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Purification and Properties of 3-*cis*-2-*trans*-Enoyl-CoA Isomerase (Dodecenoyl-CoA Δ-Isomerase) from Rat Liver Mitochondria

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Summary: The mitochondrial enzyme 3-*cis*-2-*trans*-enoyl-CoA isomerase, responsible for the positional and geometric isomerization of β,γ-unsaturated fatty acyl-CoA intermediates arising during β-oxidation of unsaturated long chain fatty acids, has been isolated from rat liver, purified to homogeneity by a heat step and a combination of gel filtration and ion-exchange chromatographic procedures. The enzyme has a molecular weight of 30000, as determined by dodecylsulfate polyacrylamide gel electrophoresis. The isomerase has a strong tendency to form a dimer. It elutes from a calibrated Sephadex G-200 column with an apparent M_r = 60000. The basic isoelectric point, pI 9.0–9.2, is due to its high content in basic amino acids. The amino acid composition determined by the ninhydrin and *o*-phthalaldehyde detection method is presented.

Reinigung und Eigenschaften der 3-cis-2-trans-Enoyl-CoA-Isomerase (Dodecenoyl-CoA-Δ-Isomerase) aus Rattenlebermitochondrien

Zusammenfassung: Das mitochondriale Enzym 3-*cis*-2-*trans*-Enoyl-CoA-Isomerase, das für die Stellungs- und geometrische Isomerisierung der β, γ-ungesättigten Fettsäure-Coenzym-A-Zwischenprodukte der β-Oxidation von ungesättigten langkettigen Fettsäuren verantwortlich ist, wurde aus Rattenleber isoliert und durch einen Hitzeschritt und eine Kombination von Gel- und Ionenaustausch-chromatographischen Verfahren bis zur Homogenität gereinigt. Das Molekulargewicht des Enzyms wurde zu 30000 mit Hilfe der Dodecylsulfat-Polyacrylamid-Gelelektrophorese bestimmt. Die Isomerase neigt zur Dimerisation. Sie eluiert von einer kalibrierten Sephadex G-200 Säule mit einem apparenten Molekulargewicht von 60000. Der isoelektrische Punkt pI = 9.0–9.2 wird durch den hohen Gehalt an basischen Aminosäuren bedingt. Die Aminosäurenzusammensetzung wurde vergleichend mit der Ninhydrin- und *o*-Phthalaldehyd-Methode bestimmt.

Enzymes:
Dodecenoyl-CoA Δ-isomerase*, dodecenoyl-CoA Δ^3-*cis*–Δ^2-*trans*-isomerase (EC 5.3.3.8);
3-Hydroxyacyl-CoA dehydrogenase, L-3-hydroxyacyl-CoA:NAD$^{\oplus}$ oxidoreductase (EC 1.1.1.35);
Enoyl-CoA hydratase, L-3-hydroxyacyl-CoA hydro-lyase (EC 4.2.1.17);
Acyl-CoA dehydrogenase, acyl-CoA:(acceptor) oxidoreductase (EC 1.3.99.3).

* This enzyme catalyzes the positional and geometric isomerization not only of substrates with 12 C-atoms, but also of shorter and longer chain homologous with β,γ-*cis*- and -*trans* double bonds. The more general term "3-*cis*-2-*trans*-enoyl-CoA isomerase" should therefore be preferred.

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Key words: β-Oxidation of mono- and poly-unsaturated fatty acids; 3-*cis*-2-*trans*-enoyl-CoA intermediates; mitochondrial 3-*cis*-2-*trans*-enoyl-CoA isomerase; enzyme purification.

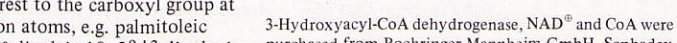
Naturally occurring mono- and poly-unsaturated (di-, tri-, tetraenoic, etc.) fatty acids are components of simple and complex eukaryotic lipids. In general their double bonds have the *cis* configuration. In poly-unsaturated fatty acids, these *cis* double bonds are arranged in polyallyl rhythm (divinylmethane rhythm, "skipped" double bonds). The unsaturated fatty acids with the *cis* double bond nearest to the carboxyl group at odd-numbered carbon atoms, e.g. palmitoleic $16:1^{9c}$, oleic $18:1^{9c}$, linoleic $18:2^{9,12}$, linolenic $18:3^{9,12,15}$ and arachidonic acid $20:4^{5,8,11,14}$, yield intermediates with 3-*cis* double bonds, while those at even carbon atoms, on repeated passages through the mitochondrial β-oxidation cycle, yield intermediates with 2-*cis* double bonds.

We have described the enzymatic steps by which the respective 3-*cis*-enoyl-CoA intermediates are transformed into the positional and geometric 2-*trans*-enoyl-CoA isomers. The 2-*cis*-enoyl-CoA intermediates are hydrated to the D(−)-3-hydroxyacyl-CoA derivative which is subsequently epimerized to the L(+) optical antipode. The former positional and geometric isomerization is catalyzed by the mitochondrial 3-*cis*-2-*trans*-enoyl-CoA isomerase, the latter reaction by the D(−)3-hydroxy acyl-CoA epimerase[1–3]. In this paper we describe the isolation and purification to homogeneity of the 3-*cis*-2-*trans*-enoyl-CoA isomerase and some properties of this mitochondrial enzyme. Rat liver mitochondria served as the enzyme source.

The isomerase has a molecular weight of 30000 as determined by dodecylsulfate polyacrylamide gel electrophoresis. It readily forms a dimeric structure. There are no sulfhydryl groups essential for the catalytic activity. *N*-Acetylcysteamine thioesters of 3-*cis*-alkenoic acids, e.g. 3-*cis*-hexenoic acid, react extremely slowly with the rat liver isomerase.

Experimental

Materials: 3-*cis* fatty acids with chain lengths C_6 to C_{12} were synthesized in this laboratory[2]. The coenzyme A ester was formed either with the mixed anhydride method with ethylchlorocarbonate[4] or the hydroxysuccinimideester method[5]. The yields ranged between 40 and 70% of theory. Similarly, *N*-acetyl-*S*-(3-*cis*-hexenoyl)cysteamine was prepared[6]. This thioester was analyzed by massspectroscopy [$M^{\oplus}$ 215; *m/e* 172 ($M^{\oplus}$−43); *m/e* 157 ($M^{\oplus}$−58)].



3-Hydroxyacyl-CoA dehydrogenase, $NAD^{\oplus}$ and CoA were purchased from Boehringer Mannheim GmbH, Sephadex G-200 and CM-Sephadex C-50 were Pharmacia products.

Methods: Rat liver cell fractionation was carried out according to Hogeboom et al.[7]. The mitochondrial fraction (20–30 mg protein/ml) was sonicated at 75 W for 4 min, with intermittant cooling in ice water. A Branson sonifier with the macrotip was used.

Enzyme assays: The two assays for enzymatic activity, (a) measuring the increase in absorbance at 263 nm and (b) measuring the reduction of $NAD^{\oplus}$ after addition of 2-enoyl-CoA hydratase and L(+)3-hydroxyacyl-CoA dehydrogenase and $NAD^{\oplus}$ to the enzyme preparation, have been described before[1]. Protein was determined according to Beisenherz et al.[8].

Conditions of column separations are given in the legends to Figures.

Dodecylsulfate polyacrylamide gel electrophoresis was carried out according to Weber and Osborn[9] and slab gel electrophoresis according to Lämmli[10] in a modified homemade electrophoresis apparatus according to Studier[11].

Amino acid analysis: Protein aliquots were hydrolyzed with 6N HCl for 24, 48, 72 and 96 h at 100 °C and the stoichiometry of serine, threonine and tyrosine determined by extrapolation of the deterioration curve. One set of aliquots was analyzed by the use of a Beckman amino acid analyzer model 121 M with the ninhydrin detection technique. These analyses were kindly carried out by *Dr. K. Beyreuther*, Institut für Genetik, Universität Köln. The other set was analyzed by the use of *o*-phthalaldehyde in the fluorimetric technique in a home built amino acid analyzer, using parts of the Durrum kit and a Knauer fluorimeter with an 8-μl flow cell. A single column (0.3 × 30 cm) with Durrum DC-4A cation exchange resin resolved all amino acids with high efficiency. Except for proline, all amino acids could be quantitated with high reliability. The two methods of detection agreed well, the fluorimetric method being 40 times more sensitive than the ninhydrin technique.

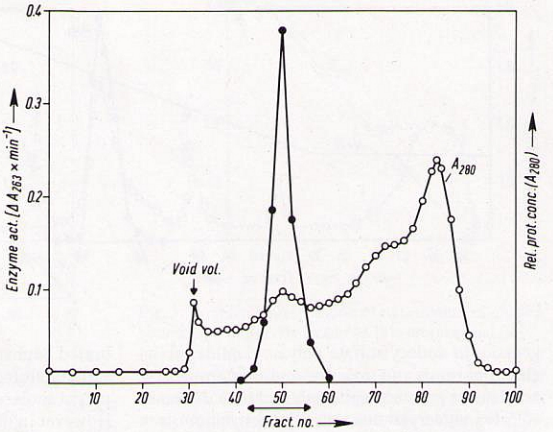


Fig. 1. Separation of 3-*cis*-2-*trans*-enoyl-CoA isomerase from rat liver mitochondrial extract after heat steps (Table 1) on Sephadex G-200. Sample size: 140 mg protein. Bed volume: 2.5 cm Ø × 65 cm. Flow rate: 12 ml/h. Eluant: 0.05M phosphate buffer, pH 7.4. Volume of fractions: 4.5 ml. Sample tested for enzyme activity: 25 µl.

Results

Isolation and purification of 3-cis-2-trans-enoyl-CoA isomerase: The 3-*cis*-2-*trans*-enoyl-CoA isomerase is located in the mitochondrial particle. The isolation of the mitochondrial fraction by standard procedures leads to the enrichment of the enzyme in the initial step. The enzyme is released by ultrasonication and then precipitated by ammonium sulfate precipitation (50–100% saturation). The precipitate is dissolved in 50mM phosphate buffer, pH 7.4, dialysed against the same buffer and then processed as summarized in Table 1. The heat stability of the 3-isomerase facilitates the enrichment of the enzyme by a heat step[1]. The enzymatic activity is determined from the increase of the absorbance at λ max = 263 nm ($\epsilon = 6.2 \times 10^6$ [$l \times mol^{-1} \times cm^{-1}$]). This requires the absence of enoyl-CoA-hydratase, which is eliminated by the heat step. Therefore up to this step the combined optical assay[1] was used for assaying the first purification steps.

The clear enzyme solution was concentrated by ultrafiltration and fractionated by gel filtration (Sephadex G-200) (Fig. 1), which leads to a four-fold increase in specific activity. The pooled and concentrated enzyme fractions were adsorbed to a CM-Sephadex column. The isomerase eluted as a rather sharp band when a buffered NaCl gradient (0–0.4M) was applied as eluant (Fig. 2). In the final purification step, the dialyzed and concentrated enzyme fractions from the CM-Sephadex chromatography were finally passed over a Sephadex G-200 column (Fig. 3). The enzyme protein proved to be homo-

Table 1. Isolation and purification of 3-*cis*-2-*trans*-enoyl-CoA isomerase.

Purification step	Total prot. [mg]	Spec. act. [U/mg]	Total act. [U]	Purification factor	Yield [%]
Heat step (70 °C for 2 min)	142	3.5	490	–	–
Sephadex G-200 chromatography	30	13.7	412	4.0	84
CM-Sephadex C-50 chromatography	6.7	43.2	287	3.13	59
Sephadex G-200 chromatography	5.05	46.9	237	1.1	48

1 U = 1 µmol 3-*cis*-dodecenoyl-CoA isomerized × mg^{-1} × min^{-1}.

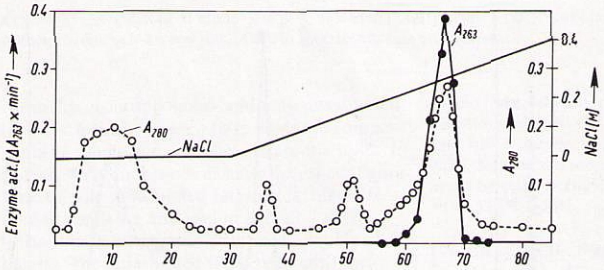


Fig. 2. Chromatographic separation on Sephadex CM-50 of isomerase from isomerase-containing fractions eluted from Sephadex G-200.
Bed volume: 1.5 cm Ø × 40 cm. Eluant: 0.05M phosphate buffer, pH 6.0; 0–0.4M NaCl gradient, started after 270 ml prewash with buffer. Volume of fractions: 3 ml. ●——●: enzyme activity (10-µl portions). ○– – –○: distribution of protein.

geneous in dodecylsulfate polyacrylamide gel electrophoresis and urea dodecylsulfate polyacrylamide gel electrophoresis and urea dodecylsulfate polyacrylamide slab gel electrophoresis (Fig. 4a,b).

Properties of the isomerase: The molecular weight was determined by chromatography over a calibrated Sephadex G-75 column. The total enzyme activity eluted in a sharp single band with an apparent molecular weight of 60000 daltons, Fig. 5. However in dodecylsulfate polyacrylamide gel

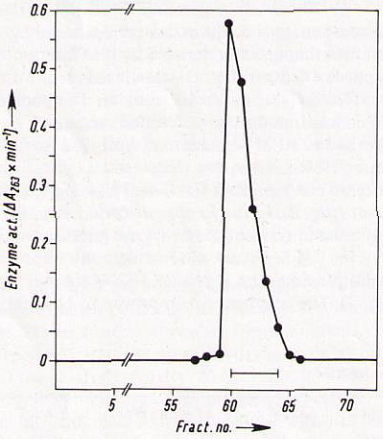


Fig. 3. Purification of isomerase on Sephadex G-200 after ion-exchange chromatography on Sephadex CM-50.
Bed volume: 1.5 cm Ø × 110 cm. Eluant: 0.05M phosphate buffer, pH 7.4. Sample size: 5 mg protein. Volume of fractions: 1.8 ml. Assay volume for enzyme activity: 10 µl.

Table 2. Amino acid analysis of 100–125 pmol isomerase was performed on a single Durrum DC 4 A ion-exchange resin bed (0.3 × 30 cm) with fluorimetric detection.
The analysis with ninhydrin detection was carried out on a Beckman amino acid analyzer. 5 nmol isomerase was required. For experimental details see Methods.

Amino acid	Detection	
	o-Phthalaldehyde [mol/mol isomerase]	Ninhydrin [mol/mol isomerase]
Asp	23	23
Thr*	14	14
Ser*	25	25
Glu	32	34
Gly	31	31
Ala	28	28
Val	20	20
Met	–	–
Ile	15	15
Leu	27	27
Tyr*	6	6
Phe	10	10
Lys	25	24
His	7	7
Arg	11	11
Pro	–	13

* Extrapolated from the time-dependent deterioration curve during HCl-hydrolysis.

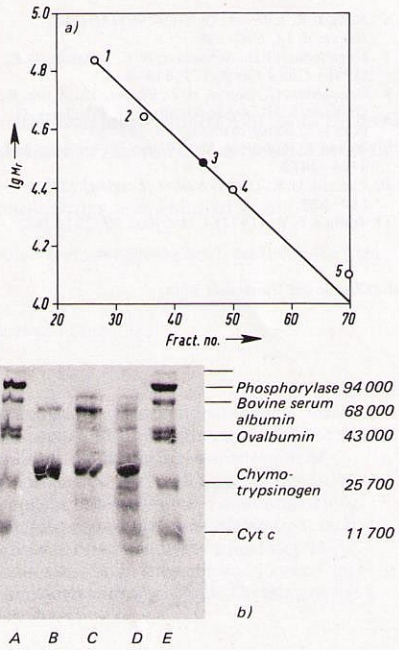


Fig. 4.
a) Graphic representation of molecular weight determination of purified 3-*cis*-2-*trans*-enoyl-CoA isomerase by sodium dodecylsulfate polyacrylamide gel electrophoresis. 1 = Bovine serum albumin (M_r = 68 000); 2 = Ovalbumin (M_r = 43 000); 3 = Isomerase (M_r = 30 000); 4 = Chymotrypsinogen (M_r = 25 700); 5 = Cytochrome *c* (M_r = 11 700).
b) Slab gel (15%) of: A = test proteins indicated on the right; B = purified isomerase; C = isomerase-active fractions after Sephadex G-200 chromatography; D = protein solution after heat denaturation step; E = test proteins.

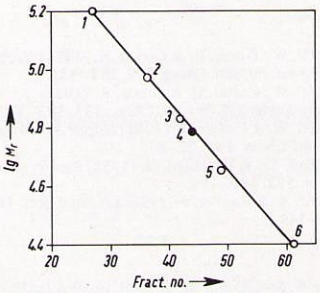


Fig. 5. Graphic representation of dependence of elution volume and molecular weight of test proteins and isomerase.
Sephadex G-75 column, bed volume 1.5 cm Ø × 110 cm. Volume of fractions: 2 ml. 2 mg of each test protein was used. Elution buffer: 0.05M phosphate buffer, pH 7.4. 1 = Aldolase (M_r = 158 000); 2 = Phosphorylase *a* (M_r = 94 000); 3 = Bovine serum albumin (M_r = 68 000); 4 = Isomerase (M_r = 60 000); 5 = Ovalbumin (M_r = 43 000); 6 = Chymotrypsinogen (M_r = 25 700).

electrophoresis[9], a molecular weight of 30000 was determined, and only a faint protein band with twice the molecular weight (60000) was visible, Fig. 4a and 4b. This dissociation of the enzyme into the two 30 000-dalton protomers is due to the presence of sodium dodecylsulfate, but independent of mercaptoethanol. Sulfhydryl reagents like *N*-ethylmaleinimide and *p*-chloromercuribenzoate did not inhibit the isomerization. Since the enzyme is fully active in the presence of dodecylsulfate, a dimeric or any multienzyme structure is not required for the enzymatic function. Higher salt concentrations do not dissociate the dimeric enzyme. The isoelectric point was determined by isoelectric focussing in a sucrose gradient and in polyacrylamide gels with Ampholine (pH 2–11) as polyamine buffer. The pI of the isomerase is 9.2–9.4.

The stoichiometry of the amino acid composition (Table 2) was derived from amino acid analyses using ninhydrin in the analysing system or *o*-phthalaldehyde for the fluorimetric determination system. The isoelectric point at alkaline pH indicates a predominance of basic amino acids which is documented by the analytical data.

This key enzyme of β-oxidation of unsaturated fatty acids is readily accessible and makes the further characterization feasible.

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