Functional Characterization of $\Delta^3,\Delta^2$-Enoyl-CoA Isomerases from Rat Liver

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SUMMARY

The degradation of unsaturated fatty acids by β-oxidation involves $\Delta^3,\Delta^2$-enoyl-CoA isomerases (enoyl-CoA isomerases) that catalyze $3$-$\text{cis} \rightarrow 2$-$\text{trans}$ and $3$-$\text{trans} \rightarrow 2$-$\text{trans}$ isomerizations of enoyl-CoAs and the $2,5 \rightarrow 3,5$ isomerization of dienoyl-CoAs. An analysis of rat liver enoyl-CoA isomerases revealed the presence of a monofunctional enoyl-CoA isomerase (ECI) in addition to mitochondrial enoyl-CoA isomerase (MECI) in mitochondria while peroxisomes contain ECI and multifunctional enzyme 1 (MFE1). Thus ECI, which previously had been described as peroxisomal enoyl-CoA isomerase, was found to be present in both peroxisomes and mitochondria. This enzyme seems to be identical with mitochondrial long-chain enoyl-CoA isomerase (Kilponen, J.M., Palosaari, P.M. and Hiltunen, J.K. 1990. Biochem. J. 269, 223-226).

All three hepatic enoyl-CoA isomerases have broad chain length specificities but are distinguishable by their preferences for one of the three isomerization reactions. MECI is most active in catalyzing the $3$-$\text{cis} \rightarrow 2$-$\text{trans}$ isomerization, ECI has a preference for the $3$-$\text{trans} \rightarrow 2$-$\text{trans}$ isomerization, and MFE1 is the optimal isomerase for the $2,5 \rightarrow 3,5$ isomerization. A functional characterization based on substrate specificities and total enoyl-CoA isomerase activities in rat liver leads to the conclusion that the $3$-$\text{cis} \rightarrow 2$-$\text{trans}$ and $2,5 \rightarrow 3,5$ isomerizations in mitochondria are catalyzed overwhelmingly by MECI while ECI contributes significantly to the $3$-$\text{trans} \rightarrow 2$-$\text{trans}$ isomerization. In peroxisomes, ECI is predicted to be the dominant enzyme for the $3$-$\text{cis} \rightarrow 2$-$\text{trans}$ and $3$-$\text{trans} \rightarrow 2$-$\text{trans}$ isomerizations of long-chain intermediates whereas MFE1 is the key enzyme in the $2,5 \rightarrow 3,5$ isomerization.
Both saturated and unsaturated fatty acids are degraded by β-oxidation. However, the degradation of unsaturated fatty acids, in contrast to the breakdown of saturated fatty acids, requires the involvement of several auxiliary enzymes that catalyze the isomerization and reduction of double bonds (reviewed in Ref. 1). During the β-oxidation of unsaturated fatty acid with even-numbered double bonds, e.g. the 12-cis double bond of linoleic acid, a 3-trans→2-trans double bond shift takes place, while three isomerizations, 3-cis→2-trans, 3-trans→2-trans, and 2,5→3,5 (see Scheme 1) occur during the β-oxidation of fatty acids with odd-numbered double bonds, e.g. the 9-cis double bond of oleic acid and linoleic acid. All of these positional and steric isomerizations of double bonds are catalyzed by Δ³,Δ²-enoyl-CoA isomerase (EC5.3.3.8) (enoyl-CoA isomerase¹). Five mammalian enzymes with enoyl-CoA isomerase activities have been described. They are mitochondrial enoyl-CoA isomerase (MECI) (2-4), mitochondrial long-chain enoyl-CoA isomerase (5), mitochondrial enoyl-CoA hydratase or crotonase (6), peroxisomal enoyl-CoA isomerase (PECI) (7), and multifunctional enzyme 1 (MFE1) (8). According to the available information, the first three enoyl-CoA isomerase activities are present in mitochondria while the last two enzymes are associated with peroxisomes. Structural information about this group of enzymes has been growing rapidly as reflected by the recent publications of crystal structures for rat crotonase (9) and yeast PECI (10). These two enzymes are hexameric proteins with similar patterns of folding even though they exhibit low sequence homology and catalyze different reactions. The specific metabolic functions of enoyl-CoA isomerases are poorly defined, especially since it was demonstrated that more than one isomerase is present in either mitochondria or peroxisomes and that collectively these enzymes catalyze three distinct reactions in β-oxidation.
Unanswered questions about the identity of enoyl-CoA isomerases, their subcellular localizations and their substrate specificities have prompted the present investigation.

**EXPERIMENTAL PROCEDURES**

*Materials* - Nycodenz, CoASH, NAD⁺, NADH, benzamidine hydrochloride, CM cellulose, di(ethylhexyl)phthalate, acyl-CoA oxidase from *Arthrobacter* sp. and most standard biochemicals were purchased from Sigma. Fatty acid free bovine serum albumin (BSA) was from Life Science Resources, Milwaukee, WI. Diketene, crotonic anhydride, hexanal, dodecanal, 3-trans-hexenoic acid, 3-hexyn-1-ol, and other standard chemicals were purchased from Aldrich. 3-Octyn-1-ol was purchased from Lancaster Synthesis Inc, Windham, NH. Matrex gel red A was purchased from Amicon, Danvers, MA. Dithiothreitol was purchased from Fisher Scientific, Fair Lawn, NJ. 5-cis-Octenoic acid, 3-cis-tetradecenoic acid, and 5-cis-tetradecenoic acid were kindly provided by Dr. Howard Sprecher, Ohio State University. Hydroxylapatite, the dye reagent for protein assays, polyacrylamide ready gels, and the materials for immunoblotting, including the goat anti-rabbit IgG conjugated with alkaline phosphatase, were bought from Bio-Rad. Sep-Pak C₁₈ cartridges were purchased from Waters. Iodixanol (Optiprep) was from Nycomed Pharma AS, Oslo, Norway. The antisera to rat peroxisomal multifunctional enzyme 1 was a kind gift of Dr. Ronald Wanders, University of Amsterdam, Netherlands. Rabbit antisera against mitochondrial and peroxisomal enoyl-CoA isomerases were raised by Pocono Rabbit Farms and Laboratory, Canadensis, PA. Male Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. Bovine liver enoyl-CoA hydratase (crotonase) (11), pig heart 3-ketoacyl-CoA thiolase (12), recombinant pig liver 3-hydroxyacyl-CoA dehydrogenase (13), and recombinant rat
mitochondrial $\Delta_{3,5}^{\Delta},\Delta_{2,4}^{\Delta}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) (14) were purified as described.

**Synthesis and Purification of Substrates** - 3-trans-Octenoic acid and 3-trans-tetradecenoic acid were synthesized from malonic acid and hexanal and dodecanal, respectively, according to Boxer and Linstead (15). 3-cis-Hexenoic acid and 3-cis-octenoic acid were synthesized from 3-hexyn-1-ol and 3-octyn-1-ol, respectively, according to Stoffel and Ecker (2). Acetoacetyl-CoA was synthesized from diketene and CoASH according to White and Jencks (16). Crotonyl-CoA was synthesized from crotonic anhydride and CoASH according to Weeks and Wakil (17). 2-trans,5-cis-Octadienoyl-CoA and 2-trans,5-cis-tetradecadienoyl-CoA were synthesized from 5-cis-octenoic acid and 5-cis-tetradecenoic acid according to Shoukry and Schulz (18). Fatty acyl-CoA derivatives of all other fatty acids were prepared by the mixed anhydride method as described by Fong and Schulz (19). All substrates for enoyl-CoA isomerase assays were purified by HPLC. A Waters µBondapak C$_{18}$ column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system was used for this purpose. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H$_2$O (9:1, v/v) content of the 50 mM ammonium phosphate buffer (pH 5.5) from 10% to 40% (for C$_6$ substrates), from 10% to 50% (for C$_8$ substrates) or from 10% to 70% (for C$_{14}$ substrates) at a flow rate of 2 ml/min. Desired fractions were collected and the organic solvent was evaporated under vacuum on a rotatory evaporator. Sep-Pak C$_{18}$ cartridges were used to concentrate the substrates after HPLC purification. After the substrates were absorbed onto the Sep-Pak columns, they were eluted with 3 ml of methanol. Methanol was evaporated under vacuum before the substrates were redissolved in H$_2$O. Concentrations of acyl-CoAs were determined spectrophotometrically by
quantifying CoASH with Ellman’s reagent (20) after cleaving the thioester bonds with NH₂OH at pH 7.0 (19).

Enzyme and Protein Assays – Enoyl-CoA isomerases were assayed spectrophotometrically by measuring the absorbance increase at either 263 nm or 280 nm on a Gilford recording spectrophotometer at 25°. A typical assay mixture contained 0.2 M KP₁ (pH 8.0), 35 µM substrate, BSA (0.2 mg/ml), and enzyme. Extinction coefficients of 6,700 M⁻¹cm⁻¹ at 263 nm or 4,400 M⁻¹cm⁻¹ at 280 nm were used to calculate rates. Enoyl-CoA isomerase activities associated with multifunctional enzyme 1 or fractions obtained by separating mitochondrial, peroxisomal, or tissue extracts were assayed by a coupled assay (21), in which the isomerization of 35 µM 3-enoyl-CoA to 2-enoyl-CoA was coupled to the hydration of the latter compound by crotonase, the NAD⁺-dependent dehydrogenation of the 3-hydroxyacyl-CoA intermediate by 3-hydroxyacyl-CoA dehydrogenase and the thiolytic cleavage of the resultant 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase. Formation of NADH (ε = 6,220 M⁻¹cm⁻¹) was the basis for calculating rates of isomerization. When 2,5-octadienoyl-CoA or 2,5-tetradecadienoyl-CoA was used as substrate in the enoyl-CoA isomerase assay, dienoyl-CoA isomerase was added as a coupling enzyme. A typical assay mixture contained 0.2 M KP₁ (pH 8.0), 35 µM substrate, dienoyl-CoA isomerase (0.25 U/ml), BSA (0.2 mg/ml), and an aliquot of enzyme. An extinction coefficient of 27,800 M⁻¹cm⁻¹ (22) was used to calculate rates. Enoyl-CoA hydratase (19), 3-hydroxyacyl-CoA dehydrogenase (21), catalase (23) and malate dehydrogenase (24) activities were determined by established procedures. Enzymes were diluted with 50 mM KP₁ (pH 7.0) containing BSA (1 mg/ml). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate to product in one minute. Protein concentrations were
determined as described by Bradford (25) with BSA as standard. For the kinetic characterizations of ECI and MECI with 3-enoyl-CoAs as substrates, absorbance changes at 280 nm were recorded because the basal absorbance at 263 nm was too high at elevated substrate concentrations. Rates were measured at five or six substrate concentrations and averages of three assays were used for each point. Preanalyses were performed to determine appropriate substrate and enzyme concentration ranges. Kinetic parameters (K_m and V_max) were obtained by nonlinear curve fitting using the SigmaPlot 2000 program. Values of k_cat, the catalytic center activity, were calculated using the reported subunit molecular masses of 40.4 kDa (7), 78.5 kDa (26), and 29 kDa (4) for ECI, MFE 1, and MECI, respectively.

Isolation and Purification of Subcellular Organelles from Rat Liver – Mitochondria and a light mitochondrial fraction were prepared from rat livers as described by Nedergaard and Cannon (27) and de Duve et al. (28), respectively. Adult male Sprague-Dawley rats (240-260 g) were used. They were kept on a standard chow and then fasted for 24 hours before their livers were removed. Mitochondria and peroxisomes were separated by Nycodenz density gradient centrifugation of a light mitochondrial fraction as described (29). 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 and 21 ml of this solution were placed in a 30 ml ultracentrifuge tube on top of 1.5 ml of a 60% sucrose cushion. A density gradient was generated by centrifugation at 60,000 x g in a T865 small angle rotor on a DuPont RC70 ultracentrifuge at 4°C for 24 h. A light mitochondrial fraction (~15 mg of protein in 1.5 ml) was layered on top of the gradient followed by 1.5 ml of a cover solution of a 3-fold diluted isolation buffer containing 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, and 10 mM Tris (pH 7.4). This was followed by centrifugation at 76,000 x g for one
hour at 4°C. Fractions of 1.6 ml each were collected from the bottom of the tube, diluted 2-fold with isolation buffer, and centrifuged at 17,500 x g for 20 minutes. Pellets from each fraction were redisolved in 100 µl of isolation buffer for further analysis. Peroxisomes were purified by centrifugation of a light mitochondrial fraction on a preformed continuous gradient of iodixanol (Optiprep) as described by van Veldhoven et al. (30). The gradient was prepared in 30 ml centrifuge tubes from equal volumes of iodixanol (20%, w/v) containing 0.41 M sucrose, 1.2 mM EDTA, 0.12% ethanol and 6 mM Mops (pH 7.2) and iodixanol (40%, w/v) containing 0.14 M sucrose, 0.8 mM EDTA, 0.08% ethanol and 4 mM Mops (pH 7.2) by use of a gradient mixer and a peristaltic pump. After 20 ml of the gradient mixture had been placed in each tube, 2 ml of iodixanol (50%, w/v) containing 25 mM sucrose, 0.5 mM EDTA, 0.05% ethanol and 2.5 mM Mops (pH 7.2) were delivered to the bottom of the tube by use of a long syringe needle. Three ml of light mitochondria (12 mg of protein/ml) were carefully layered on top of the gradient. The tubes were then centrifuged at 105,000 x g for one hour in a T865 fixed angle rotor at 4°C using the slow acceleration and deceleration mode. Fractions were collected from the bottom after slowly inserting a thin glass tube through the bottom of the tube. Marker enzyme activities for mitochondria and peroxisomes were assayed. Peroxisomal fractions were combined and diluted 2-fold with isolation buffer before they were harvested by centrifugation at 17,500 x g for 20 min.

Separation of Enoyl-CoA Isomerases on a Hydroxylapatite Column – Portions (4 g) of rat liver were homogenized in 16 ml of 10 mM KP$_i$ (pH7.4) containing 0.2M KCl, 0.5 mM EDTA, 1mM benzamidine and 0.5 mM DTT (buffer A). The homogenates were sonicated and centrifuged at 100,000 x g for 1 hour. The supernatants were dialyzed against 20 mM KP$_i$ (pH 7.0) containing 0.5mM benzamidine and 0.5 mM DTT (buffer B). After dialysis, the samples were applied to
hydroxylapatite columns (1.5 cm x 15 cm) equilibrated with buffer B at a flow rate of 10 ml/h. The proteins bound to the column were eluted with a linear gradient made up of 200 ml each of 20 mM and 500 mM KPi (pH 7.0) containing 0.5mM benzamidine and 0.5 mM DTT. Also
enoyl-CoA isomerases present in peroxisomes (15 mg of protein) and mitochondria (130 mg of protein) were separated by this procedure after sonicating and centrifuging the organelle suspensions before the resultant soluble extracts were loaded onto hydroxylapatite columns and eluted by a KPi gradient from 20 mM to 500 mM.

Purification of MECI and MFE1 - Adult Sprague-Dawley rats were fed rodent chow containing 2% (w/w) di(ethylhexyl)phthalate for two weeks before being sacrificed. For the purification of mitochondrial enoyl-CoA isomerase (MECI) (3), mitochondria isolated from rat liver (28) were sonicated in 20 mM KPi (pH 7.0) containing 0.5 mM DTT, 1 mM EDTA, 0.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride (buffer A) and centrifuged at 100,000 x g for one hour. The supernatant was applied to a Matrix Gel Red A column (2.5 x 12 cm) previously equilibrated with buffer A. The column was washed with buffer A and then was developed with a gradient made up of 100 ml of buffer A and 100 ml of buffer A containing 1.2 M KCl. Fractions containing enoyl-CoA isomerase activity were combined. After dialysis overnight against 50 mM KPi (pH 6.0) containing 0.5 mM DTT, 0.5 mM benzamidine, 10% glycerol (buffer B), the sample was applied to a CM cellulose column (1.5 x 6 cm) previously equilibrated with buffer B. Mitochondrial enoyl-CoA isomerase was eluted with a linear gradient made up of 25 ml of buffer B and 25 ml of buffer B containing 0.4 M KCl.

For the purification of MFE1 (33), a liver from a rat treated with di(ethylhexyl)phthalate was homogenized in 1:5 (w/v) of 10 mM K₃PO₄ containing 1 mM EDTA, 1 mM EGTA, 1 mM
benzamidine, 0.5 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride with a Polytron tissue homogenizer. The suspension was sonicated 10-times for 20 s each at 4°C before being centrifuged at 100,000 x g for one hour. The supernatant was adjusted to pH 7.0 before being applied to a phosphocellulose column (2.5 x 20 cm) previously equilibrated with 50 mM KP$_1$ (pH 7.0) 0.5 mM benzamidine, 0.5 mM DTT (buffer C). The column was eluted with a linear gradient made up of 200 ml of 50 mM KP$_1$ in buffer C and 200 ml of 500 mM KP$_1$ in buffer C. Active fractions were combined and fractionated with (NH$_4$)$_2$SO$_4$. The precipitate formed between 200 and 400 g/l of (NH$_4$)$_2$SO$_4$ was dialyzed overnight against buffer B before being applied to a CM cellulose column (1.5 x 8 cm) previously equilibrated with buffer B. The enzyme was eluted with a linear gradient of 100 ml of 50 mM KP$_1$ in buffer B and 100 ml of 200 mM KP$_1$ in buffer B. Active fractions were combined and concentrated.

**SDS-PAGE and Immunoblotting** – Samples were treated with equal volumes of SDS sample buffer and subjected to SDS-PAGE on either gradient (4-20%) or 10% ready gels (32). Proteins were transferred to a nitrocellulose membrane by semi-dry blotting (33) using the semi-dry transfer cell from BioRad. Proteins remaining on the gel were visualized by staining with Coomassie blue. The membrane was blocked with 5% dry milk for one hour before being incubated with a 500-fold diluted rabbit antiserum for one hour. After incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase for one and a half hour, it was developed with a staining mixture containing the alkaline phosphatase substrate until the antigen bands were visualized (34).
Expression and Purification of Recombinant Peroxisomal Enoyl-CoA Isomerase - The expression and purification of recombinant peroxisomal enoyl-CoA isomerase was done as described in principle by Geisbrecht et al. (7). For the expression of His6-PECI, freshly transformed BL21 (DE3) cells harboring the pT7His-PECI plasmid were grown at 30 °C with vigorous shaking (225 rpm) for 12-14 h in 50 ml of LB media supplemented with kanamycin sulfate (25 µg/ml) and sterile 1% dextrose. After this incubation period, 7.5 ml of the preculture cell suspension were diluted into 500 ml of 2YT media containing kanamycin sulfate (25 µg/ml) and sterile 0.2% dextrose. This culture was grown with vigorous shaking at 18 °C until the A600 reached 0.5, at which time the induction of protein expression was initiated by the addition of 1 mM isopropyl-β-D-thiogalactoside. Following growth of the induced culture for 18 h, cells were harvested and sonicated in 50 ml of buffer A (20 mM KPi (pH 7.8) containing 500 mM NaCl, and 5 mM 2-mercaptoethanol) and centrifuged at 100,000 x g for 1 hour. The supernatant was diluted to a final volume of 200 ml with buffer A and was applied at a rate of 2 ml/min to a 5 ml bed of ProBond agarose at 4 °C. The column was washed with 5 volumes of buffer A and then with 20 volumes of buffer B (20 mM KPi (pH 6.0) containing 500 mM NaCl, and 5 mM 2-mercaptoethanol). Following these washing steps, the His6-PECI was eluted from the bed using an imidazole step gradient in buffer B. One column volume each of buffers containing 50, 250, 350, and 500 mM imidazole was applied sequentially to the resin bed. The eluants were collected and each fraction was assayed for enoyl-CoA isomerase. Fractions containing high activities were pooled, and the His6-PECI present was precipitated slowly by the addition of solid ammonium sulfate to 0.4 g/ml.
RESULTS

*Characterization of Mitochondrial Long-chain Enoyl-CoA Isomerase* - The aim of this study was the characterization of all enoyl-CoA isomerases that are present in rat liver and the determination of their contributions to the total enoyl-CoA isomerase activities in both mitochondria and peroxisomes. Mitochondrial long-chain enoyl-CoA isomerase had been detected by Kilponen *et al.* (5) who separated it from mitochondrial enoyl-CoA isomerase (MECI) and peroxisomal multifunctional enzyme 1 (MFE1) by chromatography on hydroxylapatite. However, they did not purify it any further. Because this enoyl-CoA isomerase was more active with 3-*trans*-dodecenoyl-CoA than with 3-*trans*-hexenoyl-CoA as substrate, they named it long-chain enoyl-CoA isomerase. They also concluded that it had a mitochondrial localization. We repeated the separation of a soluble extract from rat liver on hydroxylapatite but assayed each fraction with 3-*trans*-octenoyl-CoA and 3-*cis*-octenoyl-CoA because the ratio of activities obtained with these two substrates aids in the identification of different enoyl-CoA isomerases. Shown in Fig. 1 is the result of this experiment. The activity pattern with 3-*trans*-octenoyl-CoA as substrate is similar to that observed by Kilponen *et al.* (5) who concluded that the enoyl-CoA isomerase eluted first from the column was a novel isomerase, which they named long-chain enoyl-CoA isomerase. The activity pattern obtained with 3-*cis*-octenoyl-CoA as substrate was quite different. The isomerase activity present in fractions 13-18 is easily missed while the existence of two isomerase activities, presumably corresponding to MFE1 and MECI, in fractions 20 to 40 is more clearly revealed than with the 3-*trans* substrate. A *trans/cis* activity ratio of close to 2 determined for fractions 13-16 is similar to that of peroxisomal enoyl-CoA isomerase (PECI), which has a *trans/cis* activity ratio of 2 in contrast to MECI and MFE1 with *trans/cis* activity ratios below 1.
The presence of PECI in fractions 13-18 was confirmed by immunoblotting (data not shown). Thus, it seems that mitochondrial long-chain enoyl-CoA isomerase is identical with PECI.

*Subcellular Localization of Enoyl-CoA Isomerases* - If mitochondrial long-chain enoyl-CoA isomerase and PECI are the same enzyme, PECI must be present in both mitochondria and peroxisomes. To confirm this prediction, a light mitochondrial fraction was prepared from a rat liver homogenate and subjected to centrifugation on a Nycodenz density gradient. Fractions were assayed for catalase and malate dehydrogenase to localize peroxisomes and mitochondria, respectively. Fractions were also analyzed by immunoblotting with antibodies to MECI and PECI. The results shown in Fig. 2 demonstrate that MECI was present only in mitochondria (fractions 7-11), whereas PECI was detected in both peroxisomes (fractions 1-5) and mitochondria (fractions 7-11). Since the dual localization of PECI contradicts the reported unique association of this enzyme with peroxisomes, further confirmation was sought. For this purpose, an extract from isolated rat liver mitochondria was subjected to chromatography on hydroxylapatite. The results shown in Fig. 3 demonstrate the presence of at least two enoyl-CoA isomerases in mitochondria. The isomerase that was eluted first had a *trans/cis* activity ratio of approximately 2 and was recognized by an antibody to PECI. Hence, this enoyl-CoA isomerase was PECI. The enoyl-CoA isomerase corresponding to the second peak was identified as MECI because it had a *trans/cis* activity ratio below 1 and was detected with antibodies raised against MECI. A similar experiment was also carried out with a soluble extract from rat liver peroxisomes, which had been purified by centrifugation on an iodixanol density gradient. The results are shown in Fig. 4. Again, two peaks of enoyl-CoA isomerase activity were detected. The isomerase that was eluted first from the column was PECI as indicated by its *trans/cis* ratio.
activity ratio of approximately 2 and because of its recognition by antibodies to PECI. The second isomerase peak was due to MFE1 as demonstrated by immunoblotting and co-elution of enoyl-CoA isomerase, enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities. The possibility that MFE1 may bind either MECI or PECI and thereby acquire enoyl-CoA isomerase activity prompted an experiment in which amounts of MFE1, MECI, and PECI having equal activities of enoyl-CoA isomerase were subjected to SDS-PAGE and immunoblotting with antibodies to MECI and PECI (data not shown). The absence of MECI and PECI from the MFE1 preparation confirmed the previous conclusion that the enoyl-CoA isomerase activity of MFE1 is an endogenous property of this enzyme (8). Overall, these data demonstrate that the enoyl-CoA isomerase with a preference for 3-trans-enoyl-CoAs as substrate has a dual localization in peroxisomes and mitochondria. This enzyme had previously been described as peroxisomal enoyl-CoA isomerase (PECI) and as mitochondrial long-chain enoyl-CoA isomerase.

Henceforth, this isomerase will be referred to as Δ³,Δ²-enoyl-CoA isomerase (ECI).

Substrate Specificities of Enoyl-CoA Isomerases - The identification of two enoyl-CoA isomerases each in mitochondria (MECI and ECI) and peroxisomes (ECI and MFE1) raised the question as to their specific functions in the β-oxidation of unsaturated fatty acids. In an attempt to answer this question we determined the catalytic efficiencies of all three isomerases with substrates of different chain lengths for all three isomerization reactions. The same substrates were used to determine the kinetic parameters (K_m, V_max) of MECI, ECI and MFE1. For the purpose of analyzing the 3-cis→2-trans and 3-trans→2-trans isomerizations, 3-hexenoyl-CoA, 3-octenoyl-CoA and 3-tetradecenoyl-CoA were used as substrates to establish a chain length spectrum from short-chain to long-chain substrates. 2,5-Octenoyl-CoA and 2,5-tetradecenoyl-
CoA served as substrates to evaluate the $2,5 \rightarrow 3,5$ isomerization. The kinetic parameters obtained with MECI are listed in Table I. This enzyme is most effective in catalyzing the $3\text{-}cis \rightarrow 2\text{-}trans$ isomerization. In fact, with 3-octenoyl-CoA as substrate, the $cis/trans$ activity ratio was observed to be 9. Although the $k_{cat}$ values decreased with increasing acyl chain length, the $K_m$ values also declined with the result that the catalytic efficiency of this enzyme varied little with changing acyl chain length. This general conclusion also applies to the $2,5 \rightarrow 3,5$ isomerization that is catalyzed by this isomerase almost as efficiently as the $3 \rightarrow 2$ isomerization.

ECI, the isomerase that is present in both mitochondria and peroxisomes, differs from MECI in that it exhibits a pronounced chain length dependency. Its catalytic efficiency in the $3 \rightarrow 2$ isomerizations increased 10-20 fold when the acyl chain length of the substrates was increased from 8 to 14 carbon atoms. This increased efficiency is the result of increased $k_{cat}$ values and lower $K_m$ values with increasing acyl chain length of the substrates. However, ECI is less effective in catalyzing the $2,5 \rightarrow 3,5$ isomerization than facilitating either the $3\text{-}trans \rightarrow 2\text{-}trans$ or $3\text{-}cis \rightarrow 2\text{-}trans$ double bond shift.

Peroxisomal MFE1 showed a different catalytic behavior than either MECI or ECI. The catalytic efficiencies determined with $3\text{-}trans$-enoyl-CoA and $3\text{-}cis$-enoyl-CoAs as substrates were generally lower than those observed with MECI and ECI and varied little with changes in the acyl chain length. However, MFE1 was the most effective of all three isomerases in catalyzing the $2,5 \rightarrow 3,5$ isomerization. In summary, the data presented in Tables I-III prompts the idea that in mitochondria ECI may contribute significantly to the $3\text{-}trans \rightarrow 2\text{-}trans$ isomerization, especially of long-chain intermediates formed during the $\beta$-oxidation of unsaturated fatty acids. In
peroxisomes, however, ECI may be the dominant enzyme for catalyzing the $3-trans \rightarrow 2-trans$ and even $3-cis \rightarrow 2-trans$ isomerization while MFE1 may play a key role in the $2,5 \rightarrow 3,5$ isomerization.

**DISCUSSION**

This study demonstrates that at least two enoyl-CoA isomerases each are present in mitochondria and peroxisomes of rat liver. The surprising conclusion reached during their characterization was that one of these isomerases, henceforth referred to as $\Delta^3,\Delta^2$-enoyl-CoA isomerase or ECI, has a dual subcellular localization in mitochondria and peroxisomes. Moreover, this isomerase is identical with peroxisomal enoyl-CoA isomerase (7), PECI, and is indistinguishable from mitochondrial long-chain enoyl-CoA isomerase (5). When initially identified and characterized, PECI was shown to be located predominantly in peroxisomes of human fibroblasts (7). It remains to be determined whether the apparent absence of this enzyme from human mitochondria is characteristic of all human cells or is a unique feature of human fibroblasts. The identification of ECI as the mitochondrial long-chain enoyl-CoA isomerase is based on the limited amount of information published about this enzyme (5). Specifically, the immunological characterization of mitochondrial long-chain enoyl-CoA isomerase after its separation from other isomerases by chromatography on hydroxylapatite and its preference for long-chain substrates support the conclusion that this isomerase and ECI are the same enzyme. It is unclear why this enzyme was not detected in peroxisomes during the previous investigation (5). It could be the consequence of its low activity compared to the isomerase activity of MFE1 in peroxisomes isolated from livers of clofibrate-treated rats. ECI was expected to be present in peroxisomes because the human isomerase has a C-terminal serine-lysine-leucine peroxisomal targeting sequence while the mouse
enzyme has a proline-lysine-leucine signal (7). The signal responsible for directing this enzyme to the mitochondrial matrix has not yet been identified. However, the N-terminal 16 residues of ECI are predicted to form an \( \alpha \)-helix with amphiphilic properties.

The activities of the mitochondrial and peroxisomal enoyl-CoA isomerases were determined to analyze how the two enzymes that are present in each organelle complement each other in catalyzing the three types of enoyl-CoA isomerization reaction that take place during the \( \beta \)-oxidation of unsaturated fatty acids. In rat liver mitochondria, where MECI and ECI coexist, the contribution of ECI to the total \( 3\text{-cis}\rightarrow 2\text{-trans} \)-octenoyl-CoA isomerase activity is negligible (5% of the total activity) as illustrated by Fig. 3. Even with \( 3\text{-cis} \)-dodecenoyl-CoA, an intermediate of oleate degradation, ECI is estimated to make only a minor contribution (25%) to the total activity. This estimate takes into account the increased catalytic efficiency of ECI with increasing acyl-chain length of the substrate and is based on the assumption that \( k_{\text{cat}}/K_m \) values for the isomerizations of \( 3\text{-cis} \)-octenoyl-CoA, \( 3\text{-cis} \)-decenoyl-CoA, \( 3\text{-cis} \)-dodecenoyl-CoA and \( 3\text{-cis} \)-tetradecenoyl-CoA increase linearly. The contribution of ECI to the \( 2,5\rightarrow 3,5 \) isomerase activity is negligible because the catalytic efficiencies of ECI and MECI with \( 2\text{-trans},5\text{-cis} \)-tetradecadienoyl-CoA, a \( \beta \)-oxidation intermediate of oleate, are similar to their catalytic efficiencies with \( 3\text{-cis} \)-octenoyl-CoA as substrate. In contrast, ECI is estimated to contribute one-third of the \( 3\text{-trans}\rightarrow 2\text{-trans} \)-octenoyl-CoA isomerase activity in mitochondria and nearly 50% of the activity with \( 3\text{-trans} \)-decenoyl-CoA, a \( \beta \)-oxidation intermediate of linoleate. The enoyl-CoA isomerase activity of crotonase was excluded from this analysis because it is 5000-times lower than the hydratase activity of this enzyme (6) and thus is insignificant compared to the activities of MECI and ECI.
In peroxisomes, ECI also contributes significantly to the 3-trans→2-trans isomerase activity. With 3-trans-octenoyl-CoA as a substrate, this contribution is estimated to be 40% of the total activity (see Fig. 4). With longer-chain β-oxidation intermediates, it is expected to be greater. Unfortunately, it is unclear which, if any, unsaturated fatty acids are degraded in peroxisomes in vivo. It is therefore not possible to make definite predictions about the contributions of ECI and MFE1 to such isomerizations even if the kinetic data for the isomerization of various β-oxidation intermediates of unsaturated and polyunsaturated fatty acids were known. Similarly, the contribution of ECI to the 3-cis→2-trans isomerizations during the peroxisomal β-oxidation of unsaturated and polyunsaturated fatty acids cannot be estimated. If long-chain and very long-chain polyunsaturated fatty acids are partially degraded in peroxisomes, ECI is expected to have a major function in the necessary 3-cis→2-trans double bond isomerizations because of its preference for long-chain substrates in contrast to MFE1, which does not exhibit such specificity. However, MFE1 will be the dominant isomerase catalyzing 2,5→3,5 isomerizations due to its high catalytic efficiency in that type of reaction.

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

Acknowledgement

We would like to thank Dr. Ronald Wanders for making antibodies to rat peroxisomal multifunctional enzyme 1 available to us.

Footnotes

* This work was supported by U.S. Public Health Service Grant HL30847 from the National Heart, Lung, and Blood Institute and by Grant RR03060 to Research Centers of Minority Institutions.

1Abbreviations used are: BSA, bovine serum albumin; dienoyl-CoA isomerase, Δ3,5,Δ2,4-dienoyl-CoA isomerase; DTT, dithiothreitol; ECI or enoyl-CoA isomerase, Δ3,Δ2-enoyl-CoA isomerase; EDTA, ethylenediaminetetraacetate; EGTA, [ethylene bis(oxyethylenenitrilo)] tetraacetate; HPLC, high-performance liquid chromatography; MECI, mitochondrial Δ3,Δ2-enoyl-CoA isomerase; MFE1, multifunctional enzyme 1; PAGE, polyacrylamide gel electrophoresis; PECI, peroxisomal Δ3,Δ2-enoyl-CoA isomerase; SDS, sodium dodecylsulfate;
## Table I

Kinetic parameters of mitochondrial enoyl-CoA isomerase (MECI)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (µM&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-trans-Hexenoyl-CoA</td>
<td>470 ± 40</td>
<td>270 ± 16</td>
<td>0.57</td>
</tr>
<tr>
<td>3-trans-Octenoyl-CoA</td>
<td>190 ± 36</td>
<td>25 ± 2.8</td>
<td>0.13</td>
</tr>
<tr>
<td>3-trans-3-Tetradecenoyl-CoA</td>
<td>48 ± 4.7</td>
<td>20 ± 1.2</td>
<td>0.42</td>
</tr>
<tr>
<td>3-cis-Hexenoyl-CoA</td>
<td>240 ± 16</td>
<td>200 ± 7.8</td>
<td>0.82</td>
</tr>
<tr>
<td>3-cis-Octenoyl-CoA</td>
<td>150 ± 24</td>
<td>180 ± 16</td>
<td>1.2</td>
</tr>
<tr>
<td>3-cis-Tetradecenoyl-CoA</td>
<td>57 ± 6</td>
<td>98 ± 4.8</td>
<td>1.7</td>
</tr>
<tr>
<td>2-trans,5-cis-Octadienoyl-CoA*</td>
<td>84 ± 18</td>
<td>45 ± 4.8</td>
<td>0.54</td>
</tr>
<tr>
<td>2-trans,5-cis-Tetradecadienoyl-CoA*</td>
<td>20 ± 5.1</td>
<td>8.3 ± 0.93</td>
<td>0.42</td>
</tr>
</tbody>
</table>

*Assayed with dienoyl-CoA isomerase as coupling enzyme
### Table II

Kinetic parameters of enoyl-CoA isomerase (ECI)

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

*Assayed with dienoyl-CoA isomerase as coupling enzyme.
### Table III

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

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

\(^a\) Assayed with 3-keto-acyl-CoA thiolase as coupling enzyme.

\(^b\) Assayed with dienoyl-CoA isomerase as coupling enzyme.
Legends to Figures

Scheme 1. Reactions catalyzed by enoyl-CoA isomerase. Shown are, from top to bottom, the isomerizations of 3-cis-enoyl-CoA to 2-trans-enoyl-CoA, 3-trans-enoyl-CoA to 2-trans-enoyl-CoA, and 2-trans,5-cis-dienoyl-CoA to 3-trans,5-cis-dienoyl-CoA.

Fig. 1. Separation rat liver enoyl-CoA isomerases by chromatography on hydroxylapatite. Fractions were assayed for enoyl-CoA isomerase activity with (●) 3-cis-octenoyl-CoA and (▼) 3-trans-octenoyl-CoA as substrates.

Fig. 2. Subcellular localization of peroxisomal enoyl-CoA isomerase (PECI) and mitochondrial enoyl-CoA isomerase (MECI). Bottom panel: (●) catalase and (▼) malate dehydrogenase activities of fractions obtained by Nycodenz gradient centrifugation of a light mitochondrial preparation. Top panel: immunoblot of equal volumes of the fractions with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI, renamed ECI) or mitochondrial enoyl-CoA isomerase (MECI).

Fig. 3. Separation of mitochondrial enoyl-CoA isomerases by chromatography on hydroxylapatite. Upper panel: Fractions assayed for enoyl-CoA isomerase activity with (●) 3-cis-octenoyl-CoA and (▼) 3-trans-octenoyl-CoA as substrates. Lower panel: immunoblot of equal aliquots of the fractions (fraction numbers are indicated above the immunoblot image) with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI, renamed ECI) or mitochondrial enoyl-CoA isomerase (MECI).
**Fig. 4. Separation of peroxisomal enoyl-CoA isomerases by chromatography on hydroxylapatite.** Upper panel: Fractions assayed for enoyl-CoA isomerase activity with (●) 3-cis-octenoyl-CoA and (▼) 3-trans-octenoyl-CoA as substrates; (♦) 2-enoyl-CoA hydratase activity; (■) 3-hydroxyacyl-CoA dehydrogenase activity. Lower panel: Immunoblot of equal aliquots of the fractions (fraction numbers are indicated above the immunoblot image) with antibodies raised against peroxisomal enoyl-CoA isomerase (PECI, renamed ECI) or peroxisomal multifunctional enzyme 1(MFE1).
SCHEME 1
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Functional Characterization of \( \delta^3,\delta^2 \)-Enoyl-CoA Isomerases from Rat Liver*  
Dongyan Zhang, Wenfeng Yu, Brian V. Geisbrecht, Stephen J. Gould, Howard Sprecher  
and Horst H. Schulz  

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