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Biochemistry

NADPH-dependent β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms

(5-enoyl-CoA/Δ^3,Δ^2-enoyl-CoA isomerase/$\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase/2,4-dienoyl-CoA reductase)

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ABSTRACT **The mitochondrial metabolism of 5-enoyl-CoAs, which are formed during the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms, was studied with mitochondrial extracts and purified enzymes of β-oxidation. Metabolites were identified spectrophotometrically and by high performance liquid chromatography. 5-*cis*-Octenoyl-CoA, a putative metabolite of linolenic acid, was efficiently dehydrogenated by medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) to 2-*trans*-5-*cis*-octadienoyl-CoA, which was isomerized to 3,5-octadienoyl-CoA either by mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) or by peroxisomal trifunctional enzyme. Further isomerization of 3,5-octadienoyl-CoA to 2-*trans*-4-*trans*-octadienoyl-CoA in the presence of soluble extracts of either rat liver or rat heart mitochondria was observed and attributed to a $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase. Qualitatively similar results were obtained with 2-*trans*-5-*trans*-octadienoyl-CoA formed by dehydrogenation of 5-*trans*-octenoyl-CoA. 2-*trans*-4-*trans*-Octadienoyl-CoA was a substrate for NADPH-dependent 2,4-dienoyl-CoA reductase (EC 1.3.1.34). A soluble extract of rat liver mitochondria catalyzed the isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 2-*trans*-4-*trans*-octadienoyl-CoA, which upon addition of NADPH, NAD^+, and CoA was chain-shortened to hexanoyl-CoA, butyryl-CoA, and acetyl-CoA. Thus we conclude that odd-numbered double bonds, like even-numbered double bonds, can be reductively removed during the β-oxidation of polyunsaturated fatty acids.**

The degradation of unsaturated fatty acids by β-oxidation involves at least two auxiliary enzymes in addition to the enzymes required for the breakdown of saturated fatty acids (1). The auxiliary enzymes acting on double bonds are 2,4-dienoyl-CoA reductase or 4-enoyl-CoA reductase (EC 1.3.1.34) and Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) (2).

Chain shortening of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms leads to the formation of 4-enoyl-CoAs, which are dehydrogenated by acyl-CoA dehydrogenase (EC 1.3.99.3) to 2,4-dienoyl-CoAs. An NADPH-dependent 2,4-dienoyl-CoA reductase, originally described by Kunau and Dommes (3), catalyzes the reduction of 2,4-dienoyl-CoAs to 3-enoyl-CoAs, which, after isomerization by Δ^3,Δ^2-enoyl-CoA isomerase to 2-enoyl-CoAs, can be completely degraded via the β-oxidation spiral.

Unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms are, according to Stoffel and Caesar (4), chain-shortened to 3-enoyl-CoAs, which, after isomerization to 2-enoyl-CoAs by Δ^3,Δ^2-enoyl-CoA isomerase, reenter the β-oxidation spiral. If so, 5-enoyl-CoAs are intermediates that would pass once more through the β-oxidation spiral before being acted upon by Δ^3,Δ^2-enoyl-CoA isomerase. This prediction, however, is contradicted by a recent observation of Tserng and Jin (5) who reported that the mitochondrial β-oxidation of 5-enoyl-CoAs is dependent on NADPH. Their analysis of metabolites by gas chromatography/mass spectrometry led them to propose that the double bond of 5-enoyl-CoAs is reduced by NADPH to yield the corresponding saturated fatty acyl-CoAs, which are then further degraded by β-oxidation.

This report addresses the question of how 5-enoyl-CoAs are chain-shortened by β-oxidation. We demonstrate that 5-enoyl-CoAs, after dehydrogenation to 2,5-dienoyl-CoAs, can be isomerized to 2,4-dienoyl-CoAs, which are reduced by the NADPH-dependent 2,4-dienoyl-CoA reductase. Thus, odd-numbered double bonds, like even-numbered double bonds, can be reductively removed during β-oxidation.

MATERIALS AND METHODS

Materials. Acetyl-CoA, *n*-butyryl-CoA, *n*-hexanoyl-CoA, *n*-octanoyl-CoA, CoA, NADPH, NAD^+, bovine serum albumin, pig heart L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), acyl-CoA oxidase from *Candida* sp., and all standard biochemicals were obtained from Sigma. 2-*trans*-4-*trans*-Octadienal and 3-*trans*-octadecenoic acid were purchased from Bedoukian Research (Danbury, CT) and Pfaltz & Bauer, respectively. 2-*trans*-4-*trans*-Octadienoic acid was prepared from 2-*trans*-4-*trans*-octadienal by oxidation with Ag_2O according to a general procedure for the oxidation of aldehydes to acids that are sensitive to strong oxidizing agents (6). 2-*trans*-4-*trans*-Octadienoic acid after crystallization from hexane had a melting point of 75–76°C [literature melting point, 76°C (7)]. The methyl esters of 5-*cis*-octenoic acid and 5-*trans*-octenoic acid were generously provided by Howard Sprecher (Ohio State University). The purities of the *cis*- and *trans*-isomers were 98% and 96%, respectively. The methyl esters were saponified with a 3-fold molar excess of aqueous 0.4 M KOH until the system became monophasic. The resultant acids were obtained after acidification and extraction with ether. The CoA derivatives of 5-*cis*-octenoic acid, 5-*trans*-octenoic acid, 3-*trans*-octenoic acid, and 2-*trans*-4-*trans*-octadienoic acid were synthesized according to the procedure of Goldman and Vagelos (8). All synthetic acyl-CoAs used in HPLC or spectrophotometric experiments were purified by HPLC. Concentrations of acyl-CoAs were determined by the method of Ellman (9) after cleaving the thioester bond with NH_2OH at pH 7. 2-*trans*-5-*cis*-Octadienoyl-CoA and 2-*trans*-5-*trans*-octadienoyl-CoA were synthesized from the corresponding 5-octenoyl-CoAs by allowing them to react with oxygen in the presence of acyl-CoA oxidase from *Candida* sp. as described (10). Bovine liver enoyl-CoA hydratase (EC 4.2.1.17) or crotonase (11), the trifunctional enzyme from rat liver peroxisomes (12, 13), and pig heart 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (14) were purified as described. Mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) was partially purified by chromatography of a soluble extract of rat liver mitochondria on

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hydroxylapatite as described by Kilponen *et al.* (15). 2,4-Dienoyl-CoA reductase (EC 1.3.1.34) was partially purified by chromatography of a soluble extract of rat liver mitochondria on agarose-heptane-adenosine-2',5'-diphosphate as described by Wang and Schulz (16). Medium-chain acyl-CoA dehydrogenase was partially purified from bovine liver mitochondria as described (17). Mitochondria, isolated from a single rat liver (18), were suspended in 0.1 M potassium phosphate (pH 8), sonicated for ten 20-s bursts at 0°C with an Ultrasonic sonifier (model W-385) equipped with a microtip, and centrifuged at 100,000 × *g* for 1 hr. The soluble mitochondrial extract (5.9 mg/ml) was stored at −70°C. Protein concentrations were determined by the method of Bradford (19).

Enzyme Assays. Acyl-CoA dehydrogenase was assayed spectrophotometrically as described (17). Δ^3,Δ^2-Enoyl-CoA isomerase was assayed spectrophotometrically at 340 nm in a coupled assay (20) with 3-*trans*-octenoyl-CoA as substrate. 2,4-Dienoyl-CoA reductase was assayed spectrophotometrically at 340 nm with 2-*trans*-4-*trans*-octadienoyl-CoA as a substrate as described (3).

Metabolic Studies. The hydration of 2,5-octadienoyl-CoA by crotonase and its isomerization to 3,5-octadienoyl-CoA were followed spectrophotometrically at 263 nm and 238 nm, respectively. Incubation mixtures contained 50 μM 2,5-octadienoyl-CoA in 0.1 M potassium phosphate (pH 8) and then purified bovine liver crotonase, purified trifunctional enzyme from rat liver peroxisomes, or partially purified Δ^3,Δ^2-enoyl-CoA isomerase from rat liver mitochondria was added to give an absorbance change of 0.08 unit/min. The conversion of 2,5-octadienoyl-CoA or 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA was followed spectrophotometrically at 300 nm. Incubation conditions were the same as described above for the formation of 3,5-octadienoyl-CoA except that the enzyme source was a soluble mitochondrial extract (30 μg of protein per ml). When the chain shortening of 2,5-octadienoyl-CoA by β-oxidation was studied, 50 μM substrate was incubated in 0.7 ml of 0.1 M potassium phosphate (pH 8) with 60 μg of soluble mitochondrial extract until the absorbance at 300 nm ceased to increase. At that point, 170 μg of soluble mitochondrial extract and 0.3 ml of 0.1 M potassium phosphate (pH 8) containing NAD^+, CoA, and NADPH were added to give final concentrations of the three coenzymes of 1 mM, 0.3 mM, and 0.1 mM, respectively. The progress of the reaction was monitored at 340 nm.

Spectrophotometric Measurements. All scans were recorded on a Gilford 2600 microprocessor-controlled UV–VIS spectrophotometer interfaced with a Hewlett Packard 7225B graphics plotter. The incubation conditions were as detailed for the metabolic studies.

HPLC Analysis. Prior to analysis by HPLC, reactions were terminated by adjusting the pH to 1–2 with concentrated HCl. Samples were filtered through 0.22-μm (pore size) membranes after which the pH was adjusted to 5 with KOH. The filtrates were applied to a Waters HPLC μBondapak C_{18} reverse-phase column (30 cm × 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H_2O, 9:1 (vol/vol), content of the 10 mM ammonium phosphate elution buffer (pH 5.5) from 10% to 50% in 30 min at a flow rate of 2 ml/min. When the chain shortening of 2,5-octadienoyl-CoA was studied, the acetonitrile/H_2O content was increased from 0 to 10% in 10 min, followed by a linear increase from 10% to 50% in 20 min.

RESULTS

Dehydrogenation of 5-Octenoyl-CoA. The mitochondrial β-oxidation of 5-enoyl-CoAs, which are presumed intermediates in the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms, was studied with 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA. The suggested reduction of 5-*cis*-enoyl-CoAs to acyl-CoAs by NADPH (5) was investigated. When 5-*cis*-octenoyl-CoA was incubated with NADPH in the presence of rat liver mitochondria, no oxidation of NADPH was observed. Thus, it seems that 5-enoyl-CoAs are not directly converted to their saturated analogs by a hypothetical NADPH-dependent 5-enoyl-CoA reductase.

Purified 5-*cis*-octenoyl-CoA, which gave a single peak on HPLC (Fig. 1*A*), was incubated with acyl-CoA oxidase either in the presence or absence of catalase. The same major reaction product was obtained under both conditions and was purified by HPLC to remove unreacted starting material as well as a more polar reaction product. The product of this enzymatic reaction was assumed to be 2-*trans*-5-*cis*-octadienoyl-CoA (Fig. 1*B*) since acyl-CoA oxidases are known to dehydrogenate acyl-CoAs to 2-*trans*-enoyl-CoAs while reducing oxygen to H_2O_2 (12). The dehydrogenation product 2-*trans*-5-*cis*-octadienoyl-CoA was clearly separated from the starting material 5-*cis*-octenoyl-CoA by HPLC (Fig. 1*C*). The absorbance spectrum of 2-*trans*-5-*cis*-octadienoyl-CoA (Fig. 2*A*) is characteristic of an acyl-CoA with a maximum close to 260 nm due to the adenine moiety of CoA. When 2-*trans*-5-*cis*-octadienoyl-CoA was incubated with crotonase, the absorbance around 260 nm decreased as expected for a 2-enoyl-CoA compound that is hydrated to 3-hydroxyacyl-CoA (Fig. 2*A*). The product of this reaction was also analyzed by HPLC and found to be eluted at a position expected for the more polar 3-hydroxy-5-*cis*-octenoyl-CoA (Fig. 1*D*). The elution time of 3-hydro-5-*cis*-octenoyl-CoA formed by crotonase-catalyzed hydration of 2-*trans*-5-*cis*-octadienoyl-CoA was identical to the elution time of a minor and more polar reaction product formed during the dehydrogenation of 5-*cis*-octenoyl-CoA by acyl-CoA oxidase. When the acyl-CoA oxidase preparation was assayed for crotonase, this enzyme was detected. Separation of crotonase and acyl-CoA oxidase by chromatography on hydroxylapatite yielded an oxidase preparation that produced little of the more polar reaction product 3-hydroxy-5-*cis*-octenoyl-CoA during the dehydrogenation of 5-*cis*-octenoyl-CoA. Thus these experiments establish that the main product formed during the dehydrogenation of 5-*cis*-octenoyl-CoA by acyl-CoA oxidase is 2-*trans*-5-*cis*-

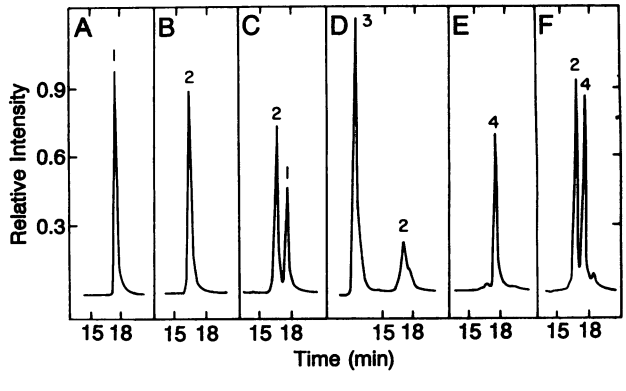

FIG. 1. HPLC analysis of metabolites formed by enzymes of β-oxidation from 5-*cis*-octenoyl-CoA. (*A*) 5-*cis*-Octenoyl-CoA (peak 1). (*B*) 2-*trans*-5-*cis*-Octadienoyl-CoA (peak 2) formed from *cis*-5-octenoyl-CoA by acyl-CoA oxidase. (*C*) 5-*cis*-Octenoyl-CoA (peak 1) and 2-*trans*-5-*cis*-octadienoyl-CoA (peak 2). (*D*) Hydration of 2-*trans*-5-*cis*-octadienoyl-CoA (peak 2) by crotonase to 3-hydroxy-5-*cis*-octenoyl-CoA (peak 3). (*E*) Isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 2-*trans*-4-*trans*-octadienoyl-CoA (peak 4) by a soluble extract of rat liver mitochondria. (*F*) 2-*trans*-5-*cis*-Octadienoyl-CoA (peak 2) and 2-*trans*-4-*trans*-octadienoyl-CoA (peak 4).

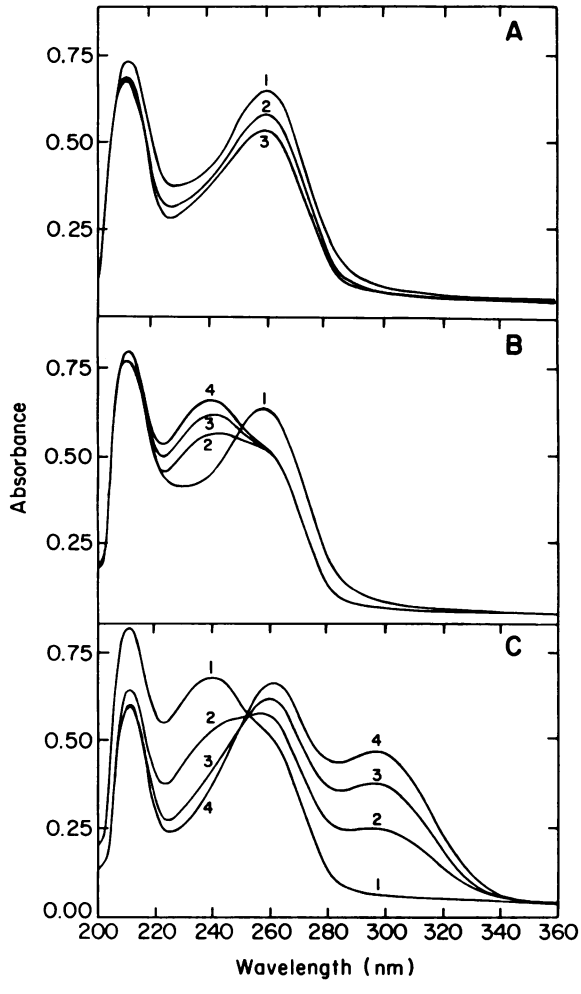

FIG. 2. Spectral changes associated with the hydration and isomerizations of 2-*trans*-5-*cis*-octadienoyl-CoA. (*A*) Hydration of 2-*trans*-5-*cis*-octadienoyl-CoA by crotonase. Traces: 1, no addition; 2, 90 sec after addition of crotonase; 3, 5 min after addition of crotonase. (*B*) Isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 3,5-octadienoyl-CoA catalyzed by the peroxisomal trifunctional enzyme. Traces: 1, no addition; 2, 90 sec after addition of enzyme; 3, 3 min after addition of enzyme; 4, 6 min after addition of enzyme. (*C*) Isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by a soluble extract of rat liver mitochondria. Traces: 1, no addition; 2, 90 sec after addition of enzyme; 3, 3 min after addition of enzyme; 4, 6 min after addition of enzyme.

octadienoyl-CoA. Virtually identical results were obtained when 5-*trans*-octenoyl-CoA was converted to 2-*trans*-5-*trans*-octadienoyl-CoA by acyl-CoA oxidase (data not shown).

The dehydrogenation of 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA by acyl-CoA dehydrogenase present in a soluble extract of a rat liver mitochondria was determined and compared with the dehydrogenation of octanoyl-CoA. Rates determined at saturating or near saturating concentrations (40–50 μM) of substrates were 0.0211 unit/mg (100%) with octanoyl-CoA vs. 0.0174 unit/mg (82%) with either 5-*cis*-octenoyl-CoA or 5-*trans*-octenoyl-CoA. Kinetic measurements with partially purified medium-chain acyl-CoA dehydrogenase from bovine liver and 5-*cis*-octenoyl-CoA as well as 5-*trans*-octenoyl-CoA as substrates yielded relative maximal velocities that were almost identical but were 25% lower than the maximal velocity obtained with octanoyl-CoA. Values of K_m for all three substrates were similar and in the low micromolar range (5–9 μM). The dehydrogenation product formed by bovine liver medium-chain acyl-CoA dehydrogenase with 5-*cis*-octenoyl-CoA as a substrate was indistinguishable from 2-*trans*-5-*cis*-octenoyl-CoA on HPLC (data not shown).

Isomerizations of 2,5-Octadienoyl-CoA. When 2-*trans*-5-*cis*-octadienoyl-CoA was incubated with a soluble extract of rat liver mitochondria, from which low molecular weight cofactors had been removed by filtration through Sephadex G-25, a single product was detected by HPLC (Fig. 1*E*). This compound, which was eluted from a reverse-phase HPLC column 1 min later than the starting material (Fig. 1*F*), was inseparable from authentic 2-*trans*-4-*trans*-octadienoyl-CoA. The same result was obtained when 2-*trans*-5-*trans*-octadienoyl-CoA was allowed to react with the soluble extract of rat liver mitochondria.

To elucidate the isomerization of 2,5-octadienoyl-CoA, the 2-*trans*-5-*cis*-isomer was incubated with purified trifunctional enzyme from rat liver peroxisomes. As is apparent from Fig. 2*B*, the absorbance around 260 nm decreased immediately whereas an absorbance maximum close to 240 nm developed more slowly. The decrease in absorbance close to 260 nm was most likely due to the instantaneous hydration of the 2,3 double bond catalyzed by the high enoyl-CoA hydratase activity of the trifunctional enzyme. The slower absorbance increase close to 240 nm is attributed to the formation of 3,5-octadienoyl-CoA catalyzed by the Δ^3,Δ^2-enoyl-CoA isomerase activity of the trifunctional enzyme (13). A partially purified preparation of mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase brought about the same absorbance changes (data not shown). Product analysis by HPLC revealed a single peak, inseparable from the starting material 2-*trans*-5-*cis*-octadienoyl-CoA (data not shown). When an equimolar mixture of starting material and product was analyzed by HPLC, a slight separation was detectable (data not shown). However, the product, in contrast to the starting material, was neither hydrated by crotonase nor by the trifunctional enzyme. The addition of a soluble extract of rat liver mitochondria to 3,5-octadienoyl-CoA resulted in the disappearance of the absorbance around 240 nm and caused a corresponding absorbance increase centered around 300 nm (Fig. 2*C*). The spectrum, upon completion of the reaction, was characteristic of a 2,4-dienoyl-CoA compound. Product analysis by HPLC revealed the presence of a single UV-absorbing compound that was coeluted with 2-*trans*-4-*trans*-octadienoyl-CoA (Fig. 1*E*) but that was clearly separated from the starting material (Fig. 1*F*).

Rates of isomerization from 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA and 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA were determined. With a soluble extract of rat liver mitochondria the 3,5 → 2,4 conversion proceeded twice as fast as the 2-*trans*,5-*trans* → 2,4-isomerization and 15 times faster than the 2-*trans*,5-*cis* → 2,4 conversion. With a fraction of the extract that was obtained by chromatography on hydroxylapatite and contained little Δ^3,Δ^2-enoyl-CoA isomerase activity, the 3,5 → 2,4 conversion was 6 times and 20 times faster than the conversions of 2-*trans*,5-*trans* → 2,4 and 2-*trans*,5-*cis* → 2,4, respectively. The isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 2,4-octadienoyl-CoA was also catalyzed by a soluble extract of rat heart mitochondria.

Characterization of 2,4-Octadienoyl-CoA. The final isomerization product formed from 2-*trans*-5-*cis*-octadienoyl-CoA, 2-*trans*-5-*trans*-octadienoyl-CoA, or 3,5-octadienoyl-CoA by a soluble extract of rat liver or rat heart mitochondria was tentatively identified as 2,4-octadienoyl-CoA based on its UV spectrum and behavior on HPLC where it was indistinguishable from synthetic 2-*trans*-4-*trans*-octadienoyl-CoA. Further proof for its structure was obtained when NADPH was added to a mixture of 2,4-octadienoyl-CoA and a soluble extract of rat liver mitochondria. As shown in Fig. 3, the absorbance at 300 nm disappeared and a decrease in absorbance at 340 nm occurred due to the NADPH-dependent

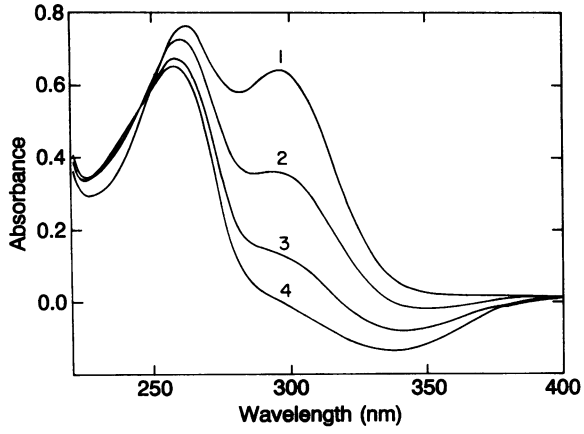

FIG. 3. Spectral changes associated with the reduction of 2,4-octadienoyl-CoA by NADPH in the presence of a soluble extract of rat liver mitochondria. Traces: 1, before addition of NADPH; 2, 3 min after addition of NADPH; 3, 9 min after addition of NADPH; 4, 20 min after the addition of NADPH. NADPH was added to both the measuring and reference cuvettes.

reduction of 2,4-octadienoyl-CoA catalyzed by 2,4-dienoyl-CoA reductase present in the extract from rat liver mitochondria. When partially purified 2,4-dienoyl-CoA reductase was used, HPLC analysis revealed the formation of 3-octenoyl-CoA upon reduction of 2,4-octadienoyl-CoA by NADPH (Fig. 4*A*). Finally, when 2-*trans*-5-*cis*-octadienoyl-CoA was first completely converted to 2,4-octadienoyl-CoA by a soluble extract of rat liver mitochondria and then incubated for 5 min in the presence of NADPH, NAD^+, and CoA, the formation of hexanoyl-CoA, butyryl-CoA, and acetyl-CoA was detected by HPLC (Fig. 4*B*). Hexanoyl-CoA and acetyl-CoA are the expected products if 2,4-octadienoyl-CoA, after reduction by NADPH-dependent 2,4-dienoyl-CoA reductase, completes one cycle of β-oxidation. Butyryl-CoA would be formed if 2,4-octadienoyl-CoA, without being reduced by 2,4-dienoyl-CoA reductase, passes twice through the β-oxidation cycle. This reaction proceeds at a significant rate when the 2,4-dienoyl-CoA intermediate has the all-*trans* configuration (21). Preliminary evidence also indicates that this mitochondrial extract has some acyl-CoA dehydrogenase activity that facilitates the complete degradation of

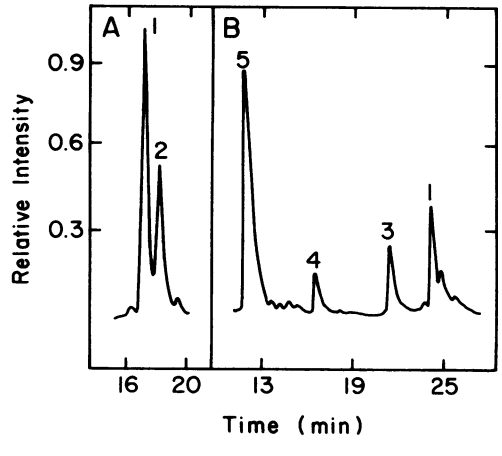

FIG. 4. HPLC analysis of metabolites formed by β-oxidation from 2-*trans*-5-*cis*-octadienoyl-CoA. 2-*trans*-5-*cis*-Octadienoyl-CoA was first converted to 2-*trans*-4-*trans*-octadienoyl-CoA by a soluble extract of rat liver mitochondria and after removal of enzymes reduced by NADPH in the presence of partially purified 2,4-dienoyl-CoA reductase (*A*) and incubated for 5 min after the addition of NADPH, NAD^+, and CoA (*B*). Peaks identified by authentic materials: 1, 2-*trans*-4-*trans*-octadienoyl-CoA; 2, 3-*trans*-octenoyl-CoA; 3, *n*-hexanoyl-CoA; 4, *n*-butyryl-CoA; 5, acetyl-CoA.

hexanoyl-CoA and butyryl-CoA to acetyl-CoA (data not shown). Since it was observed that 2-*trans*-4-*cis*-decadienoyl-CoA and 2-*trans*-4-*trans*-decadienoyl-CoA can be separated by HPLC under conditions used to identify 2,4-octadienoyl-CoA (M.N. and H.S., unpublished data), it seems that isomerizations of the two 2,5-octadienoyl-CoA isomers and of 3,5-octadienoyl-CoA yield 2-*trans*-4-*trans*-octadienoyl-CoA, because it was coeluted with authentic 2-*trans*-4-*trans*-octadienoyl-CoA.

DISCUSSION

Unsaturated fatty acids with odd-numbered double bonds, as for example oleic acid with a double bond extending from carbon atom 9 and linolenic acid with two odd-numbered double bonds extending from carbon atoms 9 and 15, are thought to be chain-shortened until the odd-numbered double bonds extend from carbon atom 3 (1). At this stage, Δ^3,Δ^2-enoyl-CoA isomerase converts 3-*cis* or 3-*trans* double bonds to a 2-*trans* double bond (4). The resultant 2-*trans*-enoyl-CoAs reenter the β-oxidation cycle beyond the first dehydrogenation step and are completely degraded. However, the observation of Tserng and Jin (5) that the effective β-oxidation of 5-*cis*-enoyl-CoAs requires NADPH raised doubts about the assumed chain shortening of 5-*cis*-enoyl-CoAs to 3-*cis*-enoyl-CoAs by a simple pass through the β-oxidation spiral. Since these authors observed the conversion of 5-*cis*-enoyl-CoAs to saturated acyl-CoAs with the same number of carbon atoms, they suggested that an NADPH-dependent 5-enoyl-CoA reductase may convert 5-enoyl-CoAs to the corresponding acyl-CoAs. Our attempt to detect such enzyme activity was unsuccessful and prompted this detailed study of the β-oxidation of 5-octenoyl-CoA, which is a metabolite of linolenic acid. Since the results obtained with 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA were qualitatively identical, only the β-oxidation of 5-*cis*-octenoyl-CoA will be discussed.

The proposed NADPH-dependent pathway by which 5-*cis*-octenoyl-CoA is chain-shortened to hexanoyl-CoA is shown in Fig. 5. All enzymes necessary for this pathway are present in a soluble extract of rat mitochondria. Mitochondrial medium-chain acyl-CoA dehydrogenase and peroxisomal acyl-CoA oxidase, which are known to introduce 2-*trans* double bonds into acyl-CoAs (1), catalyze the dehydrogenation of 5-*cis*-octenoyl-CoA (I) to 2-*trans*-5-*cis*-octadienoyl-CoA (II). The assigned structure of compound II is supported by the crotonase-catalyzed hydration of the 2-*trans* double bond observed spectrophotometrically and by HPLC. 2-*trans*-5-*cis*-Octadienoyl-CoA is acted upon by mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase and by the trifunctional enzyme of rat liver peroxisomes and converted to 3,5-octadienoyl-CoA (III). The structure assigned to compound III is supported by several facts and observations. (*i*) Δ^3,Δ^2-Enoyl-CoA isomerases are known to catalyze the shift of double bonds from the 3,4 to 2,3 position and presumably catalyze the reverse reaction; (*ii*) the inactivity of crotonase toward compound III agrees with the absence of a 2,3 double bond; and (*iii*) the observed decrease in absorbance around 260 nm and the increase in absorbance around 240 nm agree with the disappearance of the 2,3 double bond and the formation of the 3,5-diene for which an absorbance maximum at 228 nm has been observed with hexane as a solvent (22). Since the UV spectrum shown in Fig. 2 was determined with water as solvent, the λ_{max} is expected to be shifted to the red by 10–20 nm (23). Even though the configuration of the diene of compound III has not been established, it is assumed that the 5-double bond remained unaffected by the isomerization, whereas the 3-double bond may have either the *trans* or *cis* configuration. Incubation of 3,5-octadienoyl-CoA (III) with a soluble extract of rat mitochondria produced an absorbance decrease around 240 nm and a corresponding increase around

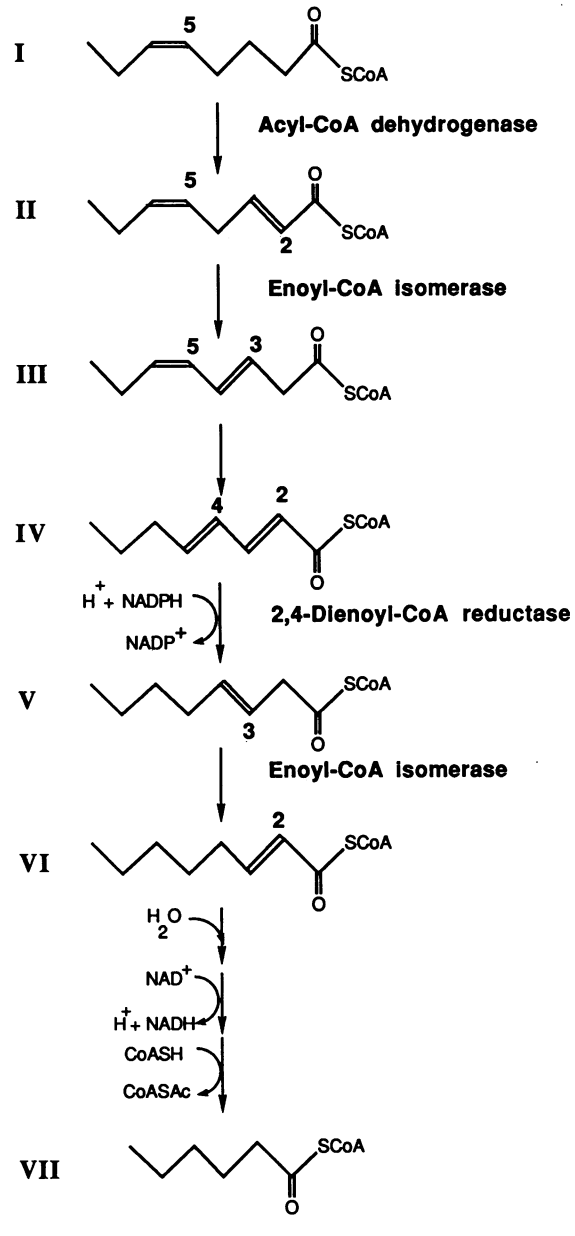

FIG. 5. Proposed pathway of the NADPH-dependent β-oxidation of 5-*cis*-octenoyl-CoA. Enoyl-CoA isomerase is Δ^3,Δ^2-enoyl-CoA isomerase. The metabolites shown are as follows: **I**, 5-*cis*-octenoyl-CoA; **II**, 2-*trans*-5-*cis*-octadienoyl-CoA; **III**, 3-*trans*-5-*cis*-octadienoyl-CoA; **IV**, 2-*trans*-4-*trans*-octadienoyl-CoA; **V**, 3-*trans*-octenoyl-CoA; **VI**, 2-*trans*-octenoyl-CoA; **VII**, *n*-hexanoyl-CoA.

300 nm in a time-dependent manner. These spectral changes are indicative of the formation of 2,4-octadienoyl-CoA (**IV**). Since the isomerization product **IV** and synthetic 2-*trans*-4-*trans*-octadienoyl-CoA could not be separated by HPLC whereas 2-*trans*-4-*trans*-decadienoyl-CoA and 2-*trans*-4-*cis*-decadienoyl-CoA can be separated (M.N. and H.S., unpublished results), the 2,4-octadienoyl-CoA most likely has the all-*trans* configuration. The isomerization of 3,5-octadienoyl-CoA (**III**) to 2,4-octadienoyl-CoA (**IV**) could be the consequence of the two double bonds shifting either simultaneously or one at a time. If the two double bonds shift one-by-one, 2,5-octadienoyl-CoA would be an intermediate in the isomerization reaction. The observation that the 3,5 → 2,4-isomerization occurred much faster than the 2,5 → 2,4-isomerization argues against a mechanism involving separate shifts of double bonds and favors the simultaneous shift of both double bonds. If so, a $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase is expected to be present in the mitochondrial extract. However, it remains to be established whether this enzymatic activity is due to an uncharacterized enzyme or is the unidentified activity of a known enzyme. The identity of 2,4-octadienoyl-CoA (**IV**) was established beyond doubt by the spectral changes observed when it was reduced by NADPH in the presence of 2,4-dienoyl-CoA reductase, by identification of the reduction product 3-octenoyl-CoA (**V**) on HPLC, and by its complete β-oxidation to hexanoyl-CoA (**VII**), butyryl-CoA, and acetyl-CoA catalyzed by a mitochondrial extract in the presence of NADPH, NAD^+, and CoA. The reported conversion of 5-*cis*-enoyl-CoAs to saturated fatty acyl-CoAs in the presence of NADPH (5) could be the consequence of 2-*trans*-enoyl-CoAs (e.g., compound **VI**) being reduced to the saturated acyl-CoAs by NADPH-dependent 2-enoyl-CoA reductase, which is present in mitochondria (24).

This study demonstrates that 5-octenoyl-CoA can be degraded by the pathway shown in Fig. 5, which requires NADPH and results in the reductive removal of the preexisting double bond. However, it is not yet clear if all 5-enoyl-CoA intermediates formed during the β-oxidation of polyunsaturated fatty acids are degraded by this pathway. It also remains to be established whether 5-enoyl-CoAs are exclusively degraded via the NADPH-dependent pathway or perhaps are metabolized by several routes, including the direct β-oxidation of 5-*cis*-enoyl-CoAs to 3-*cis*-enoyl-CoAs, which until now was thought to be their only route of β-oxidation.

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