Changes in ornithine decarboxylase and antizyme activities in developing mouse brain

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A macromolecular inhibitor to ornithine decarboxylase (ODC) present in mouse brain was identified as ODC antizyme [Fong, Heller & Canellakis (1976) *Biochim. Biophys. Acta* **428**, 456–465; Heller, Fong & Canellakis (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1858–1862] on the basis of kinetic properties, M_r and reversal of its inhibition by antizyme inhibitor. The brain antizyme, however, did not cross-react immunochemically with any of seven monoclonal antibodies to rat liver antizyme. ODC activity in mouse brain rapidly decreased after birth, in parallel with putrescine content, and almost disappeared by 3 weeks of age. Free antizyme activity appeared shortly after birth and increased gradually, whereas ODC–antizyme complex already existed at birth and then gradually decreased. Thus total amount of antizyme remained about the same throughout the developmental period in mouse brain. In addition to ODC–antizyme complex, inactive ODC protein was detected by radioimmunoassay in about the same level as the complex at 3 weeks of age. Upon cycloheximide treatment, both free ODC activity and ODC–antizyme complex rapidly disappeared, although free antizyme and the inactive ODC protein were both quite stable.

INTRODUCTION

Polyamines are thought to play essential roles not only in cell proliferation but also in tissue differentiation. Ornithine decarboxylase (ODC, EC 4.1.1.17), the first and rate-limiting enzyme in polyamine synthesis, has the shortest half-life among mammalian enzymes studied (Russell & Snyder, 1969; Tabor & Tabor, 1976). ODC activity is known to be regulated by a protein inhibitor termed ‘antizyme’, which is induced by polyamines and specifically inactivates ODC by forming a complex with it (Fong *et al.*, 1976; Heller *et al.*, 1976).

Antizyme has been detected in mouse brain under physiological conditions (Hietala, 1983). The antizyme activity increased as ODC activity decreased during maturation of mouse brain (Laitinen *et al.*, 1985a). These results suggest that antizyme plays an essential role in the regulation of ODC activity and polyamine synthesis in mouse brain. The presence of antizyme, however, seemed to be insufficiently proved in these previous studies when judged by our criteria. In the present paper we firmly demonstrate that antizyme is present in mouse brain cytosol. We also studied the developmental changes and biological half-lives of ODC activity, antizyme activity, the amount of ODC–antizyme complex and the amount of immunoreactive ODC protein in order to determine the regulatory mechanism of polyamine synthesis in mouse brain tissue.

EXPERIMENTAL

Materials

DL-[1-¹⁴C]Ornithine (54 Ci/mol) and DL- α -difluoromethyl[3,4-³H]ornithine (33.3 Ci/mmol) were obtained

from Amersham International and New England Nuclear Corp. respectively. α -Difluoromethylornithine (DFMO) was supplied by the Merrell–Dow Research Institute (Cincinnati, OH, U.S.A.), and Sephadex G-75 was from Pharmacia Fine Chemicals. All other compounds were obtained from either Nakarai Chemicals Ltd. (Kyoto, Japan) or Sigma Chemical Co.

Animals

ICR mice were purchased from CLEA Japan (Tokyo, Japan) at the age of 3–4 weeks and kept in an air-conditioned windowless room lit from 09:00 to 21:00 h. They were given laboratory chow (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. Newborn mice were obtained by breeding and kept under maternal care until use.

Preparation of crude extracts

Mice were killed by decapitation. The brains were immediately excised and homogenized with 3 vol. of ice-cold 25 mM-Tris/HCl, pH 7.4, containing 0.1 mM-EDTA, 5 mM-dithiothreitol, and 0.1 mM-pyridoxal 5'-phosphate, in a Dounce-type all-glass homogenizer. A part of the homogenate was saved for the measurement of polyamine concentrations. The remaining part was centrifuged for 45 min at 100 000 *g* at 4 °C, and the supernatant was used for the assay of ODC activity, antizyme activity, the amount of ODC–antizyme complex and the amount of immunoreactive ODC protein.

Gel-filtration chromatography

H.p.l.c. A 0.5 ml portion of the 100 000 *g* supernatant containing 1 M-NaCl was applied to a column (7.5 mm $\times$ 600 mm) of TSK gel G3000SW (Toyo Soda,

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

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Tokyo, Japan) that had been equilibrated with 25 mM-Tris/HCl (pH 7.4)/0.01% Tween 80/10 mM-2-mercaptoethanol/1 M-NaCl, and eluted with the same buffer at a flow rate of 30 ml/h. The fractions (0.43 ml each) were assayed for ODC-inhibitory activity.

Sephadex G-75 gel-filtration chromatography. A 15 ml portion of the 100 000 g supernatant, containing 1 M-NaCl, was applied to a column (22 mm × 700 mm) of Sephadex G-75 equilibrated with 25 mM-Tris/HCl (pH 7.4)/0.1 mM-pyridoxal 5'-phosphate/0.1 mM-EDTA/0.01% Tween 80/10 mM-2-mercaptoethanol/1 M-NaCl. The column was eluted with the same buffer at a flow rate of approx. 30 ml/h, and 6.0 ml fractions were collected. Fractions containing ODC-inhibitory activity were pooled and used in experiments studying kinetic properties and immunocross-reactivity with anti-(rat liver antizyme) monoclonal antibodies.

Determination of whole brain polyamine concentrations

The brain homogenate was treated with HClO₄ (final conc. 2%) and centrifuged at 5000 g for 15 min at 4 °C. The supernatant was analysed for polyamines by h.p.l.c. on a Shimadzu instrument combined with fluorimetric determination of the reaction products with *o*-phthalaldehyde/2-mercaptoethanol (Seiler & Knodgen, 1980).

Preparation of ODC, DFMO-ODC and antizyme inhibitor

ODC was partially purified by DEAE-cellulose column chromatography from livers of thioacetamide-treated rats as described previously (Kameji *et al.*, 1982). ODC inactivated by covalent binding of DFMO was prepared by incubating the partially purified rat liver ODC (300 units/mg of protein) with DFMO (20 μM) at 37 °C for 2 h and dialysing extensively against 25 mM-Tris/HCl (pH 7.4)/10 μM-pyridoxal 5'-phosphate/1 mM-dithiothreitol. Antizyme inhibitor was partially purified by DEAE-cellulose column chromatography and freed from ODC by passing the eluate through an anti-ODC-monoclonal-antibody-Affi-Gel 10 column (Matsufuji *et al.*, 1984).

Assays

ODC and antizyme activities. Assay procedures for measuring ODC activity were described previously (Hayashi & Kameji, 1983). Control assay mixture was adjusted to the same salt concentration as in test assays, since NaCl inhibits ODC activity (Obenrader & Prouty, 1977). One unit of ODC activity is defined as the amount releasing 1 nmol of CO₂ from ornithine/h at 37 °C. Antizyme activity was assayed by measuring inhibition of ODC activity as described by Hayashi & Fujita (1983). Rat liver ODC partially purified by DEAE-cellulose column chromatography was used for the assay. Mouse brain extracts often contained some ODC-inhibitory activity which was different from antizyme and inhibited a certain *proportion* (not a certain *amount*) of ODC activity. Therefore ODC-inhibitory activity in crude brain extracts was measured with various amounts of ODC and plotted on a graph with the amounts of added ODC as the abscissa; the antizyme activity was then determined by extrapolating the amount of inhibition to the ordinate (Fujita *et al.*, 1984). One unit of antizyme activity is defined as the amount inhibiting 1 unit of ODC activity.

Amount of ODC-antizyme complex. ODC-antizyme complex was determined by a previously described method in which DFMO-ODC was used to re-activate the complexed ODC (Murakami *et al.*, 1985). One unit of ODC-antizyme complex is defined as the amount consisting of 1 unit each of ODC and antizyme activities.

Amount of immunoreactive ODC protein. Polyclonal antibodies were produced in rabbits against homogeneous rat liver ODC and partially purified from the antisera as described previously (Kameji *et al.*, 1982). One mg of anti-ODC immunoglobulin caused precipitation of 0.63 μg (945 units) of rat liver ODC with the aid of *Staphylococcus aureus* Cowan I cells. For the labelling of ODC, the enzyme from testosterone-treated mouse kidney (8.5 × 10³ units) was incubated with 3 μM-DL-α-[3,4-³H]DFMO (33.3 Ci/mmol) in the presence of 40 mM-Tris/HCl pH 7.4/40 μM-pyridoxal phosphate/5 mM-dithiothreitol/0.01% Tween-80/bovine serum albumin (0.8 mg/ml) at 37 °C for 70 min. Unbound [³H]DFMO was removed by dialysis against 25 mM-Tris/HCl (pH 7.4) 10 mM-2-mercaptoethanol/0.01% Tween-80 for 72 h. The assay was carried out essentially as described by Seely & Pegg (1983b). The assay mixture contained, in 0.4 ml, 1 × 10³ c.p.m. of [³H]DFMO-labelled ODC, the test sample, 25 mM-Tris/HCl, pH 7.4, 0.1 mM-pyridoxal phosphate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 0.01% Tween-80 and an amount of antiserum that would bind about 50% of the total labelled ODC in the absence of unlabelled enzyme. A 20 μl portion of a 10% (w/v) suspension of formalin-fixed *S. aureus* Cowan I cells in 50 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/5 mM-EDTA/0.05% (v/v) Nonidet P40/0.02% (w/v) NaN₃ was used for immunoadsorption. Standard curves were set up by using fresh extracts of mouse kidney, in which ODC activity had been reported to correlate with the amount of the enzyme protein (Persson & Rosengren, 1979). One unit equivalent of the immunoreactive ODC protein was defined as the amount giving the same immunoreactivity with 1 unit of active ODC.

Others. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Immunoprecipitation of antizymes from various sources using monoclonal antibodies to rat liver antizyme

Monoclonal antibodies to rat liver antizyme were obtained by use of hybridoma technology in a manner essentially similar to that reported previously (Matsufuji *et al.*, 1984). Hybridoma cells were produced by fusing mouse myeloma cells (P3X63Ag8U.1) with spleen cells from a BALB/c mouse which had been immunized with highly purified rat liver antizyme. Seven clones of hybridoma cells secreting anti-antizyme antibody were screened, and the antibodies, each belonging to IgG₁ of the κ type, were prepared from ascites fluids of BALB/c mice which had been inoculated intraperitoneally with each clone of the hybridoma cells. Antizymes were partially purified from rat liver by DEAE-cellulose chromatography, or from rat brain, mouse liver and mouse brain by Sephadex G-75 gel-filtration chromatography. About 2 units each of the partially purified antizymes were mixed with a series of 10 μg each of the monoclonal anti-antizyme IgGs and 5 mg (wet wt.) of

formalin-fixed *S. aureus* Cowan I cells which had been bound with 0.1 mg of affinity-purified rabbit anti-(mouse IgG) antibody, in 0.4 ml of solution. The mixtures were kept overnight at 0 °C, then centrifuged at 2000 *g* for 10 min. The supernatants were assayed for antizyme activity as described above.

RESULTS

Identification of ODC inhibitor in mouse brain as antizyme

Brain extracts prepared from mice of 3 weeks of age contained substantial levels of ODC inhibitory activity. Fig. 1 shows the gel-filtration patterns of NaCl-treated brain extracts. The peaks of ODC activity and of ODC-inhibitory activity correspond to M_r values of 55000 and 26000 respectively, as estimated by h.p.l.c. on TSK gel G3000SW. The M_r of the ODC inhibitor was about the same as the originally reported value for antizymes from several kinds of cells and rat liver (Heller *et al.*, 1976). Kinetic properties of the mouse brain ODC inhibitor, partially purified by gel-filtration, are shown in Fig. 2. A fixed amount of the inhibitor caused a constant amount of ODC inhibition irrespective of the amount of ODC present (Fig. 2a), indicating that it acts by stoichiometric binding to ODC. The inhibitory action was not time-dependent (Fig. 2b), excluding the possibility that the inhibitor is a proteinase. Furthermore, the effect of the inhibitor was reversed by an addition of antizyme inhibitor (Fig. 2b) that specifically binds to

antizyme with high affinity and inhibits its activity (Fujita *et al.*, 1982). From these results the ODC inhibitor in mouse brain was identified as antizyme.

Developmental changes in polyamine concentrations, ODC activity, antizyme activity, the amount of ODC-antizyme complex and the amount of immuno-reactive ODC protein in mouse brain

Fig. 3 shows changes in polyamine concentrations in developing mouse brain. Putrescine concentration declined rapidly and by 2 weeks reached the adult level, which was about 10 % of that in the newborn. Spermidine

Fig. 1. Gel-filtration chromatography of NaCl-treated extracts from mouse brain

Pooled brain extracts were prepared from 30 male mice, 3 weeks of age, which had been treated with cycloheximide (50 μ g/g body wt.) intraperitoneally for 40 min (●), and from the same number of control mice (○). Both extracts were gel-filtered through a Sephadex G-75 column in the presence of 1 M-NaCl as described in the Experimental section. Each fraction was assayed for ODC activity in the presence of 5.5 units of partially purified ODC.

Fig. 2. Kinetic properties of the mouse brain ODC inhibitor

(a) The inhibitory activity against various amounts of ODC. Different amounts of ODC were assayed in the presence (▲) or absence (■) of a fixed amount of the inhibitor (Sephadex G-75 gel-filtered fraction) without any preincubation. (b) Time courses of the ODC reaction in the presence (●) or absence (○) of the inhibitor and in the presence of both the inhibitor and antizyme inhibitor (▲). Procedures for partial purification of ODC, the inhibitor and antizyme inhibitor are described in the Experimental section.

Fig. 3. Developmental changes in polyamine concentrations of mouse brain

Each point is the mean of three determinations for pooled brain extracts from five to ten mice of both sexes. Polyamines were analysed as described in the Experimental section. ●, Putrescine; ○, spermidine; ▲, spermine.

Fig. 4. Developmental changes in ODC activity, antizyme activity and the amount of ODC-antizyme complex in mouse brain

Each value is a mean of three determinations for pooled brain extracts from five to ten mice of both sexes. Assays were carried out as described in the Experimental section. ○, Free ODC activity; ▲, free antizyme activity; ●, amount of ODC-antizyme complex.

concentration declined gradually after birth, whereas the spermine concentration did not change significantly. These results were essentially in agreement with results previously reported (Shimizu *et al.*, 1965; Seiler & Lamberty, 1975). As shown in Fig. 4, ODC activity was relatively high (0.88 unit/mg of protein) in newborn-mouse brain, but rapidly declined and had almost disappeared by 17 days. Conversely, antizyme activity appeared soon after birth and progressively increased to the adult level (about 0.25 units/mg of protein) by 3 weeks. These results were in good accordance with those of Laitinen *et al.* (1985a). On the other hand, measurement of ODC-antizyme complex by a competitive assay (Murakami *et al.*, 1985) revealed that a substantial amount of the complex was already present in newborn-mouse brain and then gradually decreased. Consequently, the total amount of antizyme per unit weight of brain,

Fig. 5. Developmental changes in ODC activity and the amount of immunoreactive ODC protein in mouse brain

Each value is the mean of three determinations for pooled brain extracts from five to ten mice of both sexes. Procedures for assays are described in the Experimental section. ○, Total ODC (free ODC and complexed ODC) activity; ●, the amount of immunoreactive ODC protein.

calculated as the sum of free and complexed antizyme activities, remained about the same throughout the developmental period. Fig. 5 shows changes in the amount of ODC protein, determined by means of radioimmunoassay, compared with changes in total ODC activity (sum of free and complexed ODC). Although reasonably good agreement was observed between the two parameters until 17 days after birth, about twice as much ODC protein as total ODC activity was found to be present in brain cytosol on day 19 and later. The amount of immunoreactive ODC protein, however, was much smaller than that reported previously (Laitinen *et al.*, 1985a).

Immunoreactivity of mouse brain antizyme with anti-(rat liver antizyme) monoclonal antibodies

We examined immunocross-reactivity of antizymes from various sources with seven monoclonal antibodies to rat liver antizyme. Mouse brain antizyme did not cross-react significantly with any of these antibodies (Fig. 6). Rat brain antizyme also did not cross-react with either of two antibodies tested (2E9 and 3H1; results not shown), whereas mouse liver antizyme cross-reacted with three antibodies tested (2E9, 3H1 and 1C5; results not shown). These results indicate that there are tissue-specific antizymes in mouse and rat, brain antizymes being quite different immunochemically from liver antizymes.

Turnover of ODC, antizyme and their complex in mouse brain

Fig. 7 shows changes with time in ODC activity, antizyme activity and the amount of ODC-antizyme complex in the brain of 3-week-old mice upon cycloheximide treatment. Both ODC activity and the amount of ODC-antizyme complex rapidly decayed, with half-lives of about 10 min and 15 min respectively. In contrast, free antizyme activity showed a much slower decline. It is noteworthy that the level of free antizyme activity remained nearly the same until all the ODC-antizyme

Fig. 6. Immunocross-reactivity of mouse brain antizyme with monoclonal antibodies to rat liver antizyme

Partially purified antizymes from rat liver (a) and mouse brain (b) were treated with either normal IgG (C) or each of seven monoclonal antibody IgGs to rat liver antizyme (1–7), and the remaining antizyme activity was determined by measuring ODC activity after addition of a fixed amount of ODC. Details are described in the Experimental section. The broken horizontal line indicates the no-antizyme control activity.

Fig. 7. Decay of ODC, antizyme and ODC-antizyme complex upon cycloheximide treatment

Male mice, 3 weeks of age, were injected twice at the times indicated by arrows with cycloheximide (50 μ g/g body wt.) intraperitoneally and killed at the indicated times. At each point, pooled brain extract was prepared from five mice. $\circ$, Free ODC activity; $\square$, free antizyme activity; $\bullet$, amount of ODC-antizyme complex; $\blacksquare$, total antizyme (free antizyme and complexed antizyme) activity.

complex disappeared within 40 min. This might have been due to a liberation of some antizyme from the complex with the decay of the latter. As is also shown in Fig. 7, the total (sum of free and complexed) antizyme

Fig. 8. Decay of active ODC and immunoreactive ODC protein upon cycloheximide treatment

The procedure for the treatment of the animals was the same as that employed in Fig. 7. $\circ$, Total ODC (free ODC and complexed ODC) activity; $\bullet$, amount of immunoreactive ODC protein.

activity decayed with half-life of longer than 60 min. The fact that brain antizyme was much more stable than ODC was also supported by the gel-filtration experiment (Fig. 1), in which brain ODC activity was shown to disappear within 40 min after cycloheximide treatment, whereas a large portion of antizyme activity still

remained. Fig. 8 shows the decay rate of immunoreactive ODC protein upon cycloheximide treatment compared with that of the total (sum of free and complexed) ODC activity. Whereas total ODC activity completely disappeared within 40 min, immunoreactive ODC protein stopped decaying at about half the original level. It appears, therefore, that mouse brain contains a small amount of stable protein which cross-reacts with anti-ODC polyclonal antibody.

DISCUSSION

In the present study we have proved that antizyme is present in the brain cytosol of young mice. Multiple criteria are needed for the identification of ODC antizyme, since ODC activity could be affected by various kinds of macromolecular and low- M_r compounds (Ickson & Kaye, 1976; Obenrader & Prouty, 1977; Hayashi & Fujita, 1983). Our identification of mouse brain antizyme was based on the following evidence: (1) it acts in a stoichiometric and time-independent manner; (2) its inhibitory effect on ODC can be reversed by antizyme inhibitor; (3) its M_r (26000) agrees reasonably with reported values for antizymes from a variety of sources, such as rat liver (Heller *et al.*, 1976; Fujita *et al.*, 1984; Kitani & Fujisawa, 1984), mouse kidney (Hayashi *et al.*, 1986), H-35 cells (Fong *et al.*, 1976), L-1210 and mouse neuroblastoma cells (Heller *et al.*, 1976), RK13 rabbit kidney cells (Murakami *et al.*, 1986) and rat lymphoma cells (Richards *et al.*, 1986), although antizymes of M_r 50000–90000 have also been found in rat thymus (Bishop *et al.*, 1985), rat mammary gland (Hu & Brosnan, 1987) and rat lymphoma cells (Richards *et al.*, 1986). Although we did not examine another important criterion for antizyme, namely its inducibility by polyamines (Fong *et al.*, 1976), Laitinen *et al.* (1985b) observed that intraventricular administration of spermine caused some increase in the amounts of free and total antizyme activities in mouse brain. On the other hand, brain antizyme of either mouse or rat did not cross-react with monoclonal antibodies to rat liver antizyme, whereas mouse liver antizyme more or less cross-reacted with them. This indicates that brain antizymes are quite different in structure from liver antizyme, in spite of similar molecular size and the same reactivity with ODC or antizyme inhibitor. It appears, therefore, that antizymes are tissue-specific.

The present study confirmed the inverse developmental changes in ODC and antizyme activities of mouse brain previously reported (Laitinen *et al.*, 1985a). In addition, we determined the developmental changes in the amount of ODC–antizyme complex and showed that the level of total antizyme activity remained almost constant in mouse brain throughout the period. It should be noted that the size of mouse brain increases severalfold during developmental period (Seiler & Lamberty, 1975), and therefore the amount of total (free plus complexed) antizyme in whole brain also must have increased severalfold. It is generally assumed that the rate of antizyme synthesis is regulated by tissue polyamine concentrations. The apparently contradictory result, that the total amount of antizyme in whole brain increased in spite of a general decrease in polyamine concentrations during development, might have been due either to a possible decrease in the rate of antizyme degradation or to a change in cellular localization of polyamines. A

marked discrepancy between the present and previously obtained results was noted in the amount of immunoreactive ODC protein. By using a radioimmunoassay similar to ours and immunoblot detection after electrophoresis, Laitinen and her collaborators demonstrated that mouse brain cytosol contained large amounts of immunoreactive, but catalytically inactive, ODC protein (Laitinen, 1985; Laitinen *et al.*, 1985a). Although we, too, detected inactive ODC protein in mouse brain at the age of 19 days or later, its amount was only about one-fiftieth of that reported by previous workers. It remains to be clarified whether this discrepancy was due to different mouse strains studied, to different antibodies used or to some other factors. The nature of the inactive ODC protein is also unknown at the present time, except that it is quite stable *in vivo*, unlike free ODC or ODC complexed with antizyme. With what is presently known, it is conceivable that at least a part of the ODC–antizyme complex is formed during the tissue homogenization and not present *in vivo*. The exact solution of this problem should await the development of a refined technique for immunohistochemical localization of ODC and antizyme in brain tissue.

Another major finding of the present study was that both ODC and ODC–antizyme complex decayed very rapidly, whereas free antizyme decayed quite slowly in mouse brain upon cycloheximide treatment at the age of 3 weeks, when total antizyme activity far exceeded total ODC activity. Studies in our laboratory have shown that, in HTC cells, the higher the antizyme/ODC ratio, the higher the ODC decay rate, suggesting that antizyme may accelerate ODC decay in a recycling manner (Murakami *et al.*, 1985; Murakami & Hayashi, 1985). The possibility of such a mechanism was also pointed out by Seely & Pegg (1983a). Our results do not contradict that hypothesis. As noted in the Results section, liberation of antizyme from the ODC–antizyme complex was suggested from the changes in free and complexed antizyme activities upon cycloheximide treatment (Fig. 7). It is conceivable, therefore, that mouse brain antizyme participates in ODC regulation not only by inhibiting its activity but also by accelerating its degradation. There remains another possibility, however, namely that brain antizyme may participate in sequestration of ODC for future use. Candidates for such a latent ODC would be the stable, but inactive, ODC reported in the present paper and the large amounts of inactive ODC found by previous workers (Hietala, 1983; Laitinen, 1985; Laitinen *et al.*, 1985a). Liberation of active ODC from such a latent ODC might be the mechanism underlying the rapid increases in ODC activity upon various stimuli observed by several groups of workers (Casti *et al.*, 1977; Koenig *et al.*, 1983; Iqbal & Koenig, 1985; Mustelin *et al.*, 1987). The exact role of brain antizyme awaits further clarification.

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