic was the much slower progress of the degradation process as illustrated by the conversion of ~10% of the starting material to decanoyl-CoA (Scheme 3, compound X) during the first 5 min of the incubation, whereas close to 80% of the substrate was converted to decanoyl-CoA (Scheme 3, compound X) during the same time period when dienoyl-CoA isomerase was present (Fig. 8, compare A and B). All metabolites were more slowly formed and degraded in the absence of dienoyl-CoA isomerase. This observation prompts the suggestion that 3,5-tetradecadienoyl-CoA inhibits the FAO complex and hence may interfere with the efficient β-oxidation of oleic acid in intact *E. coli* cells unless this intermediate is further metabolized.

Identification of an E. coli Thioesterase Active with 3,5-Tetradecadienoyl-CoA as Substrate - An effort by André Ntamack of this research group to detect dienoyl-CoA isomerase in *E. coli* came to the conclusion that the enzyme is not present in this organism even when grown on oleate. However, André Ntamack observed that 3,5-*cis*-tetradecadienoyl-CoA, the substrate for measuring the activity of dienoyl-CoA isomerase, was metabolized by an extract from *E. coli* cells. The following experiments were prompted by the idea that a thioesterase may be expressed in *E. coli* cells for the purpose of hydrolyzing metabolites of β-oxidation when an accumulation of such intermediates would block the flux through the pathway. If such thioesterase exists, it should be highly expressed in oleate-grown *E. coli* cells that have a high capacity to oxidize fatty acids but should not be expressed or poorly expressed in glucose-grown cells with a repressed β-oxidation system. The search for such thioesterase was initiated by preparing extracts from oleate-grown and glucose-grown *E. coli* cells and by fractionating them on DEAE-cellulose. Fractions were assayed for thioesterase with tetradecanoyl-CoA as the substrate instead of 3,5-tetradecadienoyl-CoA, because the latter compound is difficult to synthesize. This experiment revealed the presence of at least two thioesterases that correspond to peaks I and II (see Fig. 9, A and B). The same two enzymes may be present in either extract, because similar elution patterns were obtained with both extracts. However, an important difference between the two extracts is the 6 times higher specific activity of thioesterase II in oleate-grown cells as compared with glucose-grown cells (compare peaks II of Fig. 9, A and B). In contrast, the specific activities of thioesterase I were nearly the same in both extracts. Thus, growth on oleate seems to induce the expression of thioesterase II but not that of the type I enzyme. An evaluation of substrate specificities revealed thioesterase I to be most active with saturated long-chain fatty acyl-CoAs like palmitoyl-CoA, myristoyl-CoA, and stearoyl-CoA (see Fig 10A). The enzyme exhibited significant activity with 3,5-*cis*-tetradecadienoyl-CoA but was less active with other long-chain intermediates of β-oxidation. Thioesterase II also had a preference for long-chain fatty acyl-CoAs. Its highest activity, however, was observed with 3,5-*cis*-tetradecadienoyl-CoA as substrate. It is noteworthy that thioesterase II effectively hydrolyzed a number of long-chain β-oxidation intermediates besides 3,5-*cis*-tetradecadienoyl-CoA. Good substrates of this enzyme were β-oxidation intermediates that have no double bond at the α-carbon, like fatty acyl-CoAs, 3-enoyl-CoA, 3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. Thus, it seems that thioesterase II would be well suited to remove a block in β-oxidation by hydrolyzing one or several intermediates that might reduce the flux through the pathway because they would either inhibit certain reactions of β-oxidation, tie up free CoA, or do both.

Identification of 3,5-Tetradecadienoic Acid in the Growth Medium of E. coli Cells Grown on Oleate - If 3,5-tetradecadienoyl-CoA is a metabolite of oleate in *E. coli* and is hydrolyzed to CoASH and 3,5-tetradecadienoic acid, the latter compound is expected to exit from cells and accumulate in the growth medium. To test for the presence of 3,5-tetradecadienoic acid in the growth medium, cells were separated from the medium by centrifugation, and the medium, after acidification, was extracted with ether. Neutral material present in the ether extract was removed by extraction with ether under alkaline conditions. The remaining acidic compounds were converted to methyl esters and analyzed by GC/MS. The analysis of this material by GC (see Fig. 11A) demonstrated the presence of many acidic compounds in the medium after growth of *E. coli* cells on oleate. Fortunately, the region of the chromatogram between 17.5 and 19.5 min, which is important for the identification of methyl tetradecanoate, methyl tetradecenoate, and methyl tetradecadienoate, was relatively uncongested. Shown in Fig. 11B is an expanded view of the region between 18 and 19.5 min where approximately 10 peaks are visible. Mass spectra corresponding to these peaks were analyzed for ions with mass/charge (*m/z*) ratios of 238 that might be due to the molecular ion of methyl 3,5-tetradecadienoate. Spectra related to four of the peaks that are marked as *1–4* in Fig. 11B were similar to each other and had apparent molecular ions at *m/z* = 238. Hence, these peaks may correspond to methyl tetradecadienoates. This view was supported by the absence of these peaks from chromatograms that were obtained with extracts of control cultures grown on either glucose or palmitate to mid-logarithmic phase or grown on oleate to early exponential phase. For the purpose of identifying the materials that gave rise to the four peaks, methyl 3,5-tetradecadienoate was prepared by hydrolyzing 3,5-*cis*-tetradecadienoyl-CoA and converting the resultant acid to the methyl ester. The material obtained by this procedure was analyzed by GC/MS. The gas chromatogram showed six significant peaks in the region between 18 and 19.5 min (see Fig. 11C). The mass spectra corresponding to four of the six peaks (see Fig. 11C) had apparent molecular ions at m/z = 238. Moreover, the positions of these four peaks were virtually identical with the positions of the four peaks tentatively attributed to methyl 3,5-tetradecadienoate in the chromatogram of the material extracted from the medium after the growth of *E. coli* cells on oleate (Fig. 11, compare B and C). In an effort to identify the compounds that gave rise to peaks 1–4 in Fig. 13B, their mass spectra were compared with the mass spectra of methyl 3,5-tetradecadienoates corresponding to peaks 1–4 in Fig. 11C. Since the mass spectrum related to the major peak (peak 3) in Fig. 11B was virtually identical with the spectrum corresponding to peak 3 in Fig. 11C (shown in Fig. 11D is the mass spectrum of the material corresponding to peak 3 in Fig. 11C), the compound that was isolated from the medium after growth of *E. coli* cells on oleate was most likely 3,5-tetradecadienoate. The stereochemistry, of this 3,5-tetradecadienoate is not certain, but it seems reasonable to assume that it may have a 5-*cis* double bond, because the authentic methyl 3,5-tetradecadienoate was prepared from 3,5-*cis*-tetradecadienoyl-

CoA. The spectra corresponding to peaks 1 and 4 in the chromatograms of Fig. 11, B and C, were very similar, and only the spectra corresponding to peaks 2 of chromatograms B and C showed significant differences. Contaminations may account for some differences between mass spectra. Also the presence of different stereoisomers of methyl 3,5-tetradecadienoate may cause spectral variations, which additionally may reflect the existence of positional isomers. Overall, the results of these experiments suggest that growth on oleate gives rise to 3,5-tetradecadienoate in the growth medium.

Purification and Characterization of E. coli Thioesterase II - The aim of this study was to characterize *E. coli* thioesterase II and to assess its contribution to the growth of *E. coli* cells on oleate. Thioesterase II was purified as described in "Experimental Procedures". When the purified enzyme was analyzed by SDS-PAGE, one single band was detected corresponding to a protein with a molecular mass around 31 kDa (Fig. 12). This result is consistent with a previous report claiming that thioesterase II is a homotetramer with a molecular weight of 122 kDa (36). The substrate specificity of purified *E. coli* thioesterase II was determined and compared with that of partially purified thioesterase II extracted from *E. coli* cells grown on oleate as the sole carbon source. To minimize the detergent effects due to long-chain acyl-CoAs, bovine serum albumin (0.2 mg/ml) was added to the reaction mixture. The substrate profile obtained with the purified thioesterase (Fig. 13) was very similar to that of the partially purified *E. coli* thioesterase II (Fig. 10 B). An exception was the relatively low activity of purified thioesterase II with myristoyl-CoA as substrate. Kinetic parameters (K_m, V_{max}) were determined for the pure enzyme with myristoyl-CoA, 3,5-*cis*-tetradecadienoyl-CoA and palmitoyl-CoA as substrates. The results shown in Table II demonstrate that the enzyme has a low K_m of 7 μM for 3,5-tetradecadienoyl-CoA but is most active with palmitoyl-CoA of the three substrates used in the kinetic evaluation. However, the catalytic efficiency (k_{cat}/K_m) of the enzyme toward 3,5-tetradecadienoyl-coA was at least 3-times higher than the catalytic efficiency with palmitoyl-CoA as substrate and 10-times higher when compared to the hydrolysis of myristoyl-CoA.

Growth Properties of E. coli Cells - The purpose of these experiments was to further evaluate the metabolic function of thioesterases. To do so, growth curves of wild-type *E. coli* and of the double thioesterase mutant were determined with M9 medium containing either glucose, oleic acid or palmitic acid as the sole carbon source in the presence or absence of chloramphenicol and kanamycin. As shown in Fig. 14A, no difference was observed between the growth behavior of wild-type and the mutant strain when grown on glucose. However on oleic acid or palmitic acid as the sole carbon source, the mutant grew slower during the early logarithmic phase but caught up with the growth of wild-type cells after the mid-logarithmic phase. Chloramphenicol and kanamycin did not affect the growth of the mutant on M9 medium plus oleic acid (data not shown).

Thioesterase Activities of Wild-type and Mutant E. coli Cells at Different Growth Stages - Since the thioesterase mutant grew on oleate as sole carbon source but grew with a more pronounced lag than wild-type cells, the question arose as to whether another thioesterase activity was expressed that supported growth or whether no thioesterase was required for growth on oleate. To answer this question, the thioesterase activity was measured at three growth stages for both wild-type and mutant cells. Growth on LB medium to mid-logarithmic phase is referred to as stage 1 in Table II. After that, cells were diluted 5-times into M9 medium with oleic acid in the presence of trypton and grown to an absorbance of 1 at 600 nm, when they were diluted 20 times into the same medium but without trypton. This was referred to as stage 2 in Table II after cells had grown to mid-logarithmic phase. At that point, cells were diluted 10-times with the same medium and grown to stationary phase which is referred to as stage 3. As shown in Table II, no thioesterase activity was detected when the mutant was grown in LB medium, in contrast to wild-type cells that contained significant thioesterase activity when grown in LB medium. However, thioesterase activity was detected in mutant cells at stage 2 after growth on oleic acid as the sole carbon source. An additional induction was observed at stage 3. However, at all growth stages, the thioesterase activity was at least 6-times higher in wild-type cells than in mutant cells. This data prompt the suggestion that a thioesterase, perhaps a thioesterase different from type II, is expressed, which allows mutant cells to grow on oleate.

DISCUSSION

The demonstration that unsaturated fatty acids with odd-numbered double bonds can be degraded by two pathways, the isomerase-dependent pathway and the reductase-dependent pathway (19), prompted the questions as to how much each pathway may contribute to the total flux through β-oxidation and what specific functions the two pathways may have. The focus of the first part of this investigation was the β-oxidation of unsaturated dietary fatty acids in mitochondria. Given that oleic acid is abundantly present in the human diet and only contains a 9-*cis* double bond, it was selected as the fatty acid best suited for this study. The oleate metabolite, 2-*trans*,5-*cis*-tetradecadienoyl-CoA, served as the substrate for the required flux measurements and product determinations. Mitochondria from rat heart were used because of their minimal contamination by peroxisomes, which contain a β-oxidation system different from the mitochondrial one. Mitochondria were solubilized with Triton X-100 to obtain a system that, in contrast to intact mitochondria, would permit rate measurements of the individual pathways. The concentration of CoASH was fixed at 0.3 mM because this is its estimated concentration in mitochondria that rapidly oxidize fatty acids (61). The concentrations of NAD^+ and NADPH were set at 1 mM and 0.5 mM, respectively, because these are saturating concentrations even though they are lower than their estimated intramitochondrial concentrations. When rates of 2-*trans*,5-*cis*-tetradecadienoyl-CoA degradation via the isomerase-dependent pathway were compared with rates of its entry into the reductase-dependent pathway, the former pathway was estimated to account for more than 85% of the β-oxidation of this metabolite of oleic acid. Similar results were obtained when the accumulation of products was determined. Decanoyl-CoA, which is formed via the isomerase-dependent pathway, accounted for 85% of the products formed from 2-*trans*,5-*cis*-tetradecadienoyl-CoA. The ratio of substrate utilization via the two pathways varied little over a significant range of 2-*trans*,5-*cis*-tetradecadienoyl-CoA concentrations and hence may reflect the relative contributions of the two pathways to β-oxidation under a variety of conditions, including conditions that exist in intact mitochondria. However, intramitochondrial conditions may change during β-oxidation, especially as NAD^+ is converted to NADH and CoASH to acetyl-CoA. Increased concentrations of NADH and acetyl-CoA may inhibit β-oxidation and thereby affect the relative contributions of the two pathways. This idea was tested by determining the effects that NADH and acetyl-CoA have on the formation of products via the two pathways. When 15% of the total NADH was in the reduced form, the relative contributions of the two pathways were unchanged, even though the total flux through β-oxidation was reduced. An increase of NADH to one-third of the total coenzyme level further reduced the rate of oxidation but only slightly increased the relative contribution of the reductase-dependent pathway from 10% to 15%. Because only 5% of the total NAD^+ is estimated to be in the reduced state during fatty acid β-oxidation in actively respiring mitochondria (61), it is unlikely that NADH would significantly change the contribution of the reductase-dependent pathway to oleate β-oxidation. The same conclusion was reached with regard to the effect of acetyl-CoA. This product of β-oxidation affected neither the rate of the process nor the contributions of the two pathways even when it comprised 80% of the total CoA content of the system. A major reason for the limited flux through the reductase-dependent pathway is the rapid and dramatic decrease in the concentration of 2-*trans*,5-*cis*-tetradecadienoyl-CoA due to its hydration. The consequence is a greatly reduced rate of its isomerization to 3,5-*cis*-tetradecadienoyl-CoA, the first metabolite of the reductase-dependent pathway. Together, the results of this study lead to the conclusion that the reductase-dependent pathway only makes a minor contribution to the total β-oxidation of oleate.

If the reductase-dependent pathway contributes little to the β-oxidation of oleate, what is its metabolic function? In an attempt to answer this question, we studied the degradation of 3,5-*cis*-tetradecadienoyl-CoA, the first metabolite of oleate with two conjugated double bonds. Although it was assumed that this oleate intermediate could be metabolized via the reductase-dependent pathway, it was uncertain whether it also could be degraded by way of the isomerase-dependent pathway. The results clearly demonstrate that 3,5-*cis*-tetradecadienoyl-CoA is rapidly converted to 2,4-tetradecadienoyl-CoA, which is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase before being degraded by β-oxidation to dodecanoyl-CoA. Because only a trace of decanoyl-CoA was detected, 3,5-*cis*-tetradecadienoyl-CoA is not a substrate of the isomerase-dependent pathway nor is its product, 2,4-tetradecadienoyl-CoA, effectively degraded by direct β-oxidation. The first observation agrees with the previous conclusion that 3,5-dienoyl-CoAs cannot be metabolized via the isomerase-dependent pathway (45). This is most likely due to the unfavorable energetics of the 3,5-dienoyl-CoA to 2,5-dienoyl-CoA conversion. Surprising was the observation that 2-*trans*,4-*trans*-tetradecadienoyl-CoA, in contrast to the medium-chain metabolite 2-*trans*,4-*trans*-octadienoyl-CoA (45), was not directly degraded by β-oxidation. It should be noted that 2-*trans*,4-*trans*-decadienoyl-CoA but not its 2-*trans*,4-*cis* isomer is a substrate, albeit a poor one, of direct β-oxidation (57). The most likely reason for the different reactivities of 2,4-tetradecadienoyl-CoA and 2,4-octadienoyl-CoA is the involvement of two different sets of β-oxidation enzymes. 2,4-Octadienoyl-CoA is presumably hydrated by crotonase and the resultant 3-hydroxyoctanoyl-CoA is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase, because both of these enzymes are more active with short-chain and medium-chain substrates than with long-chain ones (54,59,60). In contrast, 2,4-tetradecadienoyl-CoA would most likely be acted upon by long-chain enoyl-CoA hydratase and long-chain 3-hydroxyacyl-CoA dehydrogenase of the trifunctional β-oxidation complex because crotonase and 3-hydroxyacyl-CoA dehydrogenase exhibit little activity toward substrates with acyl chains having 14 carbon atoms (60). Because the equilibrium concentration of 3-hydroxy-4-enoyl-CoA formed by hydration of 2,4-dienoyl-CoAs is extremely low (equilibrium constant for the hydration is 0.003) (31), the 3-hydroxy intermediate would only be dehydrogenated at a measurable rate if the catalytic efficiency and concentration of the relevant 3-hydroxyacyl-CoA dehydrogenase are sufficiently high. These conditions seem to be met for the matrix 3-hydroxyacyl-CoA dehydrogenase acting on 3-hydroxyoct-4-enoyl-CoA but not for long-chain 3-

hydroxyacyl-CoA dehydrogenase catalyzing the dehydrogenation of 3-hydroxytetradec-4-enoyl-CoA. The general conclusion is that once 3,5-*cis*-tetradecadienoyl-CoA has been formed, it can be effectively degraded only via the reductase-dependent pathway. In the absence of this pathway, 3,5-dienoyl-CoAs would most likely accumulate and impair the oxidative function of mitochondria because of a decline of free CoA and possibly by inhibiting some of the enzymes of β-oxidation.

The reductase-dependent pathway is, however, the major pathway for the β-oxidation of unsaturated fatty acids with conjugated double bonds. Such fatty acids, specifically conjugated linoleic acid, are constituents of the human diet because they are formed in ruminants and during the partial hydrogenation of fats. The most common conjugated linoleic acid is 9-*cis*,11-*trans*-octadecadienoic acid. β-Oxidation of this fatty acid is expected to produce 3-*cis*,5-*trans*-dodecadienoyl-CoA as an intermediate, which can only be degraded by way of the reductase-dependent pathway. As previously pointed out (30), β-oxidation of 9-*cis*,11-*trans*-octadecatrienoic acid also yields 2-*trans*,5-*cis*,7-*trans*-tetradecatrienoyl-CoA as an intermediate. This metabolite might be degraded in part via the reductase-dependent pathway that requires the participation of $\Delta^{3,5,7},\Delta^{2,4,6}$-trienoyl-CoA isomerase, which is an inherent activity of dienoyl-CoA isomerase (28).

Surprising and interesting was the observed accumulation of 2,4-tetradecadienoyl-CoA during the β-oxidation of either 2-*trans*,5-*cis*-

tetradecadienoyl-CoA or 3,5-*cis*-tetradecadienoyl-CoA. This finding prompted the idea that the reaction catalyzed by 2,4-dienoyl-CoA reductase may limit the flux through the pathway even though the entry into this pathway is already restricted by competition with the dominant isomerase-dependent pathway. A previous evaluation of a possible control exerted by 2,4-dienoyl-CoA reductase over the β-oxidation of oleic acid and docosahexaenoic acid in cardiomyocytes came to the conclusion that an increase in the activity of 2,4-dienoyl-CoA reductase in response to the treatment of rats with growth hormone did not result in higher rates of β-oxidation (69). It is possible, however, that isolated and mostly quiescent cardiomyocytes are not suitable for such study because their low energy need severely restricts fatty acid oxidation with the possible result that none of the reactions of β-oxidation is limiting the rate of energy production.

Altogether the reductase-dependent pathway in rat mitochondria only makes a minor contribution to the β-oxidation of oleic acid, which is mostly degraded via the classical isomerase-dependent pathway. However, the reductase-dependent pathway is essential for the degradation of 3,5-*cis*-tetradecadienboyl-CoA, which is formed from the oleate metabolite 2-*trans*,5-*cis*-teradecadienoyl-CoA by enoyl-CoA isomerase that functions in the isomerasede-pendent pathway. The reductase-dependent pathway is also essential for the β-oxidation of conjugated linoleic acid like 9-*cis*,10-*trans*-octadecadienoic acid.

A concern regarding the study of oleate β-oxidation in mitochondria was the use of mitochondrial extracts that are devoid of enzyme organization as the result of extracting or solubilizing mitochondria. This change in structure may affect the flux pattern. The use of the FAO complex from *E. coli* alleviates this problem, because the enzymes, which catalyze the reactions that determine the entry of 2-*trans*,5-*cis*-tetradecadienoyl-CoA into the two pathways, remain associated during the isolation and purification of the complex. The demonstration that only a small amount of 2-*trans*,5-*cis*-tetradecadienoyl-CoA is diverted from the isomerase-dependent pathway in *E. coli* agrees with the hypothesis that the classical, isomerase-dependent pathway accommodates most of the flux through β-oxidation.

It has been suspected that an accumulation of fatty acid metabolites like 3,5-tetradecadienoyl-CoA might cause an inhibition of β-oxidation, because the pool of free CoA would be reduced and/or enzymes of β-oxidation would be inhibited by metabolites. This study proves this prediction to be correct. The kinetics of the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA by the FAO complex reveal a severe inhibition of the classical, isomerase-dependent pathway in the absence of dienoyl-CoA isomerase. This inhibition occurs although free CoA is available. Consequently, the inhibition of at least one enzyme of the FAO complex is most likely responsible for the reduced flux through β-oxidation. Since this inhibition is observed when dienoyl-CoA isomerase is omitted from the incubation mixture, 3,5-tetradecadienoyl-CoA accumulates and most likely inhibits the FAO complex. It is reasonable to suggest that this compound binds to the hydratase/isomerase active site of the FAO complex (68) and thereby inhibits hydration or isomerization of enoyl-CoA intermediates.

Work done by André Ntamack in this laboratory revealed that dienoyl-CoA isomerase is not present in *E. coli*, thus raising the question of how 3,5-tetradecadienoyl-CoA is metabolized to prevent a severe inhibition of oleate β-oxidation. The surprising answer was that 3,5-tetradecadienoyl-CoA is hydrolyzed by a thioesterase. This solution of a metabolic problem is simple, and the cost to the organism is only the incomplete oxidation of a small percentage of oleate that is passing through β-oxidation. The thioesterase assumed to be responsible for the hydrolysis of 3,5-tetradecadienoyl-CoA was more active with 3,5-tetradecadienoyl-CoA than with any other acyl-CoA tested as substrate, and the activity of this enzyme was induced when *E. coli* was grown on oleate. Separation of an *E. coli* extract by chromatography on DEAE-cellulose yielded two thioesterase fractions that were named thioesterase I and II according to the order of their elution from the column. Barnes *et al.* (38) introduced this nomenclature. Thioesterase I was purified by Barnes and Wakil (35) and later shown to be a periplasmic protein (34). This enzyme should not be able to hydrolyze fatty acyl-CoAs located in the cytoplasm. Thioesterase II also was purified (36) and could be the thioesterase highly active with 3,5-tetradecadienoyl-CoA as substrate. However, it remained to be determined whether the thioesterase fraction used in this study, referred to as thioesterase II, contained only thioesterase II (36) or perhaps contained more than one thioesterase. Several attempts have been made to elucidate the function of thioesterase II (33,34,70). So far, no specific function has been assigned to this enzyme, because the growth properties of *E. coli* cells seemed to be unaffected when thioesterase II was overexpressed or its gene (*tesB*) was silenced (70). The assumption underlying most of these studies was that thioesterase II might have a function in controlling or editing fatty acid synthesis that takes place with the growing acyl chain esterified to acyl carrier protein. Since thioesterase II exhibits little or no activity with fatty acyl-acyl carrier proteins (33), it is unlikely to function in fatty acid synthesis, but it could be involved in fatty acid oxidation because the substrates and intermediates of the latter process are fatty acyl-CoA thioesters.

The results presented here establish that in *E. coli* 3,5-tetradecadienoyl-CoA is not metabolized by the reductase-dependent pathway as in mammals but is hydrolyzed so that the resultant carboxylic acid can be excreted (see Scheme 3B). This is a simple but not energy-efficient solution for disposing of this metabolite. The emergence of this metabolic shortcut does not support the idea that an alternative pathway of β-oxidation is required to accommodate an increased flux through β-oxidation. It seems that in *E. coli* fatty acids with odd-numbered double bonds are efficiently degraded via the classical, isomerase-dependent pathway. It is likely that in mammals too the isomerase-dependent pathway alone assures a flux of fatty acids through β-oxidation that is sufficient to meet the energy needs of the organism. If this assumption is correct, the main function of the alternative pathway is the removal of the 3,5-intermediate. A unicellular organism like *E. coli* can easily dispose of fatty acyl-CoAs by hydrolyzing them and excreting the resultant fatty acids from the cell. In a mammal, in contrast, fatty acids that move out of cells enter the circulation, where they bind to serum albumin and are retained as long as they are hydrophobic enough and not further metabolized. The removal of fatty acids that are resistant to β-oxidation from an animal would require their partial degradation and conversion to a water-soluble product that could be excreted in the urine. Since such conversion of fatty acids is a multistep process that takes place inside of cells, the easiest disposal of a dead end fatty acyl-CoA may be its complete β-oxidation even if an additional enzyme like dienoyl-CoA isomerase is required to facilitate degradation by an alternative pathway.

A comparison of oleate β-oxidation in *E. coli* and mammals prompts the idea that the classical pathway accommodates a sufficient flux through β-oxidation when unsaturated fatty acids with odd-numbered double bonds serve as substrates. The formation of 3,5-*cis*-tetradecadienoyl-CoA may be an unavoidable side reaction due to the presence of enoyl-CoA isomerase, which catalyzes the conversion of 2,5-tetradecadienoyl-CoA to the more stable 3,5-isomer. Since 3,5-*cis*-tetradecadienoyl-CoA is an effective inhibitor of β-oxidation, it must be removed. This is achieved in *E. coli* by hydrolysis of 3,5-*cis*-tetradecadienoyl-CoA and excretion of 3,5-tetradecadienoate, whereas in mammals and perhaps in all multicellular organisms, a pathway has evolved for the β-oxidation of 3,5-*cis*-tetradecadienoyl-CoA as the most efficient way for its disposal.

The purification of recombinant thioesterase II from *E. coli* provided the opportunity to determine its substrate specificity and to compare it with that of the thioesterase, which was induced by growth of *E. coli* on oleate. Since the substrate profiles of the crude and purified thioesterase preparations were almost identical, it is concluded that thioesterase II is the enzyme responsible for hydrolyzing 3,5-*cis*-tetradecadienoyl-CoA during growth of *E. coli* on oleate. This conclusion is also supported by the kinetic data that prove 3,5-*cis*-tetradecadienoyl-coA to be the preferred substrate of thioesterase II. The proposed function of thioeasterase II in oleate β-oxidation was further assessed by comparing the growth behavior of the thioesterase mutant with that of wild-type *E. coli*. Surprisingly, the thioesterase mutant grew on oleate, although the growth lag was more pronounced than in the wild-type culture. The idea that a thioesterase may be expressed and support growth on oleate in the thioesterase mutant was confirmed. However, it remains to be established whether the thioesterase activity observed in the mutant was due to thioesterase II or reflected the presence of another enzyme. The expression of a thioesterase other than type II is more likely because the mutant was produced by deletion of part of the gene (34).

The work described herein represents the first example of a thioesterase serving an essential role in fatty acid β-oxidation coupled to oxidative phosphorylation. The enzyme hydrolyzes an intermediate that would inhibit the pathway if allowed to accumulate. Such function of thioesterases has been discussed in the past but not yet demonstrated (71). It is likely that other acyl-CoA intermediates will be identified, which are formed during fatty acid β-oxidation in mitochondria, peroxisomes, and *E. coli* and which are hydrolyzed by thioesterases to maintain a rapid flux through the pathways.

Table I

The effect of acetyl-CoA on the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA via the isomerase-dependent pathway and the reductase-dependent pathway.

Time (min)	Products of β-oxidation[1] (μM)					
	No addition		+ 0.15 mM acetyl-CoA		+ 0.24 mM acetyl-CoA	
	C_{10}-CoA	C_{12}-CoA + $\Delta^{2,4}$-C_{14}-CoA	C_{10}-CoA	C_{12}-CoA + $\Delta^{2,4}$-C_{14}-CoA	C_{10}-CoA	C_{12}-CoA + $\Delta^{2,4}$-C_{14}-CoA
0	0	0	0	0	0	0
1	1.2	1.2	1.2	1.1	0.8	1.2
3	6.9	2.3	6.3	2.2	6.9	2.9
5	14.3	2.7	12.9	2.5	12.5	2.5

[1] 20 μM 2-*trans*,5-*cis*-tetradecadienoyl-CoA was incubated with an extract of rat heart mitochondria in the presence of 0.3 mM CoASH, 1 mM NAD^+, 0.5 mM NADPH, and acetyl-CoA as indicated. Decanoyl-CoA (C_{10}-CoA) and dodecanoyl-CoA (C_{12}-CoA) plus 2,4-tetradecadienoyl-CoA ($\Delta^{2,4}$-C_{14}-CoA) were quantified to evaluate β-oxidation via the isomerase-dependent pathway and reductase-dependent pathway, respectively.

Table II

Kinetic parameters of *E. coli* thioesterase II

Substrate	V_{max} (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Myristoyl-CoA	60 ±4	50 ±6	30.5 ±2	0.6
3,5-*cis*-Tetradecadienoyl-CoA	91 ±6	7 ±1	46 ±3	6.6
Palmitoyl-CoA	166 ±17	46 ±7	84 ±9	1.8

Table III

Thioesterase activities of extracts from wild-type *E. coli* and a thioesterase double mutant

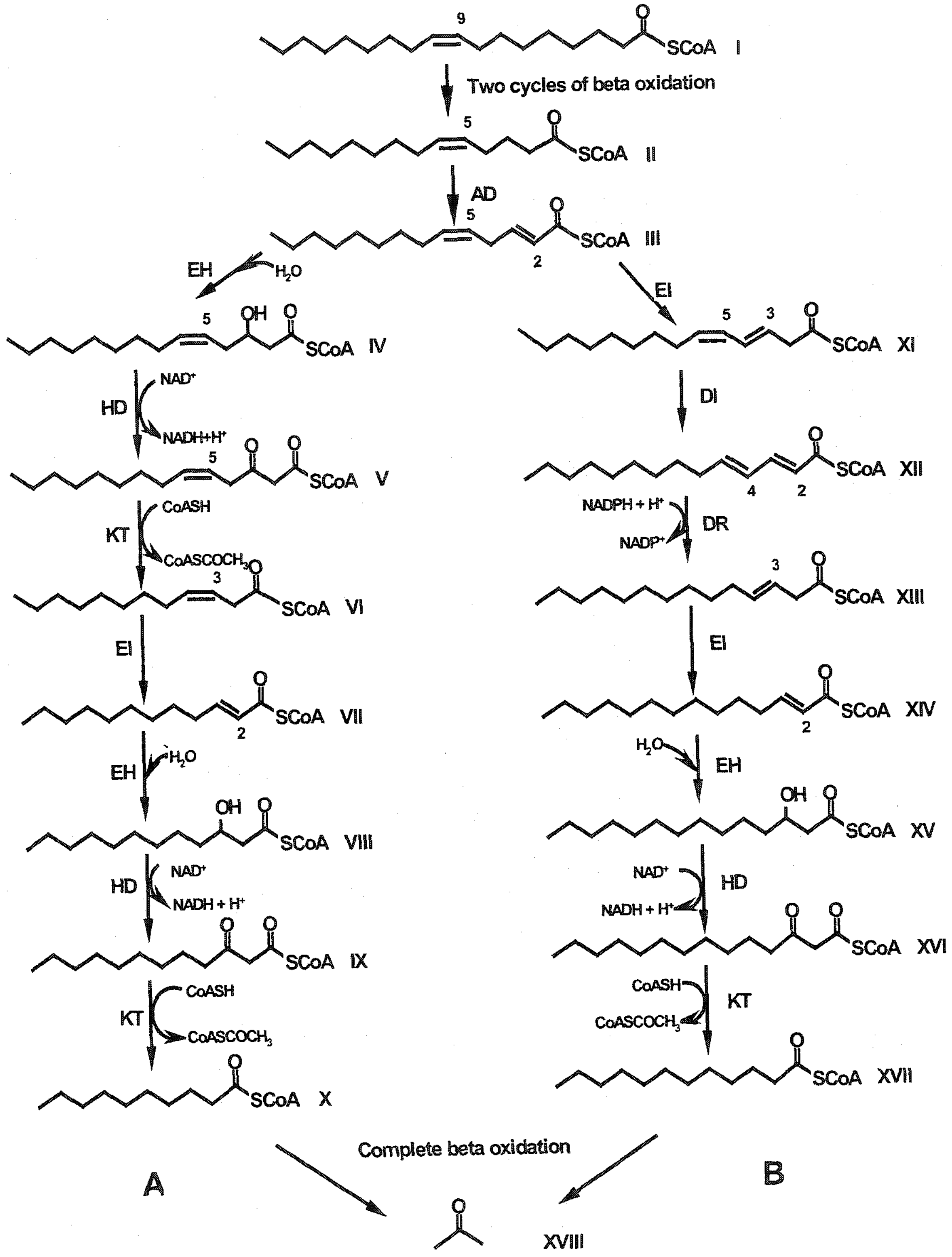
Growth Conditions*	Specific Activity (mU/mg)	
	Wild-type	Mutant
Stage 1	24 ±2.5 (3)	≤ 0.03 ±0.05 (3)
Stage 2	34 ±3.1 (3)	1.8 ±0.25 (3)
Stage 3	18 ±2 (3)	3±0.31 (3)

* Stage 1, Grown on LB medium to mid-logarithmic phase.

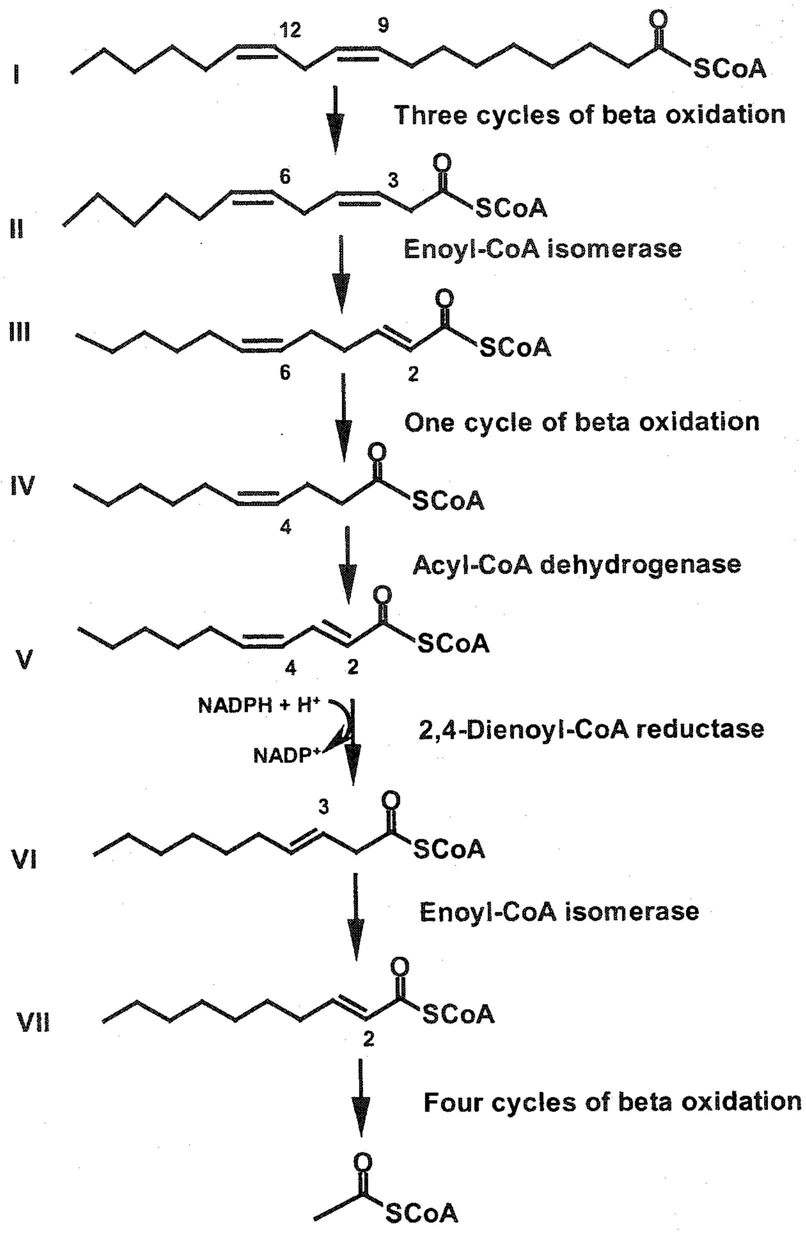
Stage 2, Cells grown as indicated in stage 1 were 5-times diluted and grown in M9 medium with oleate in the presence of trypton and again diluted 1:20 and grown in M9 with oleate to mid-logarithmic phase.

Stage 3, Cells grown as indicated in stage 2 were 10-times diluted and grown in M9 with oleate to stationary phase.

Scheme 1. **β-Oxidation of oleoyl-CoA in rat mitochondria.** A, isomerase-dependent pathway; B, reductase-dependent pathway. AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ^3,Δ^2-enoyl-CoA isomerase; DI, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase; DR, 2,4-dienoyl-CoA reductase.

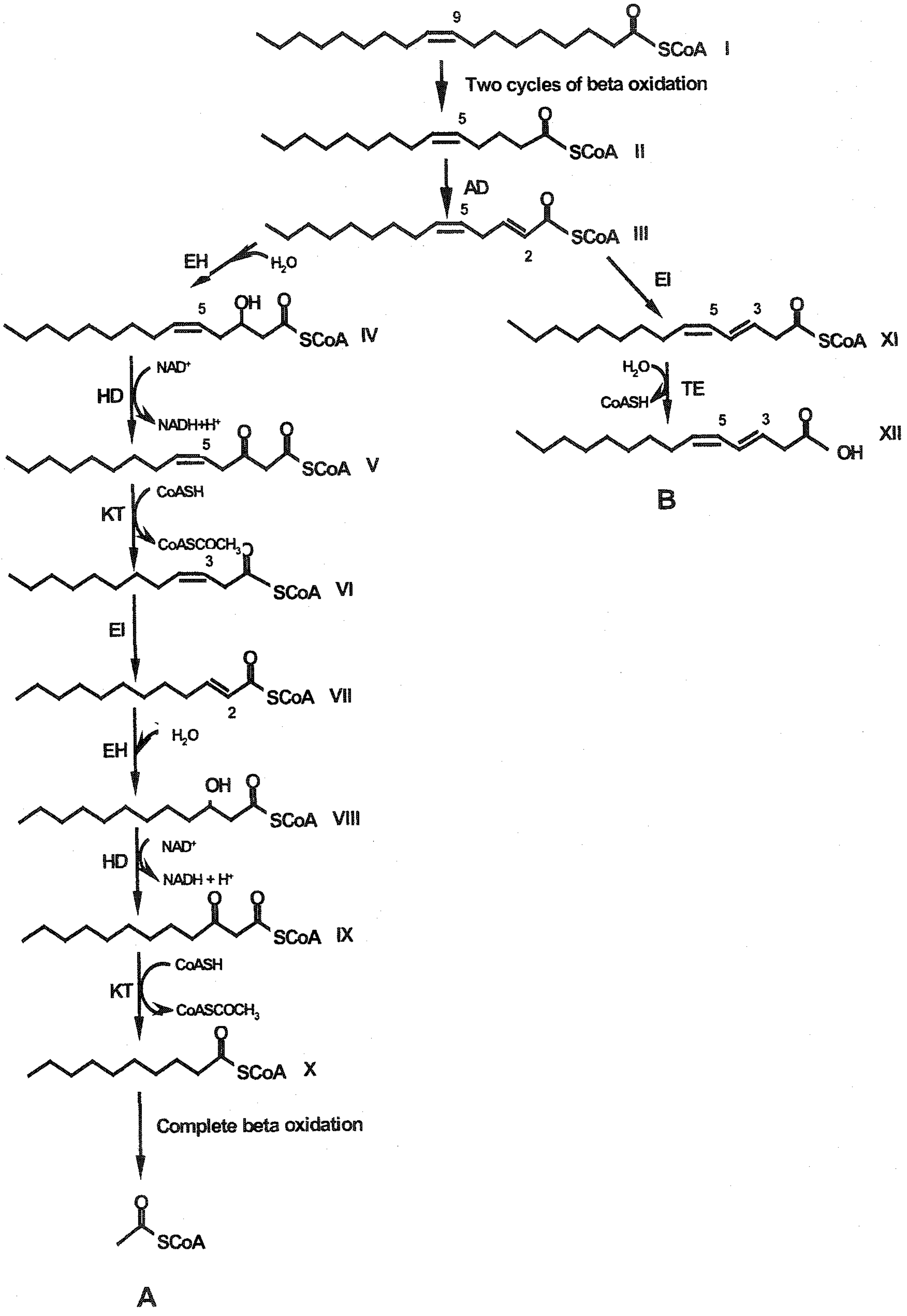

I
Two cycles of beta oxidation
II
AD
III
EH
H$_2$O
EI
IV
XI
HD
NAD$^+$
NADH+H$^+$
DI
V
XII
NADPH + H$^+$
NADP$^+$
DR
KT
CoASH
CoASCOCH$_3$
VI
XIII
EI
VII
XIV
EH
VIII
XV
HD
NAD$^+$
NADH + H$^+$
IX
XVI
KT
CoASH
CoASCOCH$_3$
X
XVII
Complete beta oxidation
A
B
XVIII
SCoA

Scheme 1



Scheme 2. **β-Oxidation of linoleoyl-CoA.**

Scheme 3. **β-Oxidation of oleoyl-CoA in *E. coli.*** A, classical or isomerase-dependent pathway; B, alternative pathway. AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ^3,Δ^2-enoyl-CoA isomerase; TE, thioesterase.


9
SCoA I
Two cycles of beta oxidation
5
SCoA II
AD
5
SCoA III
2
EH
H_2O
EI
5
OH
SCoA IV
5
3
SCoA XI
NAD⁺
HD
NADH+H⁺
H_2O
CoASH
TE
5
3
XII
OH
5
SCoA V
B
CoASH
KT
$CoASCOCH_3$
3
SCoA VI
EI
SCoA VII
2
EH
H_2O
OH
SCoA VIII
HD
NAD⁺
NADH + H⁺
SCoA IX
KT
CoASH
$CoASCOCH_3$
SCoA X
Complete beta oxidation
SCoA
A

Scheme 3

Fig. 1. **Model of the functional and physical organization of β-oxidation enzymes in mitochondria.** (A) β-Oxidation system active with long-chain (LC) acyl-CoAs; (B) β-Oxidation system active with medium-chain (MC) and short-chain (SC) acyl-CoAs. Abbreviations: T, carnitine:acylcarnitine translocase; CPT II, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; VLC, very long-chain. (Adapted from Liang, X., Le, W., Zhang, D. and Schulz, H. (2001) *Biochem. Soc. Trans.* **29**, 279-282)

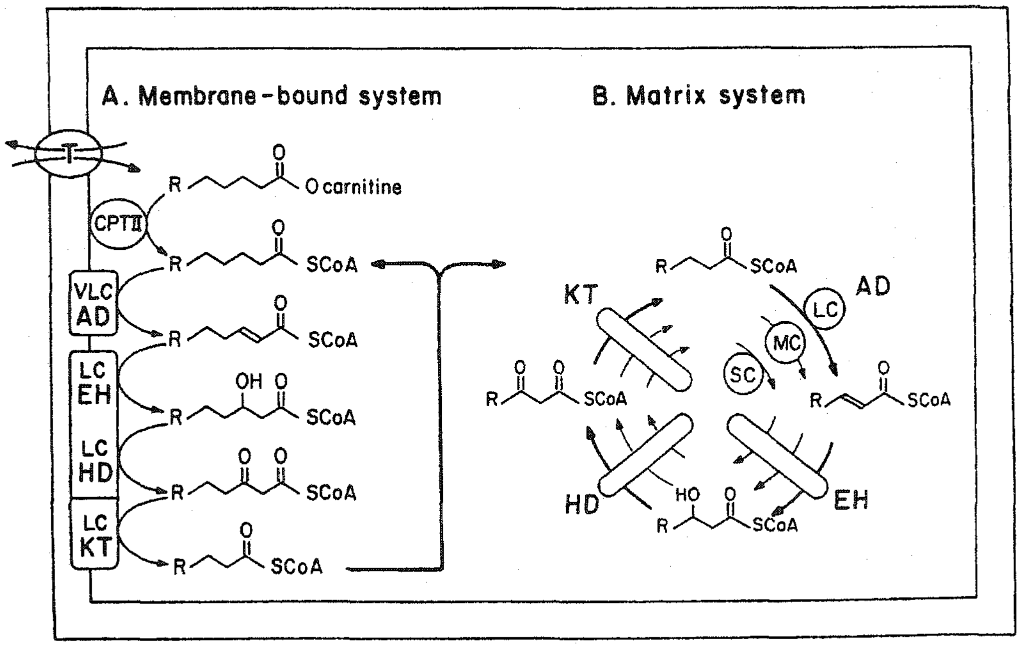

A. Membrane-bound system
B. Matrix system
T
CPTII
R
O
Ocarnitine
SCoA
VLC AD
LC EH
LC HD
LC KT
OH
KT
AD
LC
MC
SC
HD
EH
HO

Fig. 1

Fig. 2. **Rates of β-oxidation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA by solubilized rat heart mitochondria.** Rates of 2-*trans*,5-*cis*-tetradecadienoyl-CoA degradation via the isomerase-dependent pathway as a function of the substrate concentration (●). Rates were determined by measuring the formation of NADH in the presence of NAD^+ and CoASH. Rates of entry of 2-*trans*,5-*cis*-tetradecadienoyl-CoA into the reductase-dependent pathway as a function of the substrate concentration (○). Rates were determined by measuring the formation of 2,4-tetradecadienoyl-CoA in the presence of 0.1 unit of recombinant rat dienoyl-CoA isomerase but in the absence of cofactors. All rates are means of three measurements ± S.D.. Ratio of 2-*trans*,5-*cis*-tetradecadienoyl-CoA degradation via the isomerase-dependent pathway *versus* its entry into the reductase-dependent pathway (▲). For details see "Experimental Procedures."

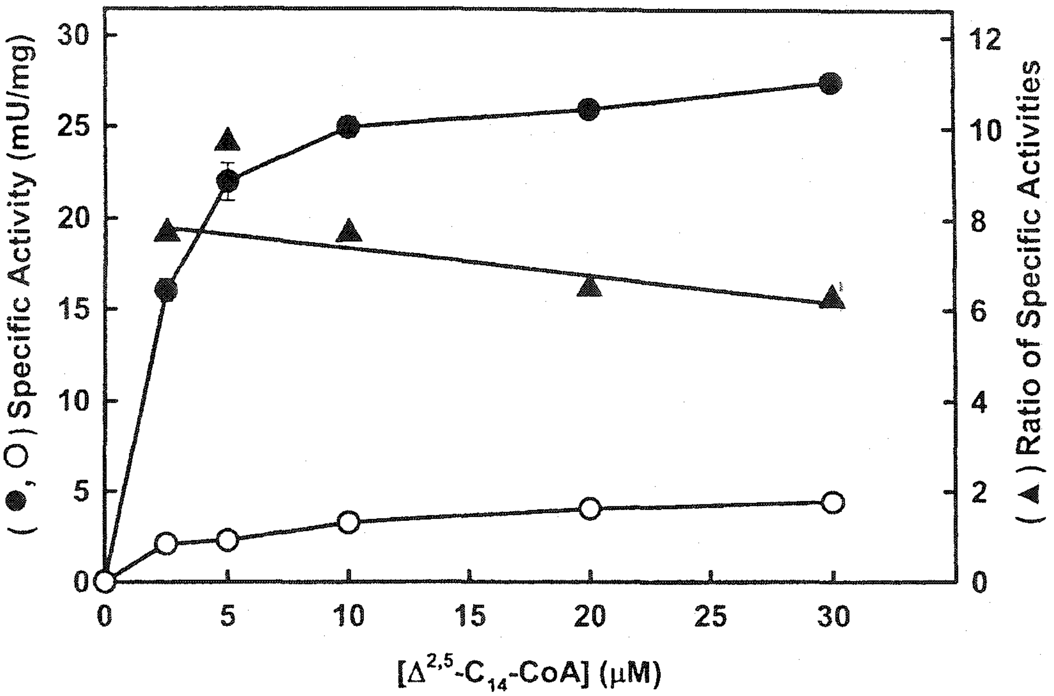

(●, ○) Specific Activity (mU/mg)
0
5
10
15
20
25
30
(▲) Ratio of Specific Activities
0
2
4
6
8
10
12
0
5
10
15
20
25
30
[$\Delta^{2,5}$-C_{14}-CoA] (μM)

Fig. 2

Fig. 3. **HPLC analysis of metabolites formed from 2-*trans*,5-*cis*-tetradecadienoyl-CoA by solubilized rat heart mitochondria in the presence of NAD$^+$, CoASH and NADPH.** Products formed 5 s (A), 1 min (B), and 5 min (C) after initiating the incubation. Peaks identified by use of authentic compound: $\Delta^{2,5}$-C_{14}-CoA, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; Δ^2-C_{12}-CoA, 2-*trans*-dodecenoyl-CoA; $\Delta^{2,4}$-C_{14}-CoA, 2-*trans*,4-*trans*-tetradecadienoyl-CoA; $3OHC_{12}$-CoA, 3-hydroxydodecanoyl-CoA; C_{10}-CoA, decanoyl-CoA; C_{12}-CoA, dodecanoyl-CoA; x, 3-keto-5-*cis*-tetradecenoyl-CoA; *, 3-dodecenoyl-CoA; +, impurity in substrate. For details see "Experimental Procedures."

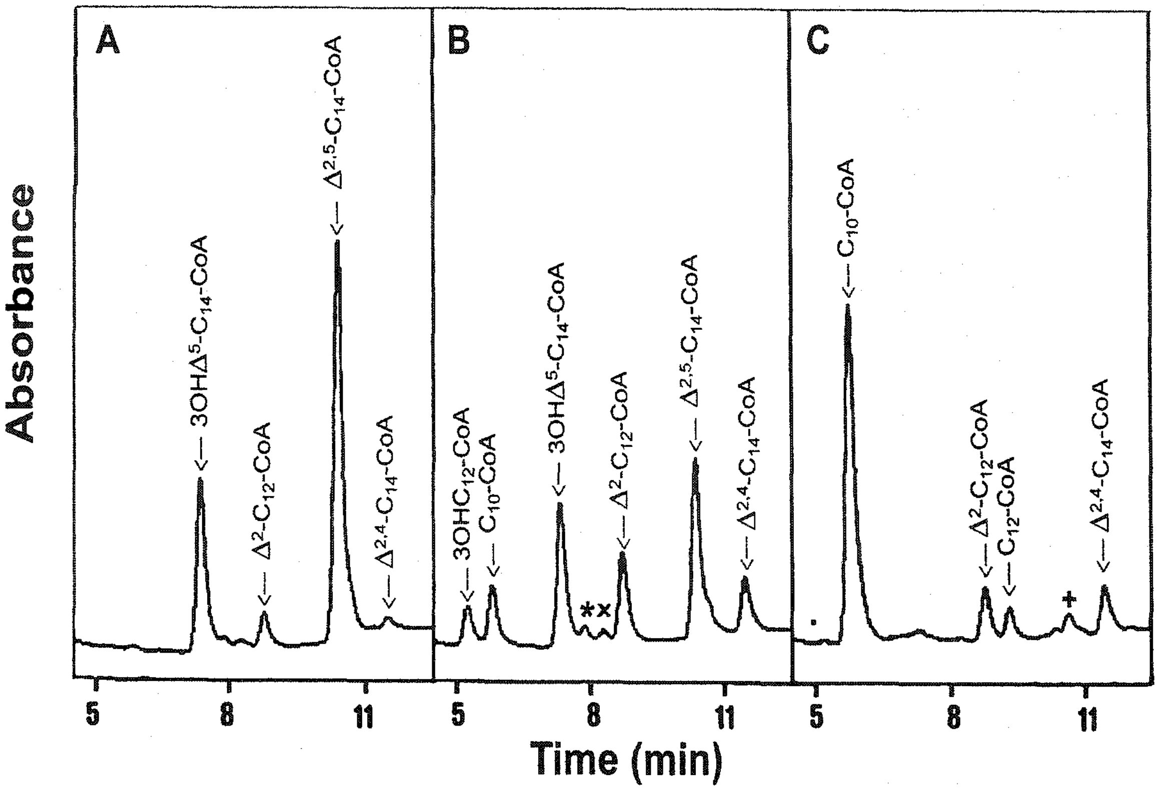

Absorbance
A
$3OH\Delta^5$-C_{14}-CoA
Δ^2-C_{12}-CoA
$\Delta^{2,5}$-C_{14}-CoA
$\Delta^{2,4}$-C_{14}-CoA
B
$3OHC_{12}$-CoA
C_{10}-CoA
$3OH\Delta^5$-C_{14}-CoA
*
x
Δ^2-C_{12}-CoA
$\Delta^{2,5}$-C_{14}-CoA
$\Delta^{2,4}$-C_{14}-CoA
C
C_{10}-CoA
Δ^2-C_{12}-CoA
C_{12}-CoA
+
$\Delta^{2,4}$-C_{14}-CoA
5
8
11
5
8
11
5
8
11
Time (min)

Fig. 3

Fig. 4. **Kinetics of 2-*trans*,5-*cis*-tetradecadienoyl-CoA utilization and metabolite formation and degradation.** ●, 2-*trans*,5-*cis*-tetradecadienoyl-CoA ($\Delta^{2,5}$-C_{14}-CoA); ○, 3-hydroxy-5-*cis*-tetradecenoyl-CoA; □, 2-*trans*,4-*trans*-tetradecadienoyl-CoA; ▼, 2-*trans*-dodecenoyl-CoA; ▽, 3-hydroxydodecanoyl-CoA; ■, decanoyl-CoA; ◆, dodecanoyl-CoA.

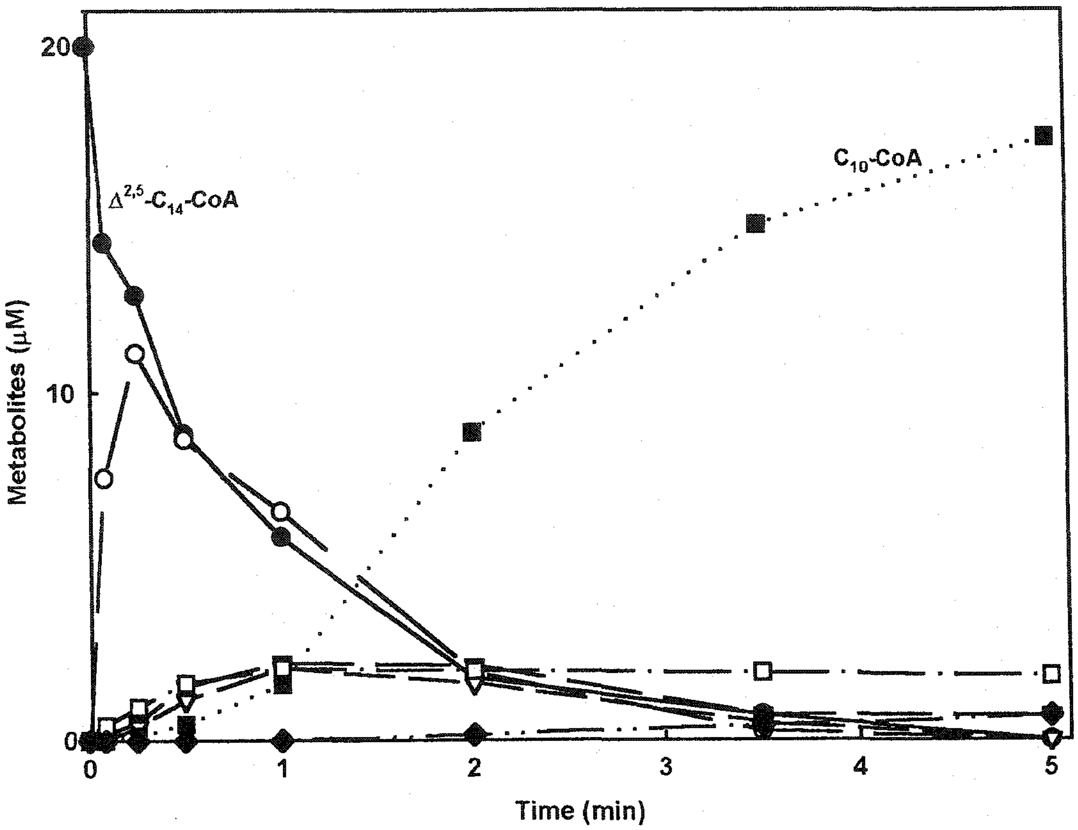

20
10
0
Metabolites (μM)
$\Delta^{2,5}$-C_{14}-CoA
C_{10}-CoA
0
1
2
3
4
5
Time (min)

Fig. 4

Fig. 5. **Metabolism of 3,5-*cis*-tetradecadienoyl-CoA by solubilized rat heart mitochondria.** A, metabolism in the presence of NAD^+ and CoASH. B, metabolism in the presence of NAD^+, CoASH, and NADPH. ●, 3,5-*cis*-tetradecadienoyl-CoA ($\Delta^{3,5}$-C_{14}-CoA); ○, 2-*trans*,4-*trans*-tetradecadienoyl-CoA ($\Delta^{2,4}$-C_{14}-CoA); ▼, dodecanoyl-CoA (C_{12}-CoA). For details see "Experimental Procedures."

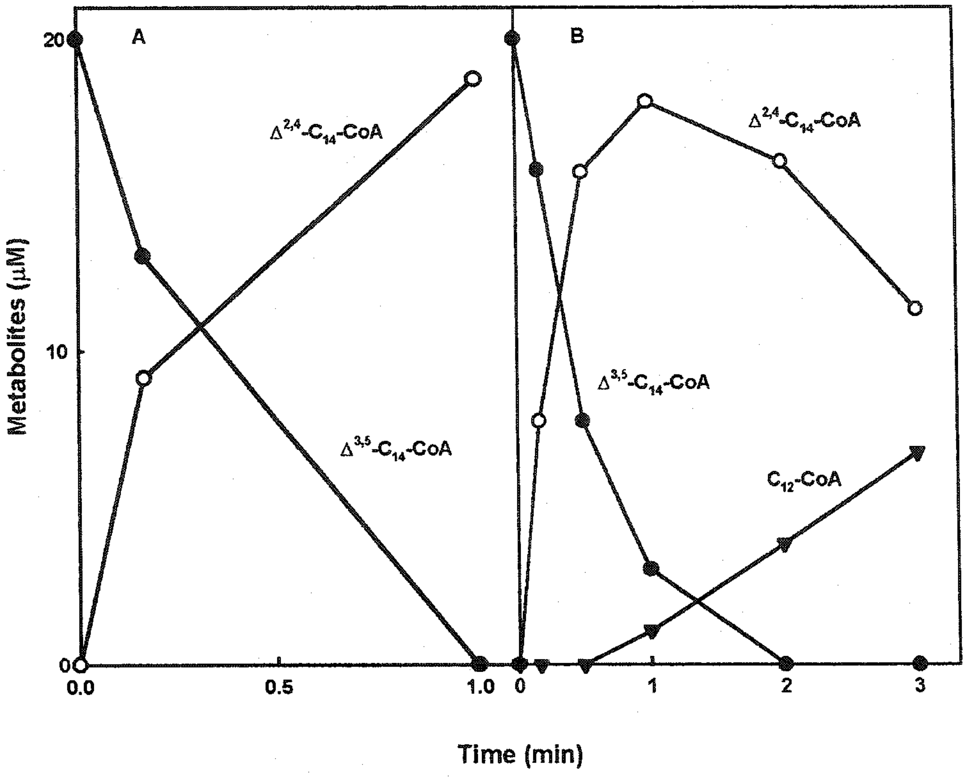

A
B
$\Delta^{2,4}$-C_{14}-CoA
$\Delta^{3,5}$-C_{14}-CoA
$\Delta^{2,4}$-C_{14}-CoA
$\Delta^{3,5}$-C_{14}-CoA
C_{12}-CoA
Metabolites (μM)
20
10
0
0.0
0.5
1.0
0
1
2
3
Time (min)

Fig. 5

Fig. 6. **The effect of NADH on the degradation of 2-*trans*,5-*cis*-tetradecadienoyl-CoA via the isomerase-dependent pathway and the reductase-dependent pathway.** 2-*trans*,5-*cis*-Tetradecadienoyl-CoA was incubated with solubilized rat heart mitochondria in the presence of 1 mM NAD^+, 0.3 mM CoASH, 0.5 mM NADPH, and no NADH (A), 0.17 mM NADH (B), 0.5 mM NADH (C), Decanoyl-CoA (C_{10}-CoA) (●) and dodecanoyl-CoA (C_{12}-CoA) plus 2,4-tetradecanoyl-CoA ($\Delta^{2,4}$-C_{14}-CoA) (○) were quantified to evaluate β–oxidation via the isomerase-dependent pathway and reductase-dependent pathway, respectively. For details see "Experimental Procedures."

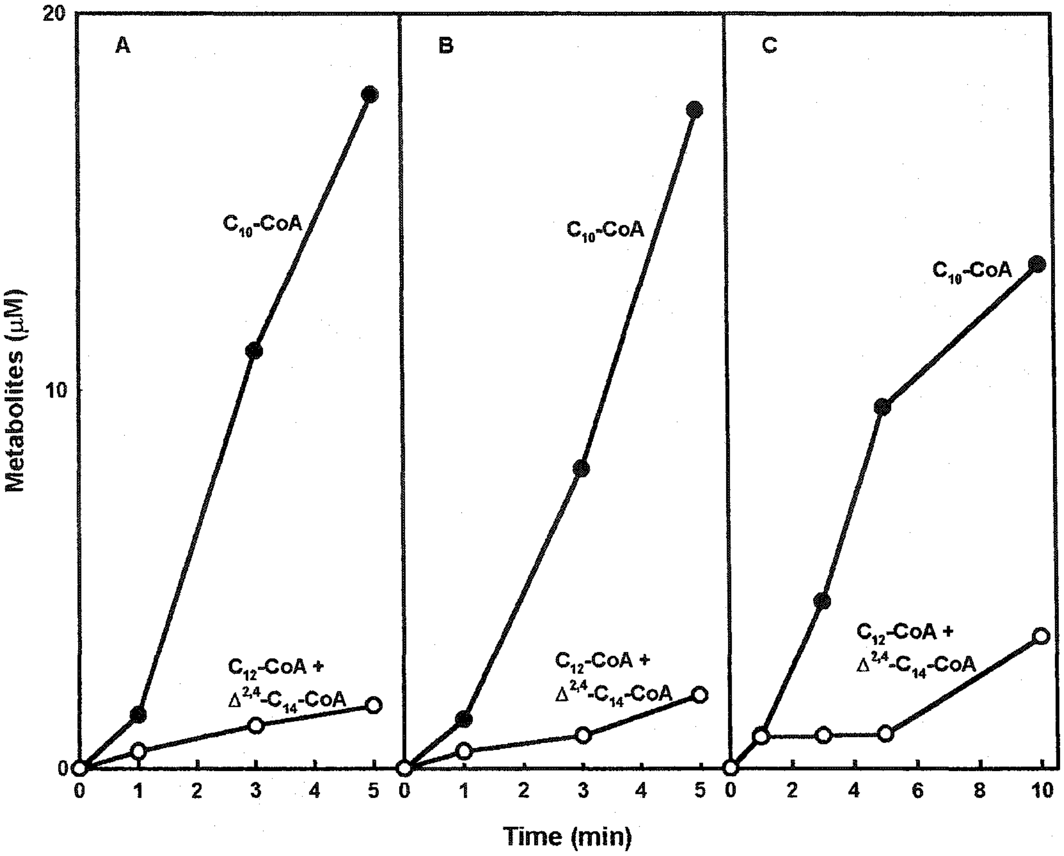
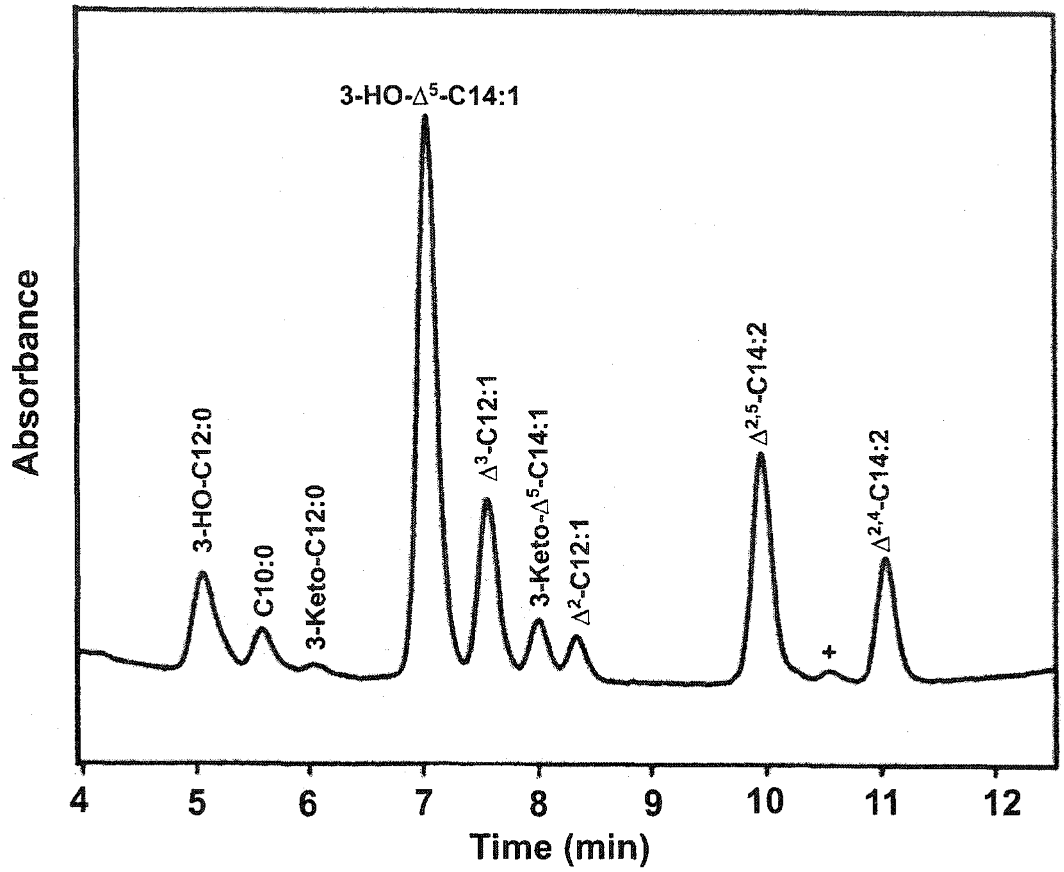

A
B
C
C10-CoA
C10-CoA
C10-CoA
C12-CoA + Δ2,4-C14-CoA
C12-CoA + Δ2,4-C14-CoA
C12-CoA + Δ2,4-C14-CoA
Metabolites (μM)
0
10
20
0
1
2
3
4
5
0
1
2
3
4
5
0
2
4
6
8
10
Time (min)

Fig. 6

Fig. 7. **HPLC analysis of metabolites formed from 2-*trans*,5-*cis*-tetradecadienoyl-CoA by the purified FAO complex from *E. coli* in the presence of NAD$^+$, CoASH, and dienoyl-CoA reductase.** Products formed 1 min after initiating the incubation. Peaks identified by use of authentic compounds (listed in the order of decreasing elution time): 2-*trans*,4-*trans*-tetradecadienoyl-CoA ($\Delta^{2,4}$-C14:2); 2-*trans*,5-*cis*-tetradecadienoyl-CoA ($\Delta^{2,5}$-C14:2*)*; 2-*trans*-dodecenoyl-CoA (Δ^{2}-C12:1); 3-keto-5-*cis*-tetradecenoyl-CoA (3-Keto-Δ^{5}-C14:1); 3-dodecenoyl-CoA (Δ^{3}-C12:1); 3-hydroxy-5-*cis*-tetradecenoyl-CoA (3-HO-Δ^{5}-C14:1); 3-ketododecanoyl-CoA (3-Keto-C12:0); decanoyl-CoA (C10:0); 3-hydroxydodecanoyl-CoA-CoA (3- HO-C12:0); (+) impurity in substrate. For details see “Experimental Procedures”.


3-HO-Δ5-C14:1
3-HO-C12:0
C10:0
3-Keto-C12:0
Δ3-C12:1
3-Keto-Δ5-C14:1
Δ2-C12:1
Δ2,5-C14:2
+
Δ2,4-C14:2
Absorbance
4
5
6
7
8
9
10
11
12
Time (min)

Fig, 7

Fig. 8. **Kinetics of 2-*trans*,5-*cis*-tetradecadienoyl-CoA utilization and metabolite formation and degradation by the FAO complex from *E. coli*.** A, in the presence of dienoyl-CoA isomerase. B, in the absence of dienoyl-CoA isomerase. ●, 2-*trans*,5-*cis*-tetradecadienoyl-CoA (2,5-C_{14}-CoA); ○, 3-hydroxy-5-*cis*-tetradecenoyl-CoA (3-OH-5-C_{14}CoA); ◆, 2-*trans*,4-*trans*-tetradecadienoyl-CoA ($\Delta^{2,4}$-C_{14}-CoA); ▼, 3-*cis*-dodecenoyl-CoA; ▽, 2-*trans*-dodecenoyl-CoA; ■, 3-hydroxydodecanoyl-CoA; □, decanoyl-CoA (C_{10}-CoA). All values are means of three or four determinations. S.D. values are displayed for the major metabolites.

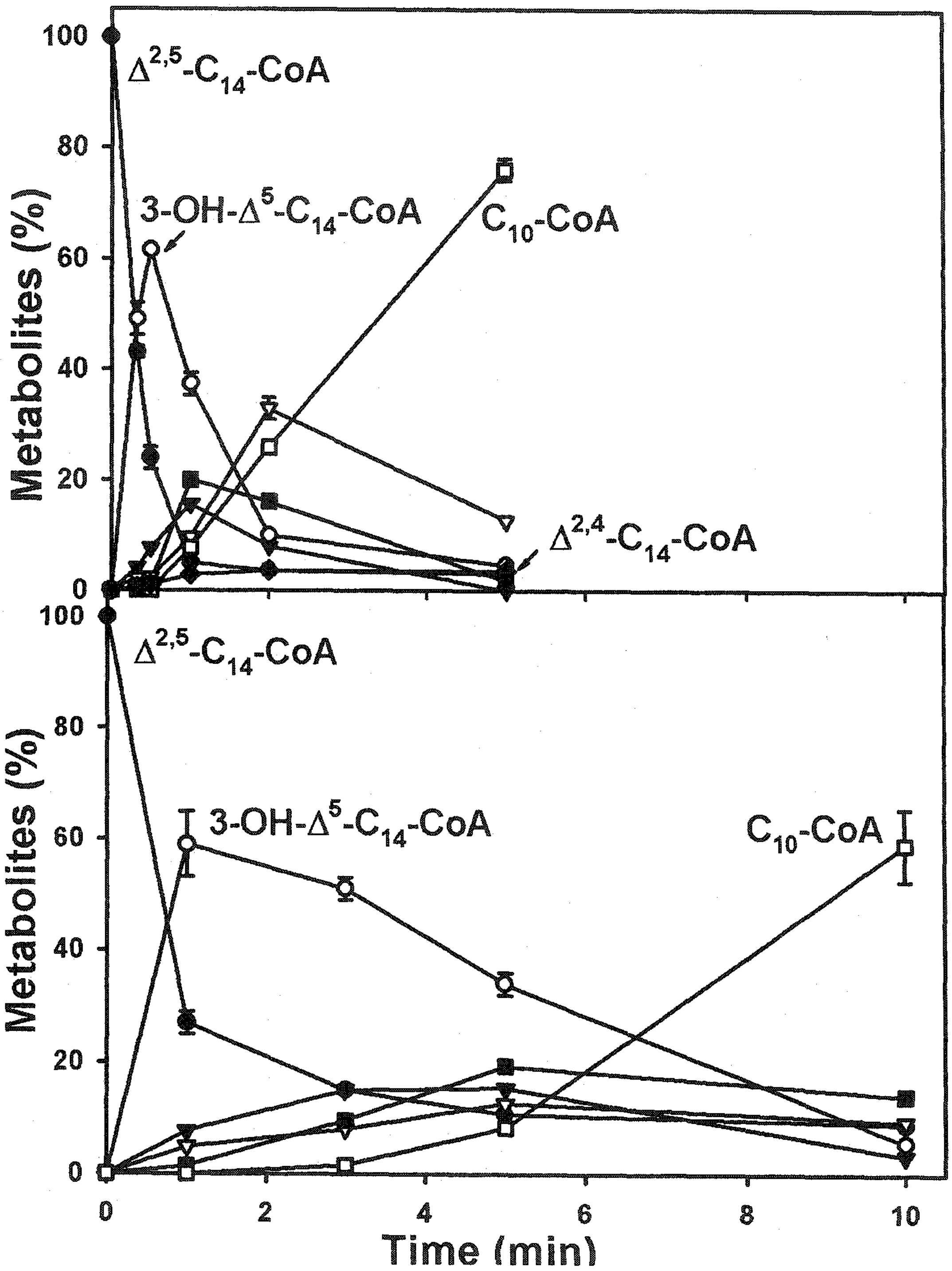

100
80
60
40
20
0
Metabolites (%)
$\Delta^{2,5}$-C_{14}-CoA
3-OH-Δ^{5}-C_{14}-CoA
C_{10}-CoA
$\Delta^{2,4}$-C_{14}-CoA
100
80
60
40
20
0
Metabolites (%)
$\Delta^{2,5}$-C_{14}-CoA
3-OH-Δ^{5}-C_{14}-CoA
C_{10}-CoA
0
2
4
6
8
10
Time (min)

Fig. 8

Fig. 9. **Separation of *E. coli* thioesterases.** Extracts of soluble proteins from *E. coli* cells grown on either oleate (panel A) or glucose (panel B) as the sole carbon source were subjected to column chromatography on DEAE-cellulose. Fractions were assayed for thioesterase with tetradecanoyl-CoA (myristoyl-CoA) as substrate.

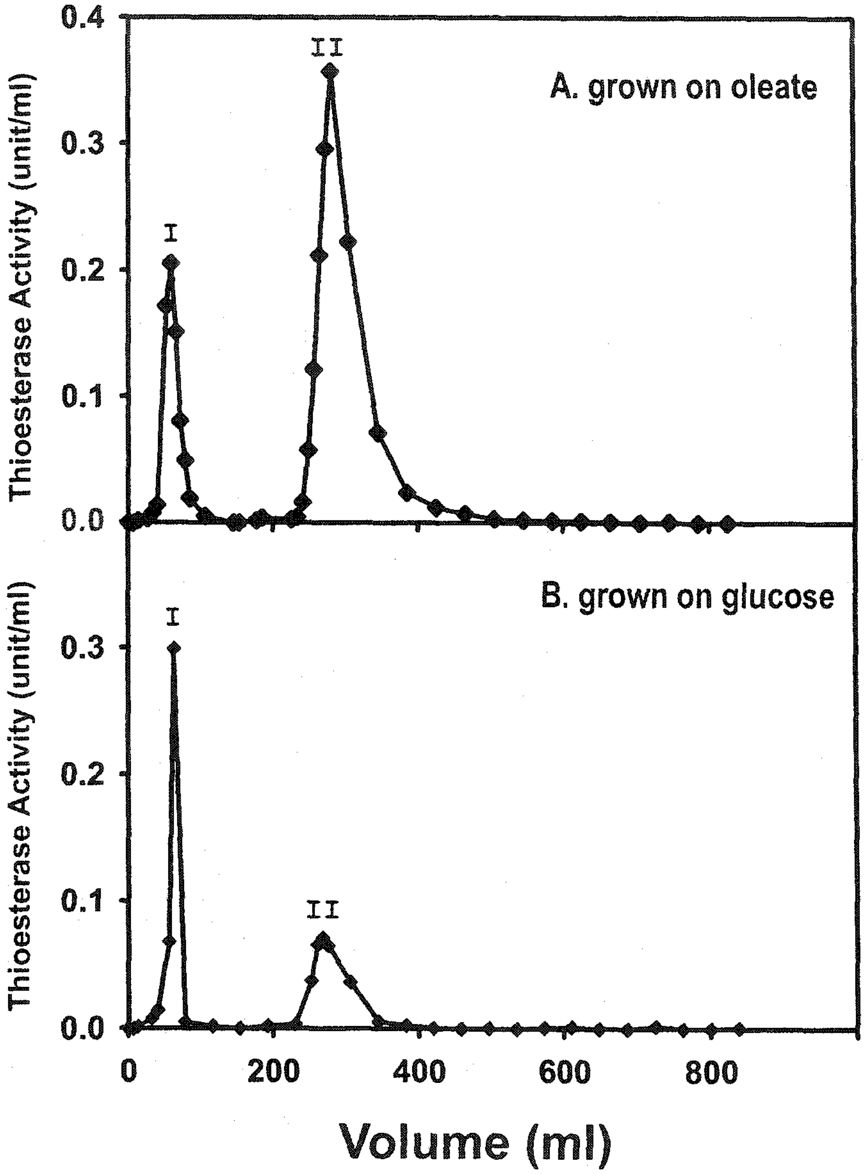

0.4
0.3
0.2
0.1
0.0
Thioesterase Activity (unit/ml)
II
I
A. grown on oleate
B. grown on glucose
I
II
0.3
0.2
0.1
0.0
Thioesterase Activity (unit/ml)
0
200
400
600
800
Volume (ml)

Fig. 9

Fig. 10. **Substrate specificities of *E. coli* thioesterase fractions.** Activities of thioesterase fractions I and II were determined at acyl-CoA concentrations of 20 μM with: C2:0, acetyl-CoA; C4:0, butyryl-CoA; C8:0, octanoyl-CoA; C14:0, tetradecanoyl-CoA (myristoyl-CoA); 2t-C14:1, 2-*trans*-tetradecenoyl-CoA; 2,5-C14:2, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3t-C14:1, 3-*trans*-tetradecenoyl-CoA; 3,5-C14:2, 3,5-*cis*-tetradecadienoyl-CoA; 3OH-C14:0, 3-hydroxytetradecanoyl-CoA; C16:0, hexadecanoyl-CoA (palmitoyl-CoA); 3Keto-C16:0, 3-ketohexadecanoyl-CoA; C18:0, octadecanoyl-CoA (stearoyl-CoA); 9c-C18:1, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of two measurements that differed by 10% or less.

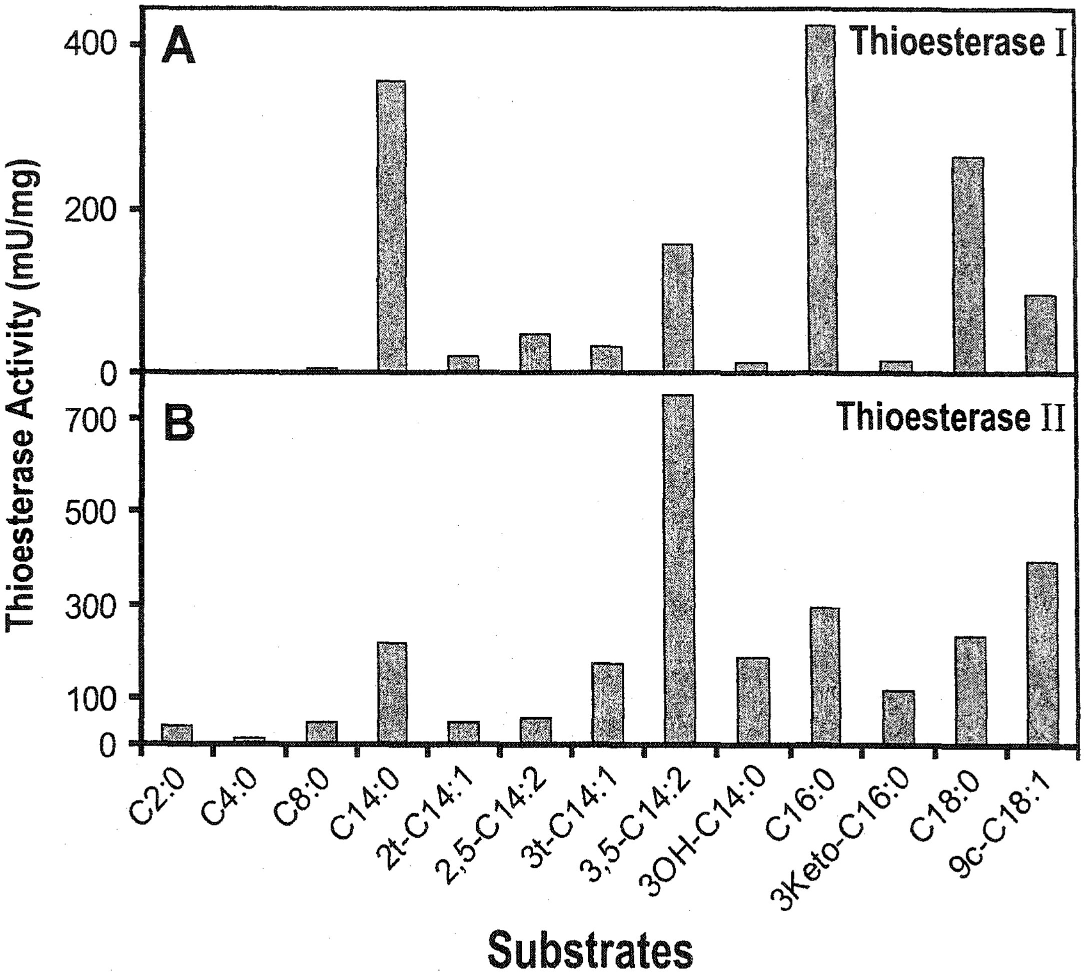

A
Thioesterase I
B
Thioesterase II
400
200
0
700
500
300
100
0
Thioesterase Activity (mU/mg)
C2:0
C4:0
C8:0
C14:0
2t-C14:1
2,5-C14:2
3t-C14:1
3,5-C14:2
3OH-C14:0
C16:0
3Keto-C16:0
C18:0
9c-C18:1
Substrates

Fig. 10

Fig. 11. **Identification of 3,5-tetradecadienoic acid in the medium after growth of *E. coli* on oleate as the sole carbon source.** A. Gas chromatogram of the methyl esters of the acidic fraction extracted from the growth medium. B. Region of the gas chromatogram where methyl 3,5-tetradecadienoate would be eluted. Peaks marked 1 through 4 have molecular ions with mass-to-charge ratios (m/z) of 238. C. Gas chromatogram of methyl 3,5-tetradecadienoate prepared from 3,5-*cis*-tetradecadienoyl-CoA. Peaks 1 through 4 are due to 3,5-tetradecadienoates or isomers with molecular ions at m/z = 238. D. Mass spectrum of the material that gave rise to peak 3 of panel C.

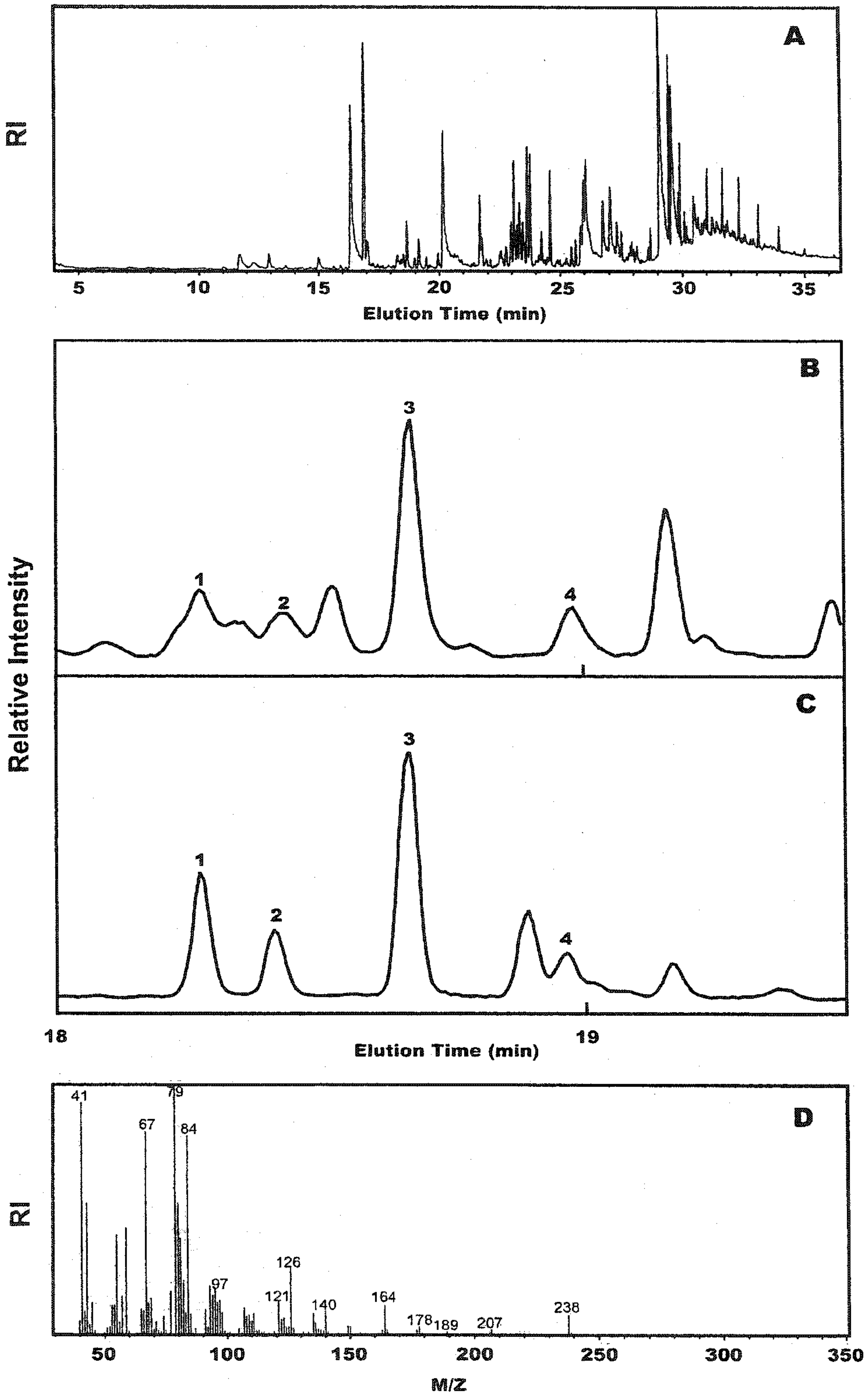

A
RI
5
10
15
20
25
30
35
Elution Time (min)
B
Relative Intensity
1
2
3
4
C
1
2
3
4
18
19
Elution Time (min)
D
RI
41
79
67
84
126
97
121
140
164
178
189
207
238
50
100
150
200
250
300
350
M/Z

Fig. 11

Fig. 12. **SDS-PAGE of purified thioesterase II from *E. coli.*** Purified thioesterase and molecular markers were applied to the right and left lanes respectively. Molecular masses are indicated next to the protein bands.

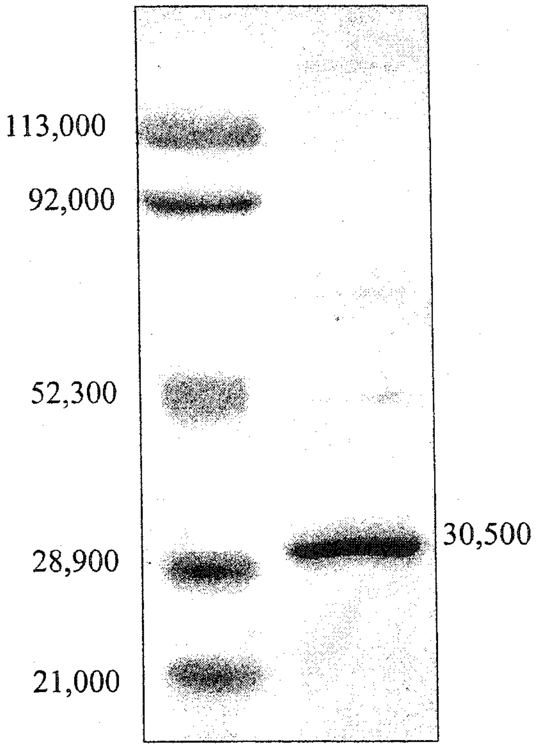


Fig. 12

Fig. 13. **Substrate specificity of purified *E. coli* thioesterase II.** Activities of thioesterase II were determined at acyl-CoA concentrations of 20 μM with: C2:0, acetyl-CoA; C4:0, butyryl-CoA; C8:0, octanoyl-CoA; C14:0, tetradecanoyl-CoA (myristoyl-CoA); 2t-C14:1, 2-*trans*-tetradecenoyl-CoA; 2,5-C14:2, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3t-C14:1, 3-*trans*-tetradecenoyl-CoA; 3,5-C14:2, 3,5-*cis*-tetradecadienoyl-CoA; 3OH-C14:0, 3-hydroxytetradecanoyl-CoA; C16:0, hexadecanoyl-CoA (palmitoyl-CoA); 3Keto-C16:0, 3-ketohexadecanoyl-CoA; C18:0, octadecanoyl-CoA (stearoyl-CoA); 9c-C18:1, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of three or four determinations ± S. D.

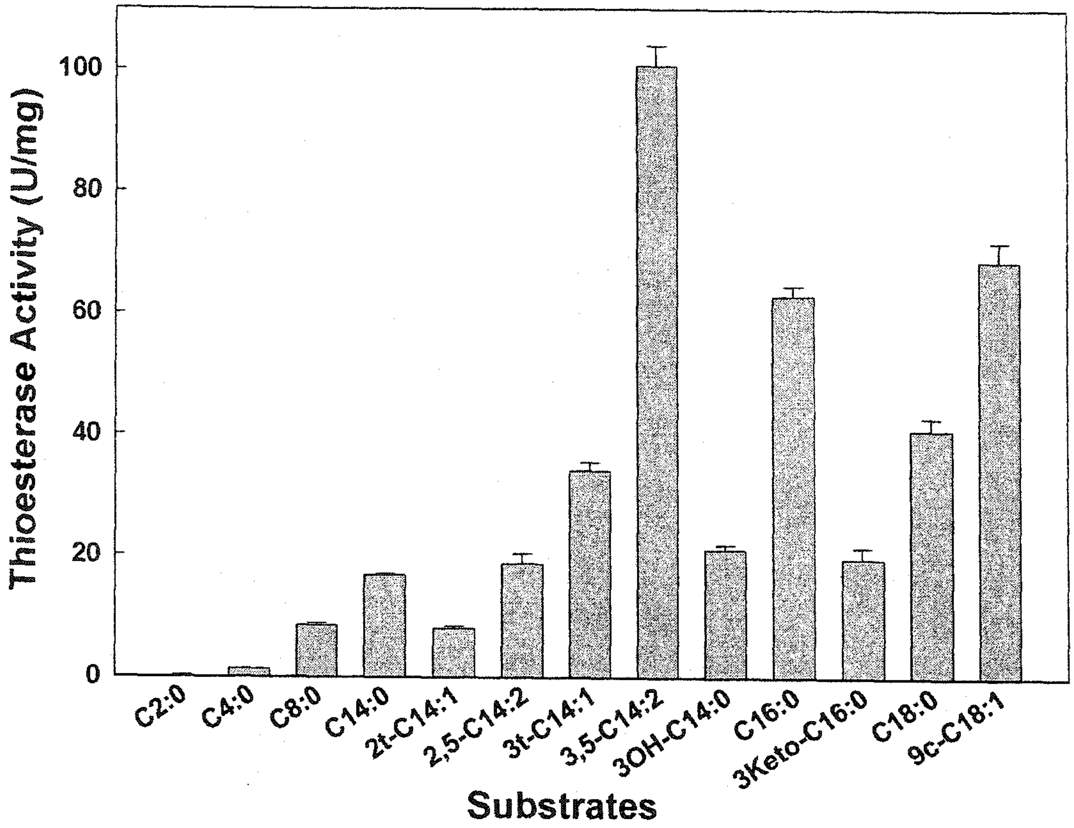

Thioesterase Activity (U/mg)
0
20
40
60
80
100
C2:0
C4:0
C8:0
C14:0
2t-C14:1
2,5-C14:2
3t-C14:1
3,5-C14:2
3OH-C14:0
C16:0
3Keto-C16:0
C18:0
9c-C18:1
Substrates

Fig. 13

Fig. 14. **Growth curves of wild-type *E. coli* and a thioesterase double mutant.** *E. coli* cells were grown on M9 minimal medium with either (A) glucose or (B) oleate or palmitate as the sole carbon source. In panel A, ● wild type; ▽ mutant; In panel B, ● wild type grown on oleate; ○ wild type grown on palmitate; ▼ mutant grown on oleate; ▽ mutant grown on palmitate. For details see "Experimental Procedures."

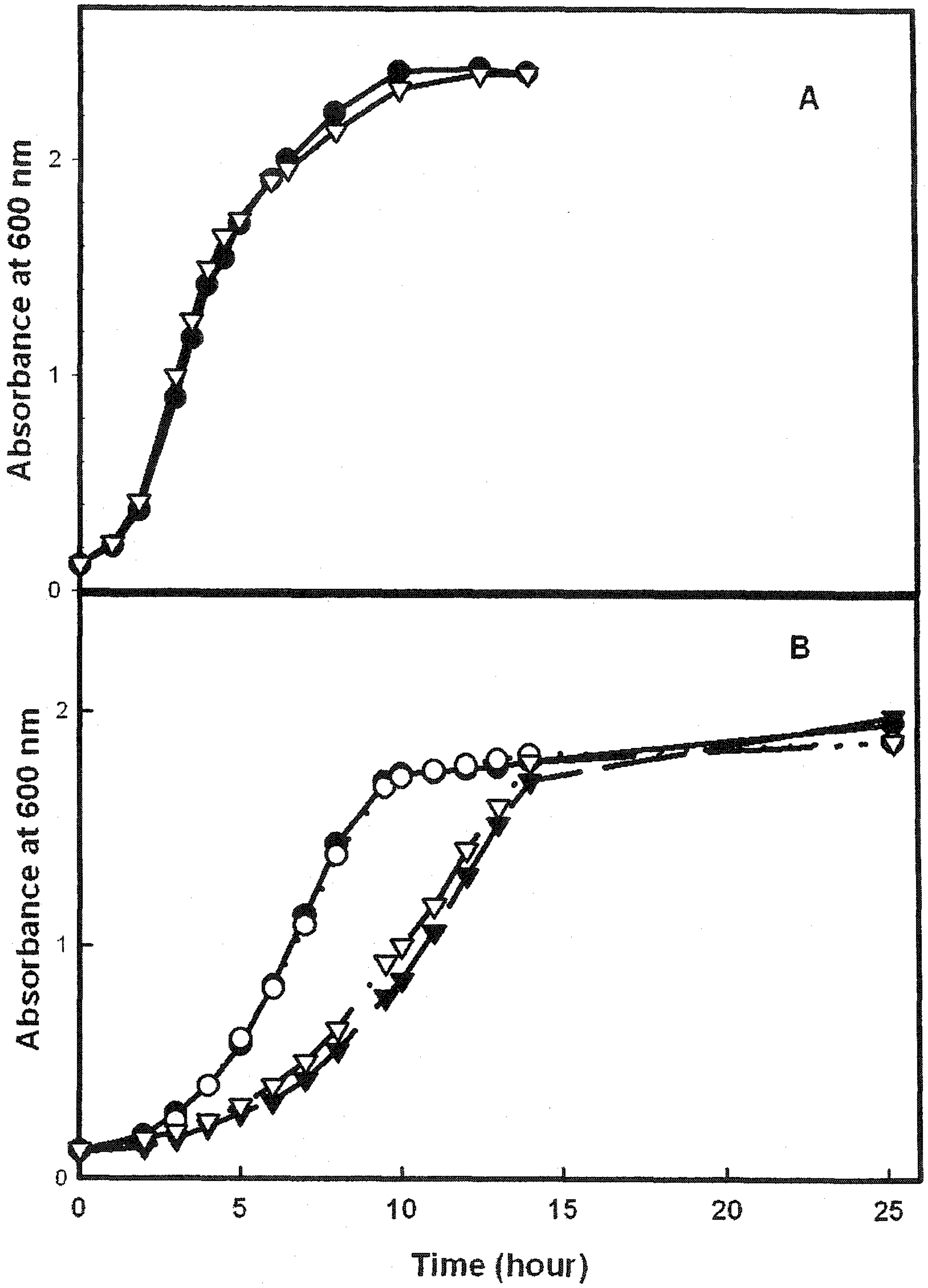

A
B
Absorbance at 600 nm
Absorbance at 600 nm
0
1
2
0
1
2
0
5
10
15
20
25
Time (hour)

Fig. 14

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