Inhibition of peroxisomal fatty acyl-CoA oxidase by antimycin A

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Peroxisomal fatty acyl-CoA oxidase was inhibited by micromolar concentrations of antimycin A, an inhibitor of mitochondrial respiration. The inhibition was observed with all three substrates tested, i.e. palmitoyl-CoA, trihydroxycoprostanoyl-CoA and hexadecanedioyl-CoA. The peroxisomal D-amino acid oxidase was also inhibited by antimycin, but the peroxisomal L- α -hydroxyacid oxidase and uric acid oxidase and the mitochondrial monoamine oxidase were not. The degree of inhibition of acyl-CoA oxidase by antimycin was strongly dependent on the amount of cellular protein present in the assay mixture: at a fixed antimycin concentration, the inhibition was gradually lost with increasing protein concentrations. At a fixed cellular protein concentration in the assay mixtures, the mitochondrial oxidation of glutamate or palmitoylcarnitine was inhibited at antimycin concentrations that were much lower than those required for the inhibition of fatty acyl-CoA oxidase. Our results, nevertheless, demonstrate that antimycin A must be used with caution, when it is added to homogenates or subcellular fractions in order to distinguish between mitochondrial and peroxisomal fatty acid oxidation.

INTRODUCTION

Mitochondria of animal cells and peroxisomes of virtually all eukaryotic cells possess the necessary enzymic equipment for the β -oxidation of fatty acids [1,2]. In the intact animal cell, mitochondria oxidize the major portion of the long-chain fatty acids (C_{16} – C_{18}) [3], which because of their abundance, are an important source of metabolic fuel. Peroxisomal β -oxidation, which in animal cells does not go to completion, but which carries out only a limited number of cycles of the β -oxidation spiral [4,5], appears to be responsible for the chain shortening of very-long-chain fatty acids ($> C_{20}$) [6,7], of long-chain dicarboxylic acids [8], and of the acyl chain of certain xenobiotics [9]. In addition, the hepatic peroxisomal β -oxidation system catalyses the oxidative cleavage of the side chain of cholesterol in bile acid synthesis [10]. Apparently, peroxisomes are capable of partially degrading a variety of fatty acids and fatty acid derivatives, transforming these hydrophobic molecules to shorter and more polar metabolites, which can be more easily excreted or which, in some cases, are further oxidized by the mitochondria.

In intact mitochondria, fatty acid oxidation is tightly coupled to the respiratory chain, so that β -oxidation stops when respiration is inhibited. Peroxisomal β -oxidation is not directly coupled to a respiratory chain. In broken-cell preparations supplemented with the appropriate cofactors, peroxisomal β -oxidation continues to proceed in the presence of respiratory inhibitors [1,3]. Since a specific inhibitor of peroxisomal β -oxidation is not known, inhibitors of mitochondrial respiration are frequently used to distinguish between peroxisomal and mitochondrial β -oxidation, when fatty acid oxidation is studied in broken-cell preparations that contain both organelles.

We now report that the respiratory inhibitor antimycin

A also inhibits acyl-CoA oxidase, the first and rate-limiting enzyme of the peroxisomal β -oxidation sequence [11]. The concentrations required for inhibition of the enzyme are much larger than those required for inhibition of the respiratory chain, but they are of the same order of magnitude as those usually added to achieve inhibition of mitochondrial respiration in studies on peroxisomal fatty acid oxidation [3,12–14].

EXPERIMENTAL

Materials

Homovanillic acid, tyramine, glycollic acid, uric acid, horseradish peroxidase (type II) and rotenone were from Sigma, St. Louis, MO, U.S.A. FAD, ATP, ADP, NAD^+ and lactic acid were from Boehringer, Mannheim, Germany. CoA, palmitoyl-CoA and palmitoylcarnitine were obtained from Pharmacia, Uppsala, Sweden. D-Alanine and L-glutamate were from Janssen Chimica, Beerse, Belgium, and from Merck, Darmstadt, Germany, respectively. Antimycin A was from Calbiochem, San Diego, CA, U.S.A.

Trihydroxycoprostanic acid was purified from alligator bile as described previously [15]. Hexadecanedioyl-CoA and trihydroxycoprostanoyl-CoA were synthesized by the method described by Webster & Killenberg [16]. Trihydroxy[26- ^{14}C]coprostanic acid was prepared by a modification of the methods described by Tserng & Klein [17] and Carlson & Fromm [18] for the synthesis of radioactive C_{24} bile acids. Trihydroxycoprostanic acid (200 mg) was first formylated by heating in a mixture of 2 ml of 89.9% (w/v) formic acid and one drop of 70% (w/v) $HClO_4$. After cooling to 40 °C, acetic anhydride was added dropwise until bubbles appeared, and the solution was then allowed to cool to room temperature. The formylated trihydroxycoprostanic acid solution was

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extracted with diethyl ether and dried. 26-Chloro-3 α ,7 α ,12 α -triformyl-oxy-norcholestan-3-one was obtained by dissolving the triformyltrihydroxycoprostanic acid in a mixture of 4.5 ml of dry benzene, 348 mg of lead tetraacetate and 34.8 mg of LiCl, with N₂ gas bubbling through, and heating this to 83 °C for 2 h. After this, 4 $\times$ 17.4 mg of LiCl was added, with 1 h intervals. The reaction mixture was then filtered and the filtrate washed and dried. The residue of the drying step was then dissolved in 2 ml of dry dimethyl sulphoxide containing 1 mCi of Na¹⁴CN (Amersham International, Amersham, Bucks., U.K.) and 11 mg of NaCN, and heated to 130 °C for 2 h. After cooling, 2 ml of 2 M-NaOH was added, and the solution was heated to 90 °C for 1 h to hydrolyse the formyl groups. The [26-¹⁴C]cyano-3 α ,7 α ,12 α -trihydroxynorcholestan-3-one thus formed was extracted with chloroform and dried. The residue was purified by t.l.c. in iso-octane/ethyl acetate/acetic acid (5:5:1, by vol.). The nitrile was then dissolved in 20 ml of ethanol plus 20 ml of aq. 12.5 (w/v) NaOH and heated at 100 °C for 24 h. After cooling, 50 ml of water was added, and the solution was extracted with diethyl ether. The solution was then acidified and the trihydroxy[¹⁴C]coprostanic acid was extracted with chloroform. Analysis by t.l.c. and by g.l.c. showed the reaction product to be pure and identical with trihydroxycoprostanic acid.

Animals

Male Wistar rats, weighing 200–300 g and maintained on a standard laboratory diet, were used.

Preparation of subcellular fractions

For the experiments in which the subcellular distribution of trihydroxy[26-¹⁴C]coprostanic acid oxidation was studied, 25 % (w/v) liver homogenates were prepared in 0.25 M-sucrose/5 mM-dithiothreitol/0.1 % (v/v) ethanol, and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and soluble (S) fractions as described by de Duve *et al.* [19].

Oxidase activities (H₂O₂ production) were measured in postnuclear supernatants or in peroxisomal fractions, purified on linear sucrose gradients as described previously [20].

Fractions enriched in mitochondria and used for the determinations of the mitochondrial oxidation of palmitoylcarnitine and of glutamate were prepared from 20 % (w/v) liver homogenates, made in 0.3 M-mannitol/1 mM-EGTA/10 mM-Hepes, pH 7.3. The homogenates were centrifuged at 1000 *g* for 10 min. The supernatants were centrifuged at 20000 *g* for 10 min to sediment most of the mitochondria. The sedimented organelles were washed by centrifugation at 20000 *g* for 10 min and resuspended in homogenization medium to a final protein concentration of 15 mg/ml.

Enzyme assays

The oxidation of trihydroxy[26-¹⁴C]coprostanic acid (conversion of trihydroxycoprostanic acid into cholate, [1-¹⁴C]propionate and their CoA derivatives) was studied in a modified Krebs–Henseleit bicarbonate buffer, containing no Ca²⁺, twice the normal amount of

MgSO₄, 4 vol. of 0.154 M-NaCl and 100 vol. of 0.154 M-KCl per 130 vol. of buffer [21]. The reaction mixture consisted of modified Krebs–Henseleit buffer containing 4 mM-ATP, 0.4 mM-CoA, 2 mM-NAD⁺, 100 μ M-trihydroxy[¹⁴C]coprostanic acid (sp. radioactivity 0.6 Ci/mol), 50 μ M defatted [22] bovine serum albumin and, when required, 50 μ M-rotenone and 7.3 μ M-antimycin A. The reaction was started by adding 0.02 ml of homogenate or subcellular fraction, suspended in 0.25 M-sucrose/5 mM-dithiothreitol/0.1 % ethanol, and containing 0.05–0.4 mg of protein, to 0.23 ml of the reaction mixture. The mixtures were incubated in a shaking water bath at 37 °C for 30 or 60 min, and the reactions were terminated by the addition of 0.03 ml of 10 M-KOH. The alkali-treated mixtures (pH 13) were kept at room temperature for 60 min in order to hydrolyse the radioactive trihydroxycoprostanoyl-CoA, which is formed as an intermediate during the oxidation of trihydroxycoprostanic acid. The hydrolysis step proved necessary for the following reasons (M. Casteels, L. Schepers & G. P. Mannaerts, unpublished work): (1) high steady-state concentrations of trihydroxycoprostanoyl-CoA (20–30 μ M) were reached during the incubations, since trihydroxycoprostanic acid activation was severalfold more active than peroxisomal oxidation; (2) approx. 20 % of the trihydroxycoprostanoyl-CoA remained in the final aqueous phase (see below), which was used for counting radioactivity of the labelled oxidation products, resulting in a serious overestimation of the oxidation rates. Then 0.03 ml of 6 % (w/v) albumin was added, and the mixtures were acidified (pH < 1) with 0.5 ml of 12 % (w/v) HClO₄. The denatured protein was sedimented by centrifugation at 2100 *g* for 10 min. Part of the radioactive trihydroxycoprostanic acid co-precipitated in this step. The remaining part was extracted from the supernatant with chloroform, and a portion of the final aqueous phase was counted for radioactivity (radioactive oxidation products). With this procedure less than 3 % of the trihydroxy[¹⁴C]coprostanic acid and more than 97 % of [¹⁴C]propionic acid were recovered in the final aqueous phase. The production of radioactive CO₂, measured after its trapping in Hyamine hydroxide [21], was negligible.

Oxidase activities were measured at room temperature by a fluorometric method as described by Vamecq & Van Hoof [20], with the following modifications. Fluorescence was continuously recorded. The reaction mixture (volume 1 ml) consisted of 50 mM-glycylglycine buffer, pH 8.3, 0.55 mM-homovanillic acid, 20 μ g of peroxidase/ml, 25 μ M-FAD and liver protein as indicated in the Tables and Figures. The reactions were started by the addition of the following substrates: palmitoyl-CoA (100 μ M), hexadecanedioyl-CoA (100 μ M), trihydroxycoprostanoyl-CoA (100 μ M), tyramine (10 mM), glycollate (10 mM), D-alanine (10 mM) and uric acid (60 μ M).

The mitochondrial oxidation of glutamate and of palmitoylcarnitine was measured by following the glutamate- and palmitoylcarnitine-dependent reduction of ferricyanide as described by Osmundsen & Bremer [23]. The final glutamate and palmitoylcarnitine concentrations were 10 mM and 40 μ M respectively. When glutamate oxidation was measured, 1 mM-malate was also present.

Glutamate dehydrogenase, catalase and protein were measured as described previously [24].

RESULTS AND DISCUSSION

In rat liver the oxidation of trihydroxycoprostanic acid to cholic acid occurs mainly, if not exclusively, in the peroxisomes [10]. In the course of experiments on the oxidation of trihydroxycoprostanic acid in rat liver homogenates, we found to our surprise that trihydroxycoprostanic acid oxidation was inhibited by a mixture of the respiratory-chain inhibitors antimycin A and rotenone, but not by 2 mM-cyanide. Subsequent cell-fractionation studies revealed that the subcellular distribution of trihydroxycoprostanic acid oxidation in the absence of inhibitors, as well as the subcellular distribution of that part of the oxidation that was inhibited by the inhibitors, closely resembled the distribution of the peroxisomal marker enzyme catalase and not that of the mitochondrial marker enzyme glutamate dehydrogenase, as could be expected for the inhibitable part of the oxidation (Fig. 1). These observations demonstrate that the inhibitable part of the oxidation does not represent mitochondrial oxidation, but that antimycin

and/or rotenone inhibit peroxisomal trihydroxycoprostanic acid oxidation.

We therefore measured acyl-CoA oxidase, the first and rate-limiting enzyme of the peroxisomal β -oxidation sequence [11], with trihydroxycoprostanoyl-CoA and palmitoyl-CoA as substrates. Figs. 2(a) and 2(b) show that acyl-CoA oxidase activity was inhibited by the mixture of antimycin and rotenone, irrespective of whether trihydroxycoprostanoyl-CoA or palmitoyl-CoA was the substrate. Hexadecanedioyl-CoA oxidase activity was also inhibited (results not shown). Fig. 2(c) demonstrates that the inhibitor of acyl-CoA oxidase was antimycin and not rotenone. The inhibition of acyl-CoA oxidase activities by antimycin was concentration-dependent (results not shown).

Among peroxisomal oxidases, antimycin inhibited not only acyl-CoA oxidase but also D-amino acid oxidase (Table 1). Tyramine oxidase (monoamine oxidase), present in the mitochondrial outer membrane, and two other peroxisomal oxidases, L- α -hydroxyacid oxidase, measured with glycollate or lactate as the substrate, and

Fig. 1. Subcellular distribution of enzyme activities and trihydroxy[26- 14 C]coprostanic acid oxidation after differential centrifugation of a rat liver homogenate

Results are expressed as relative specific activities versus cumulative percentage of total recovered protein. Relative specific activity is defined as the percentage of total recovered activity present in the individual fraction divided by the percentage of total recovered protein present in that fraction. The N fraction and the M fraction contain most of the nuclei and of the mitochondria, respectively, and the remaining fractions are most enriched in peroxisomes and lysosomes (L), microsomes (P) and soluble components (S). (a) Catalase (marker for peroxisomes); (b) glutamate dehydrogenase (marker for mitochondria); (c) trihydroxy[14 C]coprostanic acid oxidation in the absence of rotenone and antimycin; (d) rotenone- and antimycin-sensitive part of trihydroxy[14 C]coprostanic acid oxidation. Total activities were 57 units/g of liver (a), 184 units/g of liver (b), 18.5 nmol/min per g of liver (c), 5.2 nmol/min per g of liver (d). Percentage recoveries after fractionation were: 99 (a), 88 (b), 91 (c) and 81 (d). Catalase units in (a) are as defined by Baudhuin *et al.* [25].

Fig. 2. Effect of rotenone and antimycin A on acyl-CoA oxidase activities

Acyl-CoA oxidase activities were measured in peroxisomal fractions purified by sucrose-density-gradient centrifugation. (a) Trihydroxycoprostanoyl-CoA was used as the substrate. Rotenone and antimycin A were added together as indicated by the arrow (+R/A). The peroxisomal protein concentration was 0.44 mg/ml of reaction mixture. (b) Palmitoyl-CoA was used as the substrate. Curve a: palmitoyl-CoA oxidase activity in the absence of respiratory inhibitors. Curve b: rotenone and antimycin A were added as indicated by the arrow (+R/A). Curve c: rotenone and antimycin were added before the start of the reaction. The peroxisomal protein concentration was 0.29 mg/ml of reaction mixture. (c) Palmitoyl-CoA was used as the substrate. Rotenone (+R) and antimycin A (+A) were added separately, as indicated by the arrows. After the addition of antimycin, palmitoyl-CoA was added for a second time (+Pal-CoA). The peroxisomal protein concentration was 0.15 mg/ml of reaction mixture. In all experiments the rotenone and antimycin concentrations were 50 μ M and 7.3 μ M respectively.

Table 1. Effect of antimycin A on oxidase activities

Oxidase activities were measured in postnuclear supernatants in the absence and the presence of 7.3 μ M-antimycin A. The liver protein concentration in the assay mixtures was 0.6 mg/ml. Results are means $\pm$ S.E.M. for three experiments: ND, not detectable.

Substrate	Oxidase activity (nmol of H ₂ O ₂ /min per mg of protein)	
	No antimycin A	+Antimycin A
Palmitoyl-CoA	3.90 $\pm$ 0.76	1.82 $\pm$ 0.41
D-Alanine	0.86 $\pm$ 0.09	ND
Glycollate	7.50 $\pm$ 0.55	8.04 $\pm$ 0.61
Lactate	1.08 $\pm$ 0.05	1.17 $\pm$ 0.11
Urate	8.52 $\pm$ 0.96	8.59 $\pm$ 0.72
Tyramine	9.12 $\pm$ 1.01	8.54 $\pm$ 1.20

Fig. 3. Effect of protein concentration in the assay on the inhibition of acyl-CoA oxidase by antimycin A

Acyl-CoA oxidase activities were measured in postnuclear supernatants in the absence and presence of 7.3 μ M-antimycin A with palmitoyl-CoA (○), trihydroxycoprostanoyl-CoA (□) or hexadecanedioyl-CoA (△) as the substrate and at different liver protein concentrations. Results are expressed as percentage inhibition versus increasing protein concentration.

urate oxidase, were not inhibited at the concentrations used, indicating that antimycin did not interfere with a component of the assay system other than the inhibited oxidases.

The inhibition of palmitoyl-CoA oxidase could be abolished by adding sufficient amounts of albumin before or after the addition of antimycin (results not shown). The degree of inhibition was also strongly dependent on the amount of liver protein present in the assay mixtures (Fig. 3). At lower protein concentrations the inhibition was virtually complete, but it was gradually lost with increasing protein concentrations. It is likely that antimycin, which is a lipophilic compound, can be

Table 2. Effect of antimycin A on mitochondrial oxidation and on peroxisomal acyl-CoA oxidase

Mitochondrial oxidation of glutamate and of palmitoyl-carnitine and peroxisomal palmitoyl-CoA oxidase were measured in crude mitochondrial fractions, which were still contaminated by peroxisomes, in the absence and the presence of increasing antimycin A concentrations. Control rates (absence of antimycin) were 104 ± 11 and 64 ± 6 nmol of ferricyanide reduced/min per mg of protein for the mitochondrial oxidation of glutamate and of palmitoylcarnitine respectively, and 6.2 ± 1.8 nmol of H_2O_2 /min per mg of protein for palmitoyl-CoA oxidase. The liver protein concentration in the assay mixture was 2.3 ± 0.1 mg/ml ($n = 3$).

[Antimycin A] (μM)	Inhibition (%)		
	Mitochondrial oxidation		Palmitoyl-CoA oxidase
	Glut- amate	Palmitoyl- carnitine	
7.3×10^{-3}	0	0	0
2.9×10^{-3}	0	0	0
7.3×10^{-2}	100	100	0
7.3×10^{-1}	100	100	0
7.3	100	100	5 ± 1

bound by albumin and cellular proteins and that it also partitions in the phospholipid phase of membranes. Table 2 shows that, at a fixed cellular protein concentration in the assay mixtures, the mitochondrial oxidation of glutamate and of palmitoylcarnitine was inhibited at concentrations of antimycin approximately 3 orders of magnitude lower than those required for inhibition of acyl-CoA oxidase.

Our results show that antimycin A inhibits acyl-CoA oxidase and peroxisomal fatty acid oxidation at concentrations which are sometimes used to suppress the mitochondrial component of β -oxidation, when fatty acid oxidation is studied in preparations that contain peroxisomes as well as mitochondria. The degree of inhibition is strongly dependent on the assay conditions, such as the amounts of cellular protein and albumin present in the assay mixtures. It is therefore not our intention to conclude that peroxisomal fatty acid oxidation was inhibited in earlier studies [3,12–14], in which antimycin was used as 'specific' inhibitor of mitochondrial respiration and mitochondrial β -oxidation. In our own previous experiments, for instance, we failed to observe an inhibition of peroxisomal fatty acid oxidation by antimycin, because of the presence of albumin and a higher cellular protein concentration in the assay mixtures [3]. Since the antimycin-sensitivity of the respiratory chain is much higher than that of acyl-CoA oxidase, antimycin can still be used as a respiratory inhibitor in studies on peroxisomal fatty acid oxidation. Before use it is necessary, however, to determine the appropriate inhibitor concentration that inhibits mitochondrial respiration without affecting acyl-CoA oxidase activity under the proper assay conditions. The electron

carriers of the respiratory chain are embedded in the mitochondrial inner membrane, whereas acyl-CoA oxidase is a soluble enzyme of the peroxisomal matrix. Partitioning of the lipophilic inhibitor in the lipid phase of the membrane is a possible explanation for the high antimycin-sensitivity of the respiratory chain.

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