Isomerization of *trans*-2,Δ^5-Dienoyl-CoA's to Δ^3,Δ^5-Dienoyl-CoA's in the β-Oxidation of Δ^5-Unsaturated Fatty Acids†

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Received May 17, 1994; Revised Manuscript Received August 25, 1994®

ABSTRACT: The NADPH-dependent reduction pathway for the metabolism of Δ^5-unsaturated fatty acids involves the isomerization of *trans*-2,Δ^5-dienoyl-CoA, initially formed from the dehydrogenation of Δ^5-enoyl-CoA, to isomeric Δ^3,Δ^5-dienoyl-CoA. The latter intermediates were then isomerized to *trans*-2,*trans*-4-dienoyl-CoA, which then follows the NADPH-dependent pathway mediated by 2,4-dienoyl-CoA reductase. The isomerization from *trans*-2,Δ^5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA is catalyzed by Δ^3,Δ^2-enoyl-CoA isomerase. In this investigation, we identified the stereoisomers of Δ^3,Δ^5-dienoates that were formed in the reaction. Starting from *trans*-2,*cis*-5-decadienoyl-CoA, the isomerization produced *cis*-3,*cis*-5- and *trans*-3,*cis*-5-decadienoates. On the other hand, *trans*-2,*trans*-5-decadienoyl-CoA yielded *cis*-3,*trans*-5- and *trans*-3,*trans*-5-decadienoates. In addition to purified rat liver Δ^3,Δ^2-enoyl-CoA isomerase, acyl-CoA oxidase from *Arthrobacter* also catalyzed the isomerization from *trans*-2,*cis*-5-dienoyl-CoA. However, this acyl-CoA oxidase could not catalyze the similar isomerization of *trans*-2,*trans*-5-dienoyl-CoA. Δ^3,Δ^5-*t*-2,*t*-4-Dienoyl-CoA isomerase used *cis*-3,*cis*-5-, *trans*-3,*cis*-5-, and *cis*-3,*trans*-5-dienoyl-CoA's as substrates and converted them to *trans*-2,*trans*-4-dienoyl-CoA. In contrast, *trans*-3,*trans*-5-dienoyl-CoA was not a substrate for this isomerization. Extensive purification of acyl-CoA oxidase through column chromatography could not remove or diminish the isomerization activity associated with acyl-CoA oxidase. Acyl-CoA oxidases derived from *Candida* and rat liver also possess isomerization activity. In contrast, acyl-CoA dehydrogenases from beef liver could not catalyze the isomerization. The dehydrogenation and isomerization of *cis*-5-enoyl-CoA's catalyzed by commercially available acyl-CoA oxidase preparations render the preparation of Δ^3,Δ^5-dienoyl-CoA's feasible. The data obtained so far tend to rule out the possibility that the isomerase activity of acyl-CoA oxidase was due to contaminating enzymes.

The NADPH-dependent reductive metabolism of odd-numbered double bond unsaturated fatty acids proceeds from the conversion of *trans*-2,*cis*-5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA, which is then isomerized to *trans*-2,*trans*-4-dienoyl-CoA by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase as shown in Figure 1 (Smeland et al., 1992). The competition between this reduction pathway and that of the isomerase-mediated pathway described by Stoffel et al. (1964) depends on the isomerization of the common intermediate, *trans*-2,*cis*-5-dienoyl-CoA, to Δ^3,Δ^5-dienoyl-CoA. The latter compound is not a substrate for enoyl-CoA hydratase and therefore channels the substrate to the subsequent isomerization to *trans*-2,*trans*-4-dienoyl-CoA.

The isomerization of *trans*-2,*cis*-5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA was mediated by Δ^3,Δ^2-enoyl-CoA isomerase or by peroxisomal trifunctional enzyme (Smeland et al., 1992; Luo et al., 1994; Chen et al., 1994). This isomerization of *trans*-2,Δ^5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA has been implied from indirect spectrophotometric absorption and enzyme reactions. The shift to a 240 nm absorbance maximum from around 260 nm upon the addition of peroxisomal trifunctional enzyme to a solution of *trans*-2,*cis*-

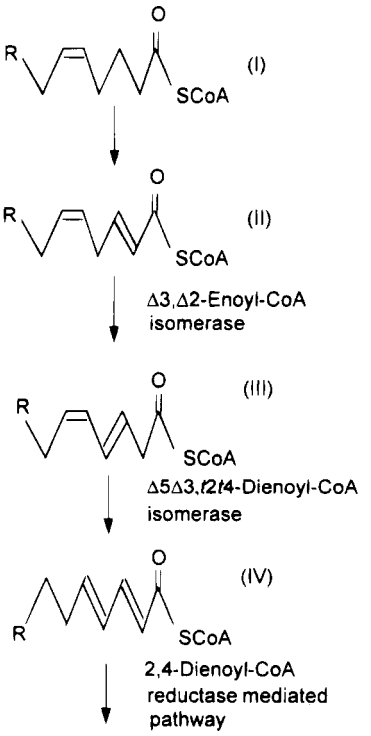

FIGURE 1: Reduction pathway for the metabolism of *cis*-5-enoyl-CoA.

† 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.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

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5-octadienoyl-CoA and the absence of hydration by subsequently added crotonase were cited as evidence that the isomerized product was 3,5-octadienoyl-CoA. Using *cis*-5- and *trans*-5-octenoyl-CoA's as substrates, Smeland et al. (1992) and Luo et al. (1994) concluded that the reactions of both substrates with acyl-CoA oxidases from *Candida* or *Arthrobacter* were identical. From their data, both *cis*-5- and *trans*-5-octenoyl-CoA's were dehydrogenated to *trans*-2,*cis*-5(*trans*-5)-octadienoyl-CoA's, but no isomerization occurred. This conclusion was based on the absorption change induced by the addition of trifunctional enzyme following the incubation with acyl-CoA oxidase. For both substrates, they observed an increase in absorption at 260 nm after incubation with acyl-CoA oxidase and a shift to the 240 nm absorption maximum only after the addition of trifunctional enzyme.

In the present investigation, we provide direct evidence that the isomerized products were Δ^3,Δ^5-dienoates. The geometric isomers of these Δ^3,Δ^5-dienoates were identified through chemical syntheses of authentic samples, capillary column gas chromatographic retentions, and mass spectrometric fragmentations. The substrate properties of isomeric Δ^3,Δ^5-dienoyl-CoA's for the subsequent isomerization, catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, to *trans*-2,*trans*-4-dienoyl-CoA were also studied. In the course of these studies, we found that acyl-CoA oxidase, used for the dehydrogenation of Δ^5-enoyl-CoA to *trans*-2,Δ^5-dienoyl-CoA, also catalyzed the subsequent isomerization of *trans*-2,*cis*-5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA's. In contrast, *trans*-2,*trans*-5-dienoyl-CoA was not isomerized by acyl-CoA oxidase preparations. The details of these isomerization reactions were studied by gas chromatography−mass spectrometry. These data were compared to spectrophotometric absorption, which was used by other investigators in previous studies (Smeland et al., 1992; Luo et al., 1994).

MATERIALS AND METHODS

Reagents and Chemicals. *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. *trans*-5-Decenoic and *cis*-5-octenoic acids were prepared by the oxidations of *trans*-5-decen-1-ol and *cis*-5-octen-1-ol, respectively (obtained from Lancaster Chemicals, Windham, NH), by Jones' reagent in acetone. Acyl-CoA esters were prepared by the modified mixed anhydride method with ethyl chloroformate and triethylamine in tetrahydrofuran (Schulz, 1974). Their concentrations were determined by Ellman's procedure (Ellman, 1959). [2,2′-$^2H_2$]-*cis*-5-Decenoic acid was synthesized as described previously (Chen et al., 1994). Δ^3,Δ^2-Enoyl-CoA isomerase and Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase were isolated from rat liver, as described in a previous communication (Chen et al., 1994).

Acyl-CoA Oxidase. Acyl-CoA oxidase (from *Arthrobacter* sp.) was purchased from Boehringer-Mannheim (Indianapolis, IN). Gel filtration through a column of Sepharose CL-6B (2.6 × 95 cm) showed a symmetrical peak (both UV absorption at 280 nm and enzyme activity). The analysis using an FPLC system (Pharmacia, Uppsala, Sweden) through a column of Superose 6 HR 10/30 (Pharmacia) showed another higher molecular weight peak (7%, based on UV absorption) with very low acyl-CoA oxidase activity (specific activity <5% of the main peak), in addition to a symmetrical peak of acyl-CoA oxidase that eluted later. Under SDS−polyacrylamide gel electrophoresis, these products (both pre- and post-FPLC separation) showed a major band of the subunit at 52 kDa molecular mass (Sztajer et al., 1993), with a trace of very diffuse bands around 23 kDa. Attempts to remove these diffuse bands by successive chromatography on Blue-Sepharose, CM-cellulose, DEAE-cellulose, hydroxylapatite, Sepharose gel filtration, and FPLC through Superose 6 HR 10/30 were unsuccessful. It is likely that the diffuse band at 23 kDa was either an integral part of the *Arthrobacter* acyl-CoA oxidase or a decomposition product of acyl-CoA oxidase formed immediately after purification or during SDS−PAGE. These products tested negative for crotonase and Δ^3,Δ^2-enoyl-CoA isomerase activities. The specific activity of prepurified acyl-CoA oxidase was 115 units/mg of protein. After purification, the specific activities were slightly higher (135 ± 18 unit/mg of protein, $n = 3$). The purified enzyme showed Δ^3,Δ^5-*t*-2,*c*-5-dienoyl-CoA isomerase activity of 0.53 ± 0.01 munit/munit of acyl-CoA oxidase; these values were not statistically different from that of the unpurified enzyme (0.52 ± 0.02 munit/munit of acyl-CoA oxidase).

Synthesis of trans-2,cis-5-Decadienoic Acid. The procedure of Heslinga et al. (1973) was used. In brief, a reaction mixture of 1-hexyne (46 mmol) and ethylmagnesium bromide (46 mmol) in tetrahydrofuran was added to 4-bromocrotonic acid (20 mmol) under the catalysis of cuprous cyanide to yield *trans*-2-decen-5-ynoic acid. This acetylenic acid was hydrogenated under the Lindlar catalyst to form *trans*-2,*cis*-5-decadienoic acid. Gas chromatographic analysis showed a single peak, and a 70 eV electron impact mass spectrum displayed a pattern identical to that of the second peak of products from the incubation of *cis*-5-decenoyl-CoA and acyl-CoA oxidase. ^1H NMR (300 MHz) showed the expected *trans* coupling (15 Hz) for $C^{2,3}$ protons and *cis* coupling (10 Hz) for $C^{5,6}$ protons (Heslinga, 1973; Tsuboi et al., 1984).

Synthesis of trans-3,cis-5-Decadienoic Acid and trans-3,trans-5-Decadienoic acid. These compounds were synthesized by three different procedures. The first procedure was adopted from that of Pfeffer and Silbert (1971) using the isomerization of *trans*-2,*trans*-4-decadienoic acid induced by lithium diisopropylamide in anhydrous tetrahydrofuran. This procedure yields a mixture of two isomers of decadienoic acid in equal proportions. Their trimethylsilyl derivatives showed RRT (relative retention time to pentadecanoate, which eluted at 34.6 min on SPB-1 and at 33.9 min on SPB-35) of 0.627/0.644 on SPB-1 and 0.667/0.684 on SPB-35. The second procedure used triethylamine-catalyzed isomerization of cyclopentyl *trans*-2,*trans*-4-decadienoate, similar to that described for *trans*-2,*trans*-4-octadienoate by Frighetto et al. (1978). This procedure also produced a 1:1 mixture of two isomers identical to that produced from the isomerization of *trans*-2,*trans*-4-decadienoate with lithium diisopropylamide. The third approach was isomerization of the ethyl ester of *trans*-2,*trans*-4-decadienoate induced by lithium diisopropylamide as described by Tsuboi et al. (1984). This reaction yielded two isomers with retention times identical to those produced from the two previous reactions, except that the earlier eluted peak was the predominant isomer. The geometry of these isomeric products has been characterized

extensively by ^1H NMR, ^{13}C NMR, and chemical conversion in these literature reports.

Enzyme Measurements. (1) Acyl-CoA oxidase activity was determined by the increase in absorption at 300 nm using *cis*-4-decenoyl-CoA or *trans*-4-decenoyl-CoA (40 μM) as substrate. The assay was performed at 37 °C, and the reaction was initiated by the addition of substrate. 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 in this laboratory.

(2) *Δ^3,Δ^5-t-2,t-5-Dienoyl-CoA isomerase* activity in acyl-CoA oxidase was estimated from the production rate of Δ^3,Δ^5-dienoates in the incubation of *cis*-5-decenoyl-CoA with acyl-CoA oxidase at 37 °C and pH 8.0. Δ^3,Δ^5-Dienoates were determined by capillary column gas chromatography. The production rates were determined only at the portion of the reaction time curve that still contained un-reacted *cis*-5-decenoyl-CoA; this usually occurred before 1−2 min of incubation. Since the intrinsic Δ^3,Δ^5-*t*-2,*t*-5-dienoyl-CoA isomerase activity is proportional to the activity of acyl-CoA oxidase, the measured activity of dienoyl-CoA isomerase was normalized to the activity of acyl-CoA oxidase toward *cis*-5-decenoyl-CoA as substrate and was expressed as milliunits/milliunit of acyl-CoA oxidase.

(3) 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 was produced 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. The reaction was started with the addition of the enzyme extract.

(4) Δ^3,Δ^2-Enoyl-CoA Isomerase. This enzyme's activity was assayed at 37 °C as described in the literature (Palosaari et al., 1990), with some modification. The assay mixture contained 17 μmol of EDTA, 2 μmol of NAD, 0.4 unit of β-hydroxyacyl-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). Crotonase was measured by the hydration of crotonyl-CoA (Wakil & Mahler, 1954), coupled with dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase and conversion of NAD to NADH. One unit of enzyme activity is defined as the conversion of 1 μmol of substrate per minute.

Other Measurements. Spectrophotometric measurements were performed with a Hewlett-Packard 8452A diode array spectrophotometer with a 1 mL cuvette maintained at 37 °C.

Metabolic Studies. The studies were carried out in 25 mL Erlenmeyer flasks in a metabolic shaking incubator (140 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 phosphate buffer. 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 an 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 dual capillary column gas chromatography (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 increased at 4 °C/min to 250 °C with a 50:1 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 an 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 as that described for GC. Electron impact (70 eV) ionization and repetitive scanning (300 atomic mass units/s) from *m/z* 49 to 550 was used for obtaining mass spectra. The criteria for identification of a compound were that the retention times on both columns in the GC and the mass spectra must be identical to those obtained from authentic samples.

Selected Ion Monitoring Determination of Deuterium Enrichments in Metabolites. Derivatized samples were separated on a column of the SPB-1 capillary gas chromatograph as described in the preceding section. The column temperature was 140 °C initially and then increased at a rate of 4 °C/min after injection. Chromatographic effluent was monitored at *m/z* 225 (d_0), 226 (d_1), and 227 (d_2) at a dwell time of 100 ms for each ion for decadienoic acids, while *m/z* 233, 234, and 235 were used for 3-hydroxy-*cis*-5-decenoic acid. 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 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

Metabolism of cis-5-Decenoyl-CoA Catalyzed by Acyl-CoA Oxidase. The incubation of *cis*-5-decenoyl-CoA with acyl-CoA oxidase was expected to yield *trans*-2,*cis*-5-decadienoate only (Smeland et al., 1992; Luo et al., 1994). However, at least three major products were obtained in these studies, as shown in Figure 2. Electron impact mass spectra (70 eV) of the trimethylsilyl derivatives of these metabolites (Figure 3) showed the molecular ions at *m/z* 240 and the M − 15 ions at *m/z* 225. This indicated that these metabolites were isomeric decadienoic acids. Metabolite A had a mass fragmentation pattern distinguishably different from those of the other two peaks. Furthermore, metabolite A was the major product when the incubation time was short. With the progress of the incubation, the intensity of metabolite A decreased, while those of the other two increased. These observations pointed to the likelihood that metabolite A was *trans*-2,*cis*-5-decadienoic acid, while the other two were geometric isomers of Δ^3,Δ^5-decadienoic acids.

Identification of Products. trans-2,*cis*-5-Decadienoic acid was synthesized from catalytic hydrogenation of the condensation product of 4-bromocrotonic acid and 1-hexyne (Heslinga et al., 1973). The mass spectrum of the synthetic

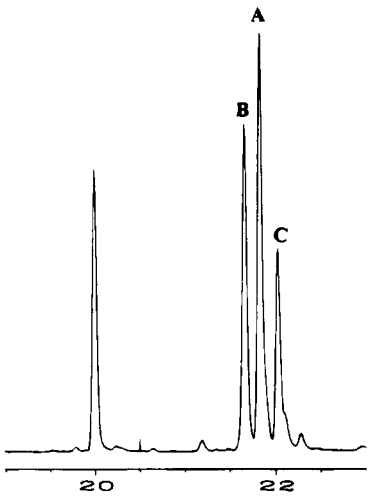

FIGURE 2: Metabolites produced from the incubation of *cis*-5-decenoyl-CoA (86 μM) with acyl-CoA oxidase (0.2 unit/mL) from *Arthrobacter*: (A) *trans*-2,*cis*-5-decadienoate; (B) *trans*-3,*cis*-5-decadienoate; (C) *cis*-3,*cis*-5-decadienoate. The peak at 20 min is *cis*-5-decenoate.

compound was identical to that of metabolite A. In addition, their retention times on two different stationary phase capillary columns of the gas chromatograph were identical. Therefore, metabolite A was identified as *trans*-2,*cis*-5-decadienoic acid.

The relative retention times (RRTs) of metabolite B/metabolite C were 0.627/0.636 on the SPB-1 column and 0.667/0.677 on the SPB-35 column. From the reaction of cyclopentyl *trans*-2,*trans*-4-decadienoate in triethylamine (Frighetto et al., 1978), a 1:1 mixture of Δ^3,Δ^5-decadienoate esters was obtained. The trimethylsilyl derivatives of these isomeric acids had relative retention times of 0.627/0.644 on SPB-1 and 0.667/0.684 on SPB-35. Their mass spectra were identical to those derived from metabolites B and C. This reaction has been shown to produce *trans*-3,*cis*-5-dienoate and *trans*-3,*trans*-5-dienoate (Frighetto et al., 1978). Furthermore, under basic catalysis by lithium diisopropylamide in tetrahydrofuran at -80 °C, ethyl *trans*-2,*trans*-4-decadienoate was reported to produce *trans*-3,*cis*-5-decadienoate and *trans*-3,*trans*-5-decadienoate in a 3:1 product ratio (Tsuboi et al., 1984). Using the same conditions, we obtained the isomeric Δ^3,Δ^5-decadienoate in a 2.3:1 product ratio of compounds, with RRT = 0.627 and RRT = 0.644 on SPB-1. Therefore, metabolite B at RRT = 0.627 must be *trans*-3,*cis*-5-decadienoate. Metabolite C was assigned as *cis*-3,*cis*-5-decadienoate on the basis of the fact that the isomerization of *trans*-2-enoyl-CoA to Δ^3-enoyl-CoA catalyzed by isomerase produces *cis*-3- and *trans*-3-isomers (Jin et al., 1992; Tserng & Jin, 1991).

The assignment of these structures to metabolites B and C is further supported by similar experiments with *trans*-5-decenoyl-CoA. Coincubation of *trans*-5-decenoyl-CoA with acyl-CoA oxidase and purified Δ^3,Δ^2-enoyl-CoA isomerase produced isomeric Δ^3,Δ^5-decadienoate with RRTs of 0.624/0.644 on the SPB-1 column and 0.662/0.683 on SPB-35. The late eluting metabolite with an RRT of 0.644 (SPB-1) was identical to *trans*-3,*trans*-5-decadienoate produced from base-catalyzed isomerization. The other metabolite, with an RRT of 0.624 (SPB-1), was assigned as *cis*-3,*trans*-5-decadienoate on the basis of the same argument that isomerization from *trans*-2 compounds catalyzed by isomerase produces *cis*-3- and *trans*-3-acids. Therefore, all isomeric Δ^3,Δ^5-decadienoates have been either synthesized or produced enzymatically and have distinguishably different retention times on the two different capillary GC columns.

Likewise, three chromatographic peaks were obtained for the incubation of *cis*-5-dodecenoyl-CoA and *cis*-5-tetradecenoyl-CoA with acyl-CoA oxidase. These products were assumed to be the corresponding *trans*-3,*cis*-5-dodecadienoate, *trans*-2,*cis*-5-dodecadienoate, and *cis*-3,*cis*-5-dodecadienoate derived from dehydrogenation and isomerization of *cis*-5-dodecenoyl-CoA. Similarly, *trans*-3,*cis*-5-tetradecadienoate, *trans*-2,*cis*-5-tetradecadienoate, and *cis*-3,*cis*-5-tetradecadienoate were obtained from *cis*-5-tetradecenoyl-CoA.

Distribution of Isomers at Different pH Values. The distribution of isomers was studied at different pH's using *cis*-5-decenoyl-CoA (85 μM) as substrate in 20 mM phosphate or Tris buffer at 37 °C for 10 min. The product ratio between *trans*-3,*cis*-5- and *cis*-3,*cis*-5- decreased from 1.78 to 0.66 with the increase in the pH value. These ratios were 1.78 (pH 6), 0.77 (pH 7), 0.76 (pH 7.4), 0.72 (pH 8), and 0.66 (pH 9).

The distribution of products was studied in more detail at pH 6 and 8 using three different substrates. As shown in Figure 4, the product *trans*-3,*cis*-5-/*cis*-3,*cis*-5-dienoate ratios were consistently lower at pH 8 than at pH 6 for all three substrates, i.e., *cis*-5-decenoyl-CoA, *cis*-5-dodecenoyl-CoA, and *cis*-5-tetradecenoyl-CoA. Therefore, high pH favored the formation of *cis*-3-isomers over *trans*-3-isomers. Furthermore, the isomer ratios stayed relatively constant between incubation times of 10 and 30 min for most of the experiments; this indicated that little interconversion took place between the two Δ^3,Δ^5-isomers once they were formed. The difference in the product ratios at pH 6 and 8 was due to the prior commitment in the isomerization from the *trans*-2,*cis*-5-precursor rather than further conversion between the two isomers after products were formed. This conclusion was further confirmed by another experiment. In this experiment, the medium at the end of a 30 min incubation at pH 6 was adjusted to pH 8 and incubation was continued for another 20 min. The product ratio was 1.77 initially at pH 6; it became 1.91 after further incubation at pH 8. In contrast, the incubation at pH 8 for 50 min yielded a product ratio of 1.03.

The isomerization from *trans*-2,*cis*-5- to Δ^3,Δ^5-dienoate required a fatty acid as the CoA derivative. When free *trans*-2,*cis*-5-decadienoate was incubated with acyl-CoA oxidase, the starting material was recovered. No isomerized products could be detected.

In contrast to acyl-CoA oxidase-catalyzed isomerization, the incubation of *cis*-5-decenoyl-CoA with acyl-CoA oxidase and purified rat liver Δ^3,Δ^2-dienoyl-CoA isomerase resulted in different product ratios. In these cases, more *trans*-3,*cis*-5-isomer was produced than *cis*-3,*cis*-5-metabolites, i.e., *trans*-3,*cis*-5-/*cis*-3,*cis*-5-decadienoate ratios were 3.11 $\pm$ 0.34 at pH 8.0.

Metabolism of trans-5-Decenoyl-CoA Catalyzed by Acyl-CoA Oxidase. The isomerization from 2,5- to 3,5-dienoyl-CoA mediated by acyl-CoA oxidase was limited only to *cis*-5-isomers. As shown in Figure 5A, when *trans*-5-decenoyl-CoA was used as the substrate, a major metabolite plus two

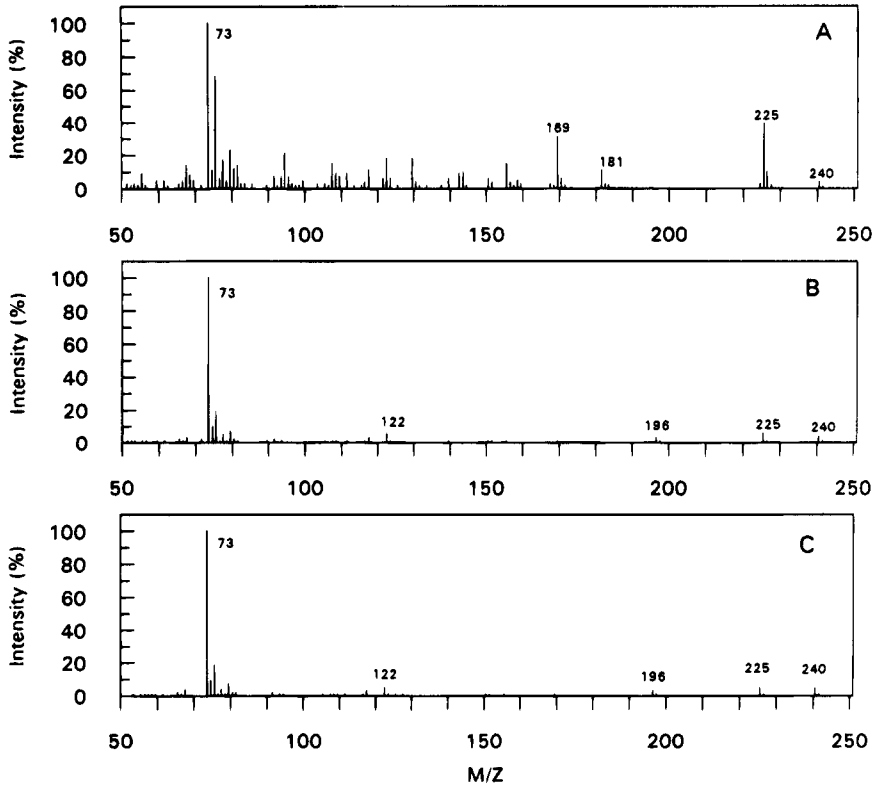

FIGURE 3: Electron impact mass spectra (70 eV) of metabolite A (A), metabolite B (B), and metabolite C (C).

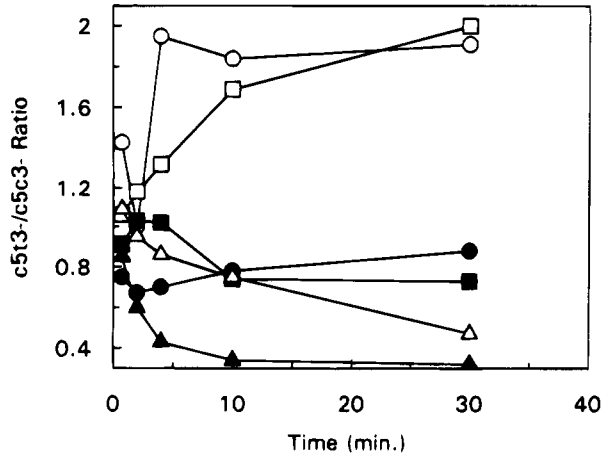

FIGURE 4: *cis*-5,*trans*-3- and *cis*-3,*cis*-5-dienoate product ratio (*c*-5,*t*-3/*c*-5,*c*-3 ratio) from the reaction of *cis*-5-enoyl-CoA (100 μM) and acyl-CoA oxidase (0.2 unit/mL) in 20 mM phosphate buffer at 37 °C and pH 6 and 8 of the incubation medium: *cis*-5-decenoyl-CoA at pH 6 (○), *cis*-5-decenoyl-CoA at pH 8 (●), *cis*-5-dodecenoyl-CoA at pH 6 (□), *cis*-5-dodecenoyl-CoA at pH 8 (■), *cis*-5-tetradecenoyl-CoA at pH 6 (△), and *cis*-5-tetradecenoyl-CoA at pH 8 (▲).

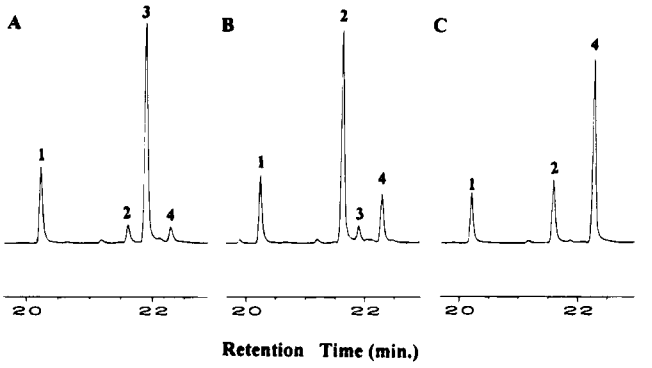

FIGURE 5: (A) Metabolites produced from the incubation of *trans*-5-decenoyl-CoA (100 μM) with acyl-CoA oxidase (0.5 unit/mL) from *Arthrobacter* in pH 8 (20 mM) phosphate buffer at 37 °C. The metabolites were (1) *trans*-5-decenoate, (2) *cis*-3,*trans*-5-decadienoate, (3) *trans*-2,*trans*-5-decadienoate, and (4) *trans*-3,*trans*-5-decadienoate. (B) Δ^3,Δ^2-Enoyl-CoA isomerase (2.6 munits/mL) was included in the incubation. *cis*-3,*trans*-5-Decadienoate was the major metabolite. (C) Δ^3,Δ^2-Enoyl-CoA isomerase (25.5 munits/mL) was included in the incubation. *trans*-3,*trans*-5-Decadienoate became the major product. The incubation time was 10 min.

minor metabolites were formed. Based on mass spectrometric fragmentation and the comparison with synthetic compounds, the major product was *trans*-2,*trans*-5-decadienoate, while the minor metabolites were *trans*-3,*trans*-5- and *cis*-3,*trans*-5-decadienoate, respectively. Further incubation of this mixture for 1−2 h did not change the metabolite profiles, indicating that acyl-CoA oxidase did not possess significant activity to isomerize *trans*-2,*trans*-5-dienoyl-CoA. In contrast, the addition of purified Δ^3,Δ^2-enoyl-CoA isomerase induced the isomerization of *trans*-2,*trans*-5-decadienoyl-CoA to Δ^3,Δ^5-decadienoyl-CoA, as shown in Figure 5B,C.

Isomerization Was Catalyzed by Enzymes, Not Chemically Induced. Under the milder conditions used in the hydrolysis of acyl-CoA esters in this investigation, i.e., room temperature and 0.1 N KOH, no isomerization of *trans*-2,*cis*-5-decadienoate or *trans*-2,*cis*-5-decadienoyl-CoA was observed. That the isomerization reported in this investigation was not mediated by chemical reaction during alkaline hydrolysis was further supported by deuterium labeling experiments. When [2,2′-$^2H_2$]-*cis*-5-decenoyl-CoA (86% d_2, 11% d_1, and 3% d_0, 100 μM) was incubated at 37 °C with acyl-CoA oxidase (0.2 unit/mL) in 20 mM (pH 8) phosphate buffer for 5 min, one deuterium label was retained in *trans*-2,*cis*-5-decadienoate (89% d_1), and 30−50% of the d_1 label was found on Δ^3,Δ^5-decadienoates, as would be expected

from enzyme-catalyzed dehydrogenation and isomerization (Ikeda et al., 1985). For a chemically catalyzed isomerization, the precursor and products are expected to lose all of their deuterium labels from a nonspecific alkaline-catalyzed reaction involving an active hydrogen adjacent to the carbonyl group. Further evidence that the isomerization was catalyzed enzymatically was provided by the subsequent addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase to the incubation mixture. In these experiments, *trans*-2,*trans*-4-decadienoate was produced. The unisomerized *trans*-2,*cis*-5-decadienoyl-CoA is not a substrate for Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (Smeland et al., 1992). Furthermore, studies performed based on the absorbance change using a spectrophotometer also indicated an isomerization catalyzed by acyl-CoA oxidase. No alkaline hydrolysis was involved in these experiments.

Reaction of Δ^3,Δ^5-Dienoyl-CoA with Δ^3,Δ^5-t-2,t-4-Dienoyl-CoA Isomerase. The substrate properties of isomeric Δ^3,Δ^5-decadienoyl-CoA's for the isomerization to *trans*-2,*trans*-4-decadienoyl-CoA were studied with purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. In one of the experiments, the substrate *t*-3,*c*-5/*c*-3,*c*-5 ratio was 1.22 ± 0.17 (n = 3) with acyl-CoA oxidase only. When Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (35 munits/mL) was added to the incubation, *trans*-2,*trans*-4-decadienoate was formed at the expense of the Δ^3,Δ^5-dienoates. However, *t*-3,*c*,5/*c*-3,*c*-5 ratio of the precursor remained unchanged (1.15 ± 0.12, not significantly different). Since there was very little interconversion between the two Δ^3,Δ^5-dienoate isomers, these data indicated that Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase did not have any preference for either of the isomers.

The substrate specificity of *cis*-3,*trans*-5-decadienoyl-CoA and *trans*-3,*trans*-5-decadienoyl-CoA toward Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase was also studied. Since acyl-CoA oxidase did not isomerize *trans*-2,*trans*-5-decadienoyl-CoA, initially formed from the reaction of *trans*-5-decenoyl-CoA (100 μM) with acyl-CoA oxidase (0.5 unit/mL), to any significant degree, the isomerization was effected using purified Δ^3,Δ^2-enoyl-CoA isomerase. When Δ^3,Δ^2-enoyl-CoA isomerase was added in dilute form (2.6 munits/mL), the predominant isomer formed was *cis*-3,*trans*-5-decadienoate (*c*-3,*t*-5/*t*-3,*t*-5 = 4.63 ± 0.53, n = 3) (Figure 5B). Prolonged incubation did not change the ratio of these two isomers; therefore, these data indicate little interconversion between the two isomers. With the addition of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (7.4 munits/mL), *trans*-2,*trans*-4-decadienoate was formed at the expense of *cis*-3,*trans*-5-decadienoate, while *trans*-3,*trans*-5-decadienoate mostly remained unchanged. The product ratio of *c*-3,*t*-5/*t*-3,*t*-5 decreased with incubation time. The average of these ratios was 2.75 ± 0.34 (p = 0.002 vs parallel experiments without Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, paired t-test). These data indicated that *trans*-3,*trans*-5-decadienoyl-CoA was not a substrate for Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, but that the *cis*-3,*trans*-5-decadienoyl-CoA substrate was similar to the *cis*-3,*cis*-5- and *trans*-3,*cis*-5-decadienoyl-CoA's. When more Δ^3,Δ^2-enoyl-CoA isomerase (25.5 munits/mL) was added to the incubation, more of the *trans*-3,*trans*-5-isomer was formed (*c*-3,*t*-5/*t*-3,*t*-5 = 0.56 ± 0.05, n = 3) (Figure 5C). The activity dependence of the Δ^3,Δ^2-enoyl-CoA isomerase-induced reaction was not observed for the reactions involving *cis*-5-isomers.

Reaction of Acyl-CoA Oxidase-Isomerized Products with Crotonase. When *cis*-5-decenoyl-CoA was incubated with acyl-CoA oxidase first, followed by the addition of crotonase, 3-hydroxy-*cis*-5-decenoate was formed in lower yields (62 ± 4%, n = 5) than in experiments in which crotonase was added together with acyl-CoA oxidase at the beginning of the study. In contrast, the yields of 3-hydroxydecanoate from decanoyl-CoA were not significantly different in parallel experiments. This indicated that crotonase acts only on *trans*-2,*cis*-5-dienoyl-CoA. When the substrate was preincubated with acyl-CoA oxidase, part of the *trans*-2,*cis*-5-dienoyl-CoA produced had already been converted to Δ^3,Δ^5-dienoyl-CoA's, which are not a substrate for crotonase. The lower concentration of *trans*-2,*cis*-5-dienoyl-CoA would lower the conversion to 3-hydroxy-*cis*-5-enoyl-CoA. These data also indicated that the reverse reaction from Δ^3,Δ^5-dienoyl-CoA back to *trans*-2,*cis*-5-dienoyl-CoA was extremely slow, practically negligible.

The irreversibility of the conversion from Δ^3,Δ^5-dienoyl-CoA's back to *trans*-2,*cis*-5-dienoyl-CoA was also supported from deuterium labeling experiments. When [2,2′-$^2H_2$]-*cis*-5-decenoyl-CoA (3% d_0, 11% d_1, and 86% d_2, 100 μM) was incubated with acyl-CoA oxidase (0.5 unit/mL) and crotonase (0.4 unit/mL), the deuterium labeling patterns in the products, i.e., *trans*-2,*cis*-5-decadienoate (11% d_0 and 89% d_1) and Δ^3,Δ^5-decadienoate (64% d_0 and 36% d_1), were distinctly different. The deuterium labeling pattern in 3-hydroxy-*cis*-5-decenoate (8% d_0 and 92% d_1) was the same as that in *trans*-2,*cis*-5-decadienoate. This indicates that 3-hydroxy-*cis*-5-decenoate was derived from the hydration of *trans*-2,*cis*-5-decadienoate, and there was very little reverse conversion of Δ^3,Δ^5-decadienoyl-CoA back to *trans*-2,*cis*-5, for if this did occur, then the d_1 isotopic enrichment of *trans*-2,*cis*-5-decadienoate would be diluted and a less enriched 3-hydroxy-*cis*-5-decenoate would be formed.

Spectrophotometric Studies on the Formation of Δ^3,Δ^5-Dienoates from cis-5-Enoyl-CoA. As shown in Figure 6, the addition of acyl-CoA oxidase to a solution of *cis*-5-decenoyl-CoA (spectrum 1) increased the absorbance at 260 nm (2), indicating the formation of a *trans*-2-double bond. However, differing from the parallel studies with decanoyl-CoA (data not shown), the absorbance between 230 and 250 nm increased by more than that at 260 nm upon prolonged incubation (spectrum 3). The increase in absorbance was stabilized about 5 min after the addition of acyl-CoA oxidase. The addition of crotonase or purified Δ^3,Δ^2-enoyl-CoA isomerase to this mixture did not change the absorption spectrum (data not shown). In contrast, the addition of crotonase to the incubation with decanoyl-CoA decreased the absorbance at 260 nm (data not shown), as would be expected from the hydration of a *trans*-2-double bond. The addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase to spectrum 3 changed the absorption, with an increased maximum at 300 nm (spectrum 4), indicating the formation of *trans*-2,*trans*-4-decadienoyl-CoA. These data indicate that *trans*-2,*cis*-5-decadienoyl-CoA formed from dehydrogenation by acyl-CoA oxidase was isomerized to Δ^3,Δ^5-dienoyl-CoA, which was not acted upon by crotonase or Δ^3,Δ^2-enoyl-CoA isomerase, but was converted to *trans*-2,*trans*-4-decadienoyl-CoA by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. The same absorbance change upon the addition of acyl-CoA oxidase was also observed when *cis*-5-octenoyl-CoA, *cis*-5-dodecenoyl-CoA, and *cis*-5-tetradecenoyl-CoA were used as

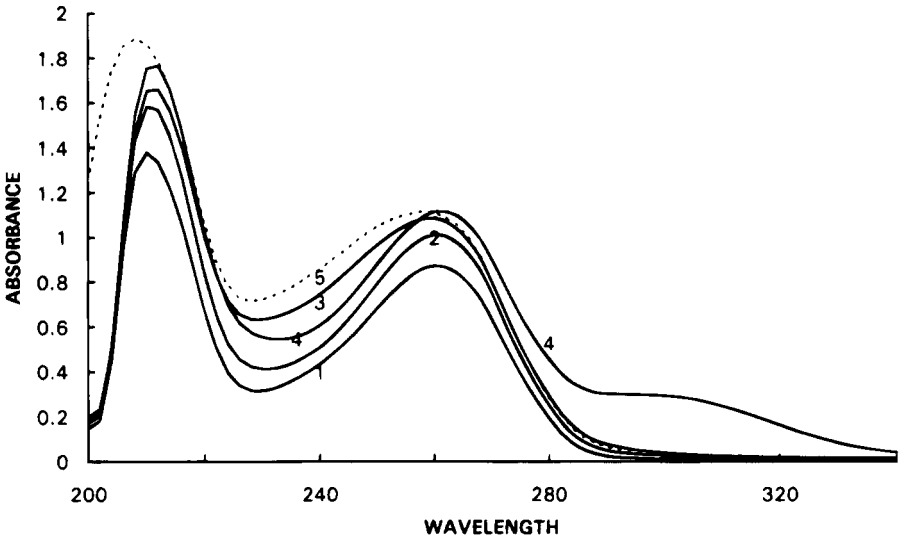

FIGURE 6: Spectral changes associated with the incubation of of *cis*-5-decenoyl-CoA (20 μM) in 0.1 M phosphate buffer (pH 8) at 37 °C with various enzymes: (1) *cis*-5-decenoyl-CoA only; (2) 10 s after the addition of acyl-CoA oxidase (0.5 unit) to spectrum 1; (3) 8 min after the addition of acyl-CoA oxidase to spectrum 1. No further change in absorption occurred with prolonged incubation. The addition of crotonase (0.4 unit) or Δ^3,Δ^2-enoyl-CoA isomerase (2.6 munits) to spectrum 3 did not change the absorption spectrum (data not shown). (4) 4 min after the addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (7.4 munits) to spectrum3. (5) The same experiment as spectrum 3, except that the reaction was done at pH 7.4.

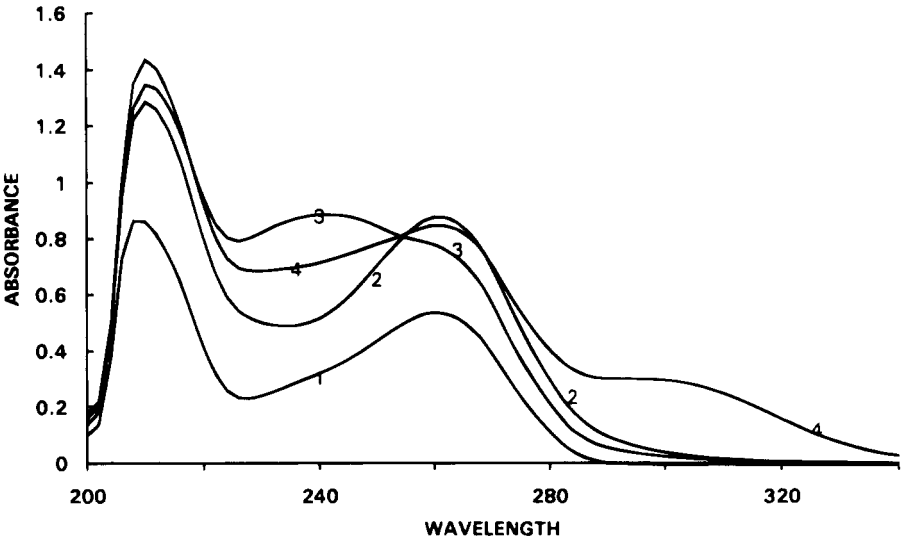

FIGURE 7: Spectral changes associated with the incubation of *trans*-5-decenoyl-CoA (20 μM) in 0.1 M phosphate buffer (pH 8) at 37 °C with various enzymes: (1) *trans*-5-decenoyl-CoA only; (2) 4 min after the addition of acyl-CoA oxidase (0.5 unit) to spectrum 1. The spectrum was not changed with prolonged incubation. However, the addition of crotonase (0.4 unit) to spectrum 2 decreased the absorption at 260 nm (data not shown). (3) 2 min after the addition of Δ^3,Δ^2-enoyl-CoA isomerase (2.6 munits) to spectrum 2. (4) 4 min after the addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase (7.4 munits) to spectrum 3.

substrates. In contrast, the saturated substrates, i.e., dodecanoyl-CoA and tetradecanoyl-CoA, behaved as expected for the formation of *trans*-2-enoyl-CoA, with increases in the absorbance at 260 nm that reached a maximum within 2 min. Furthermore, the addition of crotonase to these mixtures decreased the absorbance at 260 nm (data not shown).

At pH 8.0, the absorption maxima of the products derived from the reaction of *cis*-5-enoyl-CoA's with acyl-CoA oxidase were usually observed between 246 and 255 nm. In one series of experiments, the reaction of *cis*-5-decenoyl-CoA with acyl-CoA oxidase was studied at pH's from 6.0 to 9.0. When the absorption spectra stayed unchanged after 5−20 min of incubation, the following absorption maxima were observed: 247 (pH 6), 250 (pH 7), 250 (pH7.4), 255 (pH 8), and 260 (pH 9).

Spectrophotometric Studies with trans-5-Decenoyl-CoA and Acyl-CoA Oxidase. As shown in Figure 7, the addition of acyl-CoA oxidase alone increased the absorption at 260 nm (spectrum 1), similar to those obtained from saturated acyl-CoA's, and the increase reached a maximum within 2 min (spectrum 2). The addition of crotonase to spectrum 2 decreased the absorption at 260 nm (data not shown), indicative of the presence of a *trans*-2-enoyl-CoA. The addition of purified Δ^3,Δ^2-enoyl-CoA isomerase to spectrum 2 changed the absorption spectrum to a new maximum at 240 nm (spectrum 3), identical to that reported by Smeland et al. (1992). Further addition of purified Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase to spectrum 3 resulted in the appearance of an absorption maximum at 300 nm (spectrum 4). These experiments were consistent with the observation

from metabolite analysis that acyl-CoA oxidase alone could not isomerize *trans*-5-decenoyl-CoA to any significant extent. The isomerization of *trans*-2,*trans*-5-decadienoyl-CoA required the additional action of Δ^3,Δ^2-enoyl-CoA isomerase. Apparently, the behavior of acyl-CoA oxidase toward *cis*-5- and *trans*-5-enoyl-CoA substrates was drastically different; this is in contrast to the reports by Smeland et al. (1992) and Luo et al. (1994) that *cis*-5-octenoyl-CoA and *trans*-5-octenoyl-CoA had identical spectral changes and substrate properties toward acyl-CoA oxidase.

Other Acyl-CoA Oxidases and Acyl-CoA Dehydrogenases. Δ^3,Δ^5-*t*-2,*c*-5-Dienoyl-CoA isomerase activity was also demonstrated in purified acyl-CoA oxidases derived from *Candida* sp. (Jiang & Thorpe, 1983) and rat liver (Osumi et al., 1980). However, acyl-CoA dehydrogenases isolated from beef liver according to a modified procedure of Dommes and Kunau (1984) were found not to possess dienoyl-CoA isomerase activity. Only *trans*-2,*cis*-5-decadienoate was detected as a product when *cis*-5-decenoyl-CoA was incubated with this enzyme preparation (0.6 unit), together with phenazine ethosulfate (1.2 mM) as an artificial electron donor.

DISCUSSION

The isomerization, catalyzed by purified rat liver Δ^3,Δ^2-enoyl-CoA isomerase or *Arthrobacter* acyl-CoA oxidase preparations, from *trans*-2,*cis*-5-dienoyl-CoA produced *cis*-3,*cis*-5- and *trans*-3,*cis*-5-dienoates. The relative proportions of the two isomers depended on the pH of the incubation medium and the enzymes. Apparently, the equilibrium of the reaction favors the formation of Δ^3,Δ^5-dienoates. The reverse reaction back to *trans*-2,*cis*-5-dienoate was extremely slow, to the extent that it was almost irreversible. The further reaction of Δ^3,Δ^5-dienoyl-CoA to *trans*-2,*trans*-4-dienoyl-CoA was catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase. Both *cis*-3,*cis*-5- and *trans*-3,*cis*-5-dienoyl-CoA's were equally good substrates for the isomerization. In comparison, the isomerization of *trans*-2,*trans*-5-dienoyl-CoA, catalyzed by Δ^3,Δ^2-enoyl-CoA isomerase, produced *cis*-3,*trans*-5- and *trans*-3,*trans*-5- dienoyl-CoA's. The isomerization of *cis*-3,*trans*-5- to *trans*-2,*trans*-4- dienoyl-CoA was readily catalyzed by Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase, while *trans*-3,*trans*-5-dienoyl-CoA was not a substrate for the isomerization.

In this investigation, acyl-CoA oxidase preparations from *Arthrobacter* sp. were found to possess isomerase activity to convert *trans*-2,*cis*-5-dienoyl-CoA to isomeric Δ^3,Δ^5-dienoates. However, a similar isomerization reaction did not occur to any significant extent when *trans*-2,*trans*-5-dienoyl-CoA was used as the substrate. In contrast, Δ^3,Δ^2-enoyl-CoA isomerase readily converted *trans*-2,*trans*-5-decadienoyl-CoA to *trans*-3,*trans*-5- and *cis*-3,*trans*-5-decadienoates. This isomerase activity of acyl-CoA oxidase preparations likely was not due to the contamination of isomerase in the enzyme preparation. First, acyl-CoA oxidase, further purified by passage through Blue-Sepharose, hydroxylapatite, CM-cellulose, DEAE-cellulose, and Sepharose columns, as well as FPLC separation, showed identical isomerase activity. In addition, no isomerase activity could be detected when acyl-CoA oxidase was assayed for Δ^3,Δ^2-enoyl-CoA isomerase with *trans*-3-hexenoyl-CoA as substrate. This indicates that the isomerase activity found in acyl-CoA oxidase preparations was different from that of Δ^3,Δ^2-enoyl-CoA isomerase. The isomerase activity found in acyl-CoA oxidase preparations was high; it was about 50% of the dehydrogenation activity. If the isomerase activity was due to another enzyme other than acyl-CoA oxidase, the contaminating protein should be readily detectable or reduced by various chromatographic purification procedures. Apparently, this was not the case in the present study. The combined activities of dehydrogenation and isomerization in a single protein are also found in mammalian 3β-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase protein [Luu-The et al. (1991) and references cited therein].

The non-stereospecific nature of isomerization observed in this study is to be expected from the mechanism proposed (Chen et al., 1994). Using deuterium labeling, the result is consistent with the abstraction of a proton from the solvent instead of an intramolecular transfer, as was observed for other isomerases (Brooks & Benisek, 1994). This could indicate that the existence of the transition state during isomerization is long enough for the generation of both *cis*- and *trans*-isomers. Because the proton transferred was obtained from the solvent, a change in pH would be expected to change the product distribution. The mechanism for the change in product distribution as a function of enzyme concentration is not known. More studies are needed to address this issue.

In contrast to the observation by Schulz and co-workers (Smeland et al., 1992; Luo et al., 1994), we did not observe a shift in the absorption maximum from 260 to 240 nm for all *cis*-5-enoyl-CoA substrates, even after the addition of purified rat liver Δ^3,Δ^2-enoyl-CoA isomerase (Figure 6). In these studies, Δ^3,Δ^5-dienoates were found to be the products. The characteristic absorption maximum shift was observed only in a *trans*-5-substrate after the addition of purified enoyl-CoA isomerase together with or following acyl-CoA oxidase addition (Figure 7). It is likely that the substrates used in previous studies (Smeland et al., 1992; Luo et al., 1994) could all be *trans*-5-octenoyl-CoA in studies of supposed *cis*-5-octenoyl-CoA.

On the basis of the results of metabolite analysis from different pH incubation media, it can be concluded that the absorption maximum shift from the conversion of *trans*-2,*cis*-5-dienoyl-CoA to Δ^3,Δ^5-dienoyl-CoA was due to a mix of absorption from isomers. *trans*-3,*cis*-5-Dienoates probably have their absorption maxima at lower wavelengths than *cis*-3,*cis*-5-dienoates; therefore, the products produced at lower pH's resulted in a shift to lower maxima since *trans*-3,*cis*-5-dienoates were the predominant products. However, neither *trans*-3,*cis*-5- nor *cis*-3,*cis*-5-dienoates had lower absorption maxima than the corresponding *trans*-5-metabolites. Therefore, an even lower absorption maximum shift to 240 nm was observed for Δ^3,*trans*-5-decadienoates. In conclusion, *cis*-5- and *trans*-5-fatty acids do not behave identically in terms of isomerization induced by acyl-CoA oxidase or spectral shift as a result of transformation. The absorption maximum for *trans*-3,*cis*-5-dienoate was reported to be 233 nm, while those of *trans*-3,*trans*-5-dienoates were lower at 228−229 nm (Celmer & Solomons, 1953).

In the metabolism of unsaturated fatty acids in bacteria or fungi that use acyl-CoA oxidase as the first step in the dehydrogenation of acyl-CoA, the isomerase activity of acyl-CoA oxidase potentially could channel the reaction of *cis*-5-enoyl-CoA toward the reduction pathway. In addition, the

isomerase property of acyl-CoA oxidase preparations renders the preparation of Δ^3,Δ^5-dienoyl-CoA substrates from *cis*-5-enoyl-CoA's possible, using commercially available acyl-CoA oxidase from *Arthrobacter*. Δ^3,Δ^5-Dienoyl-CoA's are needed as substrate in the assay of Δ^3,Δ^5-*t*-2,*t*-4-dienoyl-CoA isomerase activity.

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