A macromolecular inhibitor of the antizyme to ornithine decarboxylase

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A macromolecular factor that inhibits the activity of the antizyme to ornithine decarboxylase (ODC) was found in rat liver extracts. The factor, 'antizyme inhibitor', was heat-labile, non-diffusible and of similar molecular size to ODC. The antizyme inhibitor re-activated ODC that had been inactivated by antizyme, apparently by replacing ODC in a complex with antizyme. Therefore the antizyme inhibitor can be used to assay the amount of inactive ODC–antizyme complex formed *in vitro*. When assayed by this method, the complex was shown to be eluted before ODC from a Sephadex G-100 column. Significant increase in ODC activity was observed when the antizyme inhibitor was added to crude liver extracts from rats that had been injected with 1,3-diaminopropane to cause decay of ODC activity, suggesting the presence of inactive ODC–antizyme complex in the extracts.

Ornithine decarboxylase (ODC), the first and rate-limiting enzyme of polyamine biosynthesis, has an extremely short half-life (Russell & Snyder, 1969; Russell *et al.*, 1970; Hannonen *et al.*, 1972). The activity of ODC in cells and tissues shows marked fluctuations: various growth stimuli induce ODC (Morris & Fillingame, 1974; Jänne *et al.*, 1978; Maudsley, 1979), and exogenously added diamines and polyamines cause rapid decrease of ODC activity (Schrock *et al.*, 1970; Pösö *et al.*, 1977; McCann *et al.*, 1979). Canellakis and his co-workers have found that a protein inhibitor of ODC is generated in several kinds of cultured cells and in rat liver by administration of putrescine or polyamines (Fong *et al.*, 1976; Heller *et al.*, 1976; Heller & Canellakis, 1981). The inhibitor, named 'antizyme', inactivates ODC by forming a complex with it. Although available evidence suggests that antizyme formation participates, at least in part, in the process of ODC decay caused by exogenous diamines and polyamines (Kallio *et al.*, 1979; McCann *et al.*, 1979), the physiological role of the antizyme is still unknown. One difficulty in studies on the antizyme has been lack of an appropriate method for assay of the intracellular amount of enzyme–antizyme complex (Heller & Canellakis, 1981).

In the present paper we report that a new macromolecular factor, 'antizyme inhibitor', is present in rat liver extracts. The factor not only inhibited antizyme activity but also re-activated ODC that had been inactivated by antizyme. This

suggests the reversibility of the action of antizyme *in vivo* and indicates that the factor may be useful in assay of intracellular amounts of the ODC–antizyme complex.

Experimental

Chemicals

DL-[1-¹⁴C]Ornithine (sp. radioactivity 59 Ci/mol) was obtained from New England Nuclear Corp. Dithiothreitol and bovine serum albumin were from Sigma Chemical Co. 1,3-Diaminopropane was from Tokyo Chemical Industry Co. Sephadex G-100 and Sepharose 4B were from Brown Co. Pyridoxamine phosphate–succinyl-di(aminopropyl)amine arm–Sepharose 4B was prepared by the procedure of Miller *et al.* (1972). DL- α -Hydrazino- δ -aminovaleric acid was synthesized essentially by the procedure of Sawayama *et al.* (1976).

Animals

Male Sprague–Dawley rats (5 weeks old) were purchased from Clea Japan Inc. and Sankyo Labo Service Co. They were fed *ad libitum* on laboratory chow obtained from Oriental Yeast Co., and maintained in a windowless animal room with light from 09:00 to 21:00 h. They were used for experiments at 6–8 weeks of age.

Preparation of ODC

ODC was partially purified by the procedure of Kameji *et al.* (1981) from the livers of 150 rats injected intraperitoneally with thioacetamide

Abbreviation used: ODC, ornithine decarboxylase (EC 4.1.1.17).

(150 mg/kg body wt.) and DL- α -hydrazino- δ -aminovaleric acid (75 mg/kg body wt.) 20 and 6 h respectively before death. The latter compound, a competitive inhibitor of ODC, was used to augment induction of ODC (Inoue *et al.*, 1975; Kameji *et al.*, 1981). All subsequent procedures were performed at 0–4°C. Livers were homogenized with 2 vol. of 0.2 M-sucrose in 25 mM-Tris/HCl, pH 7.5, containing 1 mM-dithiothreitol and 0.1 mM-EDTA (buffer A) in a Dounce-type all-glass homogenizer. The homogenate was centrifuged at 15 000 g for 15 min and the supernatant was centrifuged at 107 000 g for 60 min. The crude extract was directly applied to a DEAE-cellulose column (3.2 cm $\times$ 44 cm) that had been equilibrated with buffer A. The column was washed with the same buffer, and then the enzyme was eluted with a linear gradient of NaCl in buffer A (0–0.5 M; 3 litres). Active fractions were pooled and adjusted to 60% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation at 10 000 g for 10 min, dissolved in 25 mM-Tris/HCl, pH 7.2, containing 1 mM-dithiothreitol and 0.1 mM-EDTA (buffer B) and dialysed against the same buffer. We performed the purification procedure up to DEAE-cellulose chromatography twice on half scale and then combined the preparations for the next step (scales in the procedures described above are for 75 rats).

The combined preparation was applied to a pyridoxamine phosphate-succinyl-di(aminopropyl)-amine arm-Sephacryl 4B column (2.2 cm $\times$ 55 cm) that had been equilibrated with buffer B. The column was washed with the same buffer and then the enzyme was eluted with a pH gradient (buffer B containing 0.4 M-NaCl and 0.1% Tween 80 to 50 mM-Tris base containing 0.4 M-NaCl, 1 mM-dithiothreitol and 0.1% Tween 80). To prevent enzyme denaturation by high pH, 1 ml of 0.25 M-Tris/HCl, pH 7.0, was added to each collecting tube beforehand. Active fractions (12 ml each) were collected, concentrated in an Amicon ultrafiltration apparatus and dialysed against buffer A. This preparation was used as substrate in assay of antizyme or antizyme inhibitor.

Preparation of antizyme

Antizyme was partially purified from the livers of 38 rats injected twice intraperitoneally with 1,3-diaminopropane (1 mmol/kg body wt.) at 09:00 h and 11:00 h. Rats were killed by decapitation at 12:00 h. Liver extract was prepared and applied to a DEAE-cellulose column (4 cm $\times$ 27 cm) as described for preparation of ODC. Antizyme was eluted with a linear gradient of NaCl in buffer A (0–1.0 M, 2 litres). Active fractions were pooled and adjusted to 70% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation at 10 000 g for 10 min,

dissolved in buffer A, dialysed against the same buffer and used as the preparation of antizyme.

Preparation of antizyme inhibitor

Antizyme inhibitor was prepared from the livers of 50 rats treated with thioacetamide and DL- α -hydrazino- δ -aminovaleric acid as for preparation of ODC. Liver extract was prepared and applied to a DEAE-cellulose column (3 cm $\times$ 45 cm) as described for preparation of ODC. Antizyme inhibitor was eluted with a linear gradient of NaCl (0–0.5 M; 2 litres). Active fractions were pooled and stirred overnight with a gel of pyridoxamine phosphate-succinyl-di(aminopropyl)-amine arm-Sephacryl 4B that had been equilibrated with buffer B. The mixture was centrifuged at 1000 g for 10 min and the precipitated gel was washed with buffer B containing 50 mM-NaCl. The supernatant and the washings were combined, concentrated in an Amicon ultrafiltration apparatus with a PM-10 membrane, dialysed against buffer A, and used as the preparation of antizyme inhibitor.

Assays of ODC, antizyme and antizyme-inhibitor activities

ODC was assayed essentially as described by Hayashi *et al.* (1972). The reaction mixture consisted of 0.125 μCi of DL-[1- ^{14}C]ornithine, 0.4 mM-L-ornithine, 20 μM -pyridoxal phosphate, 1 mM-dithiothreitol, 40 mM-Tris/HCl buffer, pH 7.4, and enzyme solution in a final volume of 1.25 ml. Incubation was for 30 min at 37°C. One unit of enzyme activity was defined as the amount forming 1 nmol of CO_2/h under the above conditions.

Antizyme activity was assayed by measuring inhibition of ODC activity (usually about 30 units) without any preincubation. When ODC activity was inhibited by more than 50%, the assay was repeated with less antizyme. One unit of antizyme activity was defined as the amount inhibiting one unit of ODC activity.

Activity of antizyme inhibitor was assayed by measuring reversal of ODC inhibition caused by antizyme. When ODC inhibition was reversed by more than 80%, the assay was repeated with less antizyme inhibitor. One unit of antizyme inhibitor was defined as the amount reversing inhibition of one unit of ODC activity by antizyme.

Assay of ODC-antizyme complex

The amount of ODC-antizyme complex was determined as the amount of ODC activity that could be fully re-activated by antizyme inhibitor. Each sample was assayed for ODC activity in the absence and presence of a sufficient amount of antizyme inhibitor. When the increase in ODC activity after addition of antizyme inhibitor was close to the full activity of antizyme inhibitor added,

the assay was repeated with an increased amount of antizyme inhibitor.

Results

Presence of antizyme inhibitor in ODC preparations from rat liver

In preliminary experiments we confirmed that substantial activities of the antizyme to ODC could be induced in rat liver by repeated injections of 1,3-diaminopropane, an analogue of putrescine (Kallio *et al.*, 1979). We noticed, however, that scarcely any antizyme activity was detected in liver extracts when a fraction of DEAE-cellulose eluate was used as the ODC preparation in the antizyme assay. The same liver extracts showed high antizyme activities against more purified preparations of ODC. Results of mixing experiments indicated that the DEAE-cellulose fraction contained an inhibitor of antizyme.

To confirm the above observations, we performed DEAE-cellulose chromatography with a crude liver extract prepared from rats that had been treated with thioacetamide and DL- α -hydrazino- δ -aminovaleric acid. As shown in Fig. 1, the inhibitory factor was eluted as a single peak (0.30–0.37 M-NaCl), which was separated almost completely from that of ODC (0.2–0.3 M-NaCl). The total units (defined in the Experimental section) of the inhibitory factor corresponded to about 5% of

that of ODC. Since the factor had no influence on free ODC activity, we named it antizyme inhibitor.

Antizyme inhibitor activity was also found in a DEAE-cellulose eluate of liver extract from rats in which hepatic ODC activity had been induced by giving them a 50%-protein diet. However, its activity was not detected in a similar preparation from starved rats.

Physical and kinetic properties of antizyme inhibitor

Antizyme inhibitor is a macromolecular substance, since it is not diffusable and does not pass through a PM-10 membrane in an Amicon ultra-filtration apparatus. Antizyme inhibitor was eluted at about the same position as ODC on Sephadex G-100 gel-filtration analysis. Therefore the molecular weight of the antizyme inhibitor appeared to be similar to that of ODC, which is 105 000 (Kameji *et al.*, 1981). The inhibitor was completely inactivated by heating at 70°C for 15 min. It was not adsorbed to pyridoxamine phosphate-succinyl-di(aminopropyl)amine arm-Sepharose 4B, and was separated almost completely from ODC.

Fig. 2 shows that the enzymic reaction of ODC proceeded linearly for at least 60 min when the enzyme was incubated alone, or with antizyme, or

Fig. 1. Separation of antizyme inhibitor from ODC by DEAE-cellulose column chromatography

Crude liver extract was prepared from 20 rats that had been treated with thioacetamide and DL- α -hydrazino- δ -aminovaleric acid and was subjected to DEAE-cellulose chromatography as described in the Experimental section. The column size was 2.2 cm $\times$ 45 cm and the total elution volume was 760 ml. ----, [NaCl]; O, ODC activity; ■, antizyme-inhibitor activity.

Fig. 2. Time courses of the ODC reaction in the presence of antizyme or antizyme plus antizyme inhibitor

The ODC reaction was carried out at 37°C without additions (O), with antizyme (●) or with antizyme plus antizyme inhibitor (▲). Mixtures were not preincubated. Procedures for partial purification of ODC, antizyme and antizyme inhibitor are described in the Experimental section.

with antizyme plus antizyme inhibitor. This indicates that the action of antizyme inhibitor, like that of antizyme itself, is not time-dependent. Furthermore, antizyme inhibitor exhibited the same activity whether it was added to ODC before antizyme or after antizyme had been preincubated with ODC for 15 min at 37°C. This means that the antizyme inhibitor not only inhibited antizyme but also acted on inactive ODC–antizyme complex to re-activate the enzyme fully. The effect of antizyme inhibitor was proportional to its amount (Fig. 3a) and was not dependent on the amount of ODC–antizyme complex or free ODC (Figs. 3a and 3b). As shown in Fig. 3(b), when increasing amounts of antizyme were mixed with fixed amounts of ODC and antizyme inhibitor, ODC activity was not inhibited at all until the activity of antizyme added exceeded that of antizyme inhibitor present; then a linear dose–effect relationship was observed, with a slope identical with that observed in the absence of antizyme inhibitor. These results suggest that the antizyme inhibitor may act by forming a complex with antizyme and that the affinity of antizyme for antizyme inhibitor may be much higher than that for ODC.

Analysis with antizyme inhibitor of gel-filtration of the ODC–antizyme complex

A mixture of ODC and antizyme (M_r 24 500) was subjected to Sephadex G-100 gel-filtration. The remaining free ODC was eluted as a single peak, and there was no significant peak of antizyme activity (Fig. 4). To detect inactive enzyme–antizyme complex, we assayed each fraction in the presence of sufficient antizyme inhibitor and plotted the increase in ODC activity produced by antizyme inhibitor. As shown in Fig. 4, the enzyme–antizyme complex was eluted as a single peak slightly before ODC.

Use of antizyme inhibitor to detect ODC–antizyme complex formed in vivo

A preliminary experiment was performed to examine the possible formation *in vivo* of inactive ODC–antizyme complex, which can be assayed with antizyme inhibitor. 1,3-Diaminopropane was injected into rats in which hepatic ODC had been induced by treatments with thioacetamide and DL- α -hydrazino- δ -aminovaleric acid. The animals were killed 1 h later and liver extracts were assayed for ODC activity in the absence and presence of antizyme inhibitor. As shown in Table 1, 1,3-diaminopropane treatment decreased hepatic ODC activity to low values. However, significant increase in enzyme activity was observed on addition of antizyme inhibitor to the liver extracts. This suggested that the ODC–antizyme complex was present in these extracts.

Fig. 3. Titration experiments with antizyme inhibitor (a) and with antizyme (b)

(a) Increasing amounts of antizyme inhibitor were added to a series of flasks containing fixed amounts of ODC and antizyme. All mixtures were adjusted to the same final volume with buffer A and assayed for ODC activity. The broken line indicates ODC activity measured alone. (b) Increasing amounts of antizyme were added to a series of flasks containing a fixed amount of ODC with (▲) or without (●) a fixed amount of antizyme inhibitor. All mixtures were adjusted to the same volume with buffer A and assayed for ODC activity. Procedures for partial purification of ODC, antizyme and antizyme inhibitor are described in the Experimental section.

Fig. 4. *Sephadex G-100* gel-filtration of ODC-antizyme complex

Partially purified preparations of ODC (1205 units) and antizyme (539 units), both of which had been dialysed against 50 mM-Tris/HCl, pH 7.5, containing 50 mM-NaCl, 1 mM-dithiothreitol and 0.1 mM-EDTA (buffer C), were mixed and applied to a column (1.8 cm $\times$ 79 cm) of *Sephadex G-100* that had been equilibrated with buffer C. The volume of the mixture applied was 4.2 ml. The column was eluted with the same buffer. Assays were performed as described in the Experimental section. V_0 is the void volume of the column. O, ODC activity; Δ , antizyme activity; $\bullet$, amount of ODC-antizyme complex assayed with antizyme inhibitor.

Table 1. *Effect of antizyme inhibitor on ODC activity of rat liver extracts*

Three rats were each given successive intraperitoneal injections of thioacetamide (150 mg/kg body wt.), DL- α -hydrazino- δ -aminovaleric acid (75 mg/kg body wt.) and 1,3-diaminopropane (1 mmol/kg body wt.) 21, 7 and 1 h respectively before death. Crude liver extracts were prepared as described in the Experimental section for preparation of ODC. After dialysis against, buffer A, 0.5 ml portions of the extracts were assayed for ODC activity in the presence and absence of antizyme inhibitor (7 units). The average hepatic ODC activity of rats killed just before injection of 1,3-diaminopropane was 47.4 units/0.5 ml of extract.

Liver extract	ODC activity (units/0.5 ml)		Change
	No inhibitor	+ inhibitor	
Rat 1	0.8	5.0	4.2
Rat 2	0.5	3.5	3.0
Rat 3	0.5	4.2	3.7

Discussion

In the present paper we have demonstrated the presence in rat liver extracts of a factor that inhibits

the activity of the antizyme on ODC. The factor, 'antizyme inhibitor', is a heat-labile macromolecule, which is probably a protein, although this has not yet been proved. It is unlikely that the antizyme inhibitor is an artifact derived from ODC *in vitro*, since, although its molecular weight appears to be similar to that of ODC, its affinity to antizyme seems to be much higher than that of ODC, suggesting that it has a physiological role. Antizyme inhibitor was also found in liver extracts from rats in which hepatic ODC had been induced simply by feeding them on a high-protein diet. This excludes the possibility that antizyme inhibitor is formed by a chemical or pharmacological effect of thioacetamide or DL- α -hydrazino- δ -aminovaleric acid. Thus the antizyme inhibitor appears to be a natural substance and to play a physiological role in the regulation of ODC *in vivo*.

The action of antizyme inhibitor is not time-dependent. This eliminates the possibility that it is a proteinase specifically attacking antizyme. Titration experiments suggest that antizyme inhibitor acts by forming a complex with antizyme, just as antizyme inhibits ODC by forming a complex with it (Heller *et al.*, 1976; Fig. 4 in the present paper). Antizyme inhibitor not only inhibits antizyme, but also fully re-activates ODC that has been inactivated by antizyme. It appears therefore that the antizyme inhibitor releases active ODC by replacing it in a complex with antizyme. For this to occur, the antizyme inhibitor must have a much higher affinity for antizyme than does ODC. Evidence for this was provided by the titration experiment shown in Fig. 3(b). Thus, when increasing amounts of antizyme were mixed with fixed amounts of ODC and antizyme inhibitor, ODC activity was not inhibited by antizyme until all the antizyme inhibitor present had been consumed.

Kyriakidis *et al.* (1978) and Canellakis *et al.* (1981) reported that extracts of *Escherichia coli* cells contained a macromolecular ODC activator in addition to ODC and antizyme. Although their ODC activator appears to be quite different from our antizyme inhibitor in physiochemical characteristics, such as its heat stability and molecular weight, it would be interesting to examine whether it has a common mechanism of action with our antizyme inhibitor.

Since the discovery of antizyme by Canellakis and his co-workers, much attention has been paid to this unique regulatory protein. Not much progress has been made, however, in clarifying the physiological role or mode of action of antizyme. One difficulty in studies on antizyme has been the variability in antizyme activity in different species, different cells and tissues, and even different experiments with the same system. Part of the variability might have been due to the presence of antizyme inhibitor in the

ODC preparation used for antizyme assay, as shown in this study. Another serious difficulty in antizyme studies has been the lack of a simple method for assay of the ODC-antizyme complex (Heller & Canellakis, 1981). This difficulty should be overcome by use of antizyme inhibitor.

Heller *et al.* (1976) showed that inactive ODC-antizyme complex could be dissociated by 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$, and active ODC and antizyme could then be separated by Sephadex G-75 gel-filtration in the presence of 10% $(\text{NH}_4)_2\text{SO}_4$. By this method they observed that the inactive ODC-antizyme complex was eluted before ODC from a Sephadex G-75 column in the absence of a high concentration of salt, although they did not report the pattern of gel-filtration. We found in the present work by use of antizyme inhibitor that ODC inactivated by antizyme was eluted slightly before active ODC from a column of Sephadex G-100 gel. This indicated that the inactive ODC has a larger molecular size than active ODC and therefore provided evidence for formation of a complex between ODC and antizyme.

Exogenously added diamines and polyamines cause rapid decrease in cellular ODC activity, apparently by two different mechanisms: inhibition of enzyme synthesis and inactivation of preformed enzyme by antizyme (McCann *et al.*, 1979). The preliminary results reported in the present paper indicated that liver extracts from rats treated with 1,3-diaminopropane contained small but significant amounts of inactive ODC that could be re-activated by antizyme inhibitor. For determination of the relative contribution of antizyme to the process of ODC decay caused by exogenously added diamines, the following problems must be clarified: whether the inactive ODC found in liver extracts is really ODC-antizyme complex and, if so, whether the enzyme protein in the complex is stable against degradation *in vivo*. In any case, the results of the present study suggest that the action of antizyme may be reversible *in vivo*.

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