D-Aspartate oxidase in rat, bovine and sheep kidney cortex is localized in peroxisomes

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D-Aspartate oxidase (EC 1.4.3.1) was assayed in subcellular fractions and in highly purified peroxisomes from rat, bovine and sheep kidney cortex as well as from rat liver. During all steps of subcellular-fractionation procedures, D-aspartate oxidase co-fractionated with peroxisomal marker enzymes. In highly purified preparations of peroxisomes, the enrichment of D-aspartate oxidase activity over the homogenate is about 32-fold, being comparable with that of the peroxisomal marker enzymes catalase and D-amino acid oxidase. Disruption of the peroxisomes by freezing and thawing released more than 90 % of the enzyme activity, which is typical for soluble peroxisomal-matrix proteins. Our findings provide strong evidence that in these tissues D-aspartate oxidase is a peroxisomal-matrix protein and should be added as an additional flavoprotein oxidase to the known set of peroxisomal oxidases.

INTRODUCTION

D-Aspartate oxidase activity (EC 1.4.3.1) was first described in rabbit kidney extracts by Still *et al.* [1,2]. Since then, appreciable amounts of this enzyme have also been found in liver, nervous tissue, intestine, lung and thyroid gland of various mammalian species (for a review see [3]). D-Aspartate oxidase is a flavoprotein of M_r 39 000, containing one flavin moiety per subunit [4]. The enzyme catalyses the oxidative deamination of dicarboxylic D-amino acids, transferring electrons to oxygen or other terminal electron acceptors [4,5]. D-Aspartate oxidase is similar to D-amino acid oxidase, but the two enzymes can be clearly distinguished by their differences in substrate specificity and in their responses to selective inhibition. Moreover, they exhibit marked differences in phylogenetic distribution, and can be separated by differential purification (for review see [3,4]).

To our knowledge, no definitive information exists about the subcellular distribution of this enzyme. Because of some similarities with peroxisomal D-amino acid oxidase, it has been suggested that it is present in peroxisomes [3,6]. Indeed, the kidney cortex, which exhibits the highest D-aspartate oxidase activity in mammals [7,8], is especially rich in peroxisomes, which are large and abundant in the epithelial cells lining the proximal tubules (for review see [9]). Recent progress in the purification of renal peroxisomes from rodents [10–12] as well as from the renal cortex of several other mammalian species [13] prompted us to investigate the exact subcellular localization of D-aspartate oxidase.

Highly purified peroxisome preparations (98 %) were obtained from rat, bovine and sheep kidney cortex as well as from rat liver and were assayed for the activity of D-aspartate oxidase.

MATERIALS AND METHODS

Preparation of purified peroxisome fractions from rat kidney cortex

Female Sprague–Dawley rats (200–250 g) were fasted overnight and kidneys were excised under diethyl ether anaesthesia. The kidneys were decapsulated and cut in half along their longitudinal axis. The medullary region was discarded, and the renal cortices were chopped with a hand-driven commercially available grinder with rotating blades (Moulinex, Köln, Germany). The resulting pulp was mixed with ice-cold homogenization buffer (10 ml/g of tissue), containing 5 mM-Mops, pH 7.4, 250 mM-sucrose, 1 mM-EDTA and 0.1 % ethanol, and filtered through two stainless steel sieves (4- and 10-mesh) by stirring with a rubber spatula. Unbroken cells, cell debris and nuclei were pelleted by centrifugation for 5 min at 200 g, and the supernatant was taken as the homogenate A [13,14]. Differential centrifugation of A yields four main fractions: a heavy-mitochondrial fraction B (700 g, 10 min), a light-mitochondrial fraction D (25500 g, 20 min), a microsomal fraction G (170000 g, 60 min) and a final supernatant F. Fraction D exhibits the highest specific activities of peroxisomal marker enzymes. This fraction was considered a crude peroxisomal preparation and was further subfractionated by density-dependent banding on a linear Metrizamide gradient by using a vertical rotor as described previously [13,14].

Peroxisome preparations from rat liver and bovine and sheep kidney cortex

These were obtained as described recently [13,14]. Isolated peroxisome preparations were partially

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disrupted [15] by two cycles of freezing at -18°C and thawing at room temperature. Centrifugation (25000 g , 20 min) yields the readily soluble matrix enzymes in the supernatant and peroxisomal ghosts in the pellet (see also [15]).

Electron microscopy

Gradient fractions were fixed for 20 min by mixing with 7.5% (v/v) glutaraldehyde in 48% (w/v) Metrizamide buffered with 5 mM-Mops, pH 7.4. Further processing was by the method of Baudhuin *et al.* [16] as modified by Völkl & Fahimi [14].

Determination of enzyme activities

Catalase was determined as described by Baudhuin *et al.* [17], esterase as described by Beaufay *et al.* [18], cytochrome *c* oxidase as described by Cooperstein & Lazarow [19], and β -glucuronidase as described recently [14]. D-Amino acid oxidase and D-aspartate oxidase activities were determined by measuring H_2O_2 production by the method of Allain *et al.* [20]. The assays were performed in 50 mM-phosphate buffer, pH 8.2 (25°C), with 0.1% Triton X-100 and 10 or 20 mM substrate concentration. D-Proline (10 mM) and D-aspartate (20 mM) of the highest degree of purity available (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used as standard substrates for D-amino acid oxidase and D-aspartate oxidase respectively. Sodium benzoate is a potent inhibitor of D-amino acid oxidase activity, and was included routinely (10 mM concn.) in D-aspartate oxidase assays. This inhibitory effect was confirmed in preliminary experiments, with peroxisome fractions from rat kidney, rat liver, sheep and bovine kidney cortex as enzyme sources. An average inhibition of 96% was found with 10 mM-sodium benzoate for D-amino acid oxidase activity, but D-aspartate oxidase was not inhibited. On the contrary, *meso*-tartrate which is a potent inhibitor of D-aspartate oxidase activity was included (20 mM) in the assays for D-amino acid oxidase activity. The average inhibition for D-aspartate oxidase was 94%, whereas D-amino acid oxidase activity is slightly (6%) stimulated by *meso*-tartrate. Protein was determined as described by Lowry *et al.* [21]. One munit of enzyme activity corresponds to 1 nmol of H_2O_2 formed/min. Enzyme distribution along the gradients is presented in histograms in accordance with Beaufay *et al.* [22].

RESULTS AND DISCUSSION

Fig. 1 shows the distributions of D-aspartate oxidase activity as well as of the peroxisomal marker enzymes catalase and D-amino acid oxidase in the four main fractions obtained by differential centrifugation of rat and bovine kidney-cortex homogenates. Activities for each enzyme are greatest in fraction D. A purified peroxisome preparation was obtained by subfractionation of fraction D from rat kidney cortex by density-gradient centrifugation in Metrizamide [13,14]. The distribution of organelle marker enzymes in a typical experiment is shown in Fig. 2. Most of the catalase activity which represents peroxisomes is concentrated in two bottom fractions of the gradient at a mean equilibrium density of 1.23 g/cm^3 . These fractions were mixed and taken as purified peroxisome preparation. Cytochrome *c* oxidase, the marker for mitochondria,

Fig. 1. Distribution patterns of peroxisomal marker enzymes and of D-aspartate oxidase in the initial fractions B, D, F and G obtained by differential centrifugation of bovine and rat kidney-cortex homogenates

Each fraction is represented by the relative specific activity of the enzyme (percentage of total recovered activity/percentage of total recovered protein) and by the protein content, expressed as a percentage of the total recovered protein. The recoveries of peroxisomal markers and of D-aspartate oxidase are greatest in the light-mitochondrial fraction D (hatched column) (rat: catalase 45.4%, D-amino acid oxidase 35.1%, D-aspartate oxidase 43.5%. Bovine: catalase 43.7%, D-amino acid oxidase 47.7%, D-aspartate oxidase 52.5%). The distribution of other marker enzymes of cell organelles is as given in refs. [13,14,23].

mostly bands at a density range of about 1.15 g/cm^3 , corresponding to the bulk of protein, and appears well separated from catalase. β -Glucuronidase and esterase, the marker enzymes for lysosomes and microsomes respectively, remain in the upper part of the gradient. The distribution pattern of D-aspartate oxidase activity in the gradient, however, clearly coincides with that of the peroxisomal marker catalase (Fig. 2). Lesser amounts of both enzymes are found also in the uppermost part of the gradient, but no significant activity is detected at densities where other organelle marker enzymes band. The main biochemical properties of peroxisome preparations isolated from rat kidney cortex are listed in Table 1. As calculated from the specific activities of catalase and D-amino acid oxidase, peroxisomes are purified 36.6- and 29.2-fold respectively over the homogenate. In comparison, D-aspartate oxidase in the peroxisome preparation is enriched 32.5-fold. Fig. 3

Fig. 2. Typical volume distributions of protein and marker-enzyme activities after density-gradient centrifugation of rat kidney-cortex fraction D in Metrizamide

The enzyme (protein) distribution is shown as relative concentration in the different gradient fractions, which are given as percentages of the total gradient volume (relative concentration = concn. in a single gradient fraction/average concn. in all gradient fractions). The gradient was eluted in 2 ml portions from the bottom of the tube. The peroxisomal marker enzyme catalase as well as D-aspartate oxidase are concentrated in two bottom fractions (4 ml) at a mean equilibrium density of 1.23 g/cm³. Recovery rates (yields) of enzymes and protein are summarized in Table 1.

Table 1. Composition and enzyme activities of purified peroxisome fractions from rat kidney cortex

Values are means $\pm$ S.D. of *n* experiments. Yield = recovery (%) of the homogenate value. RSA = relative specific activity (percentage recovered enzyme activity/percentage recovered protein; homogenate = 100%).

	Homogenate (munits/mg of protein)	Peroxisome fraction		
		(munits/mg of protein)	Yield (%)	RSA
Catalase (<i>n</i> = 7)	31 $\pm$ 7.8	1143 $\pm$ 256	4.4 $\pm$ 1.2	36.6
D-Amino acid oxidase (<i>n</i> = 6)	57 $\pm$ 12	1668 $\pm$ 754	3.5 $\pm$ 1.3	29.2
D-Aspartate oxidase (<i>n</i> = 11)	1.42 $\pm$ 0.7	47.6 $\pm$ 19.1	3.9 $\pm$ 1.3	32.5
Cytochrome <i>c</i> oxidase (<i>n</i> = 6)	31.7 $\pm$ 18.7	4.2 $\pm$ 3.7	0.016 $\pm$ 0.008	0.13
β-Glucuronidase (<i>n</i> = 4)	8.5 $\pm$ 3.6	3.6 $\pm$ 2.4	0.05 $\pm$ 0.03	0.41
Esterase (<i>n</i> = 5)	233 $\pm$ 95	63 $\pm$ 34	0.03 $\pm$ 0.01	0.25
Protein (%) (<i>n</i> = 11)	100		0.12 $\pm$ 0.04	

shows an electron micrograph of a corresponding peroxisome preparation prepared by the filtration technique [14]. Virtually more than 98% of the isolated organelles are peroxisomes. This perfectly matches the biochemical finding of a highly purified peroxisome fraction.

To verify further the distribution of D-aspartate oxidase activity in other tissues, peroxisomes were isolated from rat liver as well as from bovine and sheep kidney cortex as described recently [13,14]. In all preparations D-aspartate oxidase was recovered in the peroxisome fractions. Table 2 summarizes the specific activities of

Fig. 3. A survey electron micrograph of a peroxisome preparation from rat kidney cortex

The organelle fraction obtained as described in the text was filtered on to a Millipore filter, and the pellicle was cut perpendicular to the filter surface. The photograph shows the whole depth of a pellicle. Magnification $\times 11500$; MP = Millipore filter.

Table 2. Specific activity of D-aspartate oxidase in peroxisome fractions obtained from different species

Values are means $\pm$ S.D. of n experiments.

	D-Aspartate oxidase (munits/mg of protein)
Rat kidney cortex ($n = 11$)	47.6 ± 19.1
Rat liver ($n = 4$)	6.3 ± 1.6
Bovine kidney cortex ($n = 7$)	174.3 ± 32
Sheep kidney cortex ($n = 4$)	121 ± 34

Table 3. Representative distributions of protein and D-aspartate oxidase in peroxisomal subfractions obtained by centrifugation of partially disrupted peroxisomes

Peroxisome preparations were disrupted by freezing and thawing, and then centrifuged (see the Materials and methods section). Protein and D-aspartate oxidase (D-ASPO) activity (m units/mg) were measured in the supernatant and pellet and expressed as a percentage of the values for the initial peroxisome preparation. Four preparations were studied from each species.

	Rat		Bovine		Sheep	
	D-ASPO (%)	Protein (%)	D-ASPO (%)	Protein (%)	D-ASPO (%)	Protein (%)
Peroxisome fraction	100	100	100	100	100	100
25000 g -20 min supernatant	94.4	61.3	96.4	58.9	81	57.2
25000 g -20 min pellet	5.6	38.7	3.6	41.1	11	42.8

D-aspartate oxidase in highly purified peroxisome preparations from these sources. There are considerable differences noted with respect to both the different species as well as the organs. By far the lowest specific activity is observed in rat liver peroxisomes, whereas the highest specific activity occurred in bovine kidney-cortex peroxisome preparations. This is in good agreement with previous biochemical studies [3,24,25]. In most attempts so far to isolate D-aspartate oxidase, bovine kidney has been used as a source, probably because of the high activity of the enzyme in this tissue [3,24,25]. Recently Negri *et al.* [4] reported the purification of D-aspartate oxidase to homogeneity from bovine kidney cortex.

To learn more about the intraperoxisomal localization of D-aspartate oxidase activity, e.g. if this enzyme is a soluble matrix protein or is associated as a peripheral or integral membrane protein with peroxisomal membranes, the organelles were partially disrupted by freezing and thawing. Matrix proteins easily leak out of peroxisomes, and can be recovered in the supernatant fraction after differential centrifugation [15]. Table 3 shows the results of a typical experiment. Some 50–60 % of total peroxisomal protein is solubilized by this procedure and is found in the supernatant. Since more than 90 % of D-aspartate oxidase activity was recovered in the supernatant, we can assume that the enzyme is a peroxisomal-matrix protein. Leakage from damaged peroxisomes probably explains the small amounts of D-aspartate oxidase activity remaining on top of Metrizamide gradients, a phenomenon well known for other peroxisomal-matrix enzymes such as catalase, thiolase or D-amino acid oxidase (see e.g. [15,26]).

The supernatants of peroxisomal preparations after freezing and thawing and centrifugation were used to study the apparent activity of D-aspartate oxidase with various substrates (Table 4). Under the conditions used the dicarboxylic amino acid D-aspartic acid and its *N*-methyl derivative are the only amino acids that are oxidized to a significant extent; indeed bovine and sheep peroxisomal D-aspartate oxidase exhibits higher specific activity with *N*-methyl-D-aspartate than with D-aspartate.

In recent years convincing evidence has accumulated that dicarboxylic amino acids such as aspartate and glutamate are major excitatory neurotransmitters in the brain (for reviews see, e.g., [27–29]). Whereas the L-isomers have been considered to be the endogenous ligands for the receptor and transport systems, the rarely occurring D-isomers exhibit also a marked excitatory

Table 4. Substrate specificity of D-aspartate oxidase from the kidney cortex of different species

The peroxisomal matrix fraction obtained by freezing and thawing is used as enzyme source. Different dicarboxylic D-amino acids with increasing carbon-chain length as well as the *N*-methyl derivative of D-aspartic acid were assayed at 20 mM concentration. Abbreviation: n.m., activity not measurable.

Substrate	D-Aspartate oxidase activity (munits/mg of protein)		
	Rat	Bovine	Sheep
D-Aspartate	43.3	186	116
<i>N</i> -Methyl-D-aspartate	20.2	215	131
D-Glutamate	n.m.	7	3
D-Aminopimelate	n.m.	n.m.	n.m.

effect [28]. D-Aspartate is readily transported in brain tissue and has been successfully used to study the high-affinity binding and transport system(s) of excitatory amino acids in the brain (e.g. [28,30,31]). *N*-Methyl-D-aspartate has proved to be a very potent agonist of dicarboxylic amino acid neurotransmitter receptors in brain. This had led to the postulation of a particular *N*-methyl-D-aspartate receptor subtype. This receptor is now one of the best pharmacologically characterized receptors in a group of three or four excitatory amino acid-receptor subtypes [27–29,32,33]. The endogenous ligands for *N*-methyl-D-aspartate receptor are supposed to be L-aspartate and L-glutamate [29].

Speculating about the function of peroxisomal D-aspartate oxidase, two alternative possibilities can be taken into consideration. D-Aspartate oxidase may have a protective function by degrading extrinsic D-isomers of dicarboxylic amino acids, which otherwise might exert metabolic disturbances, particularly as foreign neurotransmitter receptor ligands causing severe disruptions in the central nervous system. On the other hand, if such D-isomers are intrinsic metabolites, D-aspartate oxidase must play a significant role in regulating their turnover and metabolism.

The absence of peroxisomes in liver and kidney or the impairments of one or several peroxisomal enzymes are the characteristic features of a newly recognized group of genetic diseases, the peroxisomal disorders [34,35]. Most of these syndromes show a severe central-nervous-system involvement. The deficiency of peroxisomal D-aspartate oxidase could be an additional factor explaining the central-nervous-system affliction in these disorders.

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REFERENCES

- Still, J. L., Buell, M. V., Knox, W. E. & Green, D. E. (1949) *J. Biol. Chem.* **179**, 831–837
- Still, J. L. & Sperling, E. (1950) *J. Biol. Chem.* **182**, 585–589
- Hamilton, G. A. (1985) *Adv. Enzymol.* **57**, 85–178
- Negri, A., Massey, V. & Williams, C. H., Jr. (1987) *J. Biol. Chem.* **262**, 10026–10034
- Dixon, M. & Kenworthy, P. (1967) *Biochim. Biophys. Acta* **146**, 54–76
- Hruban, Z. & Rechcigl, M. (1969) *Int. Review Cytol. Suppl.* **1**, 12–13
- DeMarco, C., Rinaldi, A. & Crifo, C. (1969) *Physiol. Chem. Phys.* **1**, 473–478
- Davies, L. P. & Johnston, G. A. R. (1975) *J. Neurochem.* **25**, 299–304
- Ohno, S. (1985) *Int. Rev. Cytol.* **95**, 131–162
- Mackness, M. I. & Connock, M. J. (1985) *Comp. Biochem. Physiol.* **80B**, 161–164
- Hajra, A. K. & Wu, D. (1985) *Anal. Biochem.* **148**, 233–244
- Patel, N., Mackness, M. I. & Connock, M. J. (1987) *Biochem. J.* **244**, 443–448
- Zaar, K., Völkl, A. & Fahimi, H. D. (1986) *Eur. J. Cell Biol.* **40**, 16–24
- Völkl, A. & Fahimi, H. D. (1985) *Eur. J. Biochem.* **149**, 257–265
- Alexson, S. E. H., Fujiki, Y., Shio, H. & Lazarow, P. B. (1985) *J. Cell Biol.* **101**, 294–304
- Baudhuin, P., Evrard, P. & Berthet, J. (1967) *J. Cell Biol.* **32**, 181–191
- Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. & de Duve, C. (1964) *Biochem. J.* **92**, 179–184
- Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 188–200
- Cooperstein, S. J. & Lazarow, P. B. (1951) *J. Biol. Chem.* **189**, 665–670
- Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. & Fu, P. C. (1974) *Clin. Chem.* **20**, 470–473
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964) *Biochem. J.* **92**, 184–205
- Zaar, K., Völkl, A. & Fahimi, H. D. (1987) *Biochim. Biophys. Acta* **897**, 135–142
- Rinaldi, A., Pellegrini, M., Crifo, C. & DeMarco, C. (1981) *Eur. J. Biochem.* **117**, 635–638
- Nasu, S., Wicks, F. D. & Gholson, R. K. (1982) *Biochim. Biophys. Acta* **704**, 240–252
- Baudhuin, P. (1969) *Ann. N. Y. Acad. Sci.* **168**, 214–228
- Watkins, J. G. & Evans, R. H. (1981) *Annu. Rev. Pharmacol. Toxicol.* **21**, 165–204
- Foster, A. C. & Fagg, G. E. (1984) *Brain Res. Rev.* **7**, 103–164
- Robinson, M. B. & Coyle, J. T. (1987) *FASEB J.* **1**, 446–455
- Balcar, V. J. & Johnston, G. A. R. (1972) *J. Neurochem.* **19**, 2657–2666

31. Parsons, B. & Rainbow, T. C. (1983) *Neurosci. Lett.* **36**, 9–12
32. McLennan, H. (1982) *Prog. Neurobiol.* **20**, 251–271
33. Dingledine, R. (1986) *Trends Neurochem. Sci.* **9**, 47–49
34. Kelley, R. J. (1983) *Am. J. Med. Genet.* **16**, 503–517
35. Schutgens, R. B. H., Heymans, H. S. A., Wanders, R. J. A., van den Bosch, H. & Tager, J. M. (1986) *Eur. J. Pediatr.* **144**, 430–440

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