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β-Oxidation of unsaturated fatty acids in humans

Isoforms of Δ^3, Δ^2-enoyl-CoA isomerase

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This investigation was undertaken in order to elucidate the human enzymes which participate in metabolism of the double bonds of unsaturated fatty acids during β-oxidation. The results indicate that the human monofunctional Δ^3, Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) with the native M_r of 70,000 differed significantly from its rat counterpart [Palosaari et al. (1990) J. Biol. Chem. 265, 3347–3353]; the isoelectric point of the human isoform was over three pH-units more acidic, it showed different chromatographic behaviour, the human enzyme did not show any clear-cut substrate chain-length specificity and only a weak immunological cross-reactivity was detected with the antibody to rat liver mitochondrial short-chain enzyme. This explains the failure of attempts to apply the rat data directly to human beings. Another isomerase activity from human liver was found to be a part of the isomerase-hydratase-dehydrogenase polypeptide showing immunological cross-reactivity with the previously characterized peroxisomal multifunctional enzyme (MFE) from rat liver.

Unsaturated fatty acids; β-Oxidation; Cellular compartmentation; Δ^3, Δ^2-Enoyl-CoA isomerase; Multifunctional enzyme; Human liver and heart

1. INTRODUCTION

Our understanding of the biochemistry and physiology of both eukaryotic mitochondrial and peroxisomal β-oxidation is currently extending rapidly and is under continuous re-evaluation. Epimerization of 3-hydroxyacyl-CoAs appears to occur by the combined action of two stereospecific hydratases, employing a dehydration–hydration mechanism via *trans*-2-enoyl-CoA intermediates [1–3]. Recently, a novel pathway has been proposed for the metabolism of *Δcis*-5-enoic acids, namely the reduction of their CoA-esters directly to saturated acyl-CoAs [4]. New aspects also include the demonstration of the presence of a multifunctional β-oxidation enzyme (MFE) in mitochondria [5,6] and the finding that β-oxidation in yeasts proceeds via D-3-hydroxy-CoAs but not via L-3-hydroxyacyl-CoAs [7].

One characteristic of β-oxidation enzymes is the occurrence of multiple isoforms. This situation is exemplified by the separate inborn errors in the isoforms of three mitochondrial acyl-CoA dehydrogenases [8–11] and two peroxisomal acyl-CoA oxidases [12,13]. In line with this generalization, separate isomerases have been described in rat liver to date [14]. Only a limited number of experiments have been carried out so far regarding the auxiliary enzymes of β-oxidation in humans, however, which is surprising in view of the important nutritional role which (poly)unsaturated fatty acids play in human well-being. The only recognized inborn error in humans affecting the enzyme systems degrading unsaturated fatty acids is the reductase defect, which is described as being lethal at an early age [15].

In order to characterize the β-oxidation of unsaturated fatty acids in humans we began to purify Δ^3, Δ^2-enoyl-CoA isomerase. The present work shows that the human liver contains at least two isoforms of Δ^3, Δ^2-enoyl-CoA isomerase: a monofunctional enzyme with a native M_r of 70 000, which is apparently a mitochondrial protein and a peroxisomal isomerase forming part of a MFE.

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2. MATERIALS AND METHODS

2.1. *Reagents and chemicals*

L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), 4-chloro-1-naphthol, catalase (EC 1.11.1.6) (from bovine liver), dithiothreitol (DTT), bovine serum albumin (BSA), EDTA and EGTA were purchased from Sigma Chemical Co., St. Louis, MO, USA; NAD^+, coenzyme A, lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle), cytochrome *c* (from horse heart, type III) and malate dehydrogenase (EC 1.1.1.37) (from pig heart) from Boehringer Mannheim GmbH, Mannheim, Germany; Matrex gel red A from Amicon Corp., Lexington, MA; Sephacryl S-200 HR, protein A-Sephadex, phenylsepharose CL-4B, Blue dextran and Pharmalyte pH 3–10 from Pharmacia LKB Biotechnology AB, Uppsala, Sweden; hydroxyapatite and goat anti-rabbit IgG from BioRad Laboratories, Richmond, CA, USA; Freund's adjuvants from Difco Laboratories, Detroit, MI, USA, benzamidine hydrochloride hydrate (BA) and phenylmethylsulfonyl fluoride (PMSF) from Aldrich-Chemie, Steinheim, Germany and polyethylene glycol 20,000 from Fluka Chemie, Buchs, Switzerland. 2-Enoyl-CoA hydratase 1 (crotonase) (EC 4.2.1.17) was purified from rat liver according to Furuta et al. [16].

2.2. *Acyl-CoA esters*

Acyl-CoA esters were synthesized by the mixed anhydride method [17] and purified on thin layer cellulose plates [18]. *Trans*-3-enoyl acids which were not commercially available were synthesized according to Boxer and Linstead [19].

2.3. *Purification of human isomerases*

12 g of frozen human liver tissue was homogenized with an Ultra-Turrax tissue disruptor in a 5-fold volume of 5 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM BA, 0.5 mM DTT and 0.1 mM PMSF, pH 7.0 and subsequently ultracentrifuged at 100,000 × *g* for 1 h. Unless not stated otherwise, the supernatant was heat-treated at 60°C for 2 min. The sample was centrifuged at 10,000 × *g* for 10 min and the supernatant (total volume of 40 ml) was mixed into sucrose and applied to a 110 ml Ampholine LKB electrofocusing column to form a 5–50% sucrose density gradient containing 1% (v/v) of Pharmalyte, pH 3–10. After a run for 20 h at +4°C, the column was unloaded and the collected fractions were assayed for isomerase. The isomerase containing sample was dialyzed overnight against 2 × 2 litres of 20 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM BA, 0.5 mM DTT, pH 7.0 (buffer A). Ammonium sulphate was added to a sample to a final concentration of 1.2 M and the sample was applied to a phenyl sepharose hydrophobic column, 2.5 × 9 cm, in equilibrium with buffer A containing 1.2 M ammonium sulphate, and washed with 100 ml of the same buffer at a flow rate of 50 ml/h. A gradient from 0–60% ethylene glycol in buffer A (total volume 300 ml) was developed, followed by an additional wash with 60% ethylene glycol (50 ml). The pooled fractions were dialyzed against 2 × 2 litres of 20 mM potassium phosphate, 0.5 mM BA and 0.5 mM DTT, pH 7.0 (buffer B). The sample was applied to a Matrex gel red A column, 2.5 × 12 cm, in equilibrium with buffer B and washed with 100 ml of buffer B at a flow rate of 60 ml/h. A gradient from 0–1.5 M KCl in buffer B was developed (total volume 300 ml). The fractions containing isomerase activity were dialyzed overnight against 2 litres of buffer B before applying to a hydroxyapatite column, 1.6 × 5 cm, in equilibrium with buffer B. The column was washed with 75 ml of buffer B and a gradient from 20 to 500 mM potassium phosphate was generated (gradient volume 200 ml), the flow rate being 17 ml/h. Isomerase activity was measured, and the pooled fractions were concentrated with polyethylene glycol 20,000 to a final concentration of 3 ml. The sample was applied to a S-200-HR gel filtration column, 2.5 × 125 cm, which was washed with 350 ml of 200 mM potassium phosphate, 1 mM EDTA, 1 mM EGTA and 0.5 mM DTT, pH 7.4 at a flow rate of 18 ml/h. Finally, isomerase activity was measured from the fractions. The human myocardial isomerase was purified by the protocol used for liver counterpart. The native molecular weights of the proteins were determined on a Sephacryl S-200-HR gel filtration column [20]. Protein concentrations were assayed with the BioRad protein assay reagent. It is worth mentioning that in our experience isomerase loses its catalytic activity rapidly during purification, especially if the protein concentration is low.

When non-heat treated human liver extract was applied to a Matrex gel red A, two peaks of isomerase activity were found (eluting at 0.1 M and 0.9 M KCl). The high salt activity was purified according to Palosaari and Hiltunen [21] and proved to be a peroxisomal MFE.

2.4. *Antibodies and immunoblotting*

A protein A-Sephadex column was used to purify the IgG fraction of antiserum for rat liver short-chain isomerase [20]. In immunoblotting the proteins from the SDS-PAGE [22] were transferred electrophoretically onto nitrocellulose and detected using the anti-isomerase IgG as the primary antibody, goat anti-rabbit IgG with horseradish peroxidase as the second antibody [23] and 4-chloro-1-naphthol for staining.

2.5. *Others*

Human heart and liver tissues were obtained from forensic autopsies performed less than 12 h after death. The material for the successful purification was obtained from an accidental death due to submersion in icy water. The use of human tissues was approved by the Bureau of Medicine in Finland. Permission for the animal experiments was granted by the University of Oulu Committee on Animal Experimentation.

3. RESULTS

3.1. *Electrofocusing experiments*

Electrofocusing of the ultracentrifuged non-heat-treated human liver extract separated the isomerase activity into two peaks (Fig. 1). The immunoblotting experiment showed that the antibody to rat liver short-chain isomerase recognized a mildly acidic isomerase (pI of 6.1), but not a basic one (pI of 8.7) (Fig. 1), but the incubation time for staining had to be at least 5 times longer when the same amounts of protein from human and rat extracts were used. The heat-treatment at 60°C for 2 min inactivated the basic isomerase activity completely. The antibody to rat liver peroxisomal MFE recognized the latter isomerase, but not the acidic one (Fig. 1) indicating that the basic isomerase represents human liver MFE.

3.2. *Purification of isomerases from human liver*

The acidic human liver isomerase was purified to apparent homogeneity as described in detail in section 2. The protocol gave a yield of 0.320 mg of purified protein starting from 12 g of human liver, the specific activity being 8.3 nmol/s·mg^{-1} of protein. A native molecular weight of 70,000 was obtained by gel filtration on a S-200-HR column and SDS-PAGE analysis showed a subunit of 30,000 (Fig. 2B), indicating that the native enzyme is homodimeric.

The isomerase activity purified above eluted from the linear gradient on Matrex gel red A at 0.1 M KCl. Another isomerase activity from non-heat-treated human liver extract (20 g of frozen liver), was purified following the protocol described for the rat enzyme [21] with a yield of 0.23 mg, specific activity 5.0 nmol/s·mg^{-1} of protein. The molecular weight corresponded to that of the human bifunctional enzyme [24]. The subunit size was 78,000 on SDS-PAGE indicating that it is monomeric enzyme (Fig. 2C). The antibody to rat liver MFE cross-reacted with the corresponding human liver protein (Fig. 1). When this monomeric enzyme was incubated with *trans*-3-enoyl-CoA in the presence of NAD$^+$, but without 2-enoyl-CoA hydratase 1 or 3-hydroxyacyl-CoA dehydrogenase, NADH was generated. These data show the isomerase to be a multifunctional isomerase-hydratase-dehydrogenase enzyme. The isomerization:hydration:dehydration velocity ratio was 2:18:1, which is comparable to that of rat liver MFE when assayed with C_{10}-acyl-CoA-substrates.

3.3. *Characterization of the human myocardial Δ^3,Δ^2-enoyl-CoA isomerase*

The protein catalyzing isomerization was purified to

apparent homogeneity also from human myocardium extract (as indicated by the appearance of one band on SDS-PAGE; Fig. 2A) employing the methods used for purifying the acidic liver enzyme. The amount of purified protein obtained from 20 g of human heart tissue was 0.840 mg (specific activity 8.60 nmol/min·mg^{-1} of protein) and the subunit weight of 30,000 was determined by SDS-PAGE analyse. Gel filtration on a S-200-HR column gave a native M_r of 70,000 indicating that it is a dimer.

3.4. *Kinetic studies*

The substrate specificities for human liver and heart isomerase were measured using different chain-length acyl-CoA-esters and the velocity ratios with *trans*-3-C_6:C_{10}:C_{12}-enoyl-CoA were 1:2.1:1.2 and 1:3.4:2.3, respectively. The corresponding values for peroxisomal MFE were 1:5.8:2.7. Since these values valued from the ones described for corresponding rat enzymes [20], we repeated the rat experiments in parallel. The experiments nevertheless agreed with the published data showing that the rat and human isomerases have different kinetic properties. Furthermore, separate experiments showed that an acidic isomerase from human liver and heart was not able to catalyze the reduction of NAD^+ in the presence of *trans*-3-acyl-CoA esters alone, additions of 2-enoyl-CoA hydratase 1 and 3-hydroxyacyl-CoA dehydrogenase being required.

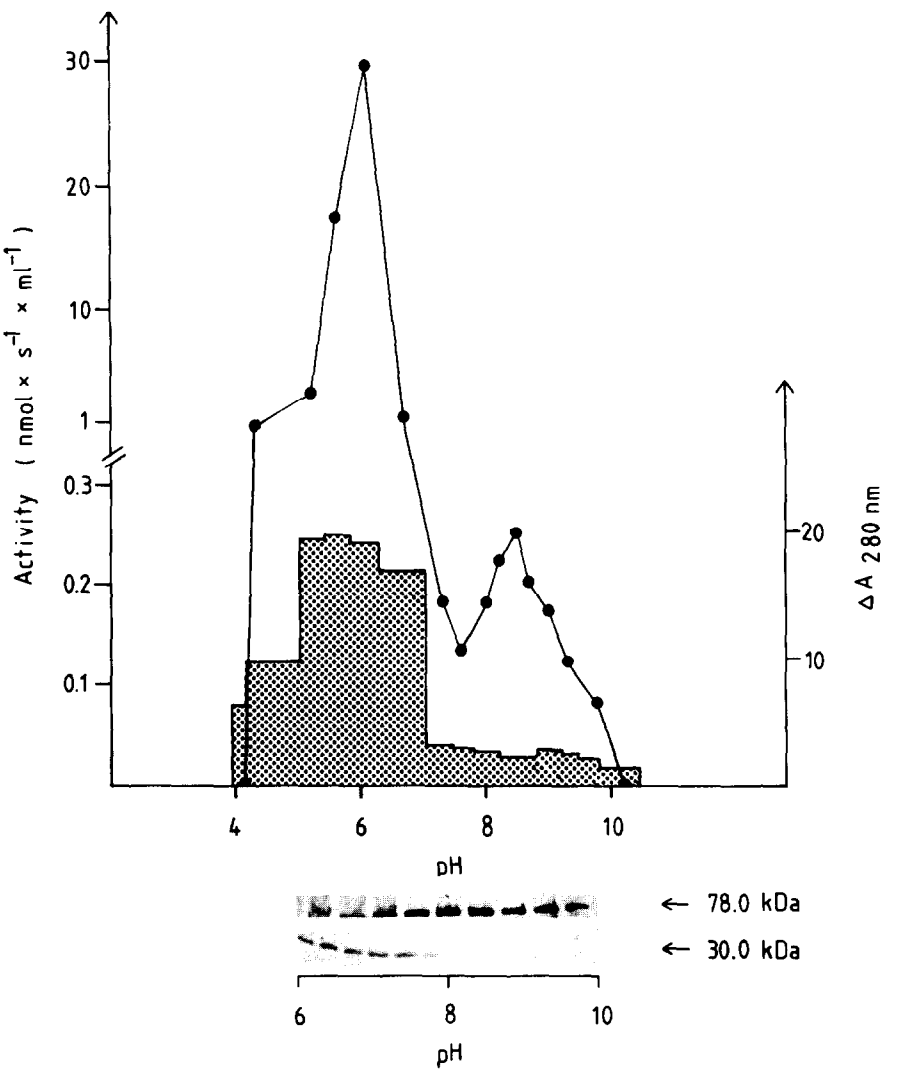

Fig. 1. Separation and immunoblotting of two human liver isomerases by electrofocusing. 6 g of frozen liver tissue was homogenized in 10 mM potassium phosphate, 0.2 M KCl, 1 mM EDTA, 0.5 mM BA, 0.5 mM DTT, and 0.1 mM PMSF, pH 7.4, 10-fold (w/v) with an Ultra-Turrax tissue disruptor and centrifuged at 100 000 × *g* for 1 h. The supernatant was dialyzed overnight with 2 litres of 5 mM potassium phosphate, 0.5 mM BA and 0.5 mM DTT, pH 7.0. Two liver Δ^3,Δ^2-enoyl-CoA isomerases were separated out in a 110 ml LKB electrofocusing column according to their isoelectric points as described in section 2. The column was unloaded by collecting fractions of 2 ml. The pH values and isomerase activity (shown by black dots) were measured from these fractions. The shaded areas denote absorbances measured at 280 nm. Immunoblottings of isomerase activity peaks are under the elution profile. Samples of 40 μl were taken from the fractions from the electrofocusing column and screened with the antibody to rat liver peroxidomal MFE (the upper panel) and the antibody to rat liver short-chain isomerase (the lower panel).

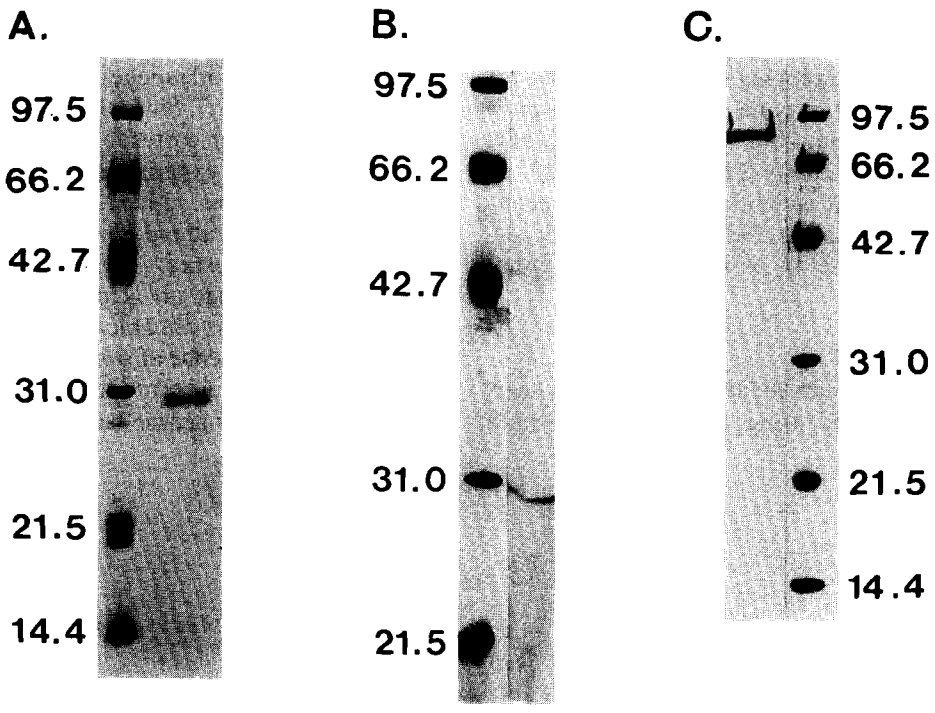

Fig. 2. Electrophoretic analysis of the purified human proteins on polyacrylamide gel. The following standards were used: phosphorylase *b* (97.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa); (2A) purified monofunctional isomerase from human myocardium (10 μg of protein), (2B) monofunctional isomerase from human liver (3 μg of protein) and (2C) multifunctional isomerase-dehydrogenase-hydratase protein from human liver (15 μg of protein).

4. DISCUSSION

The present work demonstrates that the human being has at least two isoforms of Δ^3,Δ^2-enoyl-CoA isomerase:liver and heart monofunctional isomerase (M_r 70,000) and a peroxisomal isomerase (M_r 78,000) as a part of a MFE. The observation that the human liver and heart isoforms were similar was comparable to the situation with the corresponding rat isomerases [20]. The subunit size of the monofunctional isomerase was 30,000 in both species, and they form a dimeric native enzyme. Furthermore, the antibody to the rat enzyme recognized its human counterpart (Fig. 1). There were differences between the rat and human enzymes, however, the most striking of which was in their pI values, the rat enzyme being basic (pI 9.5) and the human one mildly acidic (pI 6.1). The rat enzyme showed the highest catalytic activity with short-chain substrates, and is therefore called a short-chain isomerase [20]. The corresponding human enzyme did not show any clear cut catalytic specificity regarding substrate chain length. The enzymes also showed different chromatographic behaviour during purification. In addition to different binding and elution from ion-exchanger columns the human monofunctional isomerase eluted from Matrex gel red A at a lower KCl concentration (0.1 M) than the corresponding rat protein (0.2 M KCl) and from hydroxyapatite column at lower KP_i concentration than the rat enzyme. A tetrameric isomerase has previously been demonstrated in hog liver [25], where four isoforms were found with pI values varying from neutral to mildly acidic. We have described an isomerase activity with a native molecular weight of 200,000 by S-200-HR in rat liver [26]. When we put non-heat-treated human liver extract to a hydroxyapatite column, which was run as described [26], only two isomerase activity peaks were found. During the purification protocol these activities showed to stand for an acidic monofunctional human isomerase and a peroxisomal multifunctional isomerase-hydratase-dehydrogenase enzyme. Thus the existence of another monofunctional isomerase in human liver, the counterpart of the rat liver mitochondrial long-chain isomerase [26], remains still an open question.

The existence of several isoforms is typical of the enzymes of β-oxidation, the only exception known to have a single isoenzyme so far being peroxisomal MFE [27,28]. The isoforms are thought to allow the flexibility required for metabolism of fatty acids of variable chain-lengths. The physiological function of the human monofunctional isomerase do not fit in with this proposal. It do not show any major kinetic differences with respect to substrate chain-length at least under the conditions tested here. The human MFE must originate from the peroxisomes, in view of its similarities to the

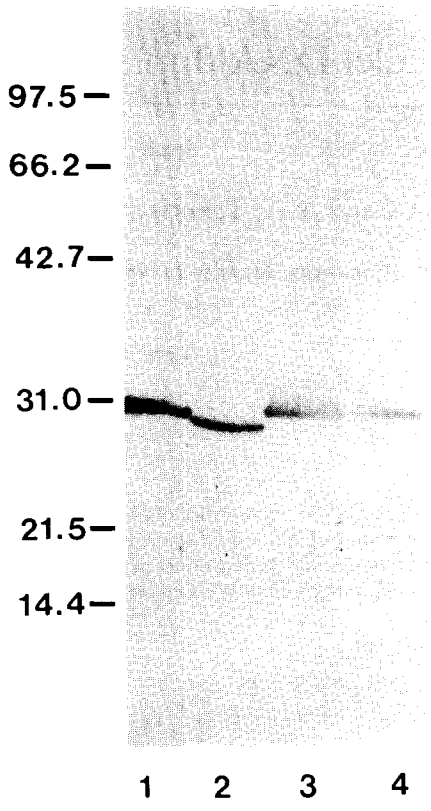

Fig. 3. Immunological cross-reactivity of human Δ^3,Δ^2-enoyl-CoA isomerases with rat antibodies. Proteins were detected by means of polyclonal antibodies as described in section 2. Immunoblotting with antibody to rat liver mitochondrial short-chain isomerase. (Lane 1) rat heart homogenate (300 μg of protein); (lane 2) rat liver homogenate (700 μg of protein); (lane 3) first isomerase activity peak from human heart on Matrex gel red A (60 μg of protein); and (lane 4) first isomerase activity peak from human liver on Matrex gel red A (45 μg of protein).

corresponding rat protein and a proposal based on the same criteria can also made regarding the monofunctional isomerase, which is likely to be mitochondrial in origin.

Our attempts to attribute the characteristics of rat isomerases to the corresponding enzymes in the human being were not very successful. Therefore the major aim of this study was to characterize these auxiliary enzymes of the β-oxidation of unsaturated fatty acids in humans. This work is required in order to deduce the primary structure of these proteins, to locate and characterize their genes and later on to screen for possible inborn errors affecting these enzyme systems in human.

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