Occurrence of a long-chain Δ^3, Δ^2 -enoyl-CoA isomerase in rat liver

Johanna M. KILPONEN, Päivi M. PALOSAARI and J. Kalervo HILTUNEN*

Department of Medical Biochemistry and Biocenter Oulu, University of Oulu, Kajaanintie 52 A, SF-90220 Oulu, Finland

Isoproteins of Δ^3, Δ^2 -enoyl-CoA isomerase (EC 5.3.3.8), an auxiliary enzyme in the β -oxidation of unsaturated fatty acids having double bonds at odd-numbered positions, were studied in livers of control and clofibrate-treated rats. When liver extracts were applied to a hydroxyapatite column at pH 7.0, the previously characterized peroxisomal trifunctional hydratase–dehydrogenase–isomerase enzyme and the mitochondrial isomerase, which shows a preference for short-chain substrates, were eluted almost in parallel. In addition to these activities, a separate isomerase was observed to elute at a lower potassium phosphate concentration in the gradient. Experiments with extracts of purified mitochondria and peroxisomes demonstrated the mitochondrial origin of this third activity. Studies on the kinetic properties of the third isomerase showed that it has a preference for C_{10} – C_{12} substrates. An M_r of 200 000 was obtained for the native protein by gel-filtration chromatography. Antibodies to mitochondrial short-chain isomerase and peroxisomal trifunctional enzyme did not recognize this novel mitochondrial isoenzyme. The immunological non-cross-reactivity can be interpreted as suggesting that the different isomerases are not closely related at the level of the primary structure of the polypeptide chain. The present data demonstrate that, similar to many other enzymes of β -oxidation, Δ^3, Δ^2 -enoyl-CoA isomerase has at least three isoenzymes in rat liver: mitochondrial short- and long-chain isomerases and an additional peroxisomal isoenzyme, which in this case is a part of a multifunctional protein.

INTRODUCTION

Even though the principles of the oxidative degradation of unsaturated fatty acids were regarded as having been solved in the early 1960s, essentially new aspects have appeared more recently. This is exemplified by the recognition of the role of peroxisomes in the β -oxidation of long-chain monounsaturated and polyunsaturated fatty acids (for review, see Vamecq & Draye, 1989) and by the identification of the 2,4-dienoyl-CoA-reductase-dependent pathway as a major route for the oxidative degradation of polyunsaturated fatty acids having double bonds at even-numbered positions (Kunau & Dommes, 1978; Schulz & Kunau, 1987). Furthermore, there is apparently no single protein capable of catalysing epimerization of 3-hydroxyacyl-CoA esters in the rat. Instead, epimerization seems to occur via dehydration–hydration reactions catalysed by the stereospecific enzymes 2-enoyl-CoA hydratase 2 and 2-enoyl-CoA hydratase 1 (Hiltunen *et al.*, 1989; Hiltunen & Kunau, 1990).

Δ^3, Δ^2 -Enoyl-CoA isomerase (dodecenoyl-CoA Δ^3 -*cis*- Δ^2 -trans-isomerase; EC 5.3.3.8) is an enzyme which is required for the metabolism of double bonds at odd-numbered positions during the β -oxidation of unsaturated fatty acids. Fractionation of rat liver subcellular organelles on a sucrose density gradient has revealed an association with isomerase activity in particles containing either glutamate dehydrogenase or catalase, suggesting the existence of both mitochondrial and peroxisomal isoenzymes (Kärki *et al.*, 1987). We have traced the peroxisomal isomerase activity in the rat to the bifunctional protein, which thus can serve as a trifunctional hydratase–dehydrogenase–isomerase enzyme (Palosaari & Hiltunen, 1990). We have also purified a mitochondrial clofibrate-inducible isomerase which shows its highest activity with short-chain Δ^3, Δ^2 -enoyl-CoA substrates (Palosaari *et al.*, 1990). This present work describes an isomerase activity which could not be explained by either of these enzymes. The further characterization of this protein

demonstrated it to be a previously unknown mitochondrial long-chain Δ^3, Δ^2 -enoyl-CoA isomerase.

MATERIALS AND METHODS

Reagents and chemicals

Crotonase (enoyl-CoA hydratase; EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and malonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), NAD⁺ and Coenzyme A from Boehringer (Mannheim, Germany), Nycodenz from Nyegaard (Oslo, Norway), Percoll, Sephacryl S-200 HR and Protein A–Sephadex from Pharmacia (Uppsala, Sweden), hydroxyapatite, the low M_r standard kit for SDS/PAGE and goat anti-(rabbit IgG) from Bio-Rad Laboratories (Richmond, CA, U.S.A.), *trans*-3-hexenoyl acid from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and benzamidinium hydrochloride (BA), octyl, decyl and tetradecyl aldehydes from Aldrich-Chemie, (Steinheim, Germany).

trans-3-Enoyl acids which were not available from other sources were synthesized according to Boxer & Linstead (1931). Acyl-CoA esters were synthesized by the mixed anhydride method (Goldman & Vagelos, 1961) and purified on cellulose thin-layer plates (Pullman, 1973).

Animals

Rats of the Sprague–Dawley strain were from the Department's own stock. Clofibrate treatment was carried out by feeding the rats on a standard pelleted diet containing 0.5% clofibrate (w/w) for 2 weeks before removal of the livers.

Chromatography on a hydroxyapatite column and isolation of subcellular organelles

Liver tissue (3 g portions) from the control and clofibrate-treated rats was homogenized (20%, w/v) using an Ultra-Turrax

Abbreviations used: BA, benzamidinium hydrochloride; DTT, dithiothreitol.

* To whom correspondence should be addressed.

tissue homogenizer in 10 mM-potassium phosphate buffer, pH 7.4, containing 0.2 M-KCl, 1 mM-EDTA, 0.5 mM-BA and 0.5 mM-dithiothreitol (DTT) (buffer A). The supernatants produced by ultracentrifugation at 100 000 *g* for 1 h were dialysed against 20 mM-potassium phosphate, pH 7.0, containing 0.5 mM-BA and 0.5 mM-DTT (buffer B). The samples were applied to a hydroxyapatite column (2.0 cm × 16.0 cm) in equilibrium with buffer B, the flow rate being 10 ml/h, and the bound proteins were eluted with a linear potassium phosphate gradient from 20 mM to 500 mM (total gradient volume 200 ml).

Mitochondria were isolated from the livers of the clofibrate-treated rats in a self-generated Percoll density gradient (Osmundsen & Bremer, 1977; Neat *et al.*, 1981). The catalase/glutamate dehydrogenase activity ratio of the mitochondrial preparation was 0.016 and that of the 10% (w/v) liver homogenate from which the mitochondria were isolated was 0.60. A light-mitochondrial fraction was prepared from the livers of the clofibrate-treated rats as described by Leighton *et al.* (1968) and the peroxisomes were further purified with 30% (w/v) Nycodenz solution using a fixed-angle rotor (Ghosh & Hajra, 1986). The catalase/glutamate dehydrogenase activity ratio of the peroxisome preparation was 212, whereas the same ratio in the initial tissue homogenate was 0.41.

The samples of isolated mitochondria (93 mg of protein) and peroxisomes (12.4 mg of protein) were extracted in buffer A containing 0.1% Triton X-100. The ultracentrifuged dialysed samples were run on a hydroxyapatite column as described above, except that the column size was 1.5 cm × 4.0 cm.

Immunoblotting

Clofibrate-inducible mitochondrial short-chain isomerase (Palosaari *et al.*, 1990) and peroxisomal trifunctional hydratase-dehydrogenase-isomerase enzyme (Osumi & Hashimoto, 1979a; Palosaari & Hiltunen, 1990) were purified from rat livers as described. Antibodies to these proteins were raised in rabbits and the IgG fraction from the sera was purified using a Protein A-Sephadex column. For immunoblotting, the proteins from the SDS/PAGE were transferred electrophoretically on to a nitrocellulose filter and were detected using goat anti-(rabbit IgG) with horseradish peroxidase conjugate as a second antibody (Towbin *et al.*, 1979) and 4-chloro-1-naphthol in the staining.

Other methods

Protein concentrations were assayed with the Bio-Rad protein assay reagent, using BSA as the standard. The *M_r* of the native protein was determined by gel filtration on Sephacryl S-200 HR, and enzyme activities were measured as described in Palosaari *et al.* (1990). The proposal for this research project was approved by the University of Oulu committee on animal experimentation.

RESULTS

Experiments on the degradation of unsaturated fatty acids in isolated subcellular organelles of the rat liver and in isolated perfused rat livers have demonstrated the dual location of Δ^3, Δ^2 -enoyl-CoA-isomerase activity in both peroxisomes and mitochondria (Hiltunen *et al.*, 1986; Kärki *et al.*, 1987). These mitochondrial and peroxisomal isomerase activities were later separated by means of a general dye-ligand chromatography step (Palosaari *et al.*, 1990). When purifying these proteins we applied liver extract to a hydroxyapatite column. As in the general dye-ligand chromatography, isomerase activity was found to elute in two peaks (Fig. 1a). In contrast with the dye-ligand column, on

which both isomerase peaks underwent multiple induction by clofibrate treatment, only the second peak was induced (Fig. 1b). Experiments with partially purified proteins demonstrated that the previously characterized mitochondrial isomerase and peroxisomal trifunctional enzyme were eluted from this column almost in parallel. Furthermore, previous works have shown that both of these enzymes are induced by clofibrate treatment (Palosaari *et al.*, 1990). Taken together, these findings may be interpreted as suggesting the existence of a third isoenzyme of the isomerase in rat liver. Isomerase activities in the fractions of the hydroxyapatite column were measured here using *trans*-3-dodecenoyl-CoA and *trans*-3-hexenoyl-CoA as substrates. The first peak always gave higher rates with *C*₁₂-CoA, whereas the reverse was true of the second peak (Fig. 1). When measured separately in samples taken from the first activity peak, the rates of isomerization were 8.1, 15.9, 24.9 and 13.5 nmol/min per mg of protein with *C*₆-, *C*₁₀-, *C*₁₂- and *C*₁₆-*trans*-3-enoyl-CoAs respectively.

To study the subcellular origin of the isomerases, extracts of highly purified mitochondria and peroxisomes were prepared and applied to the hydroxyapatite column. The experiment with the mitochondrial extract gave two activity peaks eluting at the same salt concentrations as the peaks for the total liver extract (Fig. 1c). The isomerase activity from the isolated peroxisomes gave only one peak (Fig. 1d), which eluted at the same point as the second peak in the tissue extract.

The peak isomerase activity fractions of the hydroxyapatite column originating from the clofibrate-treated rats were immunoblotted with antibodies to the peroxisomal hydratase-dehydrogenase-isomerase enzyme and to mitochondrial clofibrate-inducible isomerase. The antibody to mitochondrial clofibrate-inducible isomerase recognized a single band of *M_r* 30 000 in the second peak (Fig. 2a), whereas no reaction was

Fig. 1. Occurrence and subcellular location of a long-chain Δ^3, Δ^2 -enoyl-CoA isomerase in rat liver

Elution profile of isomerase activity from a hydroxyapatite column: control rat liver homogenate (a), clofibrate-treated rat liver homogenate (b), mitochondria isolated from clofibrate-treated rat liver (c) and peroxisomes isolated from clofibrate-treated rat liver (d). Activity was measured using either *trans*-3-dodecenoyl-CoA (○) or *trans*-3-hexenoyl-CoA (●) as substrate. Broken lines indicate potassium phosphate concentrations in the gradient.

Fig. 2. Immunoblotting of a long-chain Δ^3, Δ^2 -enoyl-CoA isomerase from rat liver with antibodies to clofibrate-inducible mitochondrial isomerase (a) and trifunctional enzyme (b) from rat liver

Lanes 1 (a and b) contain samples from the first peak of isomerase activity eluting from the hydroxyapatite column; lanes 2 (a and b) contain samples from the second peak of isomerase activity. Each lane contains isomerase activity of 1.80 nmol/min. The activities were measured using *trans*-3-dodecenoyl-CoA as a substrate for the sample in the first peak (lane 1) and *trans*-3-hexenoyl-CoA for that in the second peak (lane 2).

observed with the fraction containing long-chain isomerase activity. The M_r of the revealed band corresponded to the reported subunit size for the clofibrate-inducible isomerase (Stoffel & Grol, 1978; Palosaari *et al.*, 1990). When similar experiments were carried out with the antibody to peroxisomal trifunctional enzyme, a single band of M_r 78 000 was revealed in the second peak, whereas the fraction containing the long-chain isomerase activity gave no reaction (Fig. 2b). The M_r of the band was the same as reported previously for peroxisomal trifunctional enzyme (Osumi *et al.*, 1985; Palosaari & Hiltunen, 1990). Gel filtration on an S-200 HR column gave an M_r value of 200 000 for the native long-chain isomerase.

DISCUSSION

Hydroxyapatite column chromatography of the rat liver extract separated a novel long-chain Δ^3, Δ^2 -enoyl-CoA isomerase from the short-chain mitochondrial isomerase and the peroxisomal hydratase-dehydrogenase-isomerase enzyme characterized earlier. In addition to chromatographic separation, the characteristics of the long-chain isoenzyme were different from those of the two isomerase isoenzymes. (1) The substrate specificity showed its highest catalytic rate with C_{10-12} chain-length substrates. The activity ratio with C_6 , C_{10} , C_{12} and C_{16} substrates was 1:2:3:1.7. The velocity ratio for the short-chain mitochondrial isomerase with C_6 , C_{10} , C_{12} and C_{16} -*trans*-3-enoyl-CoA substrates was 11:5:1.3:1, values which agree with those reported earlier (Palosaari *et al.*, 1990). (2) The native M_r of the novel enzyme (200 000) is higher than that of short-chain mitochondrial isomerase (M_r 60 000; Stoffel & Grol, 1978; Palosaari *et al.*, 1990) and of the peroxisomal trifunctional enzyme (M_r 78 000; Osumi *et al.*, 1985; Palosaari & Hiltunen, 1990). (3) The long-chain isomerase was not clofibrate-inducible, unlike the other two isoenzymes (Palosaari *et al.*, 1990). (4) Antibodies to the peroxisomal trifunctional enzyme and to the mitochondrial short-chain isomerase did not recognize the long-chain isomerase, indicating their immunological differences.

Taken together, these and earlier data demonstrate that rat liver contains three isomerase isoenzymes: mitochondrial short- and long-chain isomerases and peroxisomal isomerase, as a part of a multifunctional protein. We have also tested rat heart extract, but only a minimum activity of a long-chain isomerase was observed, suggesting a tissue-specific distribution for this isoenzyme in the rat.

Struijk & Beerthuis (1966) demonstrated an isomerase of M_r 200 000 in bovine liver. In contrast with the enzyme described in this paper, this isomerase showed a preference for short-chain substrates. Miesowicz & Bloch (1979) purified a dimeric isomerase having a neutral pI from pig liver. The native protein had an M_r of 90 000 and its kinetic characteristics with regard to different substrates were dissimilar from those of the known isomerases in the rat. This means that in the light of the present literature it is not yet possible to draw conclusions about the relationship between isomerases in different mammalian species.

The enzyme activities involved in the degradation of fatty acids in β -oxidation generally have a dual location in both the mitochondria and peroxisomes. The enzyme which appears to be associated only with one compartment is 2-enoyl-CoA hydratase 2, which is required in the epimerization of 3-hydroxyacyl-CoA in peroxisomes (Chu & Schulz, 1985; Smeland *et al.*, 1989; Hiltunen *et al.*, 1989). The mitochondrial long-chain Δ^3, Δ^2 -enoyl-CoA isomerase identified here in addition to the short-chain isomerase and the peroxisomal trifunctional enzyme appears to belong to the group of enzymes which have two mitochondrial isoenzymes with different chain-length specificities and an additional peroxisomal isoenzyme, as exemplified by carnitine acyltransferase (Fritz *et al.*, 1963; Norum, 1964), 2-enoyl-CoA hydratase (Fong & Schulz, 1977; Osumi & Hashimoto, 1979a), 3-oxoacyl-CoA thiolase (Miyazawa *et al.*, 1980) and probably 3-hydroxyacyl-CoA dehydrogenase (El-Fakhri & Middleton, 1982; Osumi & Hashimoto, 1979b, 1980). The oxidation of acyl-CoA to *trans*-2-enoyl-CoA can be catalysed by three mitochondrial dehydrogenases (Ikeda *et al.*, 1985) and also by peroxisomal acyl-CoA oxidase (Osumi *et al.*, 1980). One mitochondrial isoenzyme of 2,4-enoyl-CoA reductase has been characterized so far, and the existence of an associated activity has also been demonstrated in particles containing catalase (i.e. peroxisomes) (Dommes *et al.*, 1981; Hakkola *et al.*, 1989).

The fact that the polyclonal antibodies to mitochondrial short-chain isomerase and peroxisomal trifunctional enzyme recognized only the proteins against which they were raised suggests that the isoenzymes of isomerases are not very closely related at the level of their primary polypeptide structure. The peroxisomal trifunctional enzyme is the only isomerization-catalysing protein which has been cloned and for which the primary structure and gene have been characterized (Osumi *et al.*, 1985; Ishii *et al.*, 1987). Until the other isomerases have been cloned it will not be possible to argue for an evolutionary relationship between different isomerase isoenzymes.

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