Purification and Mechanism of Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA Isomerase from Rat Liver†

Lin-Su Chen, Shiow-Jen Jin, and Kou-Yi Tserng*

Departments of Nutrition and Medicine, Case Western Reserve University School of Medicine, and Medical Research Service, Veterans Affairs Medical Center, Cleveland, Ohio 44106

Received February 25, 1994; Revised Manuscript Received May 13, 1994®

ABSTRACT: A new enzyme, i.e., Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, required in the NADPH-dependent metabolic pathway of odd-numbered double bond, unsaturated fatty acids, was isolated and purified to apparent homogeneity from rat liver. In the oxidation of odd-numbered double bond, unsaturated fatty acids, stepwise β-oxidation leads to *cis*-5-enoyl-CoA, which is then dehydrogenated and isomerized to Δ^3,Δ^5-dienoyl-CoA. Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA isomerase converts Δ^3,Δ^5-dienoyl-CoA to trans-2,trans-4-dienoyl-CoA, which is a substrate for NADPH-dependent 2,4-dienoyl-CoA reductase. This enzyme was purified through Matrex gel red A, blue Sepharose, DEAE-cellulose, CM-cellulose, hydroxylapatite, and Sepharose CL6B column chromatography of an ammonium sulfate precipitated fraction (30–80%) of rat liver homogenate. A native molecular weight of 200 000 with four subunits of 55 000 each was determined. The isoelectric point was 6.5. This enzyme was located in mitochondria and was inducible by clofibrate treatment. Using Δ^3,Δ^5-decadienoyl-CoA, Δ^3,Δ^5-dodecadienoyl-CoA, and Δ^3,Δ^5-tetradecadienoyl-CoA as substrates, the V_{max} ratio was 1:0.5:0.4 and the K_m's were 10.9, 5.9, and 1.4 μM, respectively. The specific activity of purified enzyme was 7 units/mg using Δ^3,Δ^5-decadienoyl-CoA as substrate. The mechanism of isomerization was studied by deuterium labeling. Consistent with the deuterium labeling pattern of the products, the isomerization from *trans*-2,*cis*-5-dienoyl-CoA to *trans*-2,*trans*-4-dienoyl-CoA was a two-step process through an intermediate Δ^3,Δ^5-dienoyl-CoA. The contribution of this reductive pathway to the overall oxidation of odd-numbered double bond, unsaturated fatty acids is not known, but it is likely to be a major pathway since the dehydrogenation step is also accompanied by isomerization. Once isomerized to Δ^3,Δ^5-dienoyl-CoA, the substrate is committed to the reduction pathway.

The metabolism of odd-numbered double bond, unsaturated fatty acids was thought to be exclusively from a stepwise β-oxidation until a Δ^3-double bond was reached, as shown in route B of Figure 1. A Δ^3,Δ^2-enoyl-CoA isomerase converts this Δ^3-intermediate to *trans*-2-enoyl-CoA, which is then metabolized by a regular fatty acid β-oxidation sequence (Stoffel et al., 1964). Recently, an additional pathway that reduces the double bond and depends on the presence of NADPH as a cofactor was described by us (Tserng & Jin, 1991). In that initial report, we proposed that this reaction proceeded by a direct reduction of the Δ^5-double bond. However, Smeland et al. (1992) presented evidence indicating that this reduction actually occurred through the intermediate *trans*-2,Δ^5-dienoyl-CoA. In their proposed sequence, Δ^5-enoyl-CoA is dehydrogenated to *trans*-2,Δ^5-dienoyl-CoA, which is isomerized to Δ^3,Δ^5-dienoyl-CoA by the action of Δ^3,Δ^2-enoyl-CoA isomerase, as shown in route A of Figure 1. Δ^3,Δ^5-Dienoyl-CoA is then isomerized to *trans*-2,*trans*-4-dienoyl-CoA by an as yet uncharacterized Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. The rest of the metabolic sequence from *trans*-2,*trans*-4-dienoyl-CoA proceeds by the NADPH-dependent 2,4-dienoyl-CoA reductase mediated pathway as proposed for even-numbered double bond, unsaturated fatty acids (Kunau & Dommes, 1978). Therefore, the metabolism of both odd- and even-numbered double bonds in fatty acids requires the presence of NADPH-dependent 2,4-dienoyl-CoA

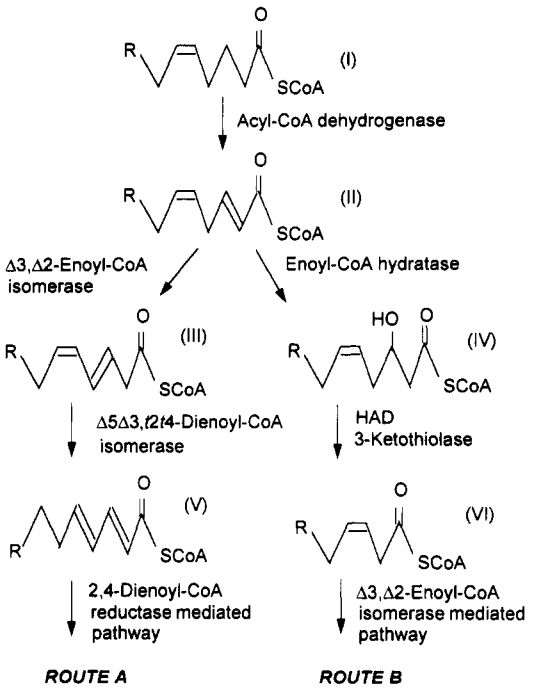

FIGURE 1: Metabolic pathways for *cis*-5 fatty acids: route A, the reduction pathway; route B, the isomerase pathway; I, *cis*-5-enoyl-CoA; II, *trans*-2,*cis*-5-dienoyl-CoA; III, *trans*-3,*cis*-5-dienoyl-CoA; IV, 3-hydroxy-*cis*-5-enoyl-CoA; V, *trans*-2,*trans*-4-dienoyl-CoA; VI, 3-keto-*cis*-5-enoyl-CoA. Abbreviations: HAD, 3-hydroxyacyl-CoA dehydrogenase; R, alkyl.

† Supported by Grant DK-45040 from the National Institute of Diabetes and Digestive and Kidney Diseases and by a Merit Review Award of the Medical Research Service of the Department of Veterans Affairs.

* Address correspondence and reprint requests to Kou-Yi Tserng, Ph.D., Medical Research Service, Veterans Affairs Medical Center, 10701 East Blvd., Cleveland, OH 44106. Telephone: 216-791-3800 (ext. 4630). Fax: 216-231-3260.

® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-10527$04.50/0

reductase. In addition, a second isomerase to isomerize Δ^3,Δ^5-dienoyl-CoA to *trans*-2,*trans*-4-dienoyl-CoA is needed for the metabolism of odd-numbered double bond fatty acids.

In order to study the NADPH-dependent reduction pathway of odd-numbered double bond fatty acids in more detail, the isolation of pure Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase is needed for reconstitution experiments and for the preparation of antibodies. We have succeeded in the isolation of apparently homogenous Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase from rat liver homogenate. Partial characterization of this purified enzyme and its mechanism of action are also described.

MATERIALS AND METHODS

Reagents and Chemicals. Matrex gel red A was purchased from Amicon Corp. (Lexington, MA); blue Sepharose CL-6B was from Pharmacia (Uppsala, Sweden); DEAE-cellulose, CM-cellulose, and Sepharose CL-6B were from Sigma (St. Louis, MO); and hydroxylapatite was from Bio-Rad (Richmond, CA). PBE 94 polybuffer exchanger and polybuffer 74 for chromatofocusing as well as PD-10 desalting columns were obtained from Pharmacia. The low molecular weight standard kit, Coomassie Blue R-250, reagents for SDS/PAGE[1] as well as Bio-Lyte 5/7 ampholyte, and standards for isoelectric focusing were purchased from Bio-Rad. Benzamidine, dithiothreitol, CoA, clofibrate, NAD^+, β-hydroxyacyl-CoA dehydrogenase (from porcine heart), crotonase (from bovine liver), NADPH, rotenone, bovine serum albumin, nagarse, and standards for gel filtration were obtained from Sigma; acyl-CoA oxidase (from *Arthrobacter* sp.) was from Boehringer Mannheim (Indianapolis, IN). *cis*-5-Decenoic, *cis*-5-dodecenoic, and *cis*-5-tetradecenoic acids were synthesized as previously described (Jin & Tserng, 1990). *trans*-2,*trans*-4-Decadienoic acid was synthesized from the oxidation of *trans*-2,*trans*-4-decadienal (Aldrich) with Jones' reagent. Acyl-CoA esters were prepared by a modified mixed anhydride method with ethyl chloroformate and triethylamine in tetrahydrofuran (Schulz, 1974). Thioester concentrations were determined by the use of Ellman's procedure after hydrolysis (Ellman, 1959). In the case of 2,4-dienoyl-CoA, spectrophotometric analysis at 300 nm gave the same results.

Synthesis of [2,2'-$^2H_2$]-cis-5-Decenoic Acid. A mixture of sodium (0.57 mmol) in CH_3OD (1 mL) and methyl *cis*-5-decenoate (1.04 mmol) was refluxed for 15 min. After cooling, the solvent was removed under dry nitrogen. The same procedure was repeated with a fresh portion of CH_3OD (1 mL). After nitrogen drying, the residue was acidified with DCl (35%) and extracted with ether. Ether was removed and the product was saponified to yield [2,2'-$^2H_2$]-*cis*-5-decenoic acid. The isotopic purity of this product was analyzed by GC/MS and found to be d_0 3%, d_1 11%, and d_2 86%.

Extraction of Isomerases from Tissues. Frozen livers (20–30 g) from male Sprague–Dawley (Charles River) rats, fed on regular Purina Rat Chow or Rat Chow containing 0.5% (w/w) clofibrate for 2 weeks, were thawed and homogenized in a Potter Elvehjem homogenizer in a 5-fold (w/v) solution of 10 mM phosphate buffer (pH 7.0) containing 0.5 mM benzamidine (BA), 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, and 0.5 mM EGTA. The homogenate was sonicated with a sonifier cell disrupter and centrifuged at 31000*g* for 20 min. The supernatant was fractionated by ammonium sulfate (30–80%) precipitation, centrifuged at 31000*g* for 20 min, and then dialyzed in buffer A (20 mM phosphate buffer, pH 7.0, containing 0.5 mM BA, 0.5 mM DTT, 0.5 mM EDTA, and 0.5 mM EGTA) overnight. The solution was centrifuged further at 111000*g* for 1 h.

Column Chromatographic Purification Procedure. (1) Matrex Gel Red A. Enzyme extract was applied to a Matrex gel red A column (1.6 × 30 cm) equilibrated with buffer A. After washing with 200 mL of buffer A, the column was eluted with a linear gradient of 0–1.2 M KCl in buffer A at a flow of 30 mL/h. Fractions of 5.5 mL were collected. The total volume of the gradients was 300 mL. Fractions containing Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity were pooled and dialyzed overnight against buffer A.

(2) Blue Sepharose. The dialysate was applied to a column of blue Sepharose CL-6B (2.6 × 19 cm) equilibrated with buffer A. This column was washed with 100 mL of buffer A and eluted with a linear gradient of 0–1.2 M KCl in buffer A at a flow rate of 30 mL/h. The total volume of the gradients was 200 mL, and fractions of 5 mL were collected. Fractions containing Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity were pooled and dialyzed overnight against buffer C (50 mM phosphate buffer, pH 6.0, containing 0.5 mM BA and 0.5 mM DTT).

(3) CM-Cellulose. The dialysate was applied to a CM-cellulose column (2.6 × 15 cm) equilibrated with buffer C. The column was washed with buffer C at a flow rate of 20 mL/h until no more UV (at 280 nm) absorbing materials were eluted (about 200 mL). Fractions of 5 mL each were collected. The fractions that contained Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity were pooled and concentrated with an Amicon ultrafiltration cell with a YM-10 membrane (Amicon, Beverly, MA) to about 10 mL. This concentrate was dialyzed overnight against buffer D (50 mM phosphate buffer, pH 6.6 containing 0.5 mM BA and 0.5 mM DTT) and then purified further with a DEAE-cellulose column. Bound proteins from the CM-cellulose column were eluted with 200 mL of a linear gradient of buffer C containing 0–0.4 M NaCl. Fractions with Δ^3,Δ^2-enoyl-CoA isomerase activity were pooled and concentrated to yield pure Δ^3,Δ^2-enoyl-CoA isomerase.

(4) DEAE-Cellulose. The dialysate was applied to a column of DEAE-cellulose (2.6 × 13 cm), equilibrated with buffer D. After the column was washed with buffer D at a flow of 15 mL/h, the adsorbed proteins were eluted with 200 mL of a linear gradient (50–400 mM) of buffer D. Fractions with Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity were pooled, concentrated, and desalted by the PD-10 column.

(5) Hydroxylapatite. The dialysate was applied to a hydroxylapatite column (2.6 × 20 cm), equilibrated with buffer H (20 mM phosphate, pH 7, containing 0.5 mM BA and 0.5 mM DTT). Elution was done at a flow rate of 20 mL/h. The column was first washed with buffer H until no more UV-absorbing materials were eluted, and then the elution was changed to a linear gradient (20–500 mM) in a total volume of 200 mL. Fractions with Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity were pooled, concentrated, and desalted by the PD-10 column.

(6) Gel Filtration with Sepharose CL-6B. The desalted solution was applied to a column of Sepharose CL-6B (2.6 × 95 cm) equilibrated with buffer G (50 mM phosphate buffer, pH 7, containing 0.5 mM BA, 0.5 mM DTT, and 0.2 M KCl). It was eluted with the same buffer at a flow rate of 20 mL/h.

Enzyme Assays. (1) Δ^3,Δ^5-t-2,t-4-Dienoyl-CoA Isomerase. The formation of *trans*-2,*trans*-4-dodecadienoyl-CoA from Δ^3,Δ^5-dienoyl-CoA catalyzed by this enzyme was measured by the increase in absorption at 300 nm. Δ^3,Δ^5-Dienoyl-CoA

[1] Abbreviations: BSA, bovine serum albumin; GC/MS, gas chromatograph/mass spectrometry; PAGE, polyacrylamide gel electrophoresis; BA, benzamidine; DTT, dithiothreitol.

was produced *in situ* from the incubation of *cis*-5-dodecenoyl-CoA (40 μM) and acyl-CoA oxidase (0.5 unit) in 1 mL of 20 mM phosphate buffer (pH 8) at 37 °C for 10 min. Acyl-CoA oxidase possesses intrinsic isomerase activity, which converted the *cis*-5,*trans*-2-dodecadienoyl-CoA initially formed to a mixture of isomeric Δ^3,Δ^5-dienoyl-CoA's in 5–6 min of incubation. The reaction was started with the addition of the enzyme extract. An extinction coefficient of 15×10^3 M^{-1} cm^{-1} for *trans*-2,*trans*-4-dienoyl-CoA at 300 nm was used for the calculation; this value was determined experimentally from a solution of *trans*-2,*trans*-4-dienoyl-CoA.

(2) *Δ^3,Δ^2-Enoyl-CoA Isomerase.* This enzyme activity was assayed at 37 °C as described in the literature (Palosaari et al., 1990) with some modifications. The assay mixture contained 17 μmol of EDTA, 2 μmol NAD, 0.4 unit of β-hydroxy-acyl CoA dehydrogenase, 0.4 unit of crotonase, and *trans*-3-hexenoyl-CoA (60 nmol) in 1 mL of 0.17 M Tris buffer (pH 9).

(3) 2,4-Dienoyl-CoA Reductase. The decrease in absorbance at 340 nm followed by the conversion of NADPH to $NADP^+$ at 37 °C was used (Kimura et al., 1984). A typical assay mixture contained NADPH (0.1 mg), BSA (0.1 mg), and the enzyme in 1 mL of 10 mM phosphate buffer (pH 7.4). The reaction was started by adding 40 nmol of *trans*-2,*trans*-4-decadienoyl-CoA.

(4) Epimerase. The activity for the epimerization of 3-hydroxyacyl-CoA by the combined action of crotonase and 3-hydroxyacyl-CoA dehydratase was measured from the formation of NADH at 340 nm. An assay mixture contained 17 μmol of EDTA, 4 nmol of rotenone, 2 μmol of NAD, 0.4 unit of 3-hydroxyacyl-CoA dehydrogenase, and 40 nmol of D,L-3-hydroxydecanoyl-CoA in 1 mL of 0.17 M Tris buffer (pH 9.0). The mixture was incubated at 37 °C to remove L-3-hydroxydecanoyl-CoA, and then the reaction was started with the addition of enzyme.

Other Measurements. (1) Native and Subunit Molecular Weights. The native molecular weight of the enzyme was determined by gel filtration through a column of Sepharose CL-6B (2.6 × 95 cm). The subunit molecular weight was determined by SDS/PAGE with 12% polyacrylamide gel using a Mini-Protean II cell (Bio-Rad). Proteins were stained with 0.1% Coomassie Blue R-250. Protein concentrations were determined by Lowry's method using the Bio-Rad reagent. Bovine serum albumin was used as the standard.

(2) Chromatofocusing and Isoelectric Focusing. The sample was applied to a chromatofocusing PBE 94 column (0.6 × 14 cm) in equilibrium with 0.025 M imidazole hydrochloride (pH 7.4). The enzyme was then eluted with Polybuffer 74 (pH 4). The enzyme activity and pH of each tube were measured. The isoelectric point was also determined by isoelectric focusing in the polyacrylamide gel with ampholyte 5/7 (pH 6–8) and a Bio-Rad Model 111 mini IEF cell.

(3) K_m and V_{max} of Enzyme. The substrates used were *cis*-5-decenoyl-CoA (0.72–43 μM), *cis*-5-dodecenoyl-CoA (0.34–41 μM), and *cis*-5-tetradecenoyl-CoA (0.33–10 μM). K_m and V_{max} were calculated using the Grafit computer program (version 3.0, Erithacus Software, Staines, U.K.).

Isolation of Subcellular Particles. Fresh rat liver was minced and homogenized in a mixture of MSM buffer (220 mM mannitol, 70 mM sucrose, and 5 mM Mops, pH 7.4) with EDTA (2 mM). The homogenate was centrifuged at 400*g* to remove debris and then centrifuged further at 7000*g* to obtain pellets of mitochondria. These mitochondria pellets were washed twice with MSM buffer and then suspended in the same solution. The supernatant from 7000*g* was centrifuged further at 32000*g* for 10 min (twice) to obtain pellets of the light mitochondrial fraction. This fraction was enriched in peroxisomes. The supernatant from 32000*g* centrifugation was further centrifuged at 105000*g* for 60 min to yield pellets of microsomes. Fresh rat heart, after it was rinsed with Chappel-Perry buffer (Tomec & Hoppel, 1975), was minced in 10-fold of the weight of the same buffer and then treated with nagarse (5 mg/g of wet weight) for 30 min. It was homogenized and centrifuged at 12000*g*. Pellets were further fractionated as described for liver to obtain mitochondria.

Metabolic Studies. The studies were carried out in 25-mL Erlenmeyer flasks in a metabolic shaking incubator (110 cycles/min) at 37 °C. Each flask contained acyl-CoA (100–160 μM), acyl-CoA oxidase (0.05–0.5 unit/mL), and purified enzymes in either 20 mM phosphate buffer (pH 8.0) or 0.1 M phosphate buffer (pH 7.4). The reaction was stopped by the addition of 1 N KOH (120 μL/mL of incubation mixture).

Metabolic Profiling of the Incubation Mixture. An aliquot (1 mL) of the incubation mixture, pretreated with KOH, was mixed with internal standard (20 μg of pentadecanoic acid) and hydrolyzed at room temperature for 50 min. The mixture was acidified and extracted with ethyl acetate:diethyl ether (1:1). After conversion to trimethylsilyl derivatives, the sample was analyzed with a dual capillary column gas chromatograph (Model 5890, Hewlett-Packard, Avondale, PA), using bonded dimethylpolysiloxane phase (SPB-1 from Supelco, Bellefonte, PA) and bonded 35% diphenyl-:65% dimethylpolysiloxane phase (SPB-35) fused silica capillary columns (also available from Supelco with custom order). The initial column temperature was 60 °C and was increased at 4 °C/min to 250 °C with a 50 to 1 split injection ratio. The quantitation was based on the relative peak area to internal standard. The amount of metabolites was calculated as the weight equivalent to that of internal standard on the basis of the area ratio.

A Hewlett-Packard 5985B gas chromatograph/mass spectrometer was used for metabolite identification and isotope enrichment determination. A shorter (12.5 m) fused silica capillary column (SPB-1) was used with the same temperature program that was described for GC. Electron-impact (70 eV) ionization and repetitive scanning (300 atomic mass units/s) from *m/z* 49 to 550 were used for obtaining mass spectra. The criteria for identification were that the retention times on both GC columns and the mass spectra were identical to those obtained from authentic samples.

Selected Ion Monitoring Determination of Deuterium Enrichments in Metabolites. Derivatized samples were separated through the SPB-1 capillary column. The column temperature was at 140 °C initially and then was increased at a rate of 4 °C/min after injection. The chromatographic effluent was monitored at *m/z* 225, 226, 227, and 228 at a dwell time of 100 ms for each ion. Peak areas were determined and normalized to the peak area of unlabeled ion (as 100). Metabolites produced from the incubation of unlabeled substrate were also analyzed to obtain natural abundance isotope enrichments. For the calculation of the percentage enrichment of each labeled species, the relative peak areas were corrected for the natural isotope abundance contribution by the technique described previously (Tserng & Kalhan, 1983).

RESULTS

Isolation and Purification of Δ^3,Δ^2-Enoyl-CoA Isomerase and Δ^3,Δ^5-t-2,t-4-Dienoyl-CoA Isomerase from Rat Liver Homogenate. Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA isomerase was iso-

Table 1: Purification of Δ^3,Δ^2-Enoyl-CoA Isomerase (Isomerase I) and Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA Isomerase (Isomerase II) from the Homogenate of 18.7 g of Frozen Rat Liver[a]

		isomerase I			isomerase II		
purification steps	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	total activity (units)	specific activity (units/mg)	yield (%)
ammonium sulfate	1420	718	0.51	100	245	0.17	100
Matrex gel red A	604	709	1.2	99	157	0.26	64
blue Sepharose gradient	92	166	1.8	23.3			
	404				124	0.31	49
CM-cellulose before gradient	240	9.6	0.04	1.3	80	0.33	33
CM-cellulose gradient	2.9	22.5	7.8	3.1			
DEAE-cellulose gradient	33.8				60	1.8	25
hydroxylapatite	6.3				48	7.6	20
gel filtration	4.2				30	7.1	13

[a] Rats were on regular Rat Chow. Isomerase I activity was measured with *trans*-3-hexenoyl-CoA as substrate; isomerase II activity was measured with Δ^3,Δ^5-dodecadienoyl-CoA as substrate. 1 unit of enzyme activity is defined as 1 μmol of substrate conversion per minute at 37 °C. Abbreviations: isomerase I, Δ^3,Δ^2-enoyl-CoA isomerase; isomerase II, Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase.

lated starting with liver homogenate from both clofibrate-treated and nontreated rats. Since clofibrate stimulated isomerase activity, the yield of these enzymes was higher when liver homogenate from clofibrate-treated rats was used. When isolated mitochondria were used, pure enzymes could be obtained in fewer steps. However, the total yields were lower due to the loss of mitochondria in isolation. Therefore, liver homogenate from untreated rats was the most convenient starting material for the isolation of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase.

Under low-salt conditions, all fatty acid enzymes were adsorbed on the Matrex gel red A column. The absorbed enzymes were then eluted with a linear gradient of potassium chloride. Δ^3,Δ^2-Enoyl-CoA isomerase and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activities were only slightly separated and were pooled together. Blue Sepharose separated the activity of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase from that of Δ^3,Δ^2-enoyl-CoA isomerase. Therefore, either Matrex gel red A or blue Sepharose can be used as the first step for purification. A combination of both Matrex gel red A and blue Sepharose columns in successive steps did not improve the subsequent purification steps.

The CM-cellulose column retained Δ^3,Δ^2-enoyl-CoA isomerase under the conditions of 50 mM phosphate buffer at pH 6.0. The majority of the fatty acid enzymes, together with Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, were not retained. Apparently homogeneous Δ^3,Δ^2-enoyl-CoA isomerase was obtained after gradient elution with a higher content of salt. When Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase is the only enzyme to be isolated, this step of CM-cellulose chromatography can be omitted.

The essential step in the purification of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was DEAE-cellulose chromatography, which separated Δ^3,Δ^2-enoyl-CoA isomerase from Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase on the basis of the large difference in their p*I* values. At pH 6.6 of the buffer eluent, Δ^3,Δ^2-enoyl-CoA isomerase was not retained. The retained Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was eluted with buffer gradient. These fractions were further purified with hydroxylapatite gel chromatography and gel filtration. The purification steps are summarized in Table 1.

Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA isomerase isolated from this procedure was apparently homogeneous under SDS/polyacrylamide gel electrophoresis (Figure 2), with a subunit molecular weight of 55 000. The native molecular weight was determined to be 200 000 from gel filtration. Using chromatofocusing column chromatography, the enzyme activity for Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was eluted at

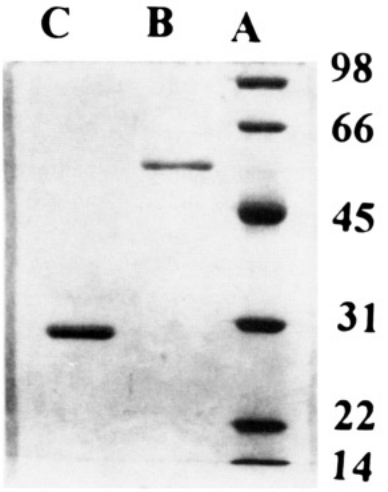

FIGURE 2: Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of purified Δ^3,Δ^2-dienoyl-CoA isomerase (1.8 μg, C), Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (1.1 μg, B), and molecular weight markers (A). The molecular weight marker enzymes were rabbit muscle phosphorylase *b* (98 000), bovine serum albumin (66 000), hen egg white ovalbumin (45 000), bovine carbonic anhydrase (31 000), soybean trypsin inhibitor (22 000), and hen egg white lysozyme (14 000).

pH 6.4. The isoelectric point (p*I*) determined using isoelectric focusing electrophoresis was 6.5. The purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was without Δ^3,Δ^2-enoyl-CoA isomerase activity. Likewise, the purified Δ^3,Δ^2-enoyl-CoA isomerase did not contain Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity. The enzyme activities were proportional to the amount of protein, at least from 0.6 to 4.6 μg/mL.

Substrate Specificity. Three substrates, i.e., Δ^3,Δ^5-decadienoyl-CoA, Δ^3,Δ^5-dodecadienoyl-CoA, and Δ^3,Δ^5-tetradecadienoyl-CoA, were generated from incubation of the corresponding *cis*-5-enoyl-CoA with acyl-CoA oxidase. Under these conditions, complete conversion to Δ^3,Δ^5-enoyl-CoA occurred. The V_{max} values of all three substrates were similar, with a ratio of 1:0.5:0.4 for the C_{10}, C_{12}, and C_{14} substrates, respectively. K_m values were 10.9 μM for C_{10}, 5.9 μM for C_{12}, and 1.4 μM for C_{14} substrates, respectively. Therefore, the enzyme seems to be more specific for the metabolism of *cis*-5-tetradecenoyl-CoA, a metabolic intermediate from the β-oxidation of oleic acid.

Location of Enzyme Activity in Rat Liver and Heart Organelles. The activity of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was measured in mitochondria, the light mitochondria fraction, microsomes, and cytosol isolated from rat liver, as well as in mitochondria isolated from rat heart. The

Table 2: Enzyme Activity (nmol/min/mg of protein, mean ± SD) in Subcellular Particles Isolated from Rat Liver and Heart[a]

	control (n = 3)			clofibrate (n = 3)			fasting (n = 3)		
enzymes	LRLM	RLM	RHM	LRLM	RLM	RHM	LRLM	RLM	RHM
isomerase I	21 ± 11	193 ± 35	400 ± 10	133 ± 12*	683 ± 47*	470 ± 17	20 ± 4	253 ± 93	333 ± 57
isomerase II	7 ± 5	53 ± 4	124 ± 5	20 ± 8*	193 ± 34*	157 ± 21	11 ± 2	82 ± 11*	99 ± 6
reductase	2 ± 1	7 ± 1	5 ± 2	7 ± 0.3*	10 ± 3	4.5 ± 0.5	4.3 ± 1.9	6 ± 1	2.9 ± 0.3
epimerase	13 ± 1	34 ± 10	7 ± 3	45 ± 18*	47 ± 4	12.3 ± 1*	25 ± 2*	30 ± 2	6 ± 2

[a] Abbreviations: isomerase I, Δ^3,Δ^2-enoyl-CoA isomerase; isomerase II, Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase; reductase, 2,4-dienoyl-CoA reductase; epimerase, 3-hydroxyacyl-CoA epimerase (combined action of crotonase and D-3-hydroxyacyl-CoA dehydratase); LRLM, rat liver light mitochondria fraction; RLM, rat liver mitochondria; RHM, rat heart mitochondria. *, significantly different ($p < 0.05$) from control values when analyzed with student's *t*-test.

activities of other enzymes related to the oxidation of unsaturated fatty acids were also determined for comparison. No Δ^3,Δ^5-*t*-2,*t*-4--dienoyl-CoA isomerase activity was found in cytosol or microsomes. The rest of the results are shown in Table 2. Like Δ^3,Δ^2-enoyl-CoA isomerase, the activity of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase appeared to be located in mitochondria in both rat liver and rat heart.

Effects of Clofibrate Treatment and Fasting on the Activities of Δ^3,Δ^5-t-2,t-4-Dienoyl-CoA Isomerase and Related Enzymes. The effects of clofibrate treatment and fasting on isomerase activity are also shown in Table 2. Fasting induced rat liver Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase slightly ($p < 0.02$). Treatment with clofibrate induced liver mitochondrial Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase 4-fold ($p < 0.002$); this compared to a 3-fold increase in Δ^3,Δ^2-enoyl-CoA isomerase activity ($p < 0.0001$). In contrast, no significant increase was observed in heart mitochondria for either enzyme. Palosaari et al. (1990) also reported a significant induction of rat liver Δ^3,Δ^2-enoyl-CoA isomerase activity by clofibrate and the absence of induction in heart enzyme. The activities of other fatty acid enzymes were either unchanged or only slightly elevated under our study conditions. Of these enzymes, 2,4-dienoyl-CoA reductase has been reported to be induced significantly in clofibrate-treated rat liver (Mizugaki et al., 1982; Dommes et al., 1981). The difference from our results could be a higher dose or longer treatment duration used in other studies. Nevertheless, it appears that the induction of isomerase activities preceded that of 2,4-dienoyl-CoA reductase when rats were treated with clofibrate. Epimerase was not induced by clofibrate; the same conclusion was reported by Mizugaki et al. (1982),

Metabolic Conversion from cis-5-Decenoyl-CoA to trans-2,trans-4-Decadienoyl-CoA. In these experiments, *trans*-2,*cis*-5-decadienoyl-CoA was formed from the incubation of *cis*-5-decenoyl-CoA with acyl-CoA oxidase isolated from *Arthrobacter*. *trans*-2,*cis*-5-Decadienoyl-CoA was gradually isomerized to *trans*-3,*cis*-5-decadienoyl-CoA and *cis*-3,*cis*-5-decadienoyl-CoA (identified by comparison with synthetic compounds after mild alkaline hydrolysis). This isomerization was catalyzed by acyl-CoA oxidase (Figure 3A).

The addition of purified Δ^3,Δ^2-isomerase to the incubation mixture together with acyl-CoA oxidase facilitated the conversion of *trans*-2,*cis*-5-decadienoyl-CoA to *trans*-3,*cis*-5-decadienoyl-CoA (Figure 3B). Only a negligible amount of *trans*-2,*trans*-4-decadienoyl-CoA was formed; this is consistent with the enzyme assay that purified Δ^3,Δ^2-enoyl-CoA isomerase did not contain Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity. The conversion to *trans*-2,*trans*-4-decadienoyl-CoA required the presence of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. When Δ^3,Δ^2-enoyl-CoA isomerase was omitted from the incubation (Figure 3C), the action of acyl-CoA oxidase and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase can produce *trans*-2,*trans*-4-decadienoyl-CoA, but the rate of formation was significantly slower than when Δ^3,Δ^2-enoyl-CoA isomerase was included in the incubation (Figure 3D). Therefore, the conversion from *cis*-5-enoyl-CoA to 2,4-dienoyl-CoA can occur without the participation of Δ^3,Δ^2-enoyl-CoA isomerase.

Table 3: Deuterium Enrichments (%) of Metabolites Produced from the Incubation of [2,2′-$^2H_2$]-*cis*-5-Decenoyl-CoA[a]

	t3c5MC10		t2c5MC10		t2t4MC10	
conditions	d_0	d_1	d_0	d_1	d_0	d_1
A	69	31	11	89		
B	67	33	11	89		
C (m/z 225)	67	33	11	89	66	34
C (m/z 169)					64	35

[a] [2,2′-$^2H_2$]-*cis*-5-Decenoyl-CoA (160 μM; enrichment: d_0, 3%; d_1, 11%; d_2, 86%) was incubated in 20 mM phosphate buffer (pH 8.0) at 37 °C in a shaking incubator for 15 min: A, with acyl-CoA oxidase (0.05 unit/mL); B, with acyl-CoA oxidase (0.05 unit/mL) and Δ^3,Δ^2-enoyl-CoA isomerase (0.17 unit/mL); C, with acyl-CoA oxidase (0.05 unit/mL), Δ^3,Δ^2-enoyl-CoA isomerase (0.17 unit/mL), and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (0.3 unit/mL). The metabolite concentrations were measured with capillary column gas chromatography, and the enrichments were determined by monitoring M − 15 ions with selected ion monitoring gas chromatography/mass spectrometry. The ions measured were m/z 225 (d_0), 226 (d_1), and 227 (d_2) for all metabolites. No m/z 227 enrichments were observed for any of these metabolites. In addition, m/z 169 (d_0), 170 (d_1), and 171 (d_2) ions were monitored for t2t4MC10. The enrichments were corrected for natural isotope abundance as described in the Materials and Methods. Abbreviations: t2c5MC10, *trans*-2,*cis*-5-decadienoate; t3c5MC10, *trans*-3,*cis*-5-decadienoate; c3c5MC10, *cis*-3,*cis*-5-decadienoate.

Deuterium Enrichments in Products from Incubation of [2,2′-$^2H_2$]-cis-5-Decenoyl-CoA. As shown in Table 3, *trans*-2,*cis*-5-decadienoic acid produced from 2,2′-d_2-labeled precursor contained only one deuterium with the same enrichment as the original d_2 label. However, the isomerized products, i.e., 3,5-decadienoic acids, contain less than half as much d_1 label as the precursor. The addition of Δ^3,Δ^2-enoyl-CoA isomerase facilitated the conversion of 2,5- to 3,5-dienoyl-CoA, but did not change the deuterium enrichment pattern of the products. This table also shows that the deuterium enrichment of *trans*-2,*trans*-4-decadienoic acid, formed after the addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, had the same pattern as 3,5-decadienoic acids.

Deuterium Enrichments in Products from the Incubation of cis-5-Decenoyl-CoA in Deuterium Oxide. *trans*-2,*cis*-5-Decadienoic acid did not contain measurable deuterium enrichment in these experiments. In contrast, 3,5-decadienoic acid contained 31–41% d_1 and 51–66% d_2. Likewise, *trans*-2,*trans*-4-decadienoic acid also contained the same deuterium enrichment pattern as 3,5-decadienoic acids (Table 4). When m/z 169 vs 170 fragments were measured for *trans*-2,*trans*-4-decadienoic acid, the enrichment pattern shifted to d_0 and d_1 with the same ratio as the d_1/d_2 measured from the m/z 225 isotope cluster. This indicated that one of the deuterium

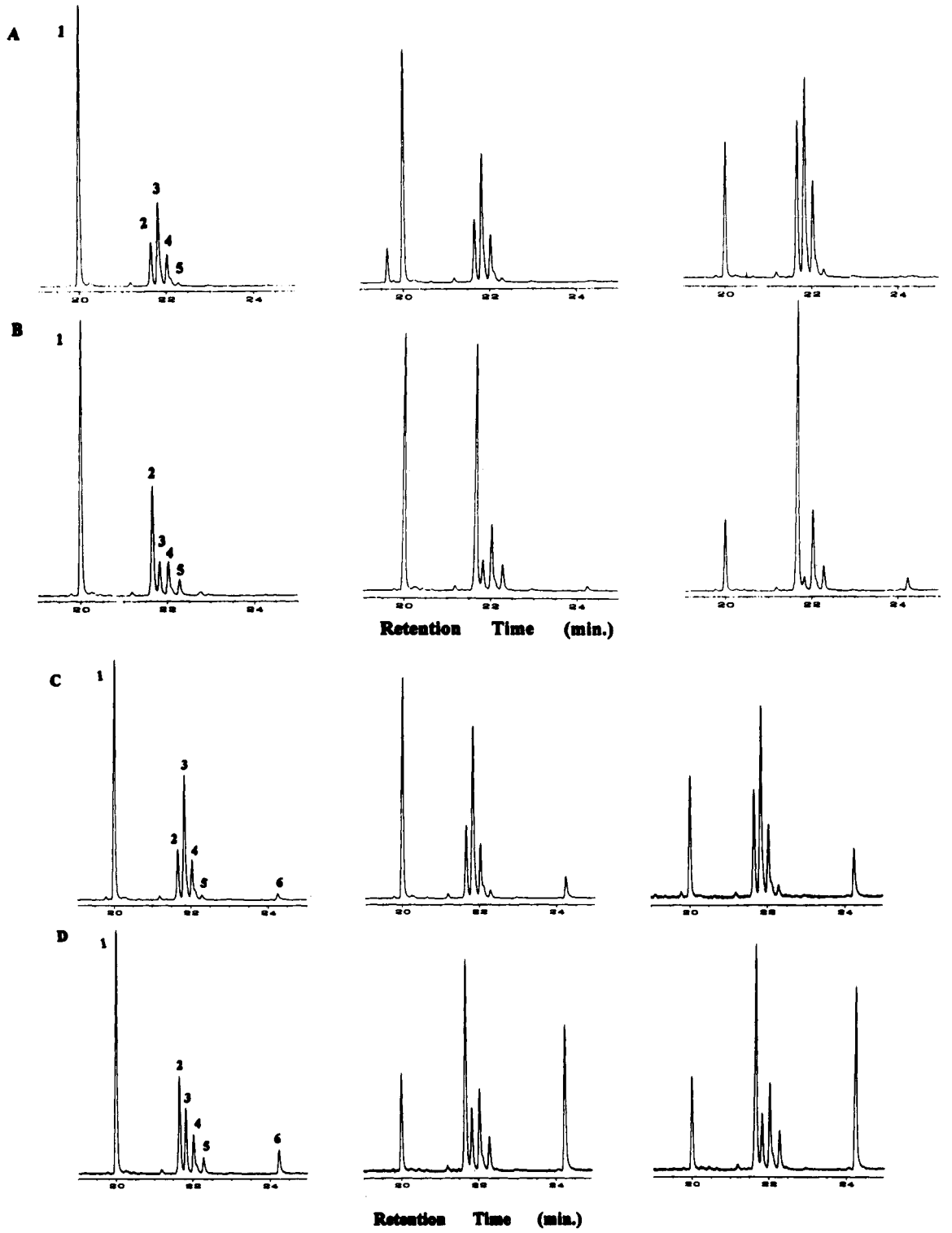

FIGURE 3: Gas chromatographic analysis of metabolites produced from the incubation of *cis*-5-decenoyl-CoA (100 μM) in 1 mL of 20 mM phosphate buffer at pH 8.0 with (A) acyl-CoA oxidase (0.05 unit), (B) acyl-CoA oxidase (0.05 unit) and Δ^3,Δ^2-dienoyl-CoA isomerase (71 munits), (C) acyl-CoA oxidase (0.05 unit) and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (35 munits), and (D) acyl-CoA oxidase (0.05 unit), Δ^3,Δ^2-dienoyl-CoA isomerase (71 munits), and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (35 munits). The incubation times were (from left to right) 5, 10, and 15 min. The substrate and metabolites are as follows: 1, *cis*-5-decenoate; 2, *trans*-3,*cis*-5-decadienoate; 3, *trans*-2,*cis*-5-decadienoate; 4, *cis*-3,*cis*-5-decadienoate; 5, *trans*-3,*trans*-5-decadienoate; and 6, *trans*-2,*trans*-4-decadienoate.

labels on *trans*-2,*trans*-4-decadienoic acid was located on a carbon other than carbons 1–5; the most likely location would be C-6 of the molecule. The location of the second deuterium on C-6 of 2,4-dienoyl-CoA is consistent with the labeling pattern of mass fragment *m/z* 169. This fragment is generated from the loss of $CH_2CH_2CH_2CH_2$ (carbon 6–9) from the M – 15 ion. Only d_0 and d_1 with the same ratio as d_1/d_2 of the M – 15 ion cluster were found on this mass fragment. This indicated that an additional deuterium on the neutral fragment (mass 56) was lost from C-6 to C-9.

DISCUSSION

A new enzyme, i.e., Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, required in the NADPH-dependent metabolic pathway of odd-numbered double bond, unsaturated fatty acids, was isolated and purified to apparent homogeneity from rat liver. Unlike

Table 4: Deuterium Enrichments (%) of Metabolites Produced from the Incubation of Unlabeled *cis*-5-Decenoyl-CoA with Acyl-CoA Oxidase Plus Isomerase I and Isomerase II in D_2O[a]

conditions	metabolites	d_0	d_1	d_2
isomerase I	t3c5MC10	4	32	55
isomerase I + isomerase II	t3c5MC10	12	55	33
	t2t4MC10 (*m*/*z* 225)	12	55	33
	t2t4MC10 (*m*/*z* 169)	59	39	0

[a] *cis*-5-Decenoyl-CoA (70 μM) was incubated with acyl-CoA oxidase (0.05 unit/mL) in 20 mM phosphate buffer (pH 8.0) at 37 °C for 15 min. For t3c5MC10, c3c5MC10, and t2t4MC10, the isotope enrichments were determined by monitoring *m*/*z* 225 (d_0), 226 (d_1), 227 (d_2), and 228 (d_3) with selected ion monitoring gas chromatography/mass spectrometry. For t2t4MC10, an additional ion cluster, *m*/*z* 169 (d_0), 170 (d_1), and 171 (d_2), was determined. Isomerase I (0.17 unit/mL) and isomerase II (0.3 unit/mL) were added singly or simultaneously. Abbreviations: see Tables 1 and 3; t2,t4MC10, *trans*-2,*trans*-4-decadienoate.

Δ^3,Δ^2-enoyl-CoA isomerase, which is required in the isomerase-mediated pathway of odd-numbered double bond, fatty acids and the 2,4-dienoyl-CoA reductase dependent pathway of even-numbered double bond, unsaturated fatty acids, this new isomerase had a much more acidic isoelectric point (p*I*) of 6.5, while that of Δ^3,Δ^2-enoyl-CoA isomerase was reported to be 9.5 (Palosaari et al., 1990) or 9.0–9.2 (Stoffel & Grol, 1978). The native molecular weight of this isomerase is 200 000, with four apparent identical subunits of 55 000. In contrast, Δ^3,Δ^2-enoyl-CoA isomerase has a native molecular weight of 60000–80000, with two identical subunits of 31 000 (9,19), while the peroxisomal trifunctional enzyme of rat liver has a native molecular weight of 78 000 with only one subunit (Palosaari & Hiltunen, 1990). Even though this new enzyme had a native molecular weight similar to the long-chain Δ^3,Δ^2-enoyl-CoA isomerase isolated by Kilponen et al. (1990), it is highly unlikely that these two enzymes are identical since our Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase did not show Δ^3,Δ^2-enoyl-CoA isomerase activity. Furthermore, the long-chain-specific Δ^3,Δ^2-enoyl-CoA isomerase was not inducible by clofibrate, while our enzyme activity was significantly induced by the treatment.

After the investigation described here was completed, another publication describing the purification of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase from rat liver appeared (Luo et al., 1994). However, the subunit molecular weight (32 000) and native molecular weight (126 000) were different from ours. The cause of the discrepancy is not known, but it is likely due to proteolytic hydrolysis. In fact, in our studies, another protein with a subunit molecular mass of 70 kDa, isolated from fresh rat liver, was found to possess the activity of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. This latter enzyme was also observed to be converted to the 55-kDa protein with repeated freezing and thawing. Further investigations to clarify these discrepancies are needed.

The NADPH-dependent pathway appears to occur in the following sequence: An odd-numbered double bond, unsaturated fatty acid is metabolized by β-oxidation to *cis*-5-enoyl-CoA, which is then dehydrogenated to *trans*-2,*cis*-5-dienoyl-CoA. *trans*-2,*cis*-5-Dienoyl-CoA is either hydrated to 3-hydroxy-*cis*-5-enoyl-CoA or isomerized to Δ^3,Δ^5-dienoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase. In the former pathway, 3-hydroxy-*cis*-5-enoyl-CoA is converted by the action of 3-hydroxyacyl-CoA dehydrogenase and 3-ketothiolase to acetyl-CoA and *cis*-3-enoyl-CoA, which is then isomerized to *trans*-2-enoyl-CoA. In the latter pathway, Δ^3,Δ^5-dienoyl-CoA is isomerized to trans-2,4-dienoyl-CoA by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. Starting with this step, the pathway is the same as that for even-numbered double bond, unsaturated fatty acids, i.e., *trans*-2,4-dienoyl-CoA is reduced by 2,4-dienoyl-CoA reductase to *trans*-3-enoyl-CoA and then isomerized to *trans*-2-enoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase.

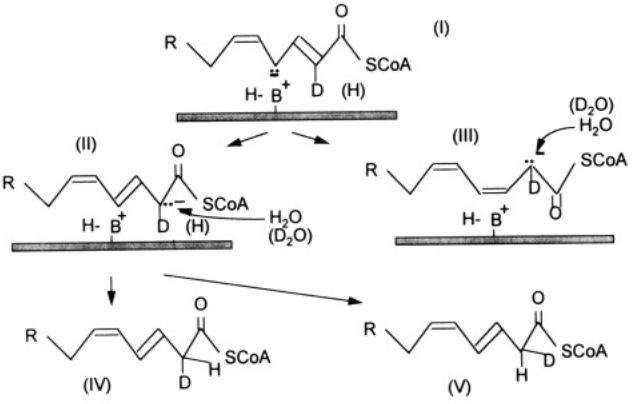

FIGURE 4: Mechanism for the isomerization of *trans*-2,*cis*-5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA catalyzed by acyl-CoA oxidase or Δ^3,Δ^2-dienoyl-CoA isomerase.

Conversion from trans-2,cis-5-Decadienoyl-CoA to trans-2,trans-4-Decadienoyl-CoA. Direct analysis of the products using capillary column gas chromatography was used in the present investigation. As shown in Figure 3, the incubation of *cis*-5-decenoyl-CoA with acyl-CoA oxidase produced not only *trans*-2,*cis*-5-decadienoate as expected but also the isomerized products, i.e., *trans*-3,*cis*-5- and *cis*-3,*cis*-5-decadienoates. The addition of purified Δ^3,Δ^2-enoyl-CoA isomerase facilitated the conversion from 2,5 to 3,5 and also changed the product distribution with significantly more *trans*-3,*cis*-5 than *cis*-3,*cis*-5 isomer. When purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was also added, the production of *trans*-2,*trans*-4-decadienoate was faster from those incubations with Δ^3,Δ^2-enoyl-CoA isomerase than those with acyl-CoA oxidase alone. This observation implied that the conversion is probably mediated through an intermediate, 5,3-dienoyl-CoA, instead of through the direct migration of a 5,6-double bond to a 4,5-double bond from Δ^2,Δ^5-dienoyl-CoA. This is consistent with the suggestion made by Smeland et al. (1992) on the basis of their experiments with spectrophotometric analysis of the isomerization of *cis*-5-octenoyl-CoA.

Mechanism of Isomerization. Consistent with the data shown in Tables 3 and 4, the mechanism of isomerization catalyzed by acyl-CoA oxidase, Δ^3,Δ^2-enoyl-CoA isomerase, and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase is proposed. Acyl-CoA oxidase abstracts a proton from C-2 to form singly labeled (d_1) *trans*-2,*cis*-5-decadienoate from d_2-labeled *cis*-5-decenoyl-CoA. In the next step, the abstraction of a proton from C-4 of *trans*-2,*cis*-5-decadienoyl-CoA (structure I) by a basic amino acid residue in the active site of either acyl-CoA oxidase or Δ^3,Δ^2-enoyl-CoA isomerase (Muller-Newton & Stoffel, 1993) forms a transit carbanion, which is stabilized by resonance structures II and III from the migration of a 2,3- to a 3,4-double bond (Figure 4). The abstraction of a proton from water to C-2 finishes the sequence to yield *trans*-3,*cis*-5-dienoyl-CoA and *cis*-3,*cis*-5-dienoyl-CoA. Apparently, the abstraction of a proton from water to C-2 can be accomplished from either side of the transit carbanion (March, 1985), resulting in racemization of the products (IV and V from II). Therefore, the *initial* products formed have *pro-R* and *pro-S* deuterium, at C-2, with a distribution ratio determined by the side of the protium abstraction, but the total d_1 enrichment is the same as that of the precursor. However, the *pro-R* deuterium immediately exchanged with water (Ikeda et al., 1985); this exchange is catalyzed by enzymes. Therefore, the

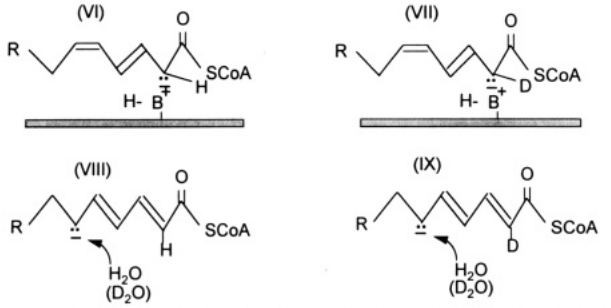

FIGURE 5: Mechanism for the isomerization of Δ^3,Δ^5-dienoyl-CoA to *trans*-2,*trans*-4-dienoyl-CoA catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase.

final products contained less deuterium than the precursor. On the other hand, when the experiments were conducted in D_2O using unlabeled substrate, the same mechanism produced d_1- and d_2-labeled 3,5-dienoyl-CoA

Mechanism of Action of Δ^3,Δ^5-t-2,t-4-Dienoyl-CoA Isomerase. The isomerization from 3,5-dienoyl-CoA to *t*-2,*t*-4-dienoyl-CoA catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase is initiated by the abstraction of a *pro-R* proton at C-2 to form carbanion, as shown in Figure 5, which is stabilized by resonance structures VIII and IX. The abstraction of a proton from water to C-6 following the migration of double bonds would not change the deuterium labeling of the product *t*-2,*t*-4-dienoyl-CoA; therefore, the d_1/d_0 ratio in the product is identical to the original d_1/d_0 ratio in 3,5-dienoyl-CoA. Likewise, unlabeled substrate in D_2O would lead to a new deuterium label at C-6, but with the same d_2/d_1 ratio as for 5,3-dienoyl-CoA from the loss of a deuterium label on C-2. Our data indicate that the proton abstracted during isomerization is derived from water in the incubation medium. In contrast to the data reported by Miesowicz and Bloch (1979) on the mechanism of isomerases isolated from pig liver, no evidence indicates an intramolecular transfer of hydrogen.

CONCLUSION

The deuterium enrichment pattern in *trans*-2,*trans*-4-decadienoate formed from the isomerization of *trans*-2,*cis*-5-decadienoyl-CoA catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase is the same as that in 3,5-decadienoate; therefore, the isomerization from *trans*-2,*cis*-5 to *trans*-2,*trans*-4 apparently is mediated through a two-step process via the intermediate 3,5-decadienoate. The direct isomerization of the 5,6-double bond from *trans*-2,*cis*-5-decadienoyl-CoA to a 4,5-double bond would be expected to result in a product an isotopic distribution similar to that of the *trans*-2,*cis*-5 precursor. These data further support the conclusion derived from the incubation experiments that the isomerization is a two-step process.

Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA isomerase activity was located mainly in mitochondria. Its activity is required for the metabolism of odd-numbered double bond fatty acids through the NADPH-dependent reduction pathway. The percentage contribution of this pathway to the total metabolic pathway of these unsaturated fatty acids is not known. However, whether isomerization to Δ^3,Δ^5-dienoyl-CoA or hydration to 3-hydroxy-*cis*-5-enoyl-CoA occurs is likely dependent on the branching point of the two pathways from *trans*-2,*cis*-5-dienoyl-CoA (Figure 1). Once isomerized to Δ^3,Δ^5-dienoyl-CoA, the substrate is no longer available for enoyl-CoA hydratase to yield 3-hydroxy-*cis*-5-enoyl-CoA. Instead, it is committed to the reduction pathway. The contribution of each pathway to the overall oxidation remains to be clarified in future studies.

REFERENCES

Dommes, V., Baumgart, C., & Kunau, W.-H. (1981) *J. Biol. Chem. 256*, 8259–8262.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Ikeda, Y., Hine, D. G., Okamura-Ikeda, K., & Tanaka, K. (1985) *J. Biol. Chem. 260*, 1326–1337.

Jin, S.-J., & Tserng, K.-Y. (1990) *Biochemistry 29*, 8540–8547.

Kilponen, J. M., Palosaari, P. M., & Hiltunen, J. K. (1990) *Biochem. J. 269*, 223–226.

Kimura, C., Kondo, A., Koeda, N., Yamanaka, H., & Mizugaki, M. (1984) *J. Biochem. (Tokyo) 96*, 1463–1469.

Kunau, W. H., & Dommes, P. (1978) *Eur. J. Biochem. 91*, 533–544.

Luo, M. J., Smeland, T. E., Shoukry, K., & Schulz, H. (1994) *J. Biol. Chem. 269*, 2384–2388.

March, J. (1985) *Advanced Organic Chemistry*, 3rd ed., p 156, John Wiley & Sons, New York.

Miesowicz, F. M., & Bloch, K. (1979) *J. Biol. Chem. 254*, 5868–5877.

Mizugaki, M., Nishimaki, T., Yamamoto, H., Sagi, M., & Yamanaka, H. (1982) *J. Biochem. (Tokyo) 92*, 2051–2054.

Muller-Newton, G., & Stoffel, W. (1993) *Biochemistry 32*, 11405–11412.

Palosaari, P. M., Kilponen, J. M., Sormunen, R. T., Hassinen, I. E., & Hiltunen, J. K. (1990) *J. Biol. Chem. 265*, 3347–3353.

Schulz, H. (1974) *J. Biol. Chem. 249*, 2704–2709

Smeland, T. E., Nada, M., Cuebas, D., & Schulz, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6673–6677.

Staack, H., Binstock, J. F., & Schulz, H. (1978) *J. Biol. Chem. 253*, 1827–1831.

Stoffel, W., & Grol, M. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1777–1782.

Stoffel, W., Ditzer, R., & Caeser, H. (1964) *Hoppe-Seyler's Z. Physiol. Chem. 339*, 167–181.

Tomec, R., & Hoppel, C. L. (1975) *Arch. Biochem. Biophys. 170*, 716–723.

Tserng, K.-Y., & Kalhan, S. C. (1983) *Am. J. Physiol. 245 (Endocrinol. Metab. 8)*, E308–E311.

Tserng, K.-Y., & Jin, S.-J. (1991) *J. Biol. Chem. 266*, 11614–11620.