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L-PIPECOLATE OXIDASE: A DISTINCT PEROXISOMAL ENZYME IN MAN

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We investigated the oxidation of L-pipecolate in human liver. The results obtained with L-pipecolate from which traces of D-pipecolate had been removed by a preincubation with D-aminoacid oxidase indicate that a distinct L-pipecolate oxidase rather than D-aminoacid oxidase is responsible for the L-pipecolate dependent H_2O_2-production in human liver. Importantly, L-pipecolate oxidase was found to be localized in peroxisomes which adds to the growing number of enzymes and metabolic functions which can be ascribed to peroxisomes.

L-pipecolic acid has long been known to be an intermediate in the degradation of L-lysine in mammals [1]. Nevertheless, information on the enzymic steps involved in the catabolism of L-pipecolic acid in mammals is sparse. Extensive studies carried out with Pseudomonas putida [2-6] indicate that in this organism L-pipecolate is converted to Δ^1-piperideine-6-carboxylate via an inducible, membrane-bound dehydrogenase associated with an electron-transport particle [5, 6]. Subsequent oxidation via α-aminoadipate δ-semialdehyde: NAD^+ oxidoreductase then produces α-aminoadipic acid [4]. In contrast, the first step in L-pipecolic acids oxidation in the yeast Rhodotorula glutinis proceeds via a L-pipecolate oxidase which in contrast to L-pipecolate dehydrogenase directly accepts molecular oxygen as substrate.

Studies in mammals have shown that L-lysine is primarily degraded via the saccharopine pathway involving the sequential action of L-lysine ketoglutarate reductase and saccharopine dehydrogenase [8, 9]. In brain, however, the activity of this pathway is negligible [9]. Indeed, Chang [10, 11] has shown that L-lysine degradation in brain proceeds predominantly via L-pipecolate as an intermediate rather than via saccharopine. The importance of the L-pipecolate pathway in man is stressed by the finding that L-pipecolate accumulates in patients suffering from the cerebro-hepato-renal

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(Zellweger) syndrome, an autosomal recessive, lethal disease characterized by the (virtual) absence of peroxisomes [12-14]. We recently presented evidence for the presence of a L-pipecolate oxidase in human liver and showed that this activity is deficient in the Zellweger syndrome [15]. Here we show that the L-pipecolate oxidase present in human liver, is localized in peroxisomes and that the enzyme is absent from rat liver.

MATERIALS AND METHODS

Enzyme activity measurements

L-pipecolate oxidase was measured fluorimetrically exactly as described before [15].

D-aminoacid: O_2 oxidoreductase was assayed at 37°C by following the increase in the absorbance at 510 nm in a medium containing 50 mM sodium pyrophosphate, 25 µM FAD, 6 mM 2,4,6-tribromohydroxybenzoate, 1 mM aminoantipyrine, 10 mM NaN_3, 0.01% (w/v) Triton X-100, 20 U/ml horseradish peroxidase and 50 mM D-alanine. The final pH was 8.3.

L-α-hydroxyacid: O_2 oxidoreductase was assayed as described for D-aminoacid oxidase with the exception that 50 mM sodium L-glycolate replaced 50 mM D-alanine. The final pH was 8.3.

Glutamate dehydrogenase was measured as described before [16].

Esterase was assayed at 37°C by following the increase in absorbance at 405 nm using a medium of the following composition: 20 mM potassium phosphate, 1 mM EDTA and 0.1% (w/v) Triton X-100, pH 6.6. Reactions were started by adding p-nitrophenylacetate in ice-cold methanol at a final concentration of 12.5 mM.

β-Hexosaminidase was assayed by adding aliquots of human liver fractions to a medium containing 2 mM β-nitrophenyl-L-acetamido-L-deoxy-β-D-glucopyranoside, 0.1 M sodium acetate and 0.1% (w/v) Triton X-100. The final pH was 5.4. After 15 min reactions were stopped by adding an equal volume of 0.6 M glycine-NaOH (pH = 10.5), and the absorbance was measured at 405 nm.

Lactate dehydrogenase was assayed by following the decrease in absorbance at 340 nm in a medium containing 50 mM potassium phosphate, 0.2 mM NADH, 0.1% (w/v) Triton X-100 and 0.5 mM pyruvate. The final pH was 7.4.

Differential centrifugation and density gradient fractionation of human liver postnuclear supernatants

Pieces of human liver obtained from patients who underwent partial liver resection, were finely minced using a medium containing 250 mM sucrose, 2 mM EDTA, 5 mM morpholinopropanesulphonic acid (MOPS)-NaOH and 0.1% (v/v) ethanol (final pH 7.4). Subsequent homogenization was carried out at 4°C using a glass potter homogenizer and a loosely fitting Teflon pestle. The resulting homogenate was subjected to differential centrifugation using a SORVALL-RC5B-superspeed centrifuge equipped with a SS-34-rotor (8 x 50 ml), as follows. The total homogenate was first centrifuged at 600 x g_{av} for 10 min. The resulting supernatant (postnuclear supernatant) was collected and centrifuged at 3600 x g_{av} for 10 min. The resulting pellet containing the bulk of the mitochondria was taken up in homogenization buffer, gently homogenized and pelletted again giving rise to the M-fraction. The 3600 x g_{av}-supernatant was subsequently centrifuged at 25.000 x g_{av} for 10 min. The pellet was taken up in sucrose-medium again, gently homogenized and pelletted by centrifugation at 25.000 x g_{av} (ML-fraction). Finally, the 25.000 x g_{av}-supernatant was centrifuged at 40.000 x g_{av} for 150 min giving rise to a microsomal fraction and a soluble fraction.

The fraction richest in peroxisomes (25.000 x g_{av}-pellet) was subjected to equilibrium density gradient centrifugation in a linear Nycodenz gradient (20% - 50%, w/v) essentially as described before for rat liver [16,17]. The gradient was formed by diffusion (24h, 4°C) after layering equal volumes (0.9 ml) of 50%, 40%, 30% and 20% (w/v) Nycodenz containg 2 mM MOPS-NaOH plus 2 mM EDTA (final pH 7.4).

Centrifugation was carried out at 4°C for 150 min at 104.000 x g_{av} in a Beckmann LC-8-70 ultracentrifuge using a SW-55 Ti-rotor. The gradient was unloaded by making use of a hypodermic needle connected to a peristaltic pump.

Materials

4-Hydroxyphenylacetic acid, D-aminoacid oxidase and L-pipecolic acid were obtained from Sigma (St. Louis, Mo., USA). Horse radish peroxidase (grade II) was from Boehringer, Mannheim, F.R.G. All other chemicals were of analytical grade.

RESULTS

We have recently shown that in human liver but not in rat liver L-pipecolate is oxidized in an H_2O_2-generating reaction catalyzed by L-pipecolate oxidase. Since the absence of L-pipecolate oxidase activity in rat liver homogenates [15] or purified rat liver peroxisomes [18], is in apparent contradiction to the conclusion by Zaar et al [18] that L-pipecolate (like D-pipecolate [19]) is oxidized by D-aminoacid oxidase, which is present both in rat liver as well as in human liver peroxisomes [20], we have reexamined this question using purified D-aminoacid oxidase from pig kidney. This was done with different concentrations of D-aminoacid oxidase of porcine kidney. The results of Fig. 1A show that H_2O_2 is, indeed, produced when D-aminoacid oxidase is allowed to act on L-pipecolate (Fig. 1A). However, the results also show that the increase in fluorescence comes to a stop after a short period of time, which is especially clear when high concentrations of D-aminoacid oxidase are added. This is not due to inactivation of D-aminoacid oxidase since subsequent addition of D-alanine elicits a steep increase in fluorescence (Fig. 1A). A similar increase in fluorescence was observed when D-pipecolic acid was added instead of D-alanine (not shown). These results suggest that D-aminoacid oxidase is not reactive with L-pipecolic acid and that the low activity seen with L-pipecolic acid is due to the reaction of D-aminoacid oxidase with D-pipecolic acid which is a minor contaminant in commercially available L-pipecolic acid preparations.

In the experiment of Fig. 1B we studied the oxidation of L-pipecolate in a human liver homogenate. Based on the results of Fig. 1A we first eliminated all D-pipecolic acid contaminating L-pipecolic acid preparations, by first adding D-aminoacid oxidase to the reaction medium. As in Fig. 1A we observed a rapid rise in fluorescence. When no further increase in fluorescence was observed, we added an aliquot of human liver homogenate which gave rise to a rapid rise in fluorescence (Fig. 1B). When rat liver homogenates were used, no H_2O_2 production occurred. These findings suggest that in human liver L-pipecolic acid is oxidized via a specific enzyme different from D-aminoacid oxidase.

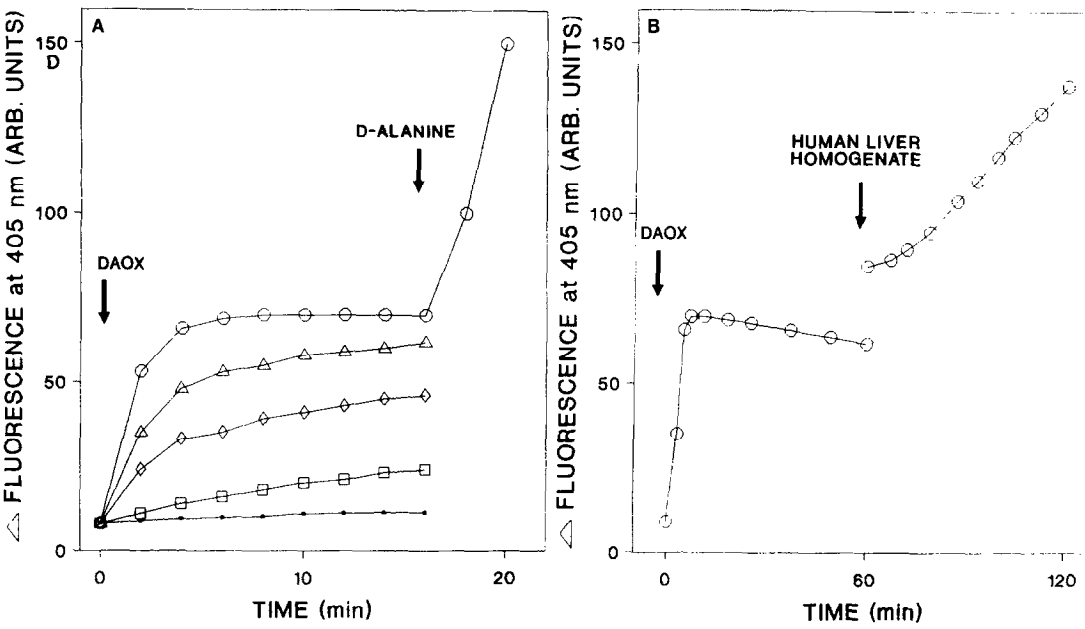

Fig. 1.
L-Pipecolate and its oxidation by D-aminoacid oxidase (A) and human liver homogenate (B). In the experiment of Fig. 1A different concentrations of D-aminoacid oxidase (0 (■), 35 (□), 70 (◇), 140 (△) and 280 (○) mU/ml, respectively) were added to the standard reaction medium containing 15 mM L-pipecolate and the increase in fluorescence measured as described [15]. In the experiment of Fig. 1B reactions were started by adding D-aminoacid oxidase (280 mU/ml) to the standard reaction medium followed by addition of an aliquot of human liver homogenate (120 µg protein/ml).

The next set of experiments was designed to determine the subcellular site of L-pipecolate oxidase. For this purpose we first prepared crude subcellular fractions (mitochondrial (M), light-mitochondrial (ML) and microsomal (Mi)) by means of differential centrifugation. In the fractions obtained the activity of different marker-enzymes was measured as well as the activity of L-pipecolate oxidase. The results depicted in Table 1 show that the relative specific activity of L-pipecolate oxidase was highest in the mitochondrial and light mitochondrial fractions and that about 80% of the total activity was present in these fractions. These results indicate that L-pipecolate oxidase is particle-bound in human liver. In order to identify the subcellular site of L-pipecolate oxidase more definitively, we subjected a light-mitochondrial fraction to density gradient centrifugation on Nycodenz. The results of Fig. 2 show that the activity of L-pipecolate oxidase closely paralleled the activity profile of D-aminoacid oxidase, a peroxisomal marker enzyme, and not that of glutamate dehydrogenase, a mitochondrial marker enzyme.

In conclusion the results described in this paper identify L-pipecolate oxidase as a distinct enzyme localized in peroxisomes in man. Interestingly,

TABLE 1: DISTRIBUTION OF L-PIPECOLIC OXIDASE IN DIFFERENT SUBCELLULAR FRACTIONS OF HUMAN LIVER

Activity measured	Relative specific activity measured in: M	ML	Mi	S
L-pipecolate oxidase	2.3(54)	2.5(27)	0.5(3)	0.25(16)
D-aminoacid oxidase	2.1(42)	4.8(44)	0.1(1)	0.25(14)
Glutamate dehydrogenase	1.45(50)	1.0(15)	0.1(1)	0.35(33)
Esterase	0.70(18)	1.8(21)	5.7(40)	0.30(21)
β-hexosaminidase	1.2(21)	6.7(54)	0.05(1)	0.50(24)
Lactate dehydrogenase	0.35(9)	0.1(1)	0.20(1)	1.2(89)

Fractions were prepared as described in the text and assayed for the various enzyme activities indicated. Results are presented from one experiment. Similar results were obtained in another experiment. Results are expressed as relative specific activity, the numbers within parentheses representing the percent of total activity in each fraction. Abbreviations: M = heavy mitochondrial fraction; ML = light mitochondrial fraction; Mi = microsomal fraction; S = soluble fraction.

Mihalik and Rhead [21] recently found that conversion of L-[^{3}H] pipecolic acid to L-α-[^{3}H] aminoadipic acid occurs in peroxisomes in cynomolgus monkey but in mitochondria in the rabbit. The results suggest that the first step

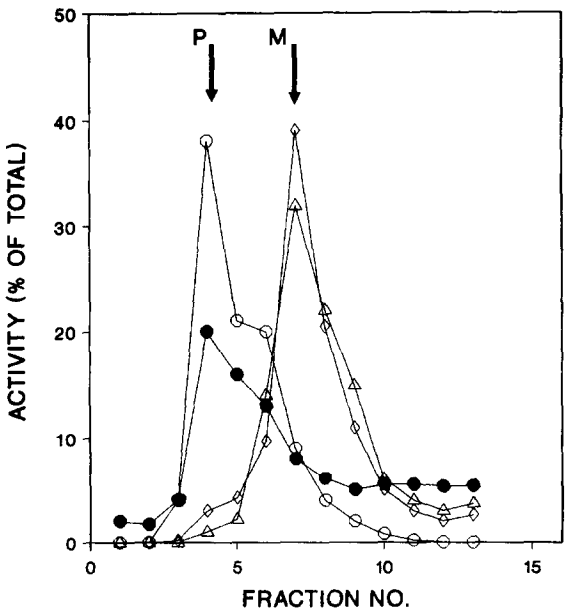

Fig. 2.
Activity of L-pipecolate oxidase in fractions prepared from fresh human liver via density gradient centrifugation on Nycodenz. A light mitochondrial fraction was prepared from human liver and subjected to density gradient centrifugation as described in Materials and Methods. The subsequent fractions were assayed for D-aminoacid oxidase (○), glutamate dehydrogenase (△), esterase (◇) and L-pipecolate oxidase (●) as described in Materials and Methods.

in the conversion of L-pipecolate to L-α-animoadipate in the cynomolgus monkey is catalyzed by a peroxisomal oxidase as in man (this paper), but by a mitochondrial dehydrogenase in the rabbit.

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