Identical catalytic-centre activity for mouse kidney and rat liver ornithine decarboxylases as determined with antizyme and affinity labelling

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Since the catalytic-centre activity of mouse kidney ornithine decarboxylase (ODC) has been assumed to be twice as high as that of rat liver ODC, we compared relative catalytic-centre activity of the two enzymes by titration with antizyme, which inhibits ODC by stoichiometric binding. In either a crude or a purified state, both enzymes were inhibited by rat liver antizyme to the same extent, indicating that they have nearly identical catalytic-centre activities. This conclusion was supported by comparison of affinity labelling of the enzymes with α -difluoromethyl[^{14}C]ornithine.

INTRODUCTION

Ornithine decarboxylase (ODC), which is a key enzyme in polyamine biosynthesis, is well known for its rapid turnover rate *in vivo* (Russell & Snyder, 1969). The content of ODC is extremely small in most animal tissues, even at a maximally induced state. We have purified ODC about 350 000-fold from the liver of thioacetamide-treated rats to apparent homogeneity, and the final specific activity was 1.1×10^6 – 1.2×10^6 nmol/h per mg of protein (Kameji *et al.*, 1982). This value was very close to that predicted by Pegg and his collaborators: the prediction was based on the affinity labelling of the enzyme in relatively crude preparations with radioactive α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor, assuming that one molecule of the reagent is needed to bind to each subunit of ODC to inactivate the enzyme (Pritchard *et al.*, 1981). We labelled purified rat liver ODC with DFMO and confirmed their result (Kameji & Hayashi, 1982). Kitani & Fujisawa (1983) also purified ODC from rat liver to a final specific activity about the same as ours. On the other hand, Pegg and his collaborators purified ODC from the kidney of androgen-treated mice, and the final specific activity that they obtained, which was close to the value determined by DFMO binding, was about twice as high as that of rat liver ODC (Seely *et al.*, 1982a). They also reported about a 2-fold difference between specific DFMO binding, and accordingly final specific activity, of purified mouse kidney and rat liver ODCs (Seely *et al.*, 1982b; Pegg *et al.*, 1987), and suggested that the difference may be due to different catalytic-centre activities of the two enzymes (Seely *et al.*, 1982b). Donato *et al.* (1986) reported an even higher final specific activity for their mouse kidney ODC preparation. On the contrary, values close to that for rat liver ODC have also been reported for apparently homogeneous mouse kidney ODC preparations (Persson, 1981; Isomaa *et al.*, 1983).

The objective of the present study was to compare relative catalytic-centre activity of rat liver and mouse

kidney ODCs by titrating the enzymes with antizyme, which inhibits ODC in a stoichiometric manner by binding to it with a high affinity (Fong *et al.*, 1976; Kitani & Fujisawa, 1984). If mouse kidney ODC catalyses twice as fast as rat liver ODC, then an equal amount of antizyme would inhibit twice as much activity of mouse kidney ODC as that of rat liver ODC. Comparison of affinity labelling of the two enzymes with DFMO was also carried out.

EXPERIMENTAL

Rat liver ODC

Sprague–Dawley rats (180–200 g) were injected intraperitoneally with 150 mg of thioacetamide/kg 20–22 h before being killed. Livers were each homogenized with 2 vol. of 0.25 M-sucrose containing 1 mM-dithiothreitol. The homogenates were centrifuged at 100 000 *g* for 60 min, and the supernatants were used as crude rat liver ODC preparations. Purification of rat liver ODC was carried out from each extract by using affinity chromatography on a HO-101 monoclonal anti-ODC antibody–Sephacrose 4B column (0.5 ml bed volume; Matsufuji *et al.*, 1984). The enzyme was eluted from the column with 3 M-MgCl₂, containing 1 mM-dithiothreitol and 0.01% Tween 80, and dialysed against 25 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-dithiothreitol and 0.01% Tween 80.

Mouse kidney ODC

Kidney ODC was induced in male ICR mice by testosterone administration (200 μg /0.1 ml of peanut oil, subcutaneously, daily for 10 days). Kidney extracts, containing ODC activity roughly 100 times as high as that of rat liver extracts, were prepared in a similar manner to that described above for liver extracts and used as crude mouse kidney ODC preparations after appropriate dilution with 25 mM-Tris buffer, pH 7.4, containing 1 mM-dithiothreitol, 0.01% Tween 80,

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, α -difluoromethylornithine.

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40 μ M-pyridoxal phosphate and 0.05% bovine serum albumin. Purified mouse kidney ODC was prepared by immunoaffinity chromatography as described above.

Rat liver antizyme

Sprague-Dawley rats (200–300 g) were injected intraperitoneally with 1 mmol of 1,3-diaminopropane or putrescine/kg 1, 2 and 3 h before being killed. An extract was prepared from pooled livers as described above and used as a crude antizyme preparation. For purification of the antizyme, the crude liver extract was applied on a column of DEAE-Cellulofine, and bound antizyme was eluted from the column with 25 mM-Tris/HCl, pH 7.4, containing 0.4 M-NaCl, 1 mM-dithiothreitol and 0.01% Tween 80, and then subjected to immunoaffinity chromatography in which a monoclonal antibody against rat liver antizyme served as a ligand. Monoclonal antibodies to rat liver antizyme have been produced in our laboratory by use of the ordinary hybridoma technology (S. Matsufuji, R. Kanamoto, Y. Murakami & S. Hayashi, unpublished work). One of them, HZ-2E9, which was IgG1 of kappa-type, was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose column chromatography and coupled to Affi-Gel 10 (18.9 mg/ml). The above antizyme preparation was applied to a column of anti-(rat liver antizyme)-Affi-Gel 10 (0.5 ml bed volume) and, after washing, bound antizyme was eluted with 3 M-MgCl₂, containing 1 mM-dithiothreitol and 0.01% Tween 80, and dialysed against 25 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol and 0.01% Tween 80.

Fig. 1. Titration of crude mouse kidney and rat liver ODCs by antizyme

Freshly prepared crude mouse kidney ODC (●) and rat liver ODC (○) were each mixed with increasing amounts of crude rat liver antizyme and assayed for the remaining ODC activity as described in the Experimental section. Similar results were obtained repeatedly.

Table 1. Inhibition of purified mouse kidney and rat liver ODCs by antizyme

Mouse kidney and rat liver ODCs were purified by immunoaffinity chromatography from each of four mice and four rats, respectively, as described in the Experimental section. Inhibition of ODC activity of each preparation by a fixed amount of affinity-purified rat liver antizyme was determined as described in the Experimental section. Values are means $\pm$ S.D. for four enzyme preparations of each group.

ODC	Amount of inhibition by antizyme (units/ml)
Mouse kidney	91.8 $\pm$ 8.6
Rat liver	92.4 $\pm$ 1.7

ODC and antizyme assays

ODC was assayed as described previously (Hayashi & Kameji, 1983), except that the concentrations of dithiothreitol and pyridoxal phosphate in the reaction mixture were 5 mM and 40 μ M respectively. One unit of ODC activity was defined as the amount releasing 1 nmol of CO₂/h at 37 °C under the specified conditions. Antizyme activity was assayed essentially as described by Hayashi & Fujita (1983). One unit of antizyme activity was defined as the amount inhibiting 1 unit of ODC activity.

Affinity labelling of ODC with [¹⁴C]DFMO

Affinity-purified mouse kidney ODC (1454 units) and rat liver ODC (1437 units) were each incubated with 6.7 μ M-DL- α -[5-¹⁴C]DFMO (60 Ci/mol), which was kindly given by Dr. A. E. Pegg, in 25 mM-Tris/HCl, pH 7.4, containing 20 μ M-pyridoxal phosphate, 5 mM-dithiothreitol and 0.01% Tween 80 in a total volume of 300 μ l, at 37 °C for 70 min. Samples (10 μ l) were taken from the reaction mixture both before and after the incubation for assay of ODC activity upon appropriate dilution. The concentration of DFMO was too low in the final assay system to affect ODC activity. For the measurement of DFMO binding, 40 μ l samples of the reaction mixture were each transferred to a piece of filter paper (1.5 cm $\times$ 1.5 cm) both before and after the incubation. The filter paper was treated in boiling 10% (w/v) trichloroacetic acid for 5 min, and washed with large volumes of hot water, ethanol and acetone in succession. After drying, the filter paper was dipped in scintillation fluid and its radioactivity was measured. The radioactivity of the sample before incubation was subtracted from that after the incubation to obtain specifically bound radioactivity.

RESULTS AND DISCUSSION

To minimize possible disturbance caused by denatured ODC which might react with antizyme, a first series of experiments was carried out with freshly prepared crude tissue extracts as enzyme and antizyme sources. A typical result is shown in Fig. 1. Both mouse kidney and rat liver ODCs were inhibited by rat liver antizyme with linear dose-effect relationships of an identical slope, indicating that both enzymes were inhibited by the antizyme to the

Table 2. Binding of [¹⁴C]DFMO to purified mouse kidney and rat liver ODCs

Freshly purified mouse kidney ODC (1454 units) and rat liver ODC (1437 units) were each incubated with α -[5-¹⁴C]DFMO at 37 °C for 70 min as described in the Experimental section. Before and after the incubation, samples were taken from the reaction mixture and assayed for ODC activity and for the binding of [¹⁴C]DFMO to the trichloroacetic acid-insoluble fraction as described in the Experimental section. Values are means of duplicate determinations. Specific activities of the enzymes were calculated by assuming a subunit M_r of 51172 for both enzymes.

ODC	Inactivated ODC (units)	Bound [¹⁴ C]DFMO		Calculated specific activity (units/mg)
		(pmol)	(fmol/unit)	
Mouse kidney	1355	18.7	13.8	1.41×10^6
Rat liver	1285	20.3	15.7	1.24×10^6

same extent. Since crude tissue extracts may contain some macromolecular and/or low- M_r compounds which affect ODC or antizyme activity (Hayashi & Fujita, 1983), the next series of experiments was carried out with affinity-purified ODC and antizyme preparations. As shown in Table 1, purified mouse kidney and rat liver ODCs were also inhibited to the same extent by antizyme. These results clearly indicate that mouse kidney and rat liver ODCs have nearly identical catalytic-centre activities. We next compared [¹⁴C]DFMO binding by affinity-purified mouse kidney and rat liver ODCs. As shown in Table 2, roughly identical amounts of DFMO were bound to each unit of both enzymes, indicating that the two enzymes have roughly identical catalytic-centre activities. Thus, in contradiction with the results of Pegg's group (Seely *et al.*, 1982a,b; Pegg *et al.*, 1987), the result of our DFMO-binding experiment supports our conclusion obtained with antizyme. Assuming a subunit M_r of 51172 for both enzymes (Gupta & Coffino, 1985), the specific activities were calculated to be 1.41×10^6 units/mg for mouse kidney ODC and 1.24×10^6 units/mg for rat liver ODC (Table 2). The value for mouse kidney ODC is less than half of the values reported by Pegg and his collaborators (Seely *et al.*, 1982a,b; Pegg *et al.*, 1987) and one-third of the value reported by Donato *et al.* (1986).

The exact reason is not clear at present for the discrepancy between our results and those of Pegg and his collaborators and of Donato *et al.* (1986). Although the DFMO-binding method is convenient for the determination of catalytic-centre activity of ODC, it may be subject to an error, owing to non-specific binding (Erwin *et al.*, 1983) and to possible loss of bound DFMO during washing by centrifugation. In fact, there are considerable variations in values reported from the same laboratory for crude or purified rat liver ODC or purified mouse kidney ODC (cf. Pritchard *et al.*, 1981; Seely *et al.*, 1982a,b; Pegg *et al.*, 1987). The inherent limitation of this method when applied to crude preparations has been pointed out (Pegg *et al.*, 1987). On the other hand, the antizyme titration method is easy and accurate for the comparison of relative catalytic-centre activity of different ODCs in either a crude or a purified state, although this method does not give an absolute catalytic-centre activity and should not be applied to any sample containing a significant amount of inactive ODC. Meanwhile, it should be pointed out that a substantial error often occurs in determination of the absolute value

for either final specific activity or catalytic-centre activity of any enzyme, because pure enzyme in minute amount tends to be inactivated, and accurate determination of protein is often difficult.

In accordance with our present conclusion, there is a general similarity in physicochemical and kinetic properties between mouse kidney and rat liver ODCs. Thus their apparent M_r values differ only slightly from each other on SDS/polyacrylamide-gel electrophoresis (Seely *et al.*, 1982a), and they are closely related immunochemically (Seely *et al.*, 1985). Moreover, K_m values of the two enzymes are not much different from each other for either ornithine or pyridoxal phosphate (Seely *et al.*, 1982a; Kitani & Fujisawa, 1983).

This study was supported in part by Grants-in-Aid for Scientific Research (61570131 and 62010071) from the Ministry of Education, Science and Culture of Japan.

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Received 27 April 1987/28 October 1987; accepted 17 November 1987