Implication of a peroxisomal enzyme in the catabolism of glutaryl-CoA

Joseph VAMECQ and François VAN HOOF

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL-75.39, Avenue Hippocrate 75, B-1200 Brussels, Belgium

(Received 24 November 1984/Accepted 13 March 1984)

A linear rate of H_2O_2 production is found when glutaryl-CoA is incubated with liver homogenates. We term this enzyme activity glutaryl-CoA oxidase. Its main characteristics are described and compared with those of glutaryl-CoA dehydrogenase (EC 1.3.99.7) and palmitoyl-CoA oxidase (EC 1.1.3.-). The latter enzyme catalyses the first step of peroxisomal β -oxidation. Glutaryl-CoA oxidase shares several properties with palmitoyl-CoA oxidase. The activities of both enzymes in mouse liver are increased by feeding the animals with a clofibrate-containing diet. Subcellular fractionation of the liver homogenates on a linear sucrose gradient indicates that glutaryl-CoA oxidase is a peroxisomal enzyme.

The classical route for the further metabolism of glutaryl-CoA, a catabolite of L-lysine, L-hydroxylysine and L-tryptophan, is dehydrogenation to glutaconyl-CoA directly followed by decarboxylation of this to crotonyl-CoA, both steps being catalysed by a single enzyme, glutaryl-CoA dehydrogenase (Scheme 1), which is a mitochondrial enzyme (Besrat *et al.*, 1969). Its activity has been assayed by measuring $^{14}CO_2$ production from [1,5- ^{14}C]glutaryl-CoA in the presence of an electron acceptor such as Methylene Blue or phenazine methosulphate (Besrat *et al.*, 1969; Christensen & Brandt, 1978). *In vivo*, a deficiency of this enzyme activity has been reported in two human autosomic recessive disorders: glutaryl-CoA dehydrogenase deficiency or glutaricaciduria type I (Goodman & Kohlhoff, 1975; Stokke *et al.*, 1975; Gregersen *et al.*, 1977), and multiple acyl-CoA dehydrogenase deficiency, also called glutaricaciduria type II (Przyrembel *et al.*, 1976; Goodman *et al.*, 1977; Gregersen *et al.*, 1977, 1980; Sweetman *et al.*, 1980).

The purpose of our investigations was to elucidate the reason for the existence of greatly different inherited disorders characterized by excessive excretion of glutaric acid in urine. We suspected the existence of distinct catabolic pathways for glutaryl-CoA by analogy with the fatty acyl-CoA β -oxidation systems, which exist both in mitochondria and in peroxisomes (Lazarow & de Duve, 1976; Lazarow, 1978; Mannaerts *et al.*, 1979; Mannaerts & Debeer, 1982). In the present

paper we demonstrate the existence of a peroxisomal glutaryl-CoA oxidation.

Materials and methods

Materials

Clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid] and CoA derivatives of palmitic acid, isobutyric acid, glutaric acid and succinic acid, as well as glucose 6-phosphate, homovanillic acid and peroxidase type II, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FAD, CoA, NADH and ATP were from Boehringer Pharma (Mannheim, Germany), 4-methylumbelliferyl derivatives were from Koch-Light Laboratories (Colnbrook, Bucks., U.K.) and other common chemicals were of analytical grade from Merck (Darmstadt, Germany).

Animals

Adult male NMRI mice, weighing at least 28 g, were used. They were fed on a standard laboratory animal chow containing less than 3% of fat. Treated animals were fed on a solid diet made by mixing the powdered animal chow with a hypolipidaemic drug. The concentration in the food was 0.5% (w/w) for neutralized clofibric acid and 0.2% (w/w) for the other two substances: nafenopin {2-methyl-2-[*p*-(1,2,3,4-tetrahydronaphth-1-yl)phenoxy]propionate, from CIBA-GEIGY, Basel, Switzerland} and POCA {ethyl 2-[5-(4-

Scheme 1. Mammalian metabolism of glutaryl-CoA

chlorophenyl)pentylloxiran-2-carboxylate, from BYK-Gulden Lomberg, Konstanz, Germany}.

Enzyme assays

Glutaryl-CoA oxidase assay was based on the fluorimetric determination of the H_2O_2 production. For the latter, the method of Guilbault *et al.* (1967) was adapted. The assay mixture (0.5 ml) contained 0.5 mM-glutaryl-CoA, 0.02 mM-FAD, 2 mM-ATP, 4 mM- MgCl_2 , 80 mM-glycylglycine buffer, pH 8.3, 10 mM-homovanillate and 0.1 mg of horseradish peroxidase. The reaction was started with the addition of an appropriate volume of tissue homogenate or of subcellular fraction, after disruption of subcellular organelles. The same method was adapted to measure L- α -hydroxy acid oxidase and palmitoyl-CoA oxidase activities, with

5 mM-glycollate (or L-lactate) and 0.5 mM-palmitoyl-CoA respectively as substrates.

Glutaryl-CoA dehydrogenase activity was assayed as described by Christensen & Brandt (1978) by measuring the $^{14}\text{CO}_2$ production from [1,5- ^{14}C]glutaryl-CoA.

The lysosomal hydrolase *N*-acetyl- β -glucosaminidase was assayed by a modification of the method of Van Hoof & Hers (1968), with 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside as substrate.

Cytochrome *c* oxidase was assayed by the method of de Duve *et al.* (1955) and glucose-6-phosphatase as described by Hers & Van Hoof (1966).

Arylsulphatase C was measured with 4-methylumbelliferyl sulphate as substrate. The assay was

made specific for the microsomal sulphatase by the ordered addition to the homogenate of imidazole buffer (63 mM, pH 7.1) and of inhibitors of the lysosomal sulphatases, 6.3 mM-barium acetate and 0.1 M-NaCl/Na₂SO₄, and finally the substrate (0.75 mM). The reaction was stopped by the addition of 0.5 M-Na₂CO₃/NaHCO₃ buffer, pH 10.7, and the fluorescence of free 4-methylumbelliferone was measured after centrifugation (F. Van Hoof, unpublished work).

Catalase was assayed by the titanium sulphate procedure, and urate oxidase by monitoring the decrease of uric acid concentration at 295 nm, as described by Baudhuin *et al.* (1964).

One unit of enzyme activity is the amount of

citrate, and examined with a Philips EM-301 electron microscope.

Results

Existence of a glutaryl-CoA oxidase

The first step in the catabolism of glutaryl-CoA is its dehydrogenation, which resembles the first step of β -oxidation of fatty acids. This step occurs both in mitochondria, catalysed by fatty acyl-CoA dehydrogenases, and in peroxisomes, catalysed by fatty acyl-CoA oxidase with the generation of H₂O₂. We have devised an assay for the glutaryl-CoA-dependent H₂O₂ production, in accordance with the following reaction:

enzyme that catalyses the conversion of 1 μ mol of substrate/min.

Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Cell fractionation

Fractionation by density-gradient equilibration was performed on postnuclear supernatants prepared in 0.25 M-sucrose/1 mM-EDTA, pH 7.4, as described by Tulkens *et al.* (1974). An 8 ml volume of sample was layered on 28 ml of a linear sucrose gradient (density limits 1.10 and 1.24 g/cm³), with 4 ml of sucrose at 1.34 g/cm³ as a cushion, and centrifuged at 49000 rev./min for 120 min in a vertical rotor (vTI 50; Beckman Instruments, Spinco Division, Palo Alto, CA, U.S.A.; operated with the 'slow-acceleration accessory', allowing smooth re-orientation of the tube contents). In all, 15–17 fractions of known densities and weights were collected. Enzyme and constituents were assayed in the presence of 0.1% Triton X-100 in order to disrupt the cell organelles.

Ultrastructural techniques

Small tissue samples were fixed with 2% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4. They were rinsed with the same buffer and post-fixed for 1 h in a 1% OsO₄/1% K₄Fe(CN)₆ mixture, dehydrated and embedded in Epon or in the mixture described by Spurr (1969). Ultrathin sections were stained with uranyl acetate and lead

H₂O₂ production occurred linearly with time and with respect to protein concentration when glutaryl-CoA oxidase was assayed in homogenates of mouse liver, kidney cortex and jejunal mucosa, but was not detectable in other organs, including lung, heart, striated and smooth muscle, brain, cerebellum and spleen. This tissue distribution suggests a peroxisomal localization of the enzyme.

Kinetic characteristics of glutaryl-CoA oxidase

Some properties of this enzymic system, and its comparison with palmitoyl-CoA oxidase and glutaryl-CoA dehydrogenase, are summarized in Table 1.

Glutaryl-CoA oxidase shares many properties with the initial step of the peroxisomal β -oxidation of fatty acids, in particular the relatively high K_m value and the restricted organ distribution. In addition, CoA and NADH are inhibitors, whereas MgATP and FAD are weak stimulators of both enzymes. L-Carnitine and saccharose are without effect.

Both enzymes can, however, be easily distinguished by the following properties. The time course of stimulation by clofibrate of the liver enzyme is different (see below). Isobutyryl-CoA and succinyl-CoA (3-carboxypropionyl-CoA) are potent inhibitors of glutaryl-CoA oxidase only. The most striking ion effect is that of Zn²⁺, which is a stimulator of glutaryl-CoA oxidase and inhibits palmitoyl-CoA oxidase (Fig. 1). In contrast, other

bivalent ions have a parallel stimulatory effect on both oxidases.

On the other hand, glutaryl-CoA dehydrogenase is clearly distinct from glutaryl-CoA oxidase. It is found in tissues where no glutaryl-CoA oxidase activity can be detected, as for instance in human fibroblasts and in mouse spleen and skeletal muscle. It is localized in mitochondria, whereas glutaryl-CoA oxidase occurs in peroxisomes. Glutaryl-CoA dehydrogenase activity in the liver

is only slightly increased by clofibrate treatment. The optimal stimulation, which occurs after 15 days, is about 1.4-fold. No effect of clofibrate could be demonstrated in kidney for this enzyme. In mouse liver, the apparent K_m of glutaryl-CoA oxidase is 50-fold higher than and the apparent V_{max} 5-fold lower than those of the dehydrogenase, although the pH optimum is similar for both enzymes.

Stimulation of glutaryl-CoA oxidase activity by hypolipidaemic agents

Like palmitoyl-CoA oxidase, the first enzyme of the peroxisomal β -oxidation, glutaryl-CoA oxidase activity is strongly stimulated in the livers of clofibrate-treated mice. The time course of this action is illustrated in Fig. 2. Although the initial stimulatory effect of clofibrate acid is approximately the same for both enzyme activities, there is an obvious difference in its later time course. After a first peak reached after 1–2 weeks of treatment, there is a decrease in both oxidase activities, which lasts for 1 month after the initial increase. This appears to result from the autophagic destruction of part of the peroxisomal population (Fig. 3). Indeed, a preliminary morphometric analysis of micrographs of liver tissue from animals treated for 0, 5 and 15 days demonstrates a highly significant increase in the proportion of autophagic vacuoles containing recognizable peroxisomes in hepatocytes.

Other hypolipidaemic agents have been less extensively studied. A similar stimulation of both oxidase activities has been obtained with nafenopin and POCA.

Fig. 1. Opposite effects of $ZnSO_4$ on the two peroxisomal enzymes glutaryl-CoA oxidase (●) and palmitoyl-CoA oxidase (○) in a liver homogenate prepared from a mouse treated for 15 days with clofibrate. For experimental details see the text.

Fig. 2. Time course of the stimulation of the activity of the two peroxisomal enzymes glutaryl-CoA oxidase (●) and palmitoyl-CoA oxidase (○) in the liver of mice continuously fed with clofibrate.

Each point represents the mean activity obtained in at least four animals, as compared with matched controls. The activity in controls remains stable at about 800 munits/g of liver for palmitoyl-CoA oxidase and at about 100–120 munits/g for glutaryl-CoA oxidase.

Fig. 3. Ultrastructure of parenchymal liver cells of a mouse fed with clofibrate for 2 weeks. The arrows point to autophagic vacuoles that contain recognizable peroxisomes (p).

Fig. 4. Isopycnic centrifugation of postnuclear supernatants of a control mouse liver homogenate
Subcellular localization of glutaryl-CoA oxidase (k) is compared with those of other peroxisomal enzymes: catalase (f), glycolate oxidase (g), L-

In the kidney, the stimulation of both oxidase activities by clofibrate (2–3-fold) is less than in the liver.

Cellular fractionation studies on a linear sucrose density gradient

As illustrated in Fig. 4, the subcellular localization of glutaryl-CoA oxidase activity in control mouse liver follows that of other known peroxisomal enzymes and is clearly distinct from that of mitochondrial, lysosomal and microsomal enzymes. Its distribution is unimodal, and closely parallels that of palmitoyl-CoA oxidase and urate oxidase, as well as the 'peroxisomal mode' (high-density equilibration) of L- α -hydroxy acid oxidase and of catalase activities.

After 15 days of treatment with clofibrate, the distribution of glutaryl-CoA oxidase becomes bimodal, like that of palmitoyl-CoA oxidase. Both these activities parallel that of catalase, which belongs to the peroxisomal matrix. Urate oxidase, an enzyme marker of the peroxisomal nucleoid, preserves its unimodal distribution (Fig. 5).

Discussion

Our results demonstrate the existence in peroxisomes of a β -oxidase acting on glutaryl-CoA, besides that acting on fatty acids, which was first reported by Lazarow & de Duve (1976). This enzyme is clearly distinct from the mitochondrial glutaryl-CoA dehydrogenase. It has a lower $V_{\max}$ and a higher K_m than the mitochondrial enzyme. Our limited knowledge about the relative abundance of glutaryl-CoA in the cell within or outside the mitochondria does not allow us to estimate the physiological importance of this novel peroxisomal enzyme, nor to ascertain the fate of glutacetyl-CoA, the end product of the reaction.

lactate oxidase (h), urate oxidase (i) and palmitoyl-CoA oxidase (j). Other markers are proteins (a) and enzymes from mitochondria, cytochrome c oxidase (b), from lysosomes, N-acetyl- β -glucosaminidase (c), and from the endoplasmic reticulum, glucose-6-phosphatase (d) and arylsulphatase C (e). Results are given as density-distribution histograms. Abscissa is the density span of the gradient, divided in 15 sections of equal density increment (0.0167) from 1.05 to 1.30 g/cm³. Ordinate is the frequency of enzymic activities or proteins in each section ($Q/\Sigma Q \cdot \Delta\rho$, where Q is the amount of activity equilibrating within the section, ΣQ the sum of the activities found in all subfractions, and $\Delta\rho$ the density increment of the section). Recovery of enzyme activity in the sum of fractions was between 78 and 134%.

Fig. 5. Comparative distribution, on a linear sucrose density gradient, of four peroxisomal enzymes of the postnuclear supernatant of liver homogenates from a control mouse (a-d) and from a mouse fed with clofibric acid for 2 weeks (e-h). Results are expressed as the relative enzyme activity in each of the 17 fractions collected, fractions being represented in increasing order of density along the abscissa. Proteins and five marker enzymes were also recorded (results not shown); their distribution was not significantly affected by the treatment. Glutaryl-CoA oxidase (a) and palmitoyl-CoA oxidase (b) acquired in the treated mouse a bimodal distribution (e and f), and their activities were stimulated 15- and 17-fold respectively. Catalase kept its bimodal distribution (c and g), and urate oxidase its unimodal density distribution (d and h). Clofibric acid treatment did not modify the activity of the last-mentioned two enzymes in this experiment.

We know that peroxisomal acyl-CoA oxidases display a broad specificity for their substrate chain length, and that other peroxisomal oxidases, including D-amino acid oxidase and L- α -hydroxy acid oxidase, also act on a wide variety of substrates. Glutaryl-CoA oxidase having not been purified, it is impossible to exclude an action of the enzyme on other dicarboxylic acids, the more so as preliminary results obtained by us clearly indicate the existence of H_2O_2 -generating oxidases, stimutable by clofibrate, that act on succinate, adipate, pimelate, suberate and sebacate. Moreover, reports in the literature (Mortensen *et al.*, 1982; Gregersen *et al.*, 1983) support the existence of the peroxisomal β -oxidation of dicarboxylic acids (C_8 to C_{12}).

Glutaryl-CoA oxidase is not restricted to rodents. We found the enzyme in human liver, with an activity about 5 times lower than in mouse liver. The question might thus be posed whether the inborn deficiency of this peroxisomal oxidase is responsible for the occurrence of a form of glutaricaciduria.

The very efficient inducibility by hypolipidaemic agents of glutaryl-CoA oxidase, at least in rodent liver, allows us to expect some benefits of these drugs in glutaricaciduria type II. This disorder has been attributed to a deficiency of the electron-transferring flavoprotein dehydrogenase necessary for the transport of H^+ and electrons from $FADH_2$ to ubiquinone (Goodman *et al.*, 1980, 1983; Rhead *et al.*, 1980; Gregersen *et al.*, 1982). Hypolipidaemic treatment could there be useful in several respects. It would first stimulate the peroxisomal glutaryl-CoA oxidase, which would be able to handle glutarate leaving the mitochondria. Further, it would greatly enhance the peroxisomal β -oxidation of fatty acids, which is deficient in the mitochondria.

We thank Professor H. S. A. Sherratt, Dr. L. Hue and Professor H. G. Hers for their continuous interest and valuable criticism. We are indebted to Mrs. B. Wallon-Lederer, Ms. B. Wantier and Mrs. D. Ghyselen-Grandfils for their skilful technical assistance, to Dr. E. Christensen for the gift of radioactive glutaryl-CoA and

to Dr. G. Ludwig for that of POCA. This investigation was supported by grants from the Belgian Fonds National de la Recherche Scientifique and U.S. Public Health Services (Grant AM 9235). J. V. is Aspirant of the Fonds National de la Recherche Scientifique.

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