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Enzymology and Mechanisms of Double Bond Isomerizations During the beta-Oxidation of Unsaturated Fatty Acids

Dongyan Zhang

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

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

November 12, 2001
Date

Chair of Examining Committee

November 12, 2001
Date

Executive Officer

Supervisory Committee

The City University of New York

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ABSTRACT

ENZYMOLOGY AND MECHANISMS OF DOUBLE BOND ISOMERIZATIONS DURING THE BETA-OXIDATION OF UNSATURATED FATTY ACIDS

by

Dongyan Zhang

Advisor: Professor Horst Schulz

$\Delta^{3,5}$, $\Delta^{2,4}$-Dienoyl-CoA isomerase (dienoyl-CoA isomerase) is required in the alternative pathway for the degradation of unsaturated fatty acids with odd-numbered double bonds. Mitochondrial and peroxisomal dienoyl-CoA isomerases are products of the same gene with slightly different N-terminal sequences. Mutations of any of the three acidic amino acid residues located at the active site (Modis et al. (1998) Structure 6, 957-970) caused activity losses. In contrast to only a 10-fold decrease in activity upon replacement of Asp176 by Ala, substitutions of Asp204 by Asn and of Glu196 by Gln resulted in 10^5-fold lower activities. Such activity losses are consistent with the direct involvement of these latter two residues in the proposed proton transfers at carbons 2 and 6 or 8 of the substrates. Probing of the wild-type and mutants forms of the enzyme with 2,5-octadienoyl-CoA as substrate revealed low Δ^2,Δ^3-enoyl-CoA isomerase and Δ^5,Δ^4-enoyl-CoA isomerase activities catalyzed by Glu196 and Asp204, respectively. Altogether, this data reveals that positional isomerizations of the diene and triene are facilitated by simultaneous proton transfers involving Glu196 and Asp204, whereas each residue alone can catalyze, albeit less efficiently, a monoene isomerization.

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Enoyl-CoA isomerase is required for the degradation of unsaturated fatty acids with double bonds at both odd-numbered and even-numbered positions. A functional analysis of rat liver Δ^3,Δ^2-enoyl-CoA isomerases (enoyl-CoA isomerases) revealed the presence of mitochondrial enoyl-CoA isomerase (MECI) and enoyl-CoA isomerase (ECI) in mitochondria while peroxisomes contain ECI and multifunctional enzyme 1 (MFE1). ECI, which previously had been described as peroxisomal enoyl-CoA isomerase (PECI), was shown to be present in both mitochondria and peroxisomes. This enzyme seems to be identical with mitochondrial long-chain enoyl-CoA isomerase. All three known hepatic enoyl-CoA isomerases, MECI, ECI and MFE1, have broad chain length specificities but are distinguishable by their catalytic efficiencies that are optimal for 3-cis→2-trans, 3-trans→2-trans, and 2,5→3,5 isomerizations, respectively. An analysis based on their total tissue activities and substrate specificities prompts the conclusion that in mitochondria, MECI is responsible for the 3-cis→2-trans and 2,5→3,5 isomerizations, while ECI is responsible for the 3-trans→2-trans isomerization. In the peroxisomes, ECI maybe the dominant enzyme for catalyzing 3-trans→2-trans and 3-cis→2-trans isomerizations of long-chain substrates, while MFE1 may play a key role in the 2,5→3,5 isomerization.

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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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ABBREVIATIONS

DECI or dienoyl-CoA isomerase	$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase;
Trienoyl-CoA isomerase	$\Delta^{3,5,7}$, $\Delta^{2,4,6}$-trienoyl-CoA isomerase;
MECI	mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase;
PECI	peroxisomal Δ^3,Δ^2-enoyl-CoA isomerase;
ECI	Δ^3,Δ^2-enoyl-CoA isomerase;
MECH or crotonase	2-trans-enoyl-CoA hydratase;
MFE1	multifunctional enzyme 1;
CD	circular dichroism;
DEHP	di(ethylhexyl)phthalate;
EDTA	ethylenediaminetetraacetate;
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;
HPLC	high-performance liquid chromatography;
PAGE	polyacrylamide gel electrophoresis;
PCR	polymerase chain reaction;
PMSF	phenylmethylsulfonyl fluoride;
SDS	sodium dodecylsulfate;
KPi	potassium phosphate;
BSA	bovine serum albumin.

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INTRODUCTION

Fatty acids are a major fuel, significant structural components and precursors of important signaling molecules in living organisms. The degradation of fatty acids in mammalian liver occurs both in mitochondria and in peroxisomes (1). Unsaturated fatty acids, including essential fatty acids such as linoleic acid and linolenic acid, also are degraded in mitochondria and possibly in peroxisomes. Investigations of the β-oxidation of saturated and unsaturated fatty acids will continue to provide invaluable information about defective fatty acid metabolism associated with enzyme deficiencies, ischemia, diabetes or obesity. The enzymes involved in β-oxidation of saturated fatty acids are acyl-CoA dehydrogenase/oxidase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. Auxiliary enzymes that are required for the β-oxidation of unsaturated fatty acids are Δ^3,Δ^2-enoyl-CoA isomerase (enoyl-CoA isomerase), $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) and 2,4-dienoyl-CoA reductase (for a review see Ref. 1).

Degradation of fatty acids in animals occurs in both mitochondria and peroxisomes. A comparison of mitochondrial and peroxisomal β-oxidation reveals common as well as distinct features (Reviewed in Ref. 2-13). A wide variety of saturated and unsaturated fatty acyl-CoAs of different chain-lengths can be degraded by β-oxidation pathways in both mitochondria and

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peroxisomes via a cyclic process consisting of 4 consecutive steps. The mitochondrial enzymes involved in this process are acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. The corresponding enzymes of β-oxidation in peroxisomes include acyl-CoA oxidase, multifunctional enzyme 1 (2-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase) and 3-ketoacyl-CoA thiolase. Another β-oxidation system that operates in peroxisomes utilizes the branched chain acyl-CoA oxidase, multifunctional enzyme 2 (2-enoyl-CoA hydratase 2 and D-3-hydroxyacyl-CoA dehydrogenase, MFE2) and SCPx-3-ketoacyl-CoA thiolase to degrade a variety of unusual fatty acids. MFE2 catalyzes the hydration of 2-enoyl-CoA to D-3-hydroxyacyl-CoA and the dehydrogenation of the latter to 3-ketoacyl-CoA (14-18). The epimerase activity observed earlier was found to be due to the combined hydratase activities of MFE1 and MFE2 in peroxisomes (19-21). The enzymes of the β-oxidation spiral in mitochondria and peroxisomes are different proteins. β-Oxidation in mitochondria is a tightly controlled metabolic process, which proceeds to completion without accumulation of significant amount of intermediates. In contrast, peroxisomal β-oxidation in animals does not go to completion, but results in chain-shortening by only a few β-oxidation cycles of very long chain fatty acids, eicosanoids, dicarboxylic acids, pristanic acid and hydroxylated cholestanoic acids (1), all of which are poor substrates for the mitochondrial pathway. The pathway through MFE2 is thought to be responsible for this function (22). Feeding of peroxisomal proliferators (10,13), such as clofibrate or di(2-

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ethylhexyl)phthalate, leads to a marked proliferation of peroxisomes in rodents, and stimulates transcription of β-oxidation enzymes in peroxisomes and mitochondria. The nuclear transcription factor, PPARα, was found to be involved in this process (1). Recent research has established that through the action of PPARα, fatty acid oxidation in both mitochondria and peroxisomes is critically up regulated to meet energy demand in fasting or diabetic subjects where fatty acid oxidation provides most of the energy (24,25).

β-Oxidation of unsaturated and polyunsaturated fatty acids leads to the formation of intermediates with double bonds close to the thioester function. It was proposed by Stoffel and Caesar in 1965 that the β-oxidation of unsaturated fatty acids with odd- numbered and even-numbered double bonds yields 3-cis-enoyl-CoA and 2-cis-enoyl-CoA intermediates, respectively (26). 3-cis-Enoyl-CoA was proposed to be isomerized to 2-trans-enoyl-CoA by enoyl-CoA isomerase and then further degraded by β-oxidation. 2-cis-Enoyl-CoA was thought to be hydrated to D-3-hydroxyacyl-CoA by enoyl-CoA hydratase and then epimerized by 3-hydroxyacyl-CoA epimerase to L-3-hydroxyacyl-CoA, which is an intermediate of normal β-oxidation. Enoyl-CoA isomerase was purified from rat liver mitochondria (26), while a 3-hydroxyacyl-CoA epimerase activity was shown to be present in rat liver (27). An alternative pathway for the degradation of unsaturated fatty acids with even-numbered double bonds was proposed by Kunau & Dommes in 1978 due to the identification of 2,4-dienoyl-CoA reductase in beef liver (28). According to the

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reductase-dependent pathway, fatty acids with even-numbered double bonds are chain-shortened to 4-cis-enoyl-CoAs which are converted to 2-trans, 4-cis-dienoyl-CoAs by acyl-CoA dehydrogenase. The reduction of 2-trans, 4-cis-dienoyl-CoAs to 3-trans-enoyl-CoAs by NADPH-dependent 2,4-dienoyl-CoA reductase facilitates their further degradation by β-oxidation. Overwhelming evidence in support of the reductase-dependent pathway has been presented (reviewed in Ref. 29). In addition, 3-hydroxyacyl-CoA epimerase was not detected in rat heart mitochondria (30). The activity was found in peroxisomes but not in mitochondria of rat liver (31). This observation disproved the operation of the epimerase-dependent pathway in mammalian mitochondria. Hence, β-oxidation of unsaturated fatty acids in mitochondria requires enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase as auxiliary enzymes and proceeds as shown in Fig. 1 for linoleoyl-CoA. Chain-shortening of linoleoyl-CoA (I) by three cycles of β-oxidation yields 3-cis,6-cis-dodecadienoyl-CoA (II) which is isomerized to 2-trans, 6-cis-dodecadienoyl-CoA (III) by enoyl-CoA isomerase. Another cycle of β-oxidation yields 4-cis-decenoyl-CoA (IV) which is dehydrogenated by acyl-CoA dehydrogenase (32) to 2-trans,4-cis-decadienoyl-CoA (V). Reduction of the latter intermediates by NADPH-dependent 2,4-dienoyl-CoA reductase produces 3-trans-decenoyl-CoA (VI). Isomerization of the double bond from the 3-trans to 2-trans position catalyzed by enoyl-CoA isomerase yields 2-trans-decenoyl-CoA (VII), which is a substrate of the β-oxidation spiral.

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The NADPH-dependent removal of odd-numbered double bonds at the stage of 5-cis-enoyl-CoA intermediates was reported in 1990 (33). A reductase-dependent alternative pathway for β-oxidation of fatty acids with odd numbered double bonds was elucidated by Smeland et al. (34). As shown in Fig. 2A, 5-cis-octenoyl-CoA (I) is dehydrogenated by medium-chain acyl-CoA dehydrogenase to 2-trans,5-cis-octadienoyl-CoA (II) which is converted to 3,5-octadienoyl-CoA (III) by enoyl-CoA isomerase. Dienoyl-CoA isomerase catalyzes the conversion of 3,5-octadienoyl-CoA (III) to 2-trans,4-trans-octadienoyl-CoA (IV) which is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-trans-octenoyl-CoA (V). Enoyl-CoA isomerase catalyzes the isomerization of 3-trans-octenoyl-CoA (V) to 2-trans-octenoyl-CoA (VI), which is an intermediate of β-oxidation. However, 2-trans,4-trans-octadienoyl-CoA (IV) intermediates can also be hydrated by enoyl-CoA hydratase (35), and the resultant 3-hydroxy,4-trans-octenoyl-CoA (VII) is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase to 3-keto,4-trans-octenoyl-CoA (VIII) which is thiolyzed to 2-trans-hexenoyl-CoA (IX) by 3-ketoacyl-CoA thiolase. However, there has been a debate as to whether the reductase-dependent and dienoyl-CoA isomerase-dependent alternative pathways (Fig. 2) or the isomerase-dependent pathway (upper part of Fig. 1) is operative in the degradation of unsaturated fatty acids with odd-numbered double bonds in mammalian cells (35-38). Previous work in this lab has demonstrated with mitochondrial extracts that the isomerase dependent pathway accounts for 80% of the flux while the alternative pathway (reductase-dependent and dienoyl-

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CoA isomerase dependent pathway) maybe responsible for the remaining 20% (35).

The pathways for the β-oxidation of polyunsaturated fatty acids with conjugated double bonds were also elucidated recently (39,40). Shown in Fig. 3 (from Ref. 40) is the alternative pathway specific for the degradation of conjugated linoleoyl-CoA. β-Oxidation of polyunsaturated fatty acids with conjugated double bonds may yield 2,5,7- or 2,4,6-trienoyl-CoA intermediates. 2,5,7-Trienoyl-CoA is either converted to 3-hydroxy-5,7-dienoyl-CoA by 2-enoyl-CoA hydratase or converted to 3,5,7-trienoyl-CoA by enoyl-CoA isomerase. 3-Hydroxy-5,7-dienoyl-CoA is further degraded to 3,5-dienoyl-CoA by the sequential actions of 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. 3,5-Dienoyl-CoA is further degraded through the alternative pathway shown in Fig. 2. The other intermediate, 3,5,7-trienoyl-CoA, is degraded through the trienoyl-CoA isomerase dependent pathway shown in Fig. 3. Trienoyl-CoA isomerase catalyzes the conversion of 3,5,7-trienoyl-CoA to 2,4,6-trienoyl-CoA. The latter intermediate is converted by the NADPH-dependent 2,4-dienoyl-CoA reductase to 3,6-dienoyl-CoA (39), which is converted to 2,6-dienoyl-CoA by enoyl-CoA isomerase. The 2,6-dienoyl-CoA is further degraded by one cycle of β-oxidation to 2,4-dienoyl-CoA, which is an intermediate for the alternative pathways in Fig. 2.

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In conclusion, five different kinds of double bond isomerizations are needed for the degradation of the naturally occurring unsaturated fatty acids and polyunsaturated fatty acids, namely, 3-trans to 2-trans, 3-cis to 2-trans, 2,5 to 3,5, 3,5 to 2,4 and 3,5,7 to 2,4,6 (Fig. 8 and 13). The first three of these reactions are catalyzed by enoyl-CoA isomerase, while the latter two are catalyzed by dienoyl-CoA isomerase.

Dienoyl-CoA isomerase (34,41) was first identified in rat liver mitochondria, but later was also detected in rat liver peroxisomes (42,43). The molecular characterization of this enzyme revealed the amino acid sequence of the unprocessed subunit, which has a peroxisomal targeting signal type I, at the C-terminus and an N-terminal sequence that is consistent with the targeting of this protein to mitochondria (44). This situation is suggestive of a dual subcellular localization and agrees with the previously observed kinetic and immunological similarities of the mitochondrial and peroxisomal forms of this enzyme (43). The N-terminal sequences of the mature dienoyl-CoA isomerase present in mitochondria and peroxisomes has been obtained (45). The mitochondrial targeting sequence consists of 34 N-terminal amino acids residues. Interestingly, the mature dienoyl-CoA isomerase in peroxisomes was found to be shorter by 2 or 4 amino acids than its mitochondrial counterpart. Recently trienoyl-CoA isomerase was isolated from rat liver (40). This isomerase activity was a component enzyme of dienoyl-CoA isomerase. The crystal structure of a recombinant form of dienoyl-CoA isomerase consisting of

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subunits without their 53 N-terminal amino acid residues was obtained at 1.5Å resolution (46). This study confirmed the proposed hexameric structure of the enzyme (44) and revealed the active site as a deeply buried hydrophobic pocket with three acidic residues, Asp176, Glu196, and Asp204. The latter two of these residues were predicted to catalyze proton transfers at carbons 2 and 6, respectively, of the 3,5-dienoyl-CoA substrate (46).

During the ß-oxidation of unsaturated fatty acid with even-numbered double bonds, e.g. the 12-cis double bond of linoleic acid, a 3-trans→2-trans double bond shift takes place (Fig. 1), while three isomerizations, 3-cis→2-trans (Fig. 1), 3-trans→2-trans, and 2,5→3,5 (Fig. 2), occur during the ß-oxidation of fatty acids with odd-numbered double bonds, e.g. the 9-cis double bond of oleic acid and linoleic acid. All of these positional and steric isomerizations of single double bonds (see Fig. 4) are catalyzed by Δ^3,Δ^2-enoyl-CoA isomerase (1). Other reactions catalyzed by this enzyme are the acetylene to allene (47,48) and 3,4 to 2,4 isomerizations (unpublished observation). Four mammalian enzymes with enoyl-CoA isomerase activities have been described. They are mitochondrial enoyl-CoA isomerase (MECI) (49-51), mitochondrial long-chain enoyl-CoA isomerase (52), peroxisomal enoyl-CoA isomerase (PECI) (53), and multifunctional enzyme 1 (MFE1) (54). Low enoyl-CoA isomerase activities were also observed in mitochondrial enoyl-CoA hydratase (MECH or crotonase) (55) and acyl-CoA oxidase (56).

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MECI was originally purified from rat liver (50,51,57) and heart (50) mitochondria. The activity of this enzyme declines with increasing chain length of the substrate (50,58) and is 5-17 times higher with 3-cis-enoyl-CoAs than with the corresponding trans substrates. Similar K_m values ranging from 10 to 30 μM have been reported for cis and trans substrates of various chain lengths (58,59). MECI has been cloned and sequenced. Slightly different molecular weights, ranging from 29,300 to 29,700, have been calculated for the mature enzyme, which is synthesized in the cytosol in a 32 to 33-kD pre-enzyme form (51,60). The gene of mouse MECI was found to span a 15-kb region and encodes 7 exons (61). Site-directed mutagenesis of rat MECI revealed that Glu-165 is indispensable for its catalytic activity. Though MECI has been crystallized (62), a crystal structure of this enzyme is still not available. However, the crystal structure of the yeast enoyl-CoA isomerase was solved recently (63). A single acidic residue, Glu158, was found to be the catalytic residue in the active site. Though there is no doubt about the mitochondrial localization of MECI, contradictory reports exist about its possible localization in peroxisomes (64,65). Long-chain enoyl-CoA isomerase was identified in rat liver mitochondria but not in peroxisomes, by a chromatographic analysis (66). No further characterization of this enzyme has been reported. Mitochondrial enoyl-CoA hydratase (MECH) has been extensively characterized and studied (1). It catalyzes the second step of the β-oxidation spiral with very high efficiency. It is a member of the low homology isomerase/hydratase superfamily, and the reaction it catalyzes is mechanistically similar to the

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reaction catalyzed by enoyl-CoA isomerase. It was found to catalyze the isomerization of 3-trans-hexenoyl-CoA at a rate of 1/5000 of that of its hydratase activity with substrates of the same chain length (67). The human and mouse peroxisomal enoyl-CoA isomerases were cloned and sequenced after identification by a homology search due to its sequence similarity to the yeast enoyl-CoA isomerase (68). A C-terminal peroxisomal targeting signal, type 1, was found in the peroxisomal enoyl-CoA isomerase. Localization of peroxisomal enoyl-CoA isomerase to the peroxisomes was confirmed (68). The association of enoyl-CoA isomerase with the hydratase and 3-hydroxyacyl-CoA dehydrogenase activities of multifunctional enzyme 1 (MFE1) was detected when an effort was made to separate enoyl-CoA isomerase activities present in a rat liver extract by dye affinity chromatography (69). The cDNA of MFE1 encodes a protein of 722 amino acids with a molecular mass of 78.5 kD (70). It has a C-terminal peroxisomal targeting signal, type 1 and is localized to peroxisomes (71). Sequence comparisons revealed that the N-terminal domain of MFE1 belongs to the isomerase superfamily (72) and is presumed to be responsible for both the enoyl-CoA hydratase and enoyl-CoA isomerase activities, while the central domain harbors the 3-hydroxyacyl-CoA dehydrogenase activity.

Structural information about the isoenzymes of enoyl-CoA isomerase has been growing rapidly as reflected by the recent publications of crystal structures for rat MECH (73) and yeast PECI (63). These two enzymes are hexameric

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proteins with similar patterns of folding even though they exhibit low sequence homology and catalyze different reactions. However, the specific metabolic functions of enoyl-CoA isomerases are poorly defined, especially since it was demonstrated that more than one isomerase is present in either mitochondria or peroxisomes and that collectively these enzymes catalyze three distinct reactions in ß-oxidation.

One aim of this study was to elucidate the catalytic mechanisms of dienoyl-CoA isomerase through mutagenesis and kinetic analysis. The second aim was to study the functional contribution and subcellular distribution of isoenzymes of enoyl-CoA isomerase in rat liver. A kinetic study of these isoenzymes was also planned to help explain the catalytic mechanisms and functional significance of different enoyl-CoA isomerases.

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EXPERIMENTAL PROCEDURES

Materials - Nycodenz, CoASH, NAD^+, NADH, NADPH, benzamidine hydrochloride, di(ethylhexyl)phthalate (DEHP), cyanogen bromide activated Sepharose, CM cellulose, acyl-CoA oxidase from Arthrobacter sp. and most standard biochemicals were purchased from Sigma. Fatty acid free bovine serum albumin (BSA) was from Life Science Resources, Milwaukee, WI. trans-3-Hexynoic acid, 3-hexyn-1-ol and other standard chemicals were purchased from Aldrich. 3-Octyn-1-ol was purchased from Lancaster Synthesis Inc, Windham, NH. Matrex gel red A was purchased from Amicon, Danvers, MA. Dithiothreitol (DTT) was purchased from Fisher Scientific, Fair Lawn, NJ. 5-cis-Octenoic acid, 3-cis-tetradecenoic acid and 5-cis-tetradecenoic acid were kindly provided by Professor Howard Sprecher, Ohio State University. Hydroxylapatite, the dye reagent for protein assays, polyacrylamide ready gels, and the materials for immunoblotting, including the goat anti-rabbit IgG conjugated with alkaline phosphatase, were bought from Bio-Rad. Sep-Pak C18 cartridges were purchased from Waters. Iodixanol (Optiprep) was from Nycomed Pharma AS, Oslo, Norway. Antibodies against rat peroxisomal multifunctional enzyme 1 were a kind gift from Dr. Wanders, University of Amsterdam, Netherlands. Rabbit antiserum against dienoyl-CoA isomerase, mitochondrial enoyl-CoA isomerase and peroxisomal enoyl-CoA isomerase were raised by Pocono Rabbit Farms and Laboratory, Canadensis, PA. Male Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. Bovine liver enoyl-CoA hydratase (crotonase) (74), pig heart 3-

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ketoacyl-CoA thiolase (75) and recombinant pig liver 3-hydroxyacyl-CoA dehydrogenase (76) were purified as described. Dienoyl-CoA isomerase cloned in the plasmid vector pND-1 was kindly provided by Dr. Yang, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY.

Analysis and Purification of Acyl-CoA Thioesters - All substrates of enoyl-CoA isomerase were purified by HPLC. A Waters uBondapak C18 column (30 cm x 3.9 mm) attached to a Waters HPLC system was used for this purpose. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H_2O (9:1, v/v) content of the 50 mM ammonium phosphate buffer (pH 5.5) for 30 minutes from 20% to 40% (for C6 substrates), 20% to 50% (for C8 substrates) or 30% to 70% (for C14 substrates) at a flow rate of 2 ml/min. Desired fractions were collected and the organic solvent was evaporated under vacuum on a flash evaporator. Sep-Pak C18 cartridges were used to concentrate the substrates after HPLC purification. After the substrates were absorbed by the Sep-Pak column, they were eluted with 3 ml of methanol. Methanol was evaporated under vacuum before the substrates were redisolved in H_2O. Concentrations of CoA derivatives of fatty acids were determined spectrophotometrically by quantifying CoASH with Ellman's reagent (77) after cleaving the thioester bonds with NH_2OH at pH 7.0 (78).

Syntheses of Substrates- Acetoacetyl-CoA was synthesized from diketene according to White and Jencks (79). Crotonyl-CoA was synthesized from crotonic

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anhydride according to Weeks and Wakil (80). Fatty acyl-CoA derivatives of all other fatty acids were prepared by the mixed anhydride method as described by Fong and Schulz (78). 3-trans-Octenoic acid and 3-trans-tetradecenoic acid were synthesized from malonic acid and hexanal and dodecanal, respectively, according to Boxer and Linstead (81). 3-cis-Hexenoic acid and 3-cis-Octenoic acid were synthesized from 3-hexyn-1-ol or 3-octyn-1-ol according to Stoffel and Ecker (82). 3,5-*cis*-Octadienoyl-CoA, 2,5-*cis*-octadienoyl-CoA and 2,5-*cis*-tetradecadienoyl-CoA were prepared as described by Shoukry and Schulz (35). 3,5,7-Decatrienoyl-CoA was prepared as described by Liang et al. (40). For the synthesis of 2,5-*cis*-octadienoyl-CoA, 10 μmol of HPLC purified 5-cis-octenoyl-CoA were incubated with 5 units of acyl-CoA oxidase in 60 ml of oxygen saturated 0.1 M KPi, pH 9.0 at room temperature with vigorous stirring. The conversion of 5-cis-octenoyl-CoA to 2,5-*cis*-octadienoyl-CoA was monitored by analyzing 10 μl of the reaction mixture at different time intervals by HPLC. When 5-cis-octenoyl-CoA was completely converted to 2,5-*cis*-octadienoyl-CoA after about one hour, 1.0 unit of recombinant dienoyl-CoA isomerase was added into the reaction mixture to convert the small amount of 3,5-*cis*-octadienoyl-CoA generated to 2,4-octadienoyl-CoA which is readily separable from 2,5-*cis*-octadienoyl-CoA by HPLC. The pH of the reaction mixture was adjusted to 1.6 with 6 N HCl to stop the reaction after allowing dienoyl-CoA isomerase to react for 5 minutes. The suspension was kept for 30 minutes before the pH was readjusted to 3-4 with 4 N KCl. The precipitated protein was removed from the reaction mixture by filtering the mixture through a 0.22 um Millex® –GP filter unit. The mixture was then passed through a Sep-Pak

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cartridge three times. Bound acyl-CoA was eluted with 3 ml of methanol into a vial containing 2 ml of H_2O. This procedure was repeated to ensure all acyl-CoA was eluted from the column. The eluates were combined and methanol was evaporated. The resultant 2,5-*cis*-octadienoyl-CoA was subjected to purification by HPLC. Contaminating 2,5-*cis*-octadienoyl-CoA and 2,5,7-decatrienoyl-CoA in preparations of 3,5-cis-octadienoyl-CoA and 3,5,7-decatrienoyl-CoA, respectively, were removed by HPLC. Preparations of 2,5-*cis*-octadienoyl-CoA and 2,5,7-decatrienoyl-CoA were purified by HPLC after converting residual amounts of 3,5-*cis*-octadienoyl-CoA and 3,5,7-decatrienoyl-CoA to their 2,4 and 2,4,6 isomers, respectively, in the presence of dienoyl-CoA/trienoyl-CoA isomerase.

Enzyme and Protein Assays – Wild-type and mutant dienoyl-CoA isomerases were assayed spectrophotometrically with 20 μM substrate in 0.2 M potassium phosphate at pH 8. The isomerase activities that were measured are indicated hereafter together with the substrates, the wavelengths and extinction coefficients of the assays: $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, 3,5-*cis*-octadienoyl-CoA, 300 nm, 27,800 $M^{-1}cm^{-1}$; $\Delta^{3,5,7},\Delta^{2,4,6}$-trienoyl-CoA isomerase, 3,5,7-decatrienoyl-CoA, 337 nm, 49,300 $M^{-1}cm^{-1}$; $\Delta^{2,5}$, $\Delta^{2,4}$-enoyl-CoA isomerase, 2,5-*cis*-octadienoyl-CoA, 300 nm, 27,800 $M^{-1}cm^{-1}$; $\Delta^{2,5}$, $\Delta^{3,5}$-enoyl-CoA isomerase, 2,5-*cis*-octadienoyl-CoA, 237 nm, 16,250 $M^{-1}cm^{-1}$; Δ^{3},Δ^{2}-enoyl-CoA isomerase, 3-*cis*-octenoyl-CoA, 263 nm, 6,700 $M^{-1}cm^{-1}$. Enoyl-CoA isomerases were assayed spectrophotometrically by measuring the absorbance increase at either 263 nm or 280 nm. A typical assay mixture contained 35 μM substrate, 0.2 mg/ml BSA, 0.2 M potassium phosphate at

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pH 8.0 and enzyme. Extinction coefficients of 6,700 $M^{-1}cm^{-1}$ at 263 nm or 4,400 $M^{-1}cm^{-1}$ at 280 nm were used to calculate rates. Enoyl-CoA isomerase activities associated with multifunctional enzyme 1 or fractions from mitochondria or peroxisomes were assayed by a coupled assay (83), in which the isomerization of 35 μM 3-enoyl-CoA to 2-enoyl-CoA was coupled to the hydration of the 2-trans double bond by crotonase, the NAD^+-dependent dehydrogenation of the 3-hydroxyacyl-CoA intermediate by 3-hydroxyacyl-CoA dehydrogenase and the thiolytic cleavage of the resultant 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase. Formation of NADH (e=6,220 $M^{-1}cm^{-1}$) was the basis for calculating rates of isomerization. When 2,5-octadienoyl-CoA or 2,5-tetradecadienoyl-CoA were used as substrates in the enoyl-CoA isomerase assay, dienoyl-CoA isomerase was used as a coupling enzyme. A typical assay mixture contained 35 μM substrate, 0.25 U/ml dienoyl-CoA isomerase, 0.2 mg/ml BSA, 0.2 M potassium phosphate at pH 8.0 and aliquot of enzyme. An extinction coefficient of 27,800 $M^{-1}cm^{-1}$ (84) was used to calculate rates. Enoyl-CoA hydratase (78), 3-hydroxyacyl-CoA dehydrogenase (83), catalase (85) and malate dehydrogenase (86) activities were assayed by established procedures. A dilution buffer of 50 mM potassium phosphate, pH 7.0, containing 1 mg/ml BSA was used to dilute the enzymes. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product in one minute. Protein concentrations were determined as described by Bradford (87) with bovine serum albumin as standard. All spectrophotometric assays were performed with a Gilford 2600 recording spectrophotometer at room temperature.

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Spectrophotometric Analyses of Reactions Catalyzed by Wild-type and Mutant Dienoyl-CoA Isomerases- The wavelength scan experiments were performed with a Hitachi U-3010 spectrophotometer at room temperature. The sample cuvette contained 20 μM substrate, 0.2 M KPi, pH 8.0, while the reference cuvette contained the same components except substrate. Spectra at desired wavelengths were obtained before 15 ng/ml of wild-type, 20 μg/ml of 196Q or 30 μg/ml of 204A was added in both the sample and the reference cuvettes. Spectra were scanned at the indicated time intervals.

Kinetic Analyses- The fact that enoyl-CoA isomerases are single substrate enzymes makes them very suitable for steady state kinetic analysis. The absorbance change at 280 nm was used for the kinetic characterization of ECI and MECI with 3-enoyl-CoA as substrates because the basal absorbance at 263 nm is too high at elevated concentrations of substrate. For kinetic analyses, assays were performed at five or six different substrate concentrations. Averages of three assays were used for each point. Preanalyses were performed to determine the proper substrate concentration ranges and enzyme usages. Kinetic parameters (K_m and V_{max}) were obtained by nonlinear curve fitting using the SigmaPlot 2000 program. Data were given by value plus minus standard error. Catalytic center activities, k_{cat}, were calculated using molecular masses of 29,256, 40,400 and 78,511 for the subunits of MECI, ECI and MFE1 (68,70,88), respectively.

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Affinity Purification of Anti-DECI - Monospecific antibodies to dienoyl-CoA isomerase were prepared from antiserum by affinity chromatography using a Sepharose-dienoyl-CoA isomerase column prepared from recombinant dienoyl-CoA isomerase and cyanogen bromide activated Sepharose. For this purpose, 1.3 mg of rDECI was diluted with 0.2 M KPi, pH 8.9 and centrifuged in a 4 ml Millipore centrifugal filter device to eliminate the DTT in the enzyme solution. This process was repeated three times. The resultant 0.65 ml of rDECI solution was mixed with 1 ml of CNBr activated Sepharose previously washed with 15 volumes of 1 mM HCl. The mixture was allowed to react for 3 hours at room temperature after adjusting the pH to 8.0. The medium was then washed with 3 ml of 1 M Tris, pH 8.0 and left overnight in 5 ml of 1 M Tris pH 8.0 to block non-reacted groups. The medium was washed with 3 volumes of 10 mM Tris-HCl pH8.8 and 3 volumes of 0.1 M glycine pH 3.0. This washing was repeated 5 times before the medium was packed into a column. The column was washed sequentially with 10 ml each of 10 mM Tris-HCl pH 7.5, 0.1 M glycine pH 3.0, 10 mM Tris-HCl pH 8.8, 0.1 M triethylamine pH 11.5 and 10 mM Tris pH 7.5. Twenty ml of antiserum to DECI was diluted 10 x with10 mM Tris-HCl pH 7.5 and passed through the column three times. The column was washed with 20 ml 10 mM Tris-HCl pH 7.5 and 20 ml of 10 mM Tris-HCl, 0.5M NaCl, pH 7.5. Antibodies to DECI were eluted with 10 ml of 0.1 M glycine pH 3.0 into a tube containing 1 ml of 1 M Tris-HCl pH 8.0. The column was washed with 10 ml of 10 mM Tris-HCl pH 8.8 before being eluted with 10 ml of 0.1 M triethylamine pH 11.5 into a tube containing 1 ml of 1 M Tris-HCl pH 8.0.

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SDS-PAGE and Immunoblotting – Samples were treated with equal volume of SDS sample buffer and subjected to SDS-PAGE on either gradient (4-20%) or 10% BioRad ready gels (89). To check for expression of wild-type and mutant dienoyl-coa isomerases, 200 μl of bacterial culture after induction for 4 hours was centrifuged on a desk-top centrifuge. The cell pellet was resuspended in 50 μl of SDS sample buffer and boiled for 3-5 minutes. Ten microliter were taken for SDS-PAGE analysis. For immunoblotting, proteins were transferred to a nitrocellulose membrane by semi-dry blotting (90) using the semi-dry transfer cell from BioRad. Proteins remaining on the gel were visualized by staining with Coomassie blue. The membrane was blocked with 5% dry milk for one hour before incubation with a 500-fold diluted rabbit antiserum for one hour. After incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase for one and a half hour, it was developed with a staining mixture containing the alkaline phosphatase substrate until the antigen bands were visible (91).

Purification of Mitochondria and Isolation of a Light Mitochondrial Fraction- Mitochondria and a light mitochondrial fraction were prepared as described by Nedergaard et al (92) and de Duve et al. (93), respectively. Adult male Sprague-Dawley rats that had been maintained on a standard chow diet were sacrificed after fasting for 24 hours. All operations thereafter were performed at 0-4°C. Livers were minced finely in isolation buffer with sharp scissors and homogenized in a Kontes Duall 24 glass tube with a loose fitting Teflon pestle. The isolation buffer

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contained 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol and 10 mM Tris-HCl (or 5 mM Mops) at pH 7.4. The homogenate was centrifuged at 30,000 x g for 10 min. Pellets were resuspended in isolation buffer and homogenized for 1-2 strokes at speed 0. The resultant suspension was centrifuged at 500 x g for 10 min. The supernatant was carefully withdrawn and centrifuged at 3000 x g for 10 min. The fluffy layer at the top of the pellet was carefully washed away before the pellet was resuspended in a small volume of buffer. This preparation was used as a source of mitochondria. The supernatant was centrifuged at 12,500 x g for 20 min. The pellet was resuspended in a small volume of isolation buffer. This preparation is referred to as light mitochondrial fraction.

Purification of Peroxisomes from Rat Liver–A preformed continuous gradient of Iodixanol (Optiprep) was used to purify peroxisomes according to Van Veldhoven et al. (94). The gradient was prepared in 30 ml centrifuge tubes by a gradient mixer and a peristaltic pump from 12 ml each of 20% Iodixanal containing 0.41 M sucrose, 1.2 mM EDTA, 0.12% ethanol and 6 mM Mops, pH 7.2 and 40% Iodixanal containing 0.14 M sucrose, 0.8 mM EDTA, 0.08% ethanol and 4 mM Mops, pH 7.2. A long-needle syringe was used to deliver 2 ml of 50% Iodixanal containing 25 mM sucrose, 0.5 mM EDTA, 0.05% ethanol and 2.5 mM Mops, pH 7.2 to the bottom of the gradient. After 3 ml of light mitochondria (12 mg of protein/ml) was carefully layered on top of the gradient, it was centrifuged at 105,000 x g for one hour in a T865 fixed angle rotor at 4°C using the slow acceleration and deceleration mode. Fractions were collected from the bottom after

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slowly inserting a thin glass tube through the bottom of the tube. Marker enzyme activities were assayed for mitochondria and peroxisomes. Peroxisomal fractions were combined and diluted 2-fold with isolation buffer before they were harvested by centrifugation at 17,500 x g for 20 min.

Separation of Enoyl-CoA Isomerases on a Hydroxylapatite Column- The tissue (4g portions) from rat liver was homogenized with a Polytron tissue homogenizer in 10 mM KPi, pH7.4, containing 0.2M KCl, 0.5 mM EDTA, 1mM BA and 0.5 mM DTT (buffer A) (20%, w/v). The extracts were sonicated and centrifuged at 100,000 x g for 1 hour. Then the supernatants were dialyzed against buffer B (20 mM KPi, pH 7.0, 0.5mM benzamidine and 0.5 mM DTT). After dialysis, the samples were applied to HAP columns (1.5 cm x 15 cm) equilibrated with buffer B at a flow rate of 10 ml/h. The proteins bound to the column were eluted with a linear gradient made up of 200 ml each of 20 mM and 500 mM KPi (pH 7.0) containing 0.5mM benzamidine and 0.5 mM DTT. Similarly, peroxisomes (15 mg of protein) and mitochondria (130 mg of protein) were sonicated and centrifuged before being loaded onto hydroxylapatite columns and eluted by a linear gradient from 20 to 500 mM KPi.

Nycodenz Gradient Separation of a Light Mitochondrial Fraction-Separation of subcellular organelles were achieved by Nycodenz density gradient centrifugation of a light mitochondrial fraction as described (95). For this purpose, a 30% (w/v) solution of Nycodenz containing 1 mM EDTA, 5 mM Hepes (pH 7.3), and 0.1%

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ethanol was prepared and 21 ml of this solution were placed in a 30 ml ultracentrifuge tube on top of 1.5 ml of a 60% sucrose cushion. A density gradient was generated by centrifugation at 60,000 x g in a T865 small angle rotor on a DuPont RC70 ultracentrifuge at 4°C for 24 h. A light mitochondrial fraction (~15 mg of protein in 1.5 ml) was layered on top of the gradient followed by 1.5 ml of a cover solution of a 3-fold diluted isolation buffer containing 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, and 10 mM Tris-HCl (pH 7.4). This preparation was centrifuged at 76,000 x g for one hour at 4°C. Fractions of 1.6 ml each were collected from the bottom of the tube. Fractions were then diluted 2 fold with isolation buffer and centrifuged at 17,500 x g for 20 minutes. Pellets from each fraction were redesolved in 100 μl of isolation buffer for further analysis.

Site-Directed Mutagenesis of Dienoyl-CoA Isomerase - Site-directed mutagenesis was carried out by use of Transformer™ Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instruction. A synthetic oligonucleotide, 5'-ATGCTTCAATAAGATTGAAAAAGGAAG-3', designed to eliminate a *Ssp*I site, was used as the selection primer. The following synthetic oligonucleotides were used as the mutagenic primers. The substituting nucleotide is underlined, and the mutant codon is in bold face type:

Asp^{176}→Ala 5'-GGAGGCGTGG**C**TCTTATTTCTG-3'

Asp^{176}→Asn 5'-GGAGGCGTG**A**ATCTTATTTCTG-3'

Glu^{196}→Ala 5'-CCAAGTCAAGG**C**GGTGGATGTG-3

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Glu[196]→Asp 5'-CCAAGTCAAGGATGTGGATGTGG-3'

Glu[196]→Gln 5'-CCAAGTCAAGCAGGTGGATGTG-3'

Asp[204]→Ala 5'-CTGGCTGCTGCTGTAGGAACGCTG-3'

Asp[204]→Asn 5'-CTGGCTGCTAATGTAGGAACGC-3'

The selection primer and one of the mutagenic primers were simultaneously annealed to one of the templates of the denatured double-stranded pNDdi and then incorporated into a new strand of DNA as a result of the elongation catalyzed by T4 DNA polymerase. After digestion with *Sspl*, the mixture of parent and newly synthesized DNA was transformed into *E. coli* BMH 71-18 *mutS*. The plasmids were isolated from the transformants and digested again with *Sspl*. The digestion mixture was transformed into *E. coli* BL21(DE3)pLysS and the desired mutant was selected for the absence of the *Sspl* restriction site. A single colony was inoculated into 2 ml of LB medium with 50 ug/ml ampicilin and grown at 37°C overnight. The culture was vortexed and frozen in a methanol/dry ice bath after addition of 0.5 ml of 50% sterile glycerol and stored at –80°C for future use. Another single colony was inoculated into 5 ml of LB medium with 50 ug/ml ampicilin and grown at 37°C overnight for isolation of plasmid. The point mutation was confirmed by sequencing of the respective mutant strain by Genemed Synthesis, Inc. Primer used in sequencing was 5'-CACTGTCATTGAGAAGTGCC-3'.

Expression and Purification of Recombinant Wild-type and Mutant Dienoyl-CoA Isomerase – To express the stored strains harboring plasmids containing wild-type

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and mutant dienoyl-CoA isomerases, a sterile wooden stick was used to streak the bacteria on an LB plate with ampicilin. The bacteria was allowed to grow at 37°C overnight. A single colony was inoculated into 5 ml of LB medium with 50 μg/ml ampicilin and grown at 37°C with vigorous shaking (225 rpm) for 16 hours. One milliliter of this culture was pipetted into 50 ml LB medium and grown at 37°C with vigorous shaking (235 rpm) for 3-4 hours to an absorbance of about 1.0 at 600 nm and then induced by 0.6 mM IPTG for 4 hours. Cells were harvested by centrifugation at 3000 x g for 5 min and stored at −80°C. The frozen pellet from approximately 350 ml of cell culture was suspended in 10 ml of 10 mM KPi (pH 8.8) containing 5 mM mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, and 0.5 mM PMSF (buffer A), and sonicated 12-times for 20 s each. The resultant suspension was centrifuged at 100,000 x g for 30 min. The supernatant was loaded onto a Q-Sepharose column (1.5 x 17 cm) previously equilibrated with buffer A. The column was extensively washed with buffer A and then eluted with a gradient made up of 120 ml of buffer A and 120 ml of buffer A containing 0.4 M KCl. The enzyme fractions were combined and concentrated in an Amicon concentrator with a PX-10 membrane. The concentrate was diluted 10-fold with 10 mM KPi (pH 6.0) containing 1 mM EDTA, 5 mM mercaptoethanol and 20% glycerol (buffer B) and applied to an S-Sepharose column (1.5 x 4 cm) previously equilibrated with buffer B. After washing extensively with buffer B, the column was developed with a gradient made up of 30 ml of buffer B and 30 ml of buffer B containing 0.4 M KCl. The enzyme fractions were combined and concentrated. Protein elution profiles obtained with the wild-type protein formed the basis for collecting

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fractions containing the mutant proteins. SDS-PAGE was performed to show that the wild-type and mutant proteins have similar molecular weights of 32 kD. The identities of wild-type and mutants D176A, D204A, D204N were further confirmed by immunoblot (data not shown).

Purification of Mitochondrial Enoyl-CoA Isomerase and Multifunctional Enzyme 1- Adult Sprague-Dawley rats were fed rodent chow containing 2% (w/w) di(ethylhexyl)phthalate for two weeks before being sacrificed. For purification of mitochondrial enoyl-CoA isomerase (50), mitochondria were isolated from the liver (92) and sonicated in 20mM KPi (pH 7.0) containing 0.5 mM dithiothreitol, 1 mM EDTA, 0.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride (buffer A) and centrifuged at 100,000 x g for one hour. The supernatant was applied to a Matrix Gel Red A column (2.5 x 12 cm) previously equilibrated with buffer A. The column was washed with buffer A and then was developed with a gradient made up of 100 ml of buffer A and 100 ml of buffer A containing 1.2 M KCl. Fractions containing enoyl-CoA isomerase activity were combined. After dialysis overnight against 50mM KPi (pH 6.0) containing 0.5 mM dithiothreitol, 0.5 mM benzamidine, 10% glycerol (buffer B), the sample was applied to a CM cellulose column (1.5 x 6 cm) previously equilibrated with buffer B. Mitochondrial enoyl-CoA isomerase was eluted with a linear gradient of 50 ml 0-0.4 M KCl in buffer B. For purification of MFE1 (96), a di(ethylhexyl)phthalate induced liver was homogenized in 1:5 (w/v) of 10 mM K_3PO_4, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl

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fluoride with a Polytron tissue homogenizer. The suspension was sonicated 10-times for 20 s each at 4°C before being centrifuged at 100,000 x g for one hour. The supernatant was adjusted to pH 7.0 before being applied to a phosphocellulose column (2.5 x 20 cm) previously equilibrated with 50 mM KPi, pH 7.0, 0.5 mM benzamidine, 0.5 mM dithiothreitol (buffer C). The column was eluted with a linear gradient of 400 ml 50 to 500 mM KPi in buffer C. Active fractions were combined and fractionated with $(NH_4)_2SO_4$, The precipitate formed between 200 and 400 g/l of $(NH_4)_2SO_4$ was dialyzed overnight against buffer B before being applied to a CM cellulose column (1.5 x 8 cm) previously equilibrated with buffer B. The enzyme was eluted with a linear gradient of 200 ml 50-200 mM KPi in buffer B.

CD Spectra of Wild-type and Mutant Dienoyl-CoA Isomerases - Far UV CD scans were acquired between 190 and 250 nm with an AVIV CD spectrophotometer equipped with temperature control. Two average scans were acquired at 20°C for each sample. The scans were normalized for protein concentration and corrected for the influence of the buffer.

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RESULTS

Mechanistic Study of Dienoyl-CoA Isomerase by Site-specific Mutagenesis - The mature mitochondrial form of dienoyl-CoA isomerase was successfully expressed in *E. coli* (Fig.5). A 20-fold purification of dienoyl-CoA isomerase, beginning with a soluble extract of such cells, yielded the pure enzyme in 70% yield (Table I). This enzyme preparation exhibited an activity of 960 units/mg (see Table II), which is significantly higher than the activity of the enzyme isolated from rat liver (41). The fact that the recombinant dienoyl-CoA isomerase also exhibited trienoyl-CoA isomerase activity, proved that both catalytic properties are associated with the same protein.

For the planned mechanistic study, mutant proteins were required in which Asp204, Glu196 and Asp176 were replaced by uncharged amino acid residues. The mutations shown in Table I were introduced by site-specific mutagenesis and the recombinant mutant proteins were purified to apparent or near homogeneity as indicated by SDS-PAGE (Fig. 6). The near-UV CD spectra of all mutant proteins were virtually indistinguishable from the spectrum of the wild-type enzyme (Fig. 7). When assayed for 3,5→2,4 dienoyl-CoA isomerase activity, the replacement of either Asp204 or Glu196 by a neutral amino acid residue resulted in an approximately 10^5-fold lower activity (see Table II). This observation agrees with the proposed functions of these two residues in the direct proton transfer to or from the substrate (46). Substitution of Glu196 by an aspartate residue produced a

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mutant enzyme that retained approximately 3% of the isomerase activity (see Table II). This lower but significant activity demonstrates that the β-carboxyl group of Asp196 can facilitate the proton transfer although less efficiently than the γ-carboxyl group of Glu196. The mutation of the third acidic group at the active site, Asp176, to Ala caused a 10-fold decrease in activity. The limited effect of this mutation argues against a direct participation of this residue in catalysis. The effects of mutating Asp204, Glu196, and Asp176 on the trienoyl-CoA isomerase activity of this enzyme were comparable to the impact on the dienoyl-CoA isomerase except that the activity losses due to the D176A and E196D mutations were more severe (see Table II). Overall this data indicates that the active site of dienoyl-CoA isomerase is identical with the active site of trienoyl-CoA isomerase and that the same acidic residues, Glu196 and Asp204, catalyze the proton transfers that result in the 3,5→2,4 and 3,5,7→2,4,6 isomerizations.

In an effort to further explore the mechanism of dienoyl-CoA/trienoyl-CoA isomerase, its activity with 2-*trans*,5-*cis*-octadienoyl-CoA as a substrate was evaluated. With the wild-type enzyme, a small but significant conversion of the 2,5 diene to the 2,4 isomer was observed (Table III). The rate of the 2,5→2,4 conversion was more than 10^4-times slower than the 3,5 →2,4 isomerization. Surprisingly, mutants of Glu196 were more active than the wild-type enzyme in catalyzing this reaction (see Table III). The E196Q mutant which was 10-times as active as the wild-type isomerase, permitted a spectroscopic analysis of the 2,5→2,4 isomerization. The time-dependent spectral changes shown in Fig. 8B are

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indicative of a direct 2,5→2,4 isomerization rather than a sequential 2,5→3,5→2,4 conversion. These spectra do not provide evidence for the formation of a 3,5 intermediate with an absorbance maximum at 238 nm nor do they reveal a 3,5→2,4 isomerization as shown in Fig.8A. Since the 2,5→2,4 conversion catalyzed by the E196Q mutant was 60-times faster than the 3,5→2,4 isomerization catalyzed by the same enzyme (see Table II and III), the 2,5→2,4 isomerization seems to be a one-step conversion.

The Asp204 mutants also catalyzed a slow but detectable 2,5→2,4 isomerization. However, as shown in Fig. 9C, the formation of the 2,4 isomer proceeded with a lag. Spectral analyses of the reactions that occurred during (see Fig. 9C, period A) and after the lag phase (see Fig.9C, period B) revealed an initial 2,5→3,5 isomerization indicated by an increase in the absorbance at 238 nm due to the formation of the 3,5 diene (see Fig. 9A) followed by the formation of the 2,4 isomer detected at 300 nm (see Fig. 9B). Overall, the product formation occurred by a 2,5→3,5→2,4 conversion that showed a pronounced lag in the formation of the 2,4 isomer because the first reaction proceeded faster than the second reaction (see Table II and III). Since the D204A mutant catalyzed the 2,5→3,5 conversion, it was expected to catalyze also the isomerization of 3-octenoyl-CoA to 2-octenoyl-CoA. This conversion was in fact observed and found to take place at a rate of 0.034 units/mg as compared to 0.4 units/mg for the 2,5→3,5 isomerization.

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The question of whether the differences between the reaction rates observed with various mutants and substrates were due to changes in K_m values, V_{max} values or both was addressed. The kinetic parameters listed in Table IV clearly show that the K_m values varied little and that differences between V_{max} values were the major cause of rate differences observed at fixed substrate concentrations of 20 μM.

Molecular Characterization of Mitochondrial and Peroxisomal Dienoyl-CoA Isomerases-In an attempt to determine if the peroxisomal dienoyl-CoA isomerase exists *in vivo* as a truncated protein, purified peroxisomes were incubated with boiling SDS-incubation buffer and subjected to SDS-PAGE followed by immunoblotting with antibodies purified by affinity chromatography on a dienoyl-CoA isomerase-Sepharose column. As shown in Fig. 10, only one band corresponding to a 32-kDa protein was observed. Thus, it seems that the native peroxisomal dienoyl-CoA isomerase is a truncated protein with a ragged N-terminus. The mature mitochondrial dienoyl-CoA isomerase was calculated to have a subunit molecular weight of 32359, while that of the preenzyme is 36174, using the Charm program.

Characterization of Mitochondrial Long-chain Enoyl-CoA Isomerase-The aim of this study was the characterization of all enoyl-CoA isomerases that are present in rat liver and the determination of their contributions to the total enoyl-CoA isomerase activities in both mitochondria and peroxisomes. Mitochondrial long-chain enoyl-CoA isomerase had been detected by Kilponen et al (52) who

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separated it from mitochondrial enoyl-CoA isomerase (MECI) and peroxisomal multifunctional enzyme 1 (MFE1) by chromatography on hydroxylapatite. However, they did not purify it any further. Since it was more active with 3-trans-dodecenoyl-CoA than with 3-trans-hexenoyl-CoA as a substrate, they named it long-chain enoyl-CoA isomerase. They also concluded that it had a mitochondrial localization. The experiment was repeated with an extract of purified mitochondria, which was subjected to chromatography on hydroxylapatite. Each fraction was assayed with 3-trans-octenoyl-CoA and 3-cis-octenoyl-CoA because the ratio of activities obtained with these two substrates aids in the identification of different enoyl-CoA isomerases. Shown in Fig. 11 is the result of this experiment. The activity pattern with 3-trans-octenoyl-CoA as substrate is similar to that observed by Kilponen et al. (52) who concluded that the enoyl-CoA isomerase eluted first from the column was a novel isomerase they named long-chain enoyl-CoA isomerase. The activity pattern obtained with 3-cis-octenoyl-CoA as substrate was quite different. The isomerase activity present in fractions 8-11 is easily missed while the existence of isomerase activities, presumably corresponding to MECI, in fractions 15 to 22 is more clearly revealed than with the 3-trans substrate. A trans/cis activity ratio of 2 determined for fractions 8-11 is indicative of peroxisomal enoyl-CoA isomerase (PECI) which has a trans/cis activity ratio of 2 in contrast to MECI and MFE1 with trans/cis activity ratios below 1. The presence of PECI in fractions 8-11 was confirmed by immunoblotting. Thus, it seems that mitochondrial long-chain enoyl-CoA isomerase is identical with PECI.

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Subcellular Localization of Enoyl-CoA Isomerases-If mitochondrial long-chain enoyl-CoA isomerase and PECI are the same enzyme, PECI must be present in both mitochondria and peroxisomes. To confirm this prediction, a light mitochondrial fraction was prepared from a rat liver homogenate and subjected to centrifugation on a Nycodenz density gradient. Fractions were assayed for catalase and malate dehydrogenase to localize peroxisomes and mitochondria, respectively. Fractions were also analyzed by immunoblotting with antibodies to MECI and PECI. The results shown in Fig. 12 demonstrate that MECI was present only in mitochondria (fractions 7-11), whereas PECI was detected in both peroxisomes (fractions 1-5) and mitochondria (fractions 7-11). Since the dual localization of PECI contradicts the reported unique association of this enzyme with peroxisomes, further confirmation was sought. For this purpose, an extract from isolated rat liver mitochondria was subjected to chromatography on hydroxylapatite. The results shown in Fig. 11 demonstrate the presence of at least two enoyl-CoA isomerases in mitochondria. The isomerase that was eluted first had a trans/cis activity ratio of approximately 2 and was detected with an antibody to PECI. Hence, this enoyl-CoA isomerase was PECI. The enoyl-CoA isomerase corresponding to the second peak was identified as MECI because it had an trans/cis activity ratio below 1 and was detected with an antibody raised against MECI. A similar experiment was also carried out with a soluble extract from rat liver peroxisomes that had been purified by centrifugation on a Iodixanol density gradient. The results are shown in Fig. 13. Again, two peaks of enoyl-CoA isomerase activity were detected. The isomerase that was eluted first from the column was PECI as indicated by its trans/cis activity

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ratio of approximately 2 and because of its recognition by antibodies to PECI. The second isomerase peak was due to MFE1 as demonstrated by immunoblotting and the co-elution of enoyl-CoA isomerase, enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities.

Overall, these data demonstrate that the enoyl-CoA isomerase with a preference for 3-trans-enoyl-CoAs as substrate has a dual localization in peroxisomes and mitochondria. This enzyme had previously been described as peroxisomal enoyl-CoA isomerase (PECI) and as mitochondrial long-chain enoyl-CoA isomerase. Henceforth, this isomerase will be referred to as Δ^3,Δ^2-enoyl-CoA isomerase (ECI).

Substrate Specificities of Enoyl-CoA Isomerases-The identification of two enoyl-CoA isomerases each in mitochondria (MECI and ECI) and peroxisomes (ECI and MFE1) raised the question as to their specific functions in the β-oxidation of unsaturated fatty acids. In an attempt to answer this question we determined the catalytic efficiencies of all three isomerases with substrates of different chain lengths for all three isomerization reactions. The same substrates were used to determine the kinetic parameters (K_m, V_{max}) of MECI, ECI and MFE1. Substrates for the 3-cis→2-trans and 3-trans→2-trans isomerization were 3-hexenoyl-CoA, 3-octenoyl-CoA and 3-tetradecenoyl-CoA to establish a chain length spectrum from short-chain to long-chain substrates. 2,5-Octenoyl-CoA and 2,5-tetradecenoyl-CoA served as substrates to evaluate the 2,5→3,5 isomerization. The kinetic parameters obtained with MECI are listed in Table V. This enzyme is most effective in

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catalyzing the 3-cis→2-trans isomerization. In fact, with 3-octenoyl-CoA as substrate, the cis/trans activity ratio was demonstrated to be 9. Although the k_{cat} values decreased with increasing acyl chain length, the K_m values also declined with the result that the catalytic efficiency of this enzyme varied little with changing acyl chain length. This general conclusion also applies to the 2,5→3,5 isomerization, which this isomerase catalyses almost as efficiently as the 3→2 isomerization.

ECI, the isomerase that is present in both mitochondria and peroxisomes, differs from MECI in that it exhibits a pronounced chain length dependency (see Table VI). Its catalytic efficiency in the 3→2 isomerizations increased 10-20 fold when the acyl chain length of the substrates was increased from 8 to 14 carbon atoms. This increased efficiency is the result of increased k_{cat} values and lower K_m values with increasing acyl chain length of the substrates. ECI is more effective in catalyzing the 3→2 than the 2,5→3,5 isomerizations.

Peroxisomal MFE1 showed a different catalytic behavior than either MECI or ECI (see Table VII). The catalytic efficiencies determined with 3-trans- and 3-cis-enoyl-CoAs as substrates were generally lower than those observed with MECI and ECI and varied little with changes in the acyl chain length. However, MFE1 was the most effective of all three isomerases in catalyzing the 2,5→3,5 isomerization. In summary, the data presented in Tables V-VII prompts the idea that in mitochondria ECI may contribute significantly to the 3-trans→2-trans

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isomerization especially of long-chain intermediates of unsaturated fatty acids. In peroxisomes, however, ECI may be the dominant enzyme for catalyzing the 3-trans→2-trans and 3-cis→2-trans isomerizations of long-chain substrates, while MFE1 may play a key role in the 2,5→3,5 isomerization.

Enoyl-CoA Isomerase Activity Associated with MFE1 Is an Intrinsic Activity - MFE1 purified from rat liver was found to have enoyl-CoA isomerase activity (69). However, it has not been excluded that the enoyl-CoA isomerase activity associated with MFE1 is due to a small amount of monofunctional enoyl-CoA isomerase that is tightly bound to it throughout the purification procedure. To address this issue, an immunoblot experiment was performed as shown in Fig. 14. Purified MFE1 containing equal or more enoyl-CoA isomerase activity than control amounts of MECI and PECI were immunoblotted with antibodies against the mitochondrial enoyl-CoA isomerase and peroxisomal enoyl-CoA isomerase. No evidence for the presence of MECI and PECI in MFE1 was obtained. Hence, enoyl-CoA isomerase activity associated with MFE1 is an intrinsic activity of this protein.

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DISCUSSION

Expression of the Mature Form of Dienoyl-CoA Isomerase-The successful expression of the mature cardiac dienoyl-CoA isomerase in *E.coli* achieved two goals. Foremost, a highly active form of this enzyme became available. In fact, the maximal specific activity of 2,450 units/mg for the recombinant isomerase was 6 times higher than the V_{max} of the enzyme isolated from rat liver (41). Although this difference may be due in part to the use of HPLC-purified substrate in this study, it also reflects the preparation of a purer enzyme. In any case , the recombinant enzyme exhibited an activity well suited for the planned mechanistic study. The second achievement was the demonstration that both dienoyl-CoA isomerase and trienoyl-CoA isomerase are associated with the same protein. This result puts to rest any existing suspicion that the two activities may be expressions of distinct but similar proteins.

Site-specific Mutagenesis Study of Dienoyl-CoA Isomerase-The successful creation and purification of several mutant forms of dienoyl-CoA isomerase permitted an analysis of its catalytic mechanism. Dramatic activity decreases were observed as the result of replacing Asp204 and Glu196 with neutral amino acids. This finding supports the proposed function of these residues in proton transfers from and to the substrate (46) because it agrees with the general prediction that the mutation of a residue that directly participates in a reaction as a general acid/base would be expected to cause a 10^5 or greater decrease in activity (97).

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Mechanistic Study of Dienoyl-CoA Isomerase with 2,5-Octadienoyl-CoA as a Substrate –In addition to the dienoyl-CoA isomerase and the trienoyl-CoA isomerase activities, this work demonstrated that dienoyl-CoA isomerase and its mutants catalyze 2,5→2,4, 2,5→3,5 and 3→2 isomerizations as well (Fig. 14). With 2,5-octadienoyl-CoA as a substrate analog a slow, but measurable 2,5→2,4 isomerization was detected. Since the different positional isomers of octadienoyl-CoA have distinct UV spectra, it was possible to analyze the mechanisms of these isomerizations. The spectral changes observed with mutant E196Q were suggestive of a direct 2,5→2,4 isomerization without the formation of an intermediate. The only alternative route, via a sequence of isomerizations with 3,5-octadienoyl-CoA as an intermediate, was ruled out because the 3,5→2,4 isomerization was much slower than the overall 2,5→2,4 isomerization. Hence the observed 2,5→2,4 isomerization must be the result of a 5→4 double bond shift as shown in Fig. 16. Asp204 is the obvious candidate to facilitate this monoene isomerization by catalyzing a 1,3-proton shift from carbon 4 to carbon 6. Such mechanism for single double bond isomerizations has been proposed for cholesterol oxidase (98) and Δ^3,Δ^2-enoyl-CoA isomerase (99) based on observed intramolecular 1,3 hydrogen shifts. If mechanistically similar to the studied isomerizations, the 1,3 proton shift catalyzed by Asp204 may not be concerted as shown in Fig. 16, but rather proceed in two steps by removal of a proton from carbon 4 and formation of a stabilized carbanion followed by addition of a proton to carbon 6. Noteworthy is the observation that the 2,5→2,4 isomerization

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catalyzed by mutant E196Q proceeded 11-times faster than the same reaction catalyzed by the wild-type isomerase. The lower rate detected with the wild-type enzyme may be due to a higher pK value of Asp204 induced by Glu196. Such electrostatic effect on Asp204 would not be effective in the E196Q mutant.

The 2,5→2,4 isomerization catalyzed by mutant D204A was more complex than the conversion brought about by the E196Q mutant. The progress curve for the D204A-catalyzed 2,5→2,4 isomerization showed a lag that was shown to correspond to the conversion of 2,5-octadienoyl-CoA to its 3,5-isomer. Since the 2,5→3,5 isomerization was faster than the subsequent 3,5→2,4 isomerization, the 3,5-intermediate accumulated and initially was detectable. The formation of 3,5-octadienoyl-CoA was the result of a double bond shift from carbon 2 to carbon 3. This double bond isomerization must have been catalyzed by Glu196 which is proposed to facilitate a 1,3 proton shift from carbon 4 to carbon 2 (see Fig. 16). Again, the proton transfers may not be concerted as shown in Fig. 15, but may be sequential resulting in the formation of a carbanion intermediate. An alternative route with a carbocationic intermediate represents an unlikely mechanism.

The analyses of the 2,5→2,4 isomerizations provides good evidence for Glu196 being close to carbon 2 and Asp204 close to carbon 6 as shown in Fig. 17. Both residues are necessary for the 3,5→2,4 isomerization that proceeds by a simultaneous shift of both double bonds (41). A similar mechanism is envisioned for the triene isomerization except that Asp204 must be close to carbon 8 (see Fig.

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17). Such dual role of Asp204 suggests a certain flexibility of the residue and/or requires different positioning of the dienoyl-CoA and trienoyl-CoA substrates at the active site. Either way, the function of Asp204 in the isomerization of the triene comes at a price which is reflected by the almost 50-fold lower activity of trienoyl-CoA isomerase as compared to dienoyl-CoA isomerase.

Altogether, this study demonstrates the need for two acidic residues to facilitate the proton transfers that result in positional isomerizations of dienes and trienes. In contrast, proton transfers that cause monoenes to shift by one carbon only require a single acidic residue as previously documented for other isomerases (98, 99).

Subcellular Localization and Characterization of Enoyl-CoA Isomerases-This study demonstrates that at least two enoyl-CoA isomerases each are present in mitochondria and peroxisomes of rat liver. The surprising conclusion reached during their characterization was that one of these isomerases, referred to as Δ^3,Δ^2-enoyl-CoA isomerase or ECI, has a dual subcellular localization in mitochondria and peroxisomes. Moreover, this isomerase is identical with peroxisomal enoyl-CoA isomerase, PECI, and is indistinguishable from mitochondrial long-chain enoyl-CoA isomerase. When initially identified and characterized, PECI was shown to be located predominantly in peroxisomes of human fibroblasts (7). It remains to be determined whether the apparent absence of this enzyme from human mitochondria or its presence in human mitochondria at only low levels is characteristic of human cells or is a unique feature of human fibroblasts. The

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identification of ECI as the mitochondrial long-chain enoyl-CoA isomerase is based on the limited amount of information published about this enzyme (6). Specifically, the immunological characterization of mitochondrial long-chain enoyl-CoA isomerase after its separation from other isomerases by chromatography on hydroxylapatite and its preference for long-chain substrates support the conclusion that this isomerase and ECI are the same enzyme. It is unclear why this enzyme was not detected in peroxisomes during the previous investigation (6). But it could be the consequence of its low activity compared to the isomerase activity of MFE1 in peroxisomes isolated from livers of clofibrate-treated rats. ECI was expected to be present in the peroxisomes because the human isomerase has a C-terminal serine-lysine-leucine peroxisomal targeting sequence while the mouse enzyme has a proline-lysine-leucine signal (7). The signal responsible for directing this enzyme to the mitochondrial matrix has not yet been identified. However, the N-terminal 16 residues of ECI are predicted to form an α-helix with amphiphilic properties characteristic of a mitochondrial targeting sequence.

Functional Contribution of Isoenzymes of Enoyl-CoA Isomerase Activities-The activities of enoyl-CoA isomerases in mitochondria and peroxisomes were evaluated to determine if and how the two enzymes present in each organelle complement each other in catalyzing the three types of enoyl-CoA isomerization reaction that take place during the β-oxidation of unsaturated fatty acids. In rat liver mitochondria, where MECI and ECI coexist, the contribution of ECI to the total 3-cis→2-trans-octenoyl-CoA isomerase activity is negligible (5% of the total

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activity) as illustrated by Fig. 3. Even with 3-cis-dodecenoyl-CoA, an intermediate of oleate degradation, ECI is estimated to make only a minor contribution (25%) to the total activity. This estimate takes into account the increased catalytic efficiency of ECI with increasing acyl-chain length of the substrate and is based on the assumption that k_{cat}/K_m values for the isomerizations of 3-cis-octenoyl-CoA, 3-cis-decenoyl-CoA, 3-cis-dodecenoyl-CoA and 3-cis-tetradecenoyl-CoA increase linearly. The contribution of ECI to the 2,5→3,5 isomerase activity is negligible because the catalytic efficiencies of ECI and MECI with 2,5-cis-tetradecadienoyl-CoA, a β-oxidation intermediate of oleate, are similar to their catalytic efficiencies with 3-cis-octenoyl-CoA as substrate. In contrast, ECI is estimated to contribute one-third of the 3-trans→2-trans-octenoyl-CoA isomerase activity in mitochondria and nearly 50% of the activity with 3-trans-decenoyl-CoA, a β-oxidation intermediate of linoleate.

In peroxisomes, ECI also contributes significantly to the 3-trans→2-trans isomerase activity. With 3-trans-octenoyl-CoA as a substrate, this contribution is estimated to be 40% of the total activity (see Fig. 4). With longer-chain β-oxidation intermediates, it is expected to be greater. Unfortunately, it is unclear which, if any, unsaturated fatty acids are degraded in peroxisomes. It is therefore not possible to make a prediction about the contributions of ECI and MFE1 to such isomerizations even if the kinetic data for the isomerization of various β-oxidation intermediates of unsaturated and polyunsaturated fatty acids were known. Similarly, the importance of ECI for the 3-cis→2-trans isomerizations during the peroxisomal β-

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oxidation of unsaturated and polyunsaturated fatty acids cannot be estimated. If long-chain and very long-chain polyunsaturated fatty acids are partially degraded in peroxisomes, ECI is expected to have a major function in the required 3-cis→2-trans double bond isomerizations because of its preference for long-chain substrates in contrast to MFE1, which does not exhibit such specificity. However, MFE1 will be the dominant isomerase catalyzing the 2,5→3,5 isomerization due to its high catalytic efficiency in that type of reaction.

In summary, in mitochondria MECI is the dominant enzyme for catalyzing 3-cis→2-trans and 2,5→3,5 isomerizations while ECI contributes significantly to 3-trans→2-trans isomerizations. The contributions of ECI and MFE1 in peroxisomal β-oxidation cannot be estimated due to a lack of information about the degradation of unsaturated fatty acids in this organelle. An exception is the 2,5→3,5 isomerization which in peroxisomes most likely is catalyzed by MFE1 because of its high catalytic efficiency in that type of reaction.

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Table I

Purification of wild-type recombinant dienoyl-CoA isomerase

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Extract	55	1200	22	100
Q-Sepharose	6.1	860	140	72
S-Sepharose	1.7	840	490	70

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Table II

Activities of wild-type and mutant dienoyl-CoA isomerases with 3,5-octadienoyl-CoA and 3,5,7-decatrienoyl-CoA as substrates

Mutant	3,5 → 2,4		3,5,7 → 2,4,6	
	Specific*	Relative	Specific*	Relative
WT	9.6×10^{2}	1	21	1
D176A	98	0.1	3.3×10^{-2}	1.6×10^{-3}
E196D	32	3.3×10^{-2}	0.12	5.7×10^{-3}
E196Q	6.5×10^{-3}	6.8×10^{-6}	$\leq 2 \times 10^{-4}$	$\leq 9.6 \times 10^{-6}$
D204A	1.8×10^{-2}	1.9×10^{-5}	$\leq 2 \times 10^{-4}$	$\leq 9.6 \times 10^{-6}$
D204N	3.8×10^{-3}	4.0×10^{-6}	$\leq 3 \times 10^{-4}$	$\leq 1.4 \times 10^{-5}$

*Specific activities in μmol/min/mg of protein were obtained with 20 μM substrate as described under Experimental Procedures.

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Table III

Activities of wild-type and mutant dienoyl-CoA isomerases with 2,5-octadienoyl-CoA as a substrate

Mutant	2,5 → 2,4		2,5 → 3,5
	Specific*	Relative	Specific*
WT	3.7×10^{-2}	1	
D176A	5.6×10^{-2}	1.5	
E196D	0.11	3	
E196Q	0.41	11	
D204A	5.4×10^{-3}	0.15	0.40
D204N	$\leq 7 \times 10^{-4}$	$\leq 1.9 \times 10^{-2}$	0.10

*Specific activities in μmol/min/mg of protein were obtained with 20 μM substrate as described under Experimental Procedures.

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Table IV

Kinetic parameters of wild-type and mutant dienoyl-CoA isomerases.

Mutant and substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
WT with 3,5-octadienoyl-CoA	30±1.8	1300±34	44
WT with 3,5,7-decatrienoyl-CoA	13±2.7	26±1.8	2
D176A with 3,5-octadienoyl-CoA	21±2.8	150±7.5	7.1
E196D with 3,5-octadienoyl-CoA	21±1.8	27±0.9	1.3
E196Q with 2,5-octadienoyl-CoA	37±4.0	0.65±0.032	0.017

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Table V

Kinetics parameters of the mitochondrial enoyl-CoA isomerase (MECI)

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
3-trans-Hexenoyl-CoA	470 ± 40	270 ± 16	0.57
3-trans-Octenoyl-CoA	190 ± 36	25 ± 2.8	0.13
3-trans-Tetradecenoyl-CoA	48 ± 4.7	20 ± 1.2	0.42
3-cis-Hexenoyl-CoA	240 ± 16	200 ± 7.8	0.82
3-cis-Octenoyl-CoA	150 ± 24	180 ± 16	1.2
3-cis-Tetradecenoyl-CoA	57 ± 6.0	98 ± 4.8	1.7
2-trans,5-cis-Octadienoyl-CoA*	84 ± 18	45 ± 4.8	0.54
2-trans,5-cis-Tetradecadienoyl-CoA*	20 ± 5.1	8.3 ± 0.93	0.42

* Assayed with dienoyl-CoA isomerase as coupling enzyme.

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Table VI

Kinetic parameters of the enoyl-CoA isomerase (ECI)

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
3-trans-Hexenoyl-CoA	1600 ± 79	100 ± 39	0.063
3-trans-Octenoyl-CoA	120 ± 12	210 ± 11	1.8
3-trans-Tetradecenoyl-CoA	29 ± 3.8	540 ± 38	19
3-cis-Hexenoyl-CoA	1200 ± 157	58 ± 5.6	0.049
3-cis-Octenoyl-CoA	100 ± 6.8	47 ± 1.4	0.45
3-cis-Tetradecenoyl-CoA	21 ± 5.9	210 ± 22	10
2-trans,5-cis-Octadienoyl-CoA*	170 ± 29	26 ± 2.6	0.15
2-trans,5-cis-Tetradecadienoyl-CoA*	29 ± 5.9	15 ± 1.4	0.52

* Assayed with dienoyl-CoA isomerase as coupling enzyme.

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Table VII

Kinetic parameters of the enoyl-CoA isomerase of the multifunctional enzyme 1 (MFE1)

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
3-trans-Hexenoyl-CoA[a]	38 ± 1.3	1.8 ± 0.039	0.047
3-trans-Octenoyl-CoA[a]	28 ± 1.5	2.0 ± 0.052	0.071
3-trans-Tetradecenoyl-CoA[a]	32 ± 11	1.4 ± 0.26	0.044
3-cis-Hexenoyl-CoA[a]	31 ± 4	1.6 ± 0.13	0.052
3-cis-Octenoyl-CoA[a]	32 ± 6.3	2.4 ± 0.21	0.075
3-cis-Tetradecenoyl-CoA[a]	45 ± 2.5	2.7 ± 1.1	0.060
2-trans,5-cis-Octadienoyl-CoA[b]	4.4 ± 1.2	11 ± 0.92	2.5
2-trans,5-cis-Tetradecadienoyl-CoA[b]	9.6 ± 0.71	12 ± 0.46	1.3

[a]Assayed with 3-keto-acyl-CoA thiolase as coupling enzyme.

[b]Assayed with dienoyl-CoA isomerase as coupling enzyme.

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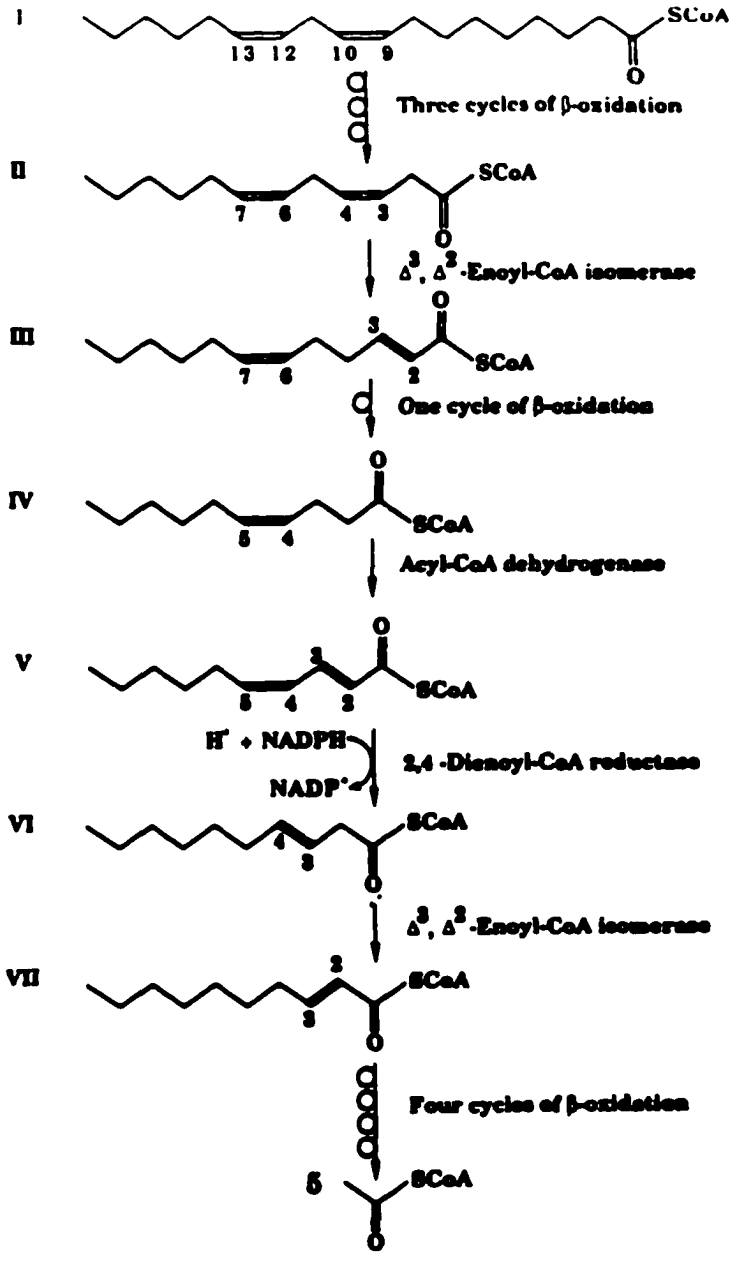

Fig. 1. **β-Oxidation of linoleoyl-CoA** (from Ref. 1).

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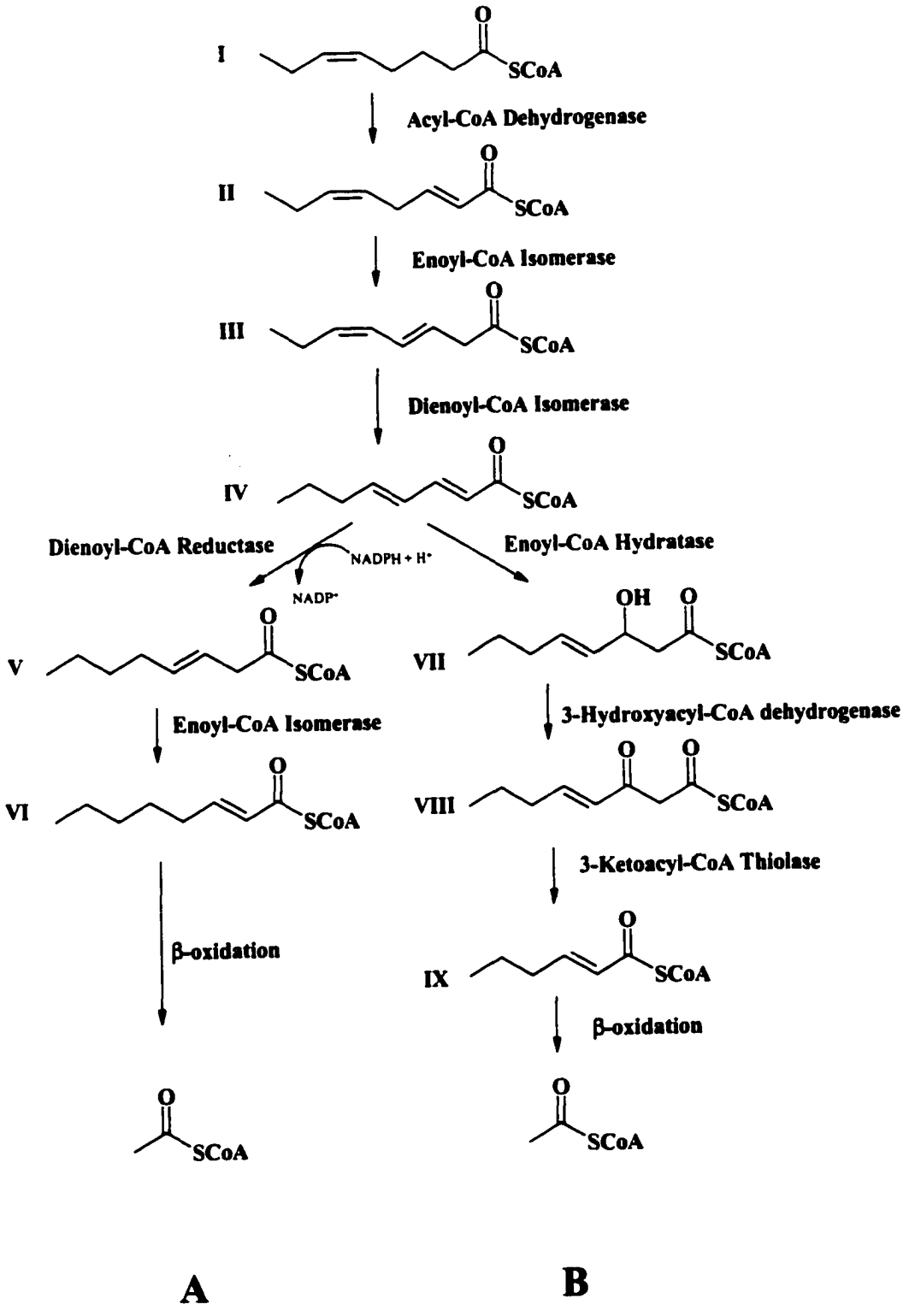

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Fig. 2. **Alternative pathway for the β-oxidation of unsaturated fatty acids with double bonds at odd-numbered positions**

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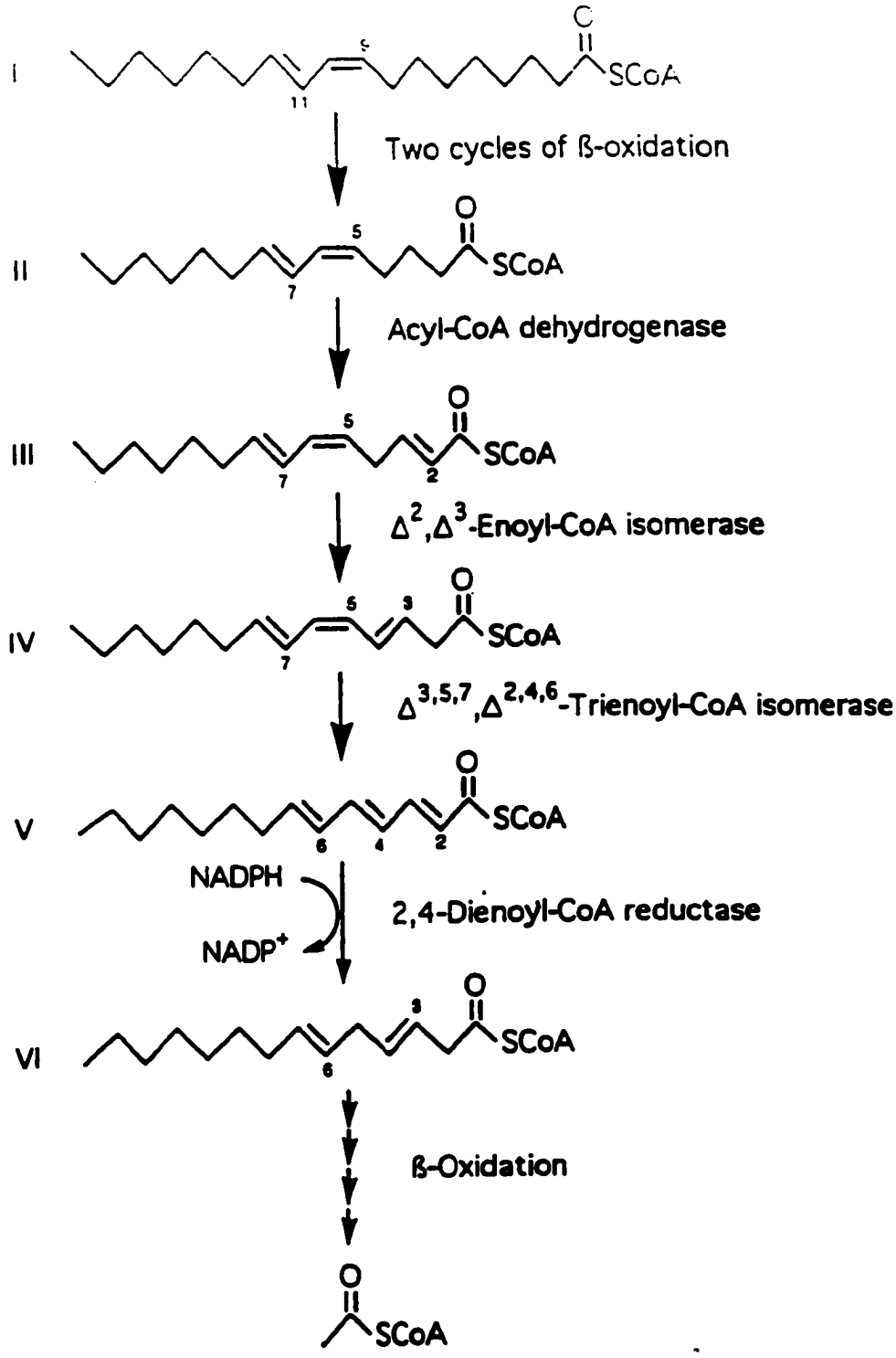

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Fig. 3. **Trienoyl-CoA isomerase-dependent pathway for the β-oxidation of 9-cis,11-trans-octadecadienoyl-CoA (conjugated linoleoyl-CoA)** (from Ref. 40)

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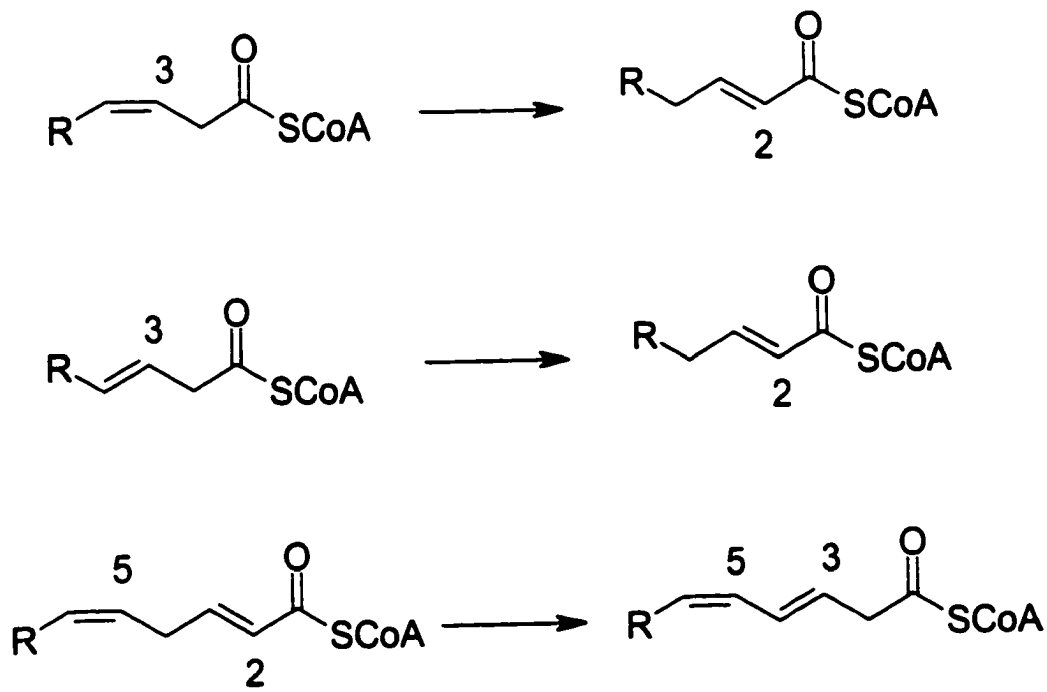

Figure 4. **Reactions catalyzed by enoyl-CoA isomerase**

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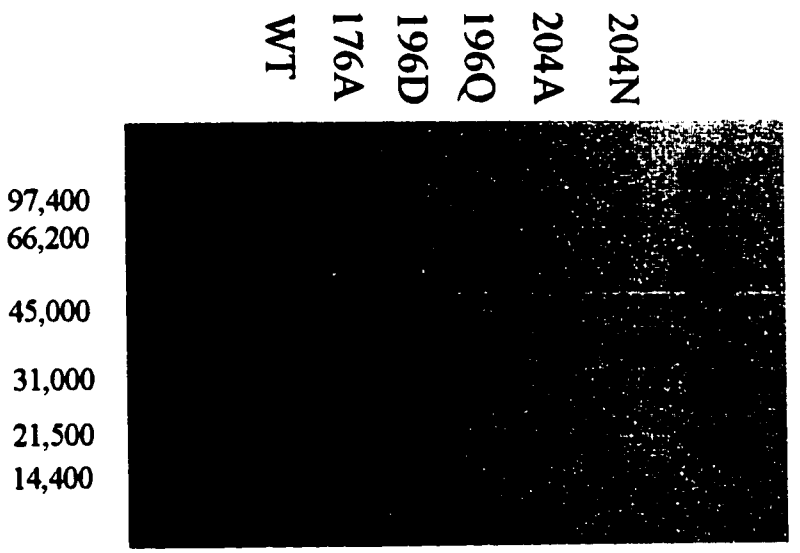

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Fig. 6. **SDS-PAGE of purified wild-type and mutant dienoyl-CoA isomerases stained with Coomasie blue.** The lanes at left and right are non-prestained and prestained molecular markers, respectively. The molecular weight of the non-prestained markers are shown to the left. Lanes in the middle are purified wild-type, D176A, E196D, E196Q, D204A and D204N mutants of dienoyl-CoA isomerase from left to right, respectively.

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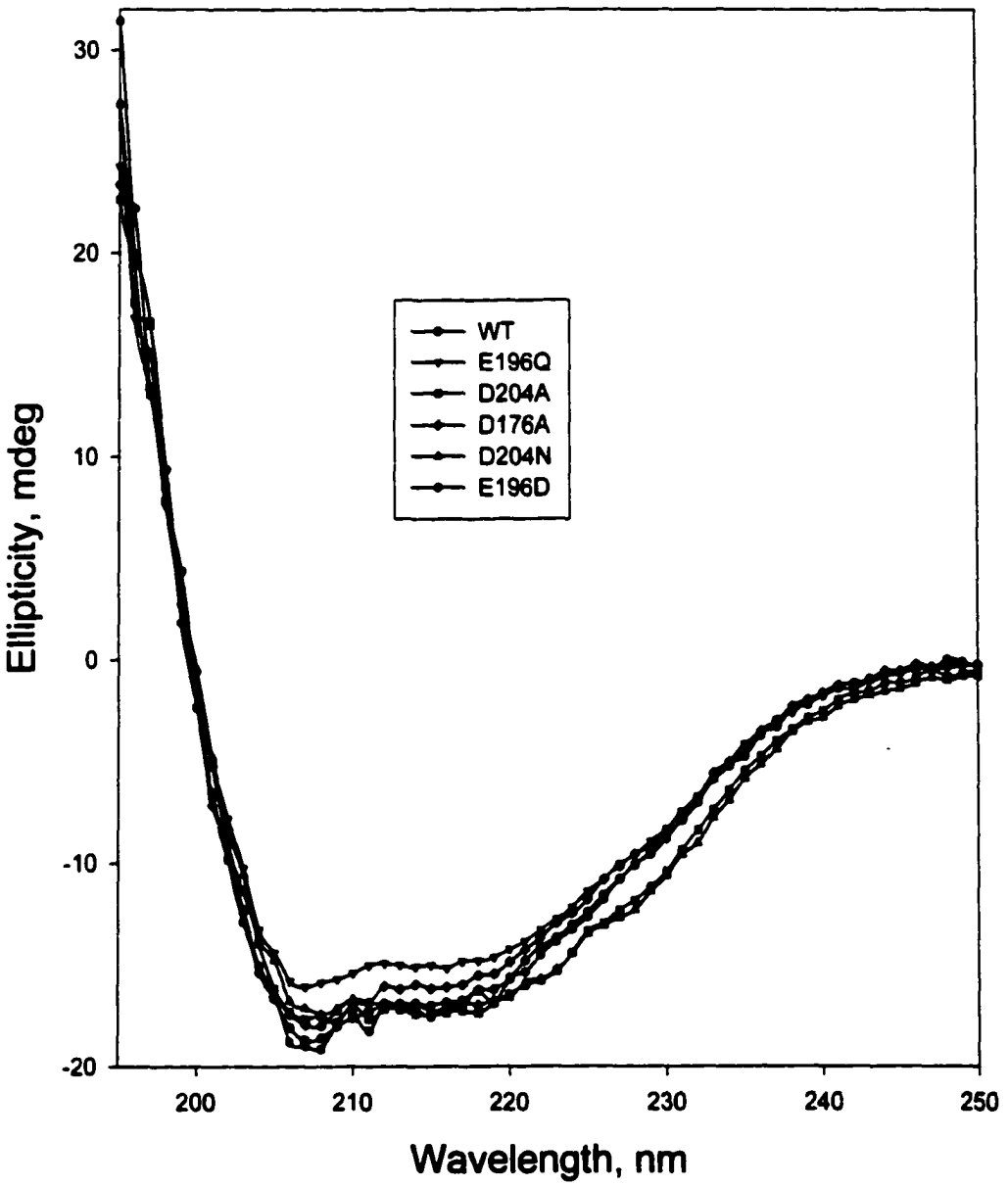

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Figure 7. **CD spectra of wild-type and mutant dienoyl-CoA isomerases.** Data were collected every one nm and labeled as circles for wild-type, triangles for E196Q, squares for D204A, diamonds for D176A, inverted triangles for D204N and hexagons for E196D.

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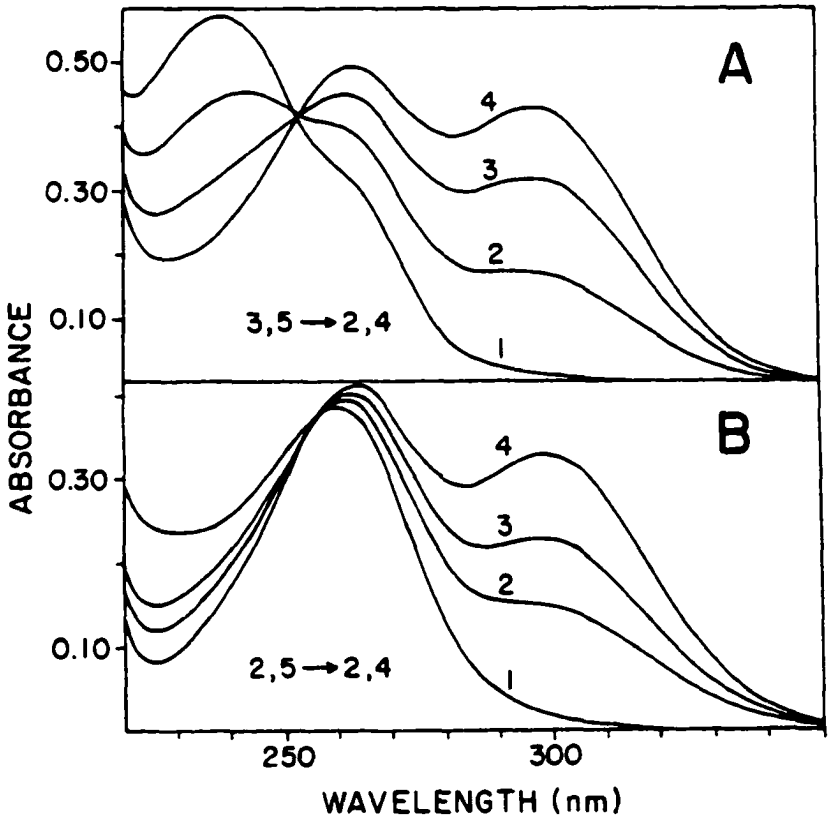

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Figure 8. **Spectrophotometric analyses of the isomerizations of 3,5-octadienoyl-CoA and 2,5-octadienoyl-CoA catalyzed by dienoyl-CoA isomerase.** A. Spectral changes associated with the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (3,5→2,4) catalyzed by wild-type dienoyl-CoA isomerase. Spectrum 1 at time 0; spectra 2 to 4 were recorded 20s, 1.5min, and 10 min after the addition of enzyme. B. Spectral changes associated with the isomerization of 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (2,5→2,4) catalyzed by the E196Q mutant of dienoyl-CoA isomerase. Spectrum 1 at time 0; spectra 2 to 4 were recorded 20s, 1.5min, and 20 min after the addition of enzyme.

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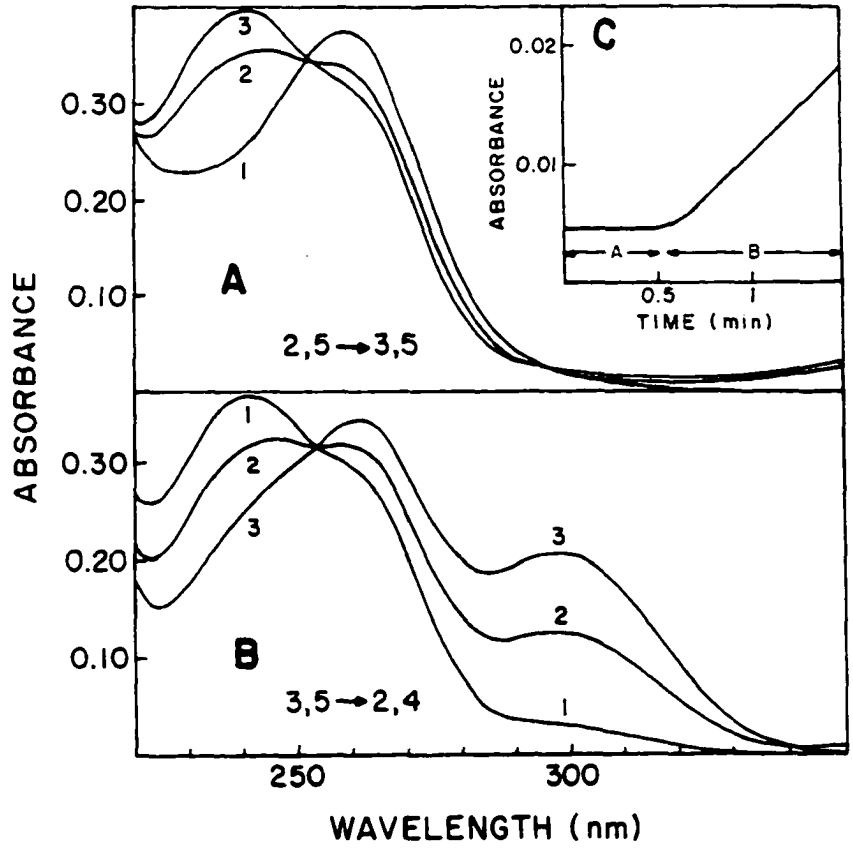

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Figure 9. **Spectrophotometric analysis of the isomerization of 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by the D204A mutant of dienoyl-CoA isomerase.** A, spectral changes within 2.5 min after mixing 2,5-octadienoyl-CoA with 204A. Spectrum 1, 2,5- octadienoyl-CoA before adding 204A; Spectrum 2 and 3, 30 s and 2.5 min after adding 204A. B, spectral changes at 3 to 25 min after adding 204A. Spectrum 1, 2 and 3, 3 min, 10 min and 25 min after adding 204A. C, absorbance changes at 300 nm observed in the reaction of 2,5-octadienoyl-CoA catalyzed by 204A. Phase a, a lag in absorbance change observed after adding 204A to 2,5-octadienoyl-CoA. Phase b, a steady increase of absorbance at 300 nm observed 0.6 min after adding 204A.

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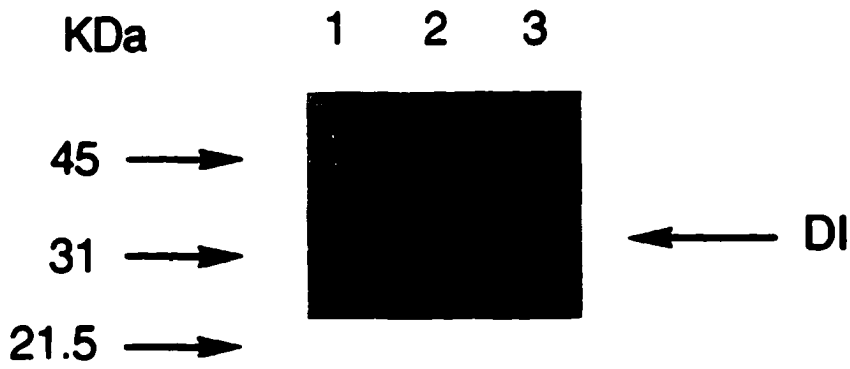

Figure 10. **Immunoblotting of rat liver peroxisomes with monospecific antibodies against dienoyl-CoA isomerase.** Lane 1, recombinant dienoyl-CoA isomerase; lane 2 and 3, puried peroxisomes.

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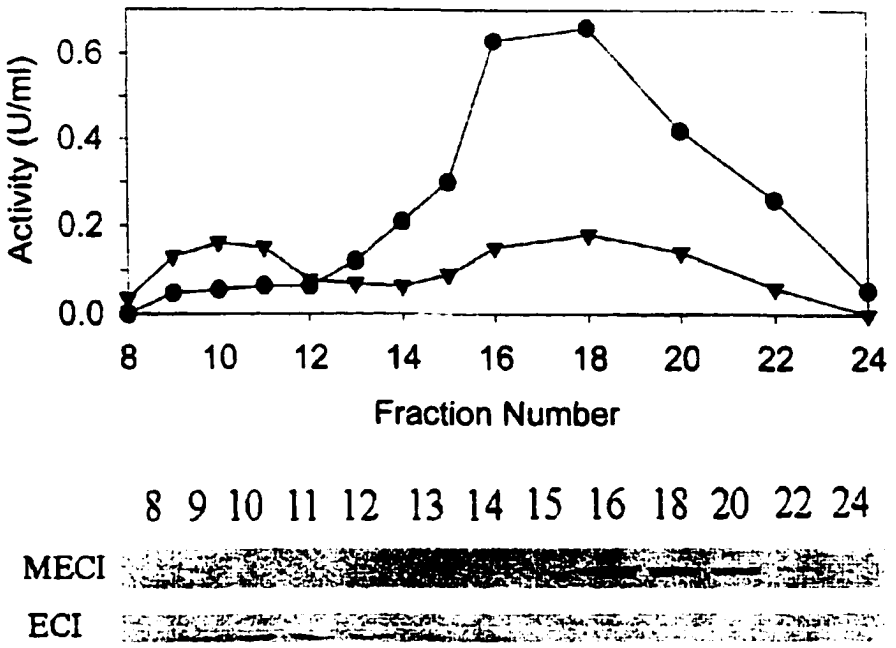

Figure 11. **Separation of mitochondrial enoyl-CoA isomerases by hydroxylapatite column chromatography.** Upper panel. Fractions were assayed for, (•) enoyl-CoA isomerase activity with cis-octenoyl-CoA as substrate; (▼) enoyl-CoA isomerase activity with trans-octenoyl-CoA as substrate. Lower panel, immunoblotting of equal portions of the fractions (fraction numbers are indicated above the immunoblot image) with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI) or the mitochondrial enoyl-CoA isomerase (MECI).

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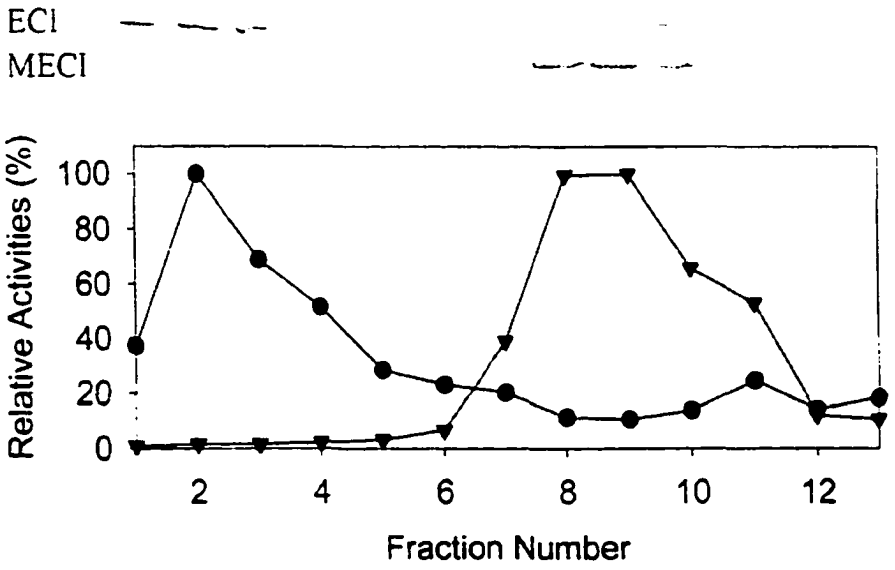

Figure 12. **Subcellular localization of peroxisomal enoyl-CoA isomerase (PECI) and mitochondrial enoyl-CoA isomerase (MECI).** Bottom panel, marker enzyme activities from fractions obtained by nycodenz gradient centrifugation of a light mitochondria preparation. (•), catalase activity; (▼) malate dehydrogenase activity. Top panel, immunoblotting of equal volumes of the fractions with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI) or the mitochondrial enoyl-CoA isomerase (MECI).

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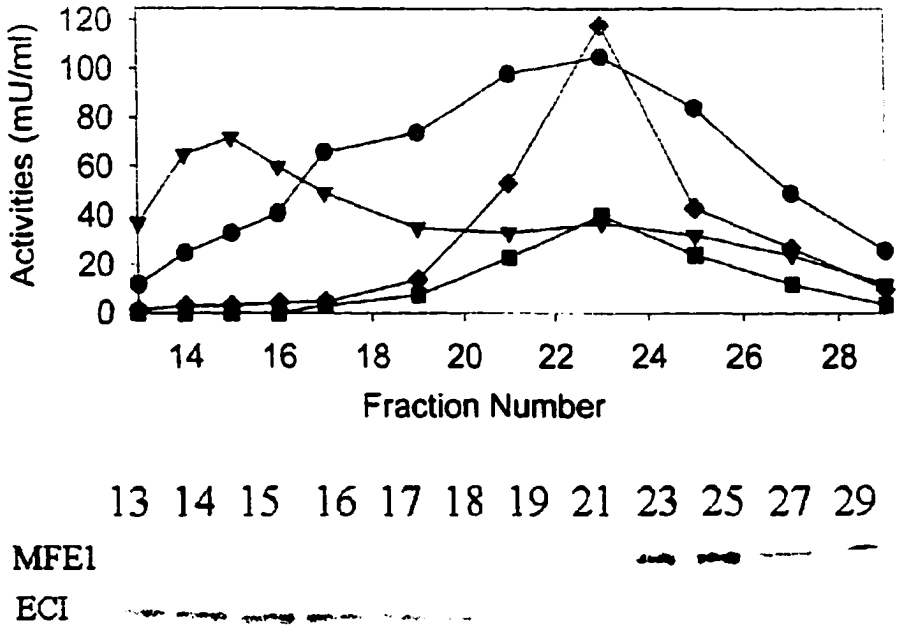

Figure 13. **Separation of peroxisomal enoyl-CoA isomerases by hydroxylapatite column chromatography.** Upper panel. Fractions were assayed for, (•) enoyl-CoA isomerase activity with cis-octenoyl-CoA as substrate; (▼)enoyl-CoA isomerase activity with trans-octenoyl-CoA as substrate; (♦) 2-enoyl-CoA hydratase activity; (■) 3-hydroxyacyl-CoA dehydrogenase activity. Lower panel. Immunoblotting of equal portions of the fractions (fraction numbers are indicated above the immunoblot image) with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI) or peroxisomal multifunctional enzyme 1(MFE1).

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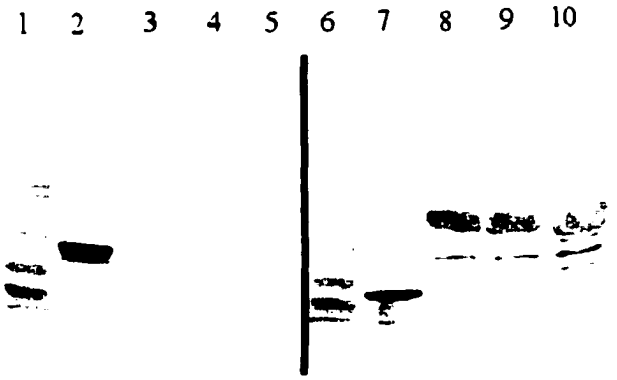

Figure 14. **Immunoblotting of MFE1 with antibodies against ECI and MECI.** Lanes 1 and 6 are molecular markers. Lanes 1 through 5 were probed with antibodies to PECI, lanes 6 through 10 were probed with antibodies to MECI. Lane 2 was loaded with 3 mU of PECI; lanes 3, 4 and 5, with MFE1 containing 6 mU, 12 mU and 24 mU of enoyl-CoA isomerase activity. Lane 7 was loaded with 7 mU of MECI and lanes 8, 9 and 10 with MFE1 containing 7mU, 14 mU and 28 mU of enoyl-CoA isomerase activity.

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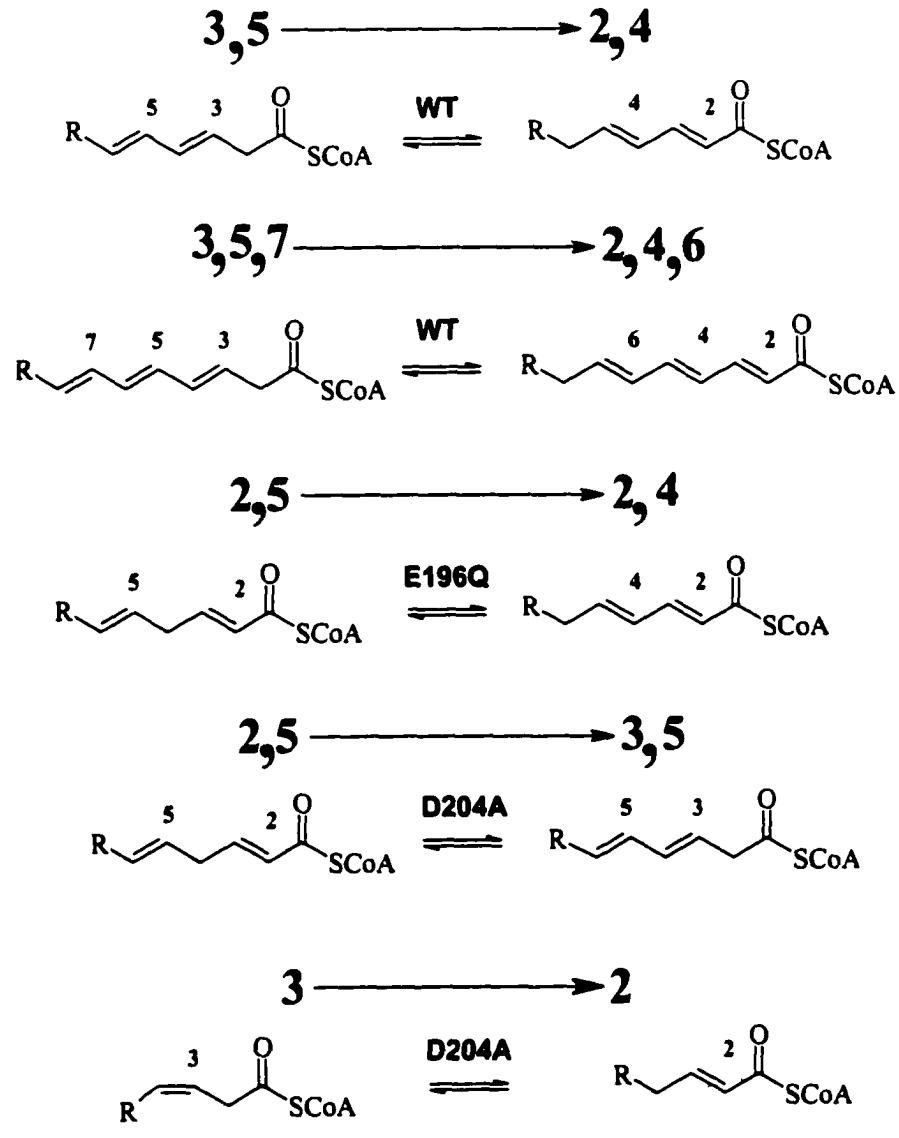

Figure 15. **Reactions catalyzed by dienoyl-CoA isomerase**

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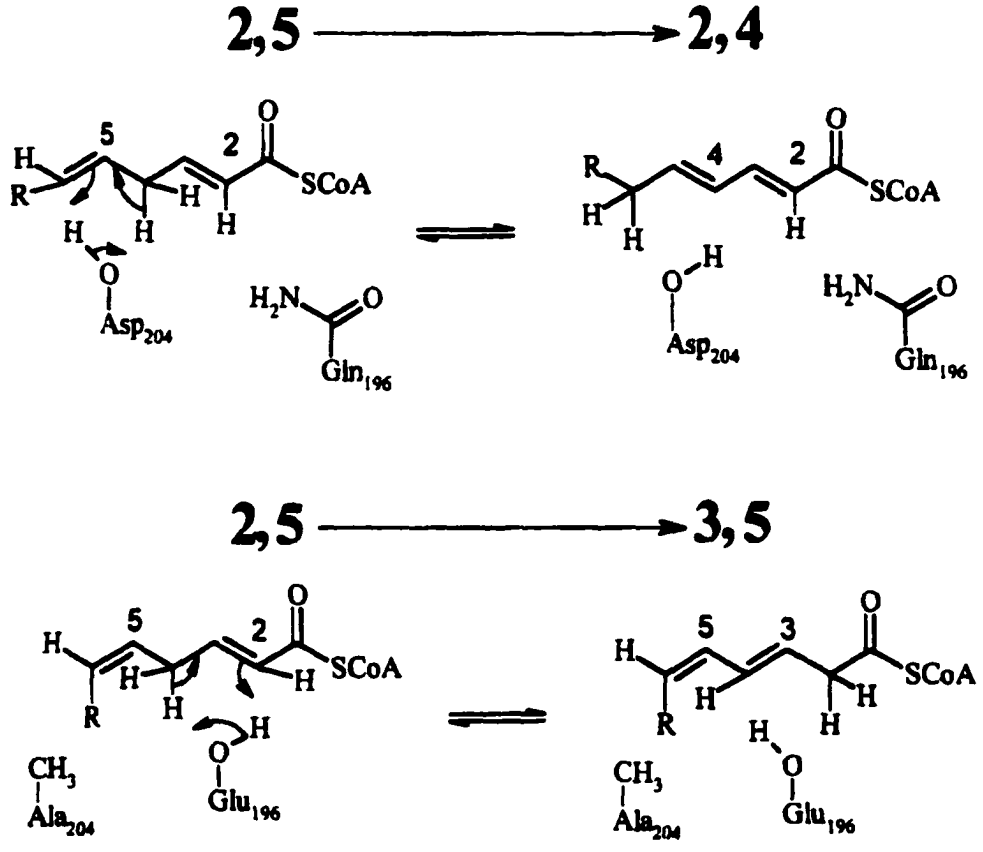

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Figure 16. **Proposed catalytic mechanisms of mutant E196Q for the isomerizations of 2,5-dienoyl-CoA (2,5) to 2,4-dienoyl-CoA (2,4) and of mutant D204A for the isomerizations of 2,5-dienoyl-CoA (2,5) to 3,5-dienoyl-CoA (3,5)**

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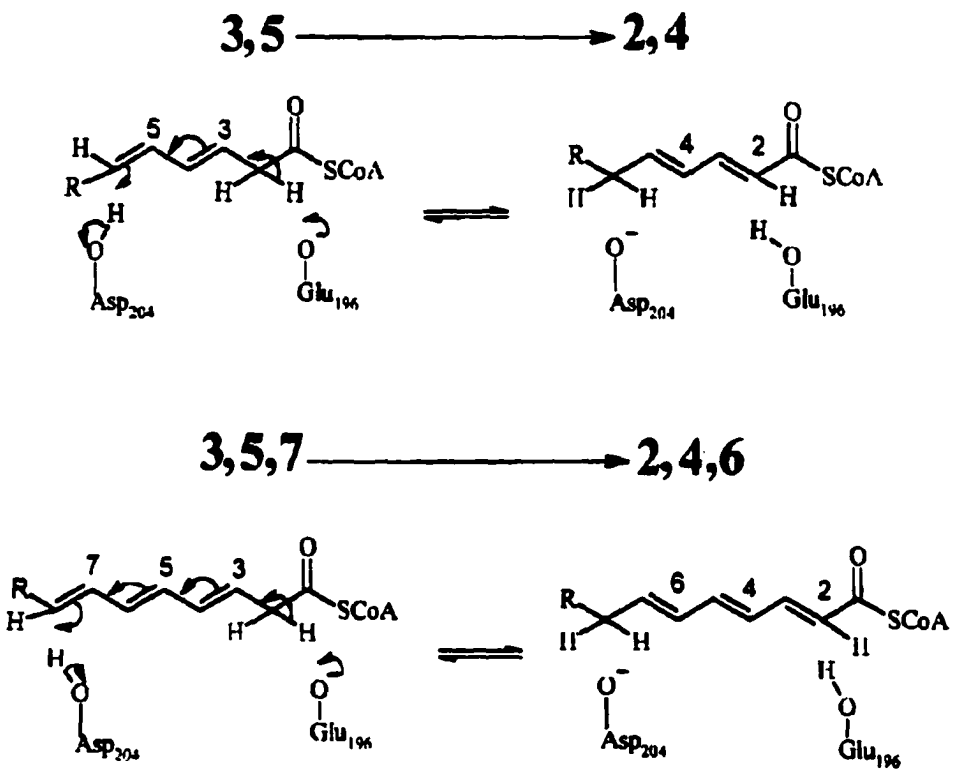

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Figure 17. **Proposed catalytic mechanisms of wild-type dienoyl-CoA/trienoyl-CoA isomerase for the isomerizations of 3,5-dienoyl-CoA (3,5) to 2,4-dienoyl-CoA (2,4) and for 3,5,7-trienoyl-CoA (3,5,7) to 2,4, 6-trienoyl-CoA (2,4,6)**

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