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Purification of mouse brain ornithine decarboxylase reveals its presence as an inactive complex with antizyme

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Mouse brain ornithine decarboxylase (ODC) was purified to near-homogeneity by using $(NH_4)_2SO_4$ precipitation and chromatography on heparin–Sepharose, pyridoxamine phosphate–agarose and DEAE-cellulose. On SDS/polyacrylamide-gel electrophoresis, the final preparation gave one protein band similar to that obtained for purified mouse kidney enzyme, corresponding to an M_r of 53000. The overall yield of the purification exceeded about 50-fold the total activity of the enzyme in the starting material. By affinity chromatography on ODC-bound Sepharose, the extra enzyme activity was shown to originate, at least partly, from the enzyme–antizyme complex. These results demonstrate that ODC in mouse brain occurs mainly in an inactive form and is activated during purification.

INTRODUCTION

ODC is the first and rate-limiting enzyme in polyamine biosynthesis in eukaryotes (Tabor & Tabor, 1976; Jänne *et al.*, 1978; Heby, 1981; Pegg & McCann, 1982). It has been purified from a variety of sources, including rat liver (Pegg & McGill, 1979; Kameji *et al.*, 1982; Kitani & Fujisawa, 1983), rat heart (Flamigni *et al.*, 1984) and mouse kidney (Seely *et al.*, 1982; Isomaa *et al.*, 1983). Compared with these tissues, ODC activity is very low in adult brain (Laitinen *et al.*, 1982; Hietala, 1983; Hietala *et al.*, 1983). Our more-recent results, however, show that mouse brain contains a high amount of immunoreactive enzyme protein (Laitinen *et al.*, 1985). This can be explained by the fact that brain cytosol fraction is rich in ODC–antizyme complex (Hietala, 1983), which also displays antigenicity (Seely & Pegg, 1983; Laitinen *et al.*, 1985). The antizyme is a polypeptide and inhibits the enzyme by non-covalent binding (Canellakis *et al.*, 1979). The enzyme–antizyme complex can be dissociated by treatment with high salt concentrations, and at least part of the enzyme is released as catalytically active (Clark & Fuller, 1976; Heller *et al.*, 1976; McCann *et al.*, 1977, 1979; Hietala, 1983).

In the present paper we show that the purification yield of the mouse brain ODC is about 50-fold as compared with the total activity of the enzyme in the starting material, and that at least part of the extra enzyme activity originates from the enzyme–antizyme complex.

METHODS

The assay conditions for ODC activity were essentially as described by Jänne & Williams-Ashman (1971). Immunoreactive enzyme protein was determined by the method of Isomaa *et al.* (1983). ODC–antizyme activity was measured as described previously (Hietala, 1983). Polyacrylamide-gel electrophoresis under denaturing conditions was conducted essentially by the method of Laemmli (1970) and the electrophoretic transfer by that of Towbin *et al.* (1979). Immunoblot detection was carried out with rabbit antiserum against purified renal ODC as described by De Blas & Cherwinski (1983). Protein measurements were performed by the method of Bradford (1976), with bovine serum albumin as the standard.

Purification of mouse brain ODC was performed as described for the renal enzyme (Isomaa *et al.*, 1983). Brains (usually 50 g of tissue) of NMRI mice were homogenized with 3 vol. of TED buffer (25 mM-Tris/HCl, 0.01 mM-EDTA, 5 mM-dithiothreitol, pH 7.4 at 20 °C), and the homogenate was centrifuged at 105000 *g* for 60 min at 4 °C. After $(NH_4)_2SO_4$ precipitation (30–50% satn.), the pellet was dissolved in TED buffer containing 0.02% Brij 35 (TEDB) and dialysed against this buffer for 4 h at 4 °C. The sample was adsorbed to heparin–Sepharose (1.6 cm × 22 cm column) with a flow rate of 25 ml/h. The matrix was washed with 5 vol. of TEDB buffer with a flow rate of 10 ml/h and eluted with a linear (0–0.4 M) KCl gradient in TEDB buffer; 5 ml fractions were collected. Peak enzyme fractions were pooled and dialysed against TEDB buffer containing 0.1 mM-ornithine. After dialysis the sample was applied to a pyridoxamine 5′-phosphate–agarose column (Isomaa *et al.*, 1983) equilibrated with TEDB buffer. The matrix was washed with 10 vol. of TEDB buffer containing 15 mM-KCl and eluted with a linear pyridoxal 5′-phosphate gradient (0–70 μM) in TEDB buffer; 2.5 ml fractions were collected. Peak enzyme fractions were pooled and applied to a DEAE-cellulose (DE 52) column (0.9 cm × 2 cm) equilibrated with TEDB containing 20 mM-KCl. The matrix was washed with 10 vol. of the equilibration buffer and eluted with a linear KCl gradient (0.02–0.3 M) in TEDB buffer; 1 ml fractions were collected.

ODC-bound Sepharose was prepared by linking the enzyme to Sepharose via antibody against the enzyme (Kitani & Fujisawa, 1984). Rabbit antiserum against ODC was obtained as described previously (Isomaa *et al.*, 1983).

Abbreviation used: ODC, ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17).

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Table 1. Purification of ODC from mouse brain

The data refer to 49.5 g wet wt. of brain (approx. 100 mice) as starting material. See the Methods section for experimental details.

	Total protein (μg)	Total activity (μmol of CO_2/h)	Specific activity (μmol of CO_2/h per mg of protein)	Purification (fold)	Yield (%)
Cytosol	782000	14.6×10^{-3}	18.7×10^{-6}	1	100
Dialysed $(NH_4)_2SO_4$ precipitate	217000	12.1×10^{-3}	55.8×10^{-6}	3	83
Heparin–Sepharose eluate	17000	53.4×10^{-3}	3.14×10^{-3}	168	366
Pyridoxamine phosphate–agarose eluate	100	732×10^{-3}	7.32	3.9×10^{5}	5014
DEAE-cellulose eluate	9	735×10^{-3}	81.7	4.4×10^{6}	5034

RESULTS AND DISCUSSION

ODC was purified from mouse brain with a yield that exceeded about 50 times the total activity of the enzyme in the starting material (Table 1). On SDS/polyacrylamide-gel electrophoresis the final preparation gave one protein band of M_r 53000, a value similar to that obtained for purified mouse kidney enzyme (Fig. 1). The specific activity of purified brain enzyme was of the same order of magnitude as that of the purified rat heart enzyme (Flamigni *et al.*, 1984), but less than those obtained for rat liver (Pegg & McGill, 1979; Kameji *et al.*, 1982; Kitani & Fujisawa, 1983) and mouse kidney enzymes (Seely *et al.*, 1982; Isomaa *et al.*, 1983). However, the facts that the protein content of the final preparation was only slightly higher than the amount of immunoreactive enzyme protein (9 rather than 7 μg) and it gave only one band on SDS-polyacrylamide-gel electrophoresis suggest the preparation to be nearly homogeneous. The reason for the lower specific activity of the final preparation than that obtained for mouse kidney enzyme is unknown.

The dramatic increase in the total activity of the enzyme occurred in the affinity-chromatography steps. This can be explained by the dissociation of enzyme–antizyme complex during the $(NH_4)_2SO_4$ precipitation and the salt-gradient elution of the heparin–Sepharose column, and, since the antizyme is very labile (Heller & Canellakis, 1981), all the antizyme dissociated is not able to re-form complex with the enzyme. To ascertain that the extra enzyme activity originates from the complex, we passed the brain cytosol sample over a column of ODC-bound Sepharose, which binds the antizyme (Kitani & Fujisawa, 1984). Table 2 shows that the total activity in the brain sample increased about 50-fold, supporting the purification result. On the other hand, the enzyme activity of mouse kidney cytosol did not change when passed over an identical column (Table 2). This can be explained by the finding that there is no antizyme-related control in the mouse kidney (Persson & Rosengren, 1979). We could not demonstrate any antizyme activity in the kidney cytosol sample (Table 2). When the brain antizyme was eluted with buffered 250 mM-NaCl (Kitani & Fujisawa, 1984), the inhibitor recovery was about one-eighth of the enzyme activity released, supporting the above-mentioned possibility that the antizyme partly loses its inactivating capability during the dissociation process.

Brain extract, and also the dialysed preparation after $(NH_4)_2SO_4$ precipitation, gave on immunoblotting one

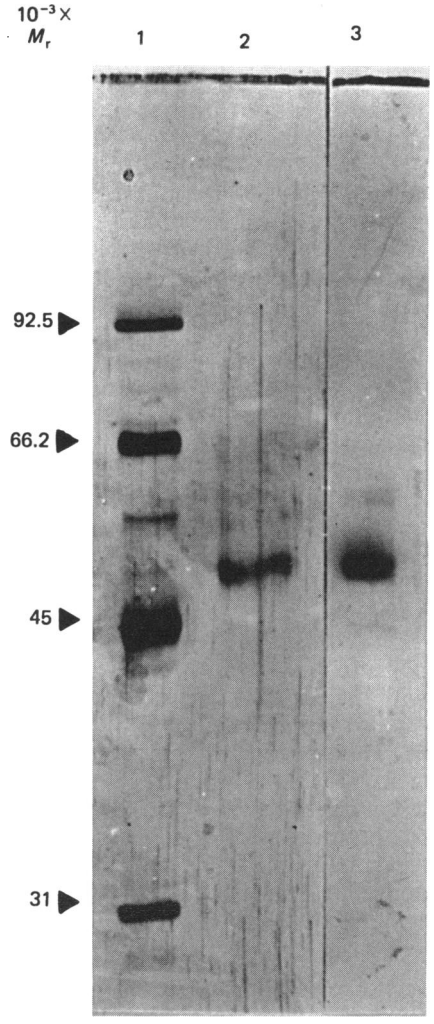

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified mouse kidney (lane 2) and brain (lane 3) ODC (100 ng)

The proteins were precipitated with 12% trichloroacetic acid, washed three times with acetone and dissolved in 0.125 M-Tris/HCl (pH 6.8)/4% SDS/20% glycerol/2.5% dithiothreitol by heating in a boiling-water bath for 90 s. The samples were then subjected to electrophoresis in 10%-acrylamide gel at 30 mA for about 4 h before fixing and staining with silver stain. Standard protein markers (lane 1) were phosphorylase *b* (M_r 92500), bovine serum albumin (66200), ovalbumin (45000) and carbonic anhydrase (31000).

Table 2. Chromatography of cytosol fractions on ODC-bound Sepharose column

The fresh brains from 10 NMRI mice were homogenized in 2 vol. of a solution consisting of 10 mM-sodium phosphate buffer, pH 7.0 (at 20 °C), 5 mM-dithiothreitol, 0.1 mM-pyridoxal phosphate, 1 mM-EDTA and 0.01% Tween 20. The homogenate was centrifuged at 105000 *g* for 1 h and the resulting supernatant applied to a column of ODC-bound Sepharose. The column was washed with the homogenization buffer containing 100 mM-NaCl and eluted with the same buffer containing 250 mM-NaCl. As the control, the extract from 10 kidneys of male NMRI mice was treated identically. Abbreviation: ND, not detectable.

	Brain		Kidney	
	ODC activity (nmol of CO_2/30 min)	Antizyme activity (nmol of CO_2/30 min)	ODC activity (nmol of CO_2/30 min)	Antizyme activity (nmol of CO_2/30 min)
Cytosol	67	ND	214	ND
Cytosol passed over a column	3566	ND	207	ND
Eluate	ND	418	ND	ND

major band, corresponding to a M_r of about 36000 (Fig. 2), which is the main degradation product of the enzyme in brain (Pulkka *et al.*, 1985). This band almost disappeared in the affinity chromatography on heparin–Sepharose and pyridoxamine-phosphate–agarose, whereas the bands of the purified enzyme appeared in the immunoblot (M_r 53000 and 51000). An ODC band of lower M_r is presumably due to proteolytic degradation, which, however, does not affect the enzyme activity (Persson *et al.*, 1984). The peak fractions from the DEAE-cellulose column gave only these two bands. Thus the affinity-chromatography steps were the key steps in the purification.

It is generally accepted that the regulation of ODC activity occurs via modulation of the amount of the enzyme protein (Tabor & Tabor, 1976; Jänne *et al.*, 1978; Heby, 1981; Pegg & McCann, 1982). The physiological significance of the enzyme–antizyme complex for the regulation is still unknown. These results demonstrate that the brain extract contains, in contrast with the other tissue extracts studied, a high amount of the enzyme, which can be released from the enzyme–inhibitor complex as catalytically active. Thus the complex-formation in the brain tissue does not lead immediately to the irreversible inactivation of the enzyme, or the enzyme is exposed during tissue homogenization to the inhibitor, and only complex formed *in vitro* releases catalytically active enzyme when dissociated. If the former assumption holds true, the brain tissue contains a large enzyme pool in the complex form, which could serve as a physiological storage form of the enzyme in brain. On the other hand, if the inhibition by the antizyme takes place during the tissue homogenization, so the real enzyme activity *in vivo* is much higher than was formerly assumed.

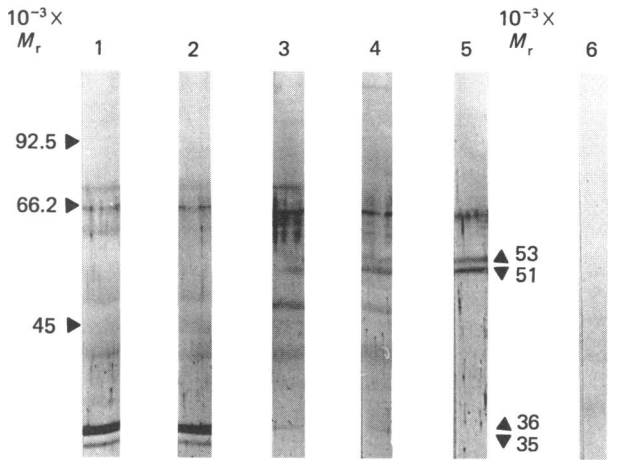

Fig. 2. Immunoblots of samples obtained at different stages during the purification of mouse brain ODC

Lane 1, crude brain extract; lane 2, $(NH_4)_2SO_4$ fraction after desalting; lane 3, heparin–Sepharose eluate; lane 4, pyridoxamine phosphate–agarose eluate; lane 5, DEAE-cellulose eluate; lane 6, brain extract, paper exposed to preimmune serum.

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