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PEROXISOMES CONTAIN $\Delta^{3,5},\Delta^{2,4}$-DIENOYL-CoA ISOMERASE AND THUS POSSESS ALL ENZYMES REQUIRED FOR THE β-OXIDATION OF UNSATURATED FATTY ACIDS BY A NOVEL REDUCTASE - DEPENDENT PATHWAY

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SUMMARY. The presence of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase in peroxisomes was demonstrated by determining the subcellular distribution of this enzyme in rat liver. The peroxisomal and mitochondrial forms of the isomerase exhibit similar chain length specificities and they are homologous as indicated by the recognition of the peroxisomal 66-kDa enzyme by an antiserum raised against the mitochondrial 32-kDa isomerase. This report demonstrates that peroxisomes contain all enzymes required for the beta oxidation of unsaturated fatty acids with odd-numbered double bonds by a novel pathway in which double bonds are reductively removed by the NADPH-dependent 2,4-dienoyl-CoA reductase.

The degradation of unsaturated and polyunsaturated fatty acids requires auxiliary enzymes in addition to the enzymes necessary for the β-oxidation of saturated fatty acids (1). 2,4-Dienoyl-CoA reductase (EC 1.3.1.34) and Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) facilitate the reductive removal of even-numbered double bonds, whereas only the latter enzyme was thought to be necessary for the isomerization of odd-numbered double bonds resulting in the formation of intermediates that can reenter the β-oxidation spiral (2). Recently, a novel pathway for the reductive removal of odd-numbered double bonds was described (3). A key reaction of this pathway is a novel double bond isomerization catalyzed by $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. This isomerase together with 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase facilitates the reduction of odd-numbered double bonds (3). $\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA isomerase, which has been purified from rat liver (4,5), was detected in mitochondria, but not in peroxisomes (4). However, the recent observation that odd-numbered double bonds are reductively removed during the β-oxidation of arachidonic acid in peroxisomes, is indicative of the presence of

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$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase in this organelle (6). This situation prompted a reinvestigation of the cellular distribution of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase

MATERIALS AND METHODS

Nycodenz, CoASH, NADPH, arachidonic acid, and most biochemicals were purchased from Sigma. Bio-Rad was the supplier of the dye reagent for protein assays and of the materials for immunoblotting including the goat anti-rabbit IgG conjugated with alkaline phosphatase. $\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA isomerase was purified from rat liver and pig heart by the same procedure (4). Rabbit antiserum to the rat liver dienoyl-CoA isomerase was raised by Pocono Rabbit Farms and Laboratory, Canadensis, PA. Male Sprague-Dawley rats were obtained from Taconic Farms, Germantown, N.Y. and maintained on rodent chow or rodent chow containing 2% (w/w) di(ethylhexyl)phthalate. 5-*cis*-Tetradecenoic acid was synthesized by reacting 4-pentyn-1-ol (Farchan Laboratories), blocked at its hydroxyl group with dihydropyran, with 1-bromooctane in liquid ammonia in the presence of sodamide. After removal of the blocking group by acid hydrolysis in methanol, the acetylenic alcohol was purified by vacuum distillation and reduced with Lindlars catalyst to 4-*cis*-tridecen-1-ol. The alcohol was converted to its mesylate which was reacted with NaCN in dimethylsulfoxide. Hydrolysis of the nitrile with anhydrous 25% HCl in methanol yielded methyl 5-*cis*-tetradecenoate which after purification yielded a product of 94% purity. 5-*cis*-Tetradecenoic acid was obtained by hydrolysis of its methyl ester with 4N NaOH. The following substrates were prepared according to the indicated procedures: 2-*trans*-4-*cis*-decadienoyl-CoA (7), sorboyl-CoA (8), 3,5-octadienoyl-CoA (4). 5-*cis*-Tetradecenoyl-CoA and arachidonoyl-CoA were synthesized by the mixed anhydride method as detailed by Fong and Schulz (9) and were converted enzymatically to 3,5-tetradecadienoyl-CoA and 3,5,8,11,14-eicosapentaenoyl-CoA, respectively, by the procedure used to prepare 3,5-octadienoyl-CoA (4).

$\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA isomerase was assayed by measuring spectrophotometrically the increase in absorbance at 300 nm as described (4). The assay of 2,4-dienoyl-CoA reductase is based on the substrate-dependent oxidation of NADPH as described (10). Malate dehydrogenase (11), catalase (12), and esterase (13) were assayed by established procedures. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per min. Protein concentrations were determined by the dye binding assay as described by Bradford (14).

Heavy and light mitochondrial fractions from rat liver were isolated as described by de Duve et al. (15). A soluble extract of heavy mitochondria was prepared by sonicating the mitochondrial suspension 10 x 10 s at 4°C with an Ultrasonic sonifier (model W-385) equipped with a microtip and centrifuging it at 100,000 x g for 60 min. Subcellular fractions were prepared by density gradient centrifugation of a light mitochondrial fraction. For this purpose, a 30% (w/v) solution of Nycodenz containing 1 mM EDTA, 5 mM Hepes (pH 7.3) and 0.1% ethanol was prepared at 4°C and 7.6 ml of this solution were placed in a 10 ml ultracentrifuge tube on top of 0.5 ml of a 60% sucrose cushion. A density gradient was generated by centrifugation at 60,000 x g in a T865.1 small angle rotor on a DuPont RC70 ultracentrifuge at 4°C for 24 hours. A light mitochondrial fraction from rat liver (4 mg of protein in 0.5 ml) was layered on top of the gradient followed by 0.5 ml of a cover solution of a 3-fold diluted isolation buffer containing 250 mM sucrose , 1 mM EDTA, 0.1% ethanol, 10 mM Tris-HCl (pH 7.4). The sample was centrifuged at 76,000 x g for 60 min at 4°C. Sixteen fractions were collected from the

bottom of the tube. Peroxisomes, microsomes, and mitochondria were localized by assaying the marker enzymes catalase, esterase, and malate dehydrogenase, respectively. Fractions were also assayed for 2,4-dienoyl-CoA reductase and $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase activities. For assaying the isomerase, fractions were dialyzed overnight against 0.1 M KP_i containing 0.25 M sucrose, 1 mM EDTA, 10 mM leupeptin, 10 mM pepstatin, 0.1% (v/v) hexamethylphosphoramide, and 5 mM mercaptoethanol and then sonicated 5 x 20 s as desribed above. All assay mixtures, except for the isomerase assay, contained 0.1% (v/v) Triton X-100 or 0.15% Tween 40.

Proteins present in rat liver mitochondria and peroxisomes were separated by electrophoresis on gradient (4-20%) polyacrylamide gels in the presence of sodium dodecylsulfate (17) and either stained with Coomassie blue or transferred to a nitrocellulose membrane by semi-dry blotting (18). Included as control samples were purified rat liver dienoyl-CoA isomerase and partially purified dienoyl-CoA isomerase from pig heart. Proteins were probed with diluted (1:250) rabbit antiserum raised against the rat liver dienoyl-CoA isomerase. After incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase , the antigen bands were visualized by incubating the membrane in a staining mixture containing the alkaline phosphatase substrate (19).

RESULTS

The presence of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) in peroxisomes was reinvestigated because this enzyme was implicated in the peroxisomal metabolism of arachidonic acid (6) even though it was not detected when peroxisomes were directly assayed for it (4). Since peroxisomes had been isolated by centrifugation on a Nycodenz density gradient and assayed for dienoyl-CoA isomerase in the presence of Triton X-100, the effects of Nycodenz and Triton X-100 on the activity of purified dienoyl-CoA isomerase from rat liver were determined. Both compounds, at concentrations that were achieved in the assay mixture, strongly inhibited dienoyl-CoA isomerase. Given this situation, the subcellular distribution of dienoyl-CoA isomerase was redetermined except that fractions obtained by Nycodenz density gradient centrifugation were extensively dialyzed and sonicated before being assayed for dienoyl-CoA isomerase activity in the absence of Triton X-100. Shown in Fig. 1 are the results of such experiment with a light mitochondrial or L-fraction isolated from the liver of a rat fed rodent chow containing 2% di(ethylhexyl)phthalate to induce high levels of peroxisomal dienoyl-CoA isomerase. Panels A-C illustrate the separation and banding of peroxisomes, mitochondria, and microsomes, respectively. When the subcellular distribution of dienoyl-CoA isomerase, shown in Fig. 1D, is compared with the locations of marker enzymes (see Fig. 1A-C), it is apparent that dienoyl-CoA isomerase is present both in mitochondria and peroxisomes. The dienoyl-CoA isomerase activity detected in fractions 12-14, where microsomes are concentrated, is attributed to contaminating mitochondria and peroxisomes. The same subcellular fractions also were assayed for 2,4-dienoyl-CoA reductase with sorboyl-CoA and 2,4-decadienoyl-CoA as substrates. As expected, 2,4-dienoyl-CoA reductase activity was found to be present in peroxisomes and mitochondria (16), whereas the activity

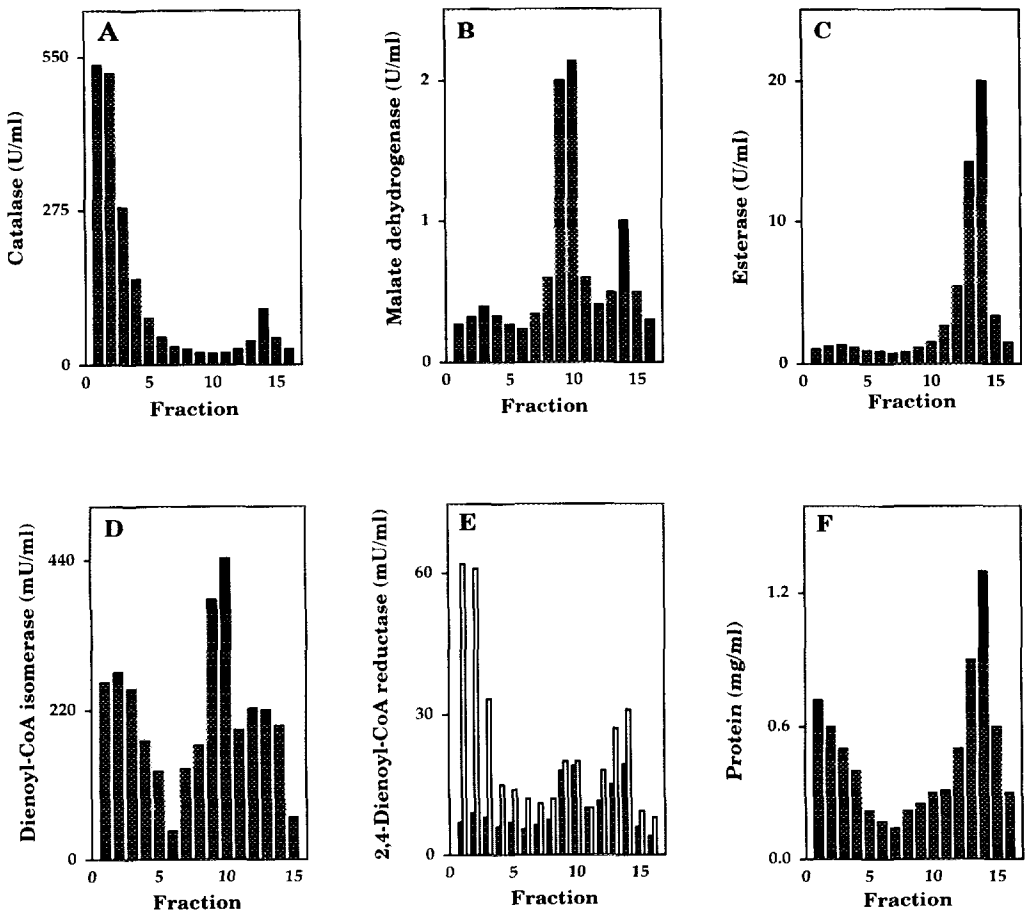

Fig. 1. Subcellular distribution of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) and 2,4-dienoyl-CoA reductase. A light mitochondrial fraction from rat liver was fractionated by Nycodenz density gradient centrifugation and fractions were assayed for marker enzymes, dienoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase with sorboyl-CoA (solid bars) and 2,4-decadienoyl-CoA (open bars) as described under MATERIALS AND METHODS.

associated with the microsomal fractions most likely was due to contaminating peroxisomes and mitochondria. Interestingly, the ratio of activities measured with the two substrates in peroxisomes was different from the mitochondrial ratio. The relatively low sorboyl-CoA reductase activity in peroxisomes as compared to mitochondria agrees with the presumed function of the former organelle in the chain-shortening of fatty acids, in contrast to mitochondria which completely degrade fatty acids.

The further characterization of peroxisomal dienoyl-CoA isomerase required the use of purified peroxisomes, most importantly peroxisomes with only a minor mitochondrial contamination. Using the specific activity of catalase relative to that of malate dehydrogenase as a criterion, the best preparation was a peroxisomal

fraction obtained by Nycodenz density gradient centrifugation of light mitochondria that had been isolated from the livers of rats fed a diet without the peroxisomal proliferator. The mitochondrial contamination of such peroxisomal fraction was well below 10%. The specific activities of dienoyl-CoA isomerase with three different substrates were determined with purified rat liver peroxisomes and an extract from heavy mitochondria (see Table I). The chain length specificities of the peroxisomal and mitochondrial dienoyl-CoA isomerase were similar and not sufficiently different to distinguish between these two forms of the enzyme. When the specific activities measured with organelles from normal rats (see Table I) are compared with those from rats fed di(ethylhexyl)phthalate (see Fig. 1), it is apparent that the activity of dienoyl-CoA isomerase was induced less in peroxisomes (3-fold) than in mitochondria (<9-fold).

The presence of dienoyl-CoA isomerase in peroxisomes and mitochondria and their structural relationship were probed by immunoblotting with antiserum raised against the purified mitochondrial isomerase from rat liver (see Fig. 2A, lane 3). This antiserum reacted with the purified 32-kDa isomerase from rat liver (see Fig. 2B, lane 8) and cross-reacted with the partially purified pig heart isomerase of equal molecular mass (see Fig. 2A, lane 4 and Fig. 2B, lane 9). When rat liver mitochondria were probed, the antiserum recognized three proteins with molecular masses of 32 kDa, 50 kDa, and 66 kDa (see Fig. 2B, lane 7). The strongest response was with the 32-kDa protein presumed to be identical with the purified isomerase. Three proteins with molecular masses of 66 kDa, 50 kDa, and 32 kDa also were recognized when rat liver peroxisomes were probed with the antiserum to mitochondrial isomerase (see Fig. 2B, lane 6). However, the strongest response was with the 66-kDa protein. This experiment demonstrates that antibodies raised against the mitochondrial dienoyl-CoA isomerase cross-react with the peroxisomal form of the isomerase which may be highly homologous or identical with the mitochondrial isozyme. Moreover, several forms of the isomerase seem to be present in either organelle. The smaller forms of the isomerase may be proteolytic

Table I: Specific activities of $\Delta^{3,5}, \Delta^{2,4}$-dienoyl-CoA isomerase in peroxisomes and in a mitochondrial extract from rat liver

Enzyme	Substrate*	Specific Activity (nmol/min/mg)	
		Peroxisomes	Mitochondrial extract
$\Delta^{3,5}, \Delta^{2,4}$-Dienoyl-CoA isomerase	C_8	180 (100%)	190 (100%)
	C_{14}	29 (16%)	36 (19%)
	C_{20}	22 (12%)	13 (7%)

*C_8, 3,5-octadienoyl-CoA; C_{14}, 3,5-tetradecadienoyl-CoA; C_{20}, 3,5,8,11,14-eicosapentaenoyl-CoA.

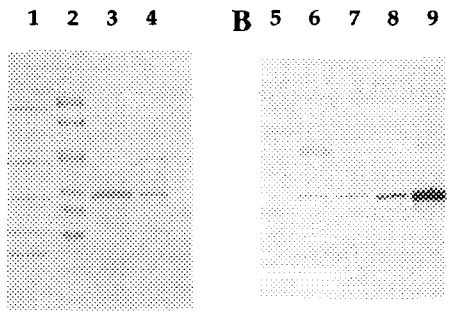

Fig. 2. Polyacrylamide gel electrophoresis of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (isomerase) in the presence of sodium dodecylsulfate. **A.** Gel after staining with Coomassie blue. **B.** Immunoblot after probing with antibodies to rat liver isomerase. Lane 1, molecular size standards (top to bottom in kDa: 97.4, 66, 45, 31, 21.3, 14.5); lanes 2 & 5, prestained molecular size standards (top to bottom in kDa: 112, 84, 53.2, 34.9, 28.7, 20.5); lanes 3 & 9, purified isomerase from rat liver (1.2 μg); lanes 4 & 8, partially purified isomerase from pig heart (1.5 μg); lane 6, rat liver peroxisomes (27 μg); lane 7, heavy mitochondria from rat liver (27 μg).

degradation products of the large form. Especially, the 66-kDa form may be cleaved once to yield two fragments of approximately 32 kDa each. Such proteolytic processing would explain the presence of two closely spaced bands which can be detected when mitochondrial dienoyl-CoA isomerase is subjected to gel electrophoresis and stained with Coomassie blue (see Fig. 2A, lane 3).

DISCUSSION

The evidence presented in this study demonstrates that dienoyl-CoA isomerase is present in peroxisomes and mitochondria. Both organelles also contain 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase in addition to the enzymes necessary for the β-oxidation of saturated fatty acids (1). Thus, peroxisomes, besides mitochondria, contain all enzymes required for the degradation of fatty acids with odd-numbered double bonds by the novel pathway outlined in Fig. 3. This pathway involves the reductive removal of the 5 double bond during one cycle of β-oxidation. After the conversion of 5-*cis*-enoyl-CoA (compound I) to 2-*trans*,5-*cis*-dienoyl-CoA (compound II) in the first reaction of the β-oxidation cycle, two isomerization reactions produce a 2,4-dienoyl-CoA intermediate (compound IV), which is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to yield first 3-*trans*-enoyl-CoA (compound V) and then 2-*trans*-enoyl-CoA (compound VI). The net result of the three isomerization and one reduction reactions is the removal of the 5 double bond (compare the structures of compounds II and VI). The enzyme unique to this pathway is dienoyl-CoA isomerase, which previously was shown to be present in mitochondria, but which also exists in peroxisomes as shown in this study. This enzyme was purified from rat liver by two groups which reported the subunit molecular mass of this enzyme

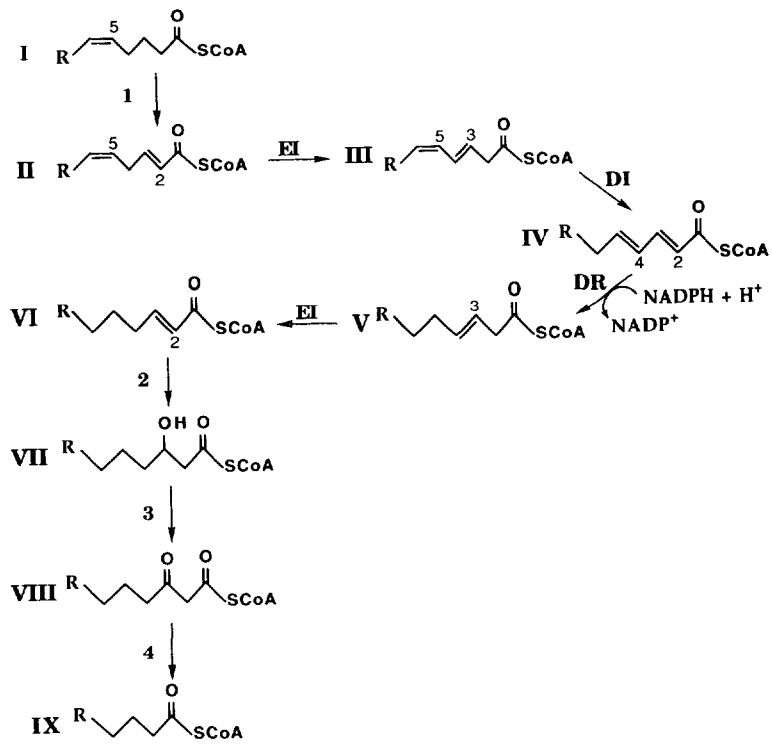

Fig. 3. Beta oxidation of 5-*cis*-enoyl-CoA. Abbreviations: EI, Δ^3,Δ^2-enoyl-CoA isomerase; DI, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase; DR, 2,4-dienoyl-CoA reductase.

to be 32 kDa (4) and 55 kDa (5). This difference may reflect the isolation of the two isoforms from mitochondria and peroxisomes or may be due to the isolation of different proteolytic forms of the mitochondrial enzyme. The purified 32-kDa isomerase used in this study is most likely of mitochondrial origin because an enzyme with the same subunit molecular mass was isolated from pig heart which contains few peroxisomes and because an antiserum raised against it recognized most strongly a 32-kDa protein in a mitochondrial extract. Elucidation of the structural relationship between various isomerase forms requires the molecular characterization of all forms.

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