Antizyme to ornithine decarboxylase is present in the liver of starved rats

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Antizyme to ornithine decarboxylase (ODC) and ODC–antizyme complex were both present in liver cytosols of starved rats. The antizyme was identified by its molecular weight, kinetic properties, formation of a complex with ODC, and reversal of its inhibition by antizyme inhibitor. The average amount of antizyme in liver cytosols of starved rats was 0.1 unit/mg of protein, roughly corresponding to basal hepatic ODC activity in rats fed *ad libitum*. The presence of ODC–antizyme complex was detected by using antizyme inhibitor. These results indicate that antizyme participates in the regulation of ODC activity *in vivo* under physiological conditions.

ODC, the first and rate-limiting enzyme in polyamine biosynthesis (Tabor & Tabor, 1976), has the shortest half-life of any enzyme yet studied (Russell & Snyder, 1969), and its activity fluctuates remarkably with diet (Hayashi *et al.*, 1972; Noguchi *et al.*, 1979), hormones and other stimuli (Morris & Fillingame, 1974; Maudsley, 1979). Such a rapid change in enzyme activity suggests the involvement of post-translational regulation. Polyamines have been proposed as regulators of ODC, since their administration decreases ODC activity rapidly in rat liver (Jänne & Hölttä, 1974) and a number of cell lines (Kay & Lindsay, 1973; Clark & Fuller, 1975). Administration of polyamines or diamines induces a regulatory protein, antizyme, which binds to ODC stoichiometrically to form an inactive ODC–antizyme complex (Fong *et al.*, 1976; Heller *et al.*, 1976). Several lines of evidence suggest that antizyme participates in the process of rapid decay of ODC activity caused by exogenous diamines and polyamines (McCann *et al.*, 1977; Branca & Herbst, 1980; Heller & Canellakis, 1981).

Antizyme has been detected in mouse brain (Hietala, 1983) and rat mammary glands (Brosnan *et al.*, 1983) without any treatment with exogenous polyamines, suggesting possible roles of antizyme under physiological conditions. However, the evidence for identification as antizyme seems to be insufficient in those studies as judged by our criteria.

In the present paper, we firmly demonstrate that

antizyme is present in the liver cytosols of starved rats. Furthermore, we show that a small amount of ODC–antizyme complex is also present in similar liver cytosols by using 'antizyme inhibitor', another regulatory protein that can re-activate ODC of inactive ODC–antizyme complex (Fujita *et al.*, 1982, 1983). These results indicate that antizyme is a physiological factor involved in the regulation of ODC activity in rat liver.

Experimental

Materials

DL-[1-¹⁴C]Ornithine (54 Ci/mol) was obtained from Amersham. Dithiothreitol, bovine serum albumin and ribonuclease A were obtained from Sigma Chemical Co. Ovalbumin and chymotrypsinogen were from Boehringer Mannheim G.m.b.H. DEAE-cellulose was from Brown Co. Sephadex G-75, Sepharose 4B and Sephacryl S-200 were from Pharmacia Fine Chemicals. Sprague-Dawley rats were purchased from Sankyo Labo Service Co. and bred in a windowless animal room with light from 09:00 to 21:00 h. They were given laboratory chow (Oriental Yeast Co.) *ad libitum* and water. Male rats weighing 180–300 g were used in all experiments.

Preparation of ODC, antizyme and antizyme inhibitor

ODC was partially purified, by DEAE-cellulose chromatography and affinity chromatography with pyridoxamine phosphate–succinyl di(aminopropyl)amine–Sephacryl 4B, from livers of rats injected intraperitoneally with thioacetamide as des-

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

cribed by Hayashi & Kameji (1983). Antizyme was partially purified, by DEAE-cellulose chromatography and Sephadex G-75 gel filtration, from livers of rats injected twice intraperitoneally with 1,3-diaminopropane as described by Hayashi & Fujita (1983). Antizyme inhibitor was partially purified as described by Hayashi & Fujita (1983) by DEAE-cellulose chromatography and affinity chromatography with pyridoxamine phosphate-succinyl-di(aminopropyl)amine-Sephrose 4B, which was used for removing ODC from the preparation, followed by gel filtration with a Sephacryl S-200 column (2.6 cm $\times$ 93 cm) equilibrated with 50 mM-Tris/HCl, pH 7.4, containing 0.1 M-NaCl, 1 mM-dithiothreitol and 0.1 mM-EDTA (buffer A). Active fractions were pooled, concentrated with an Amicon ultrafiltration apparatus with a PM 10 membrane, dialysed against 25 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol and 0.1 mM-EDTA (buffer B), and used as the preparation of antizyme inhibitor.

Partial purification of ODC inhibitor from livers of starved rats

Eight male rats were starved overnight and killed by decapitation at 11:00 h. All subsequent procedures were performed at 0–4°C. Livers were homogenized with 3 vol. of 0.2 M-sucrose containing 1 mM-dithiothreitol in a Dounce-type all-glass homogenizer. The homogenate was centrifuged at 105 000 g for 60 min, and the supernatant was applied to a DEAE-cellulose column (3.2 cm $\times$ 23 cm) equilibrated with 25 mM-Tris/HCl, pH 7.4, containing 0.1 mM-EDTA. The column was washed with the equilibrating buffer and 0.1 M-NaCl in the same buffer, successively. ODC inhibitor was eluted with 280 ml of buffer B containing 0.4 M-NaCl and concentrated with an Amicon ultrafiltration apparatus with a PM 10 membrane. The concentrate was applied to a column (3 cm $\times$ 65 cm) of Sephadex G-75 equilibrated with buffer A. Active fractions were pooled, concentrated by ultrafiltration, dialysed against buffer B, and used as the preparation of ODC inhibitor.

Preparation of columns of anti-ODC-Sephrose 4B and ODC-anti-ODC-Sephrose 4B

A hybridoma cell line, HODC-1-3C, secreting a monoclonal antibody against rat liver ODC, was recently obtained in our laboratory (S. Matsufuji, R. Kanamoto & S. Hayashi, unpublished work). The antibody was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (0–40% saturation) from ascites fluid of mice inoculated with the hybridoma cells and coupled to CNBr-activated Sepharose 4B in 0.1 M-NaHCO₃ buffer, pH 9.0 (0.55 mg of protein/ml of gel). Columns (0.1–0.2 ml) of the antibody-Seph-

rose 4B were washed with 1 M-Tris/HCl, pH 7.4, containing 1 M-NaCl and equilibrated with buffer B containing 0.2 M-NaCl. Crude liver extract (10.2 ml) of thioacetamide-treated rats containing 485 units of ODC was applied to the column. The column was washed with 1.7 ml of buffer B containing 0.2 M-NaCl and then with 2.0 ml of buffer B containing 1 M-NaCl. This column, which had adsorbed 405 units of ODC, was equilibrated with buffer B containing 0.2 M-NaCl and used as the ODC-anti-ODC-Sephrose 4B column.

Assays of ODC and antizyme activities

Procedures for assays of ODC and antizyme activities were as described by Hayashi & Kameji (1983) and Hayashi & Fujita (1983), except that the concentrations of dithiothreitol and pyridoxal phosphate in the reaction mixture were 5 mM and 0.1 mM respectively, and that bovine serum albumin was replaced with 0.1% Tween 80 in the reaction mixture. One unit of ODC activity was defined as the amount forming 1 nmol of CO₂/h. One unit of antizyme activity was defined as the amount inhibiting one unit of ODC activity. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Presence of inhibitory activity to ODC in livers of starved rats

Cytosol fractions of livers of starved rats had a weak inhibitory activity towards ODC, even after being dialysed. Fig. 1 shows the inhibitory activity of four liver cytosols each against various amounts of ODC. The result indicates that the extracts contained at least two kinds of inhibitors, one inhibiting a constant proportion of enzyme activity ($16.6 \pm 2.2\%$ /ml of extract) and the other inhibiting a constant amount of enzyme activity (3.44 ± 0.36 units/ml of extract). The inhibition by these cytosols was partially reversed by addition of antizyme inhibitor (results not shown). This finding suggests that, of the two kinds of inhibitors, the latter was antizyme.

To confirm the above observation, we partially purified the antizyme-like inhibitor from the liver cytosol by DEAE-cellulose chromatography and Sephadex G-75 gel filtration as described in the Experimental section and examined it by various criteria for antizyme, as follows.

Molecular weight of the inhibitor

Fig. 2 shows the gel-filtration pattern of the inhibitor (Fig. 2b) compared with that of antizyme induced by treatment of rats with 1,3-diaminopropane (Fig. 2a), indicating that the apparent molecular weight of the inhibitor is about the same

Fig. 1. Titration experiment with ODC and liver extracts of starved rats

Four rats (body wt. 280–300g) starved for 24 h were killed at 11:00 h. Liver cytosols were prepared as described in the Experimental section and dialysed against buffer B. Various amounts of partially purified ODC were assayed in the presence or in the absence of 1 ml of each liver cytosol (34.8 ± 2.3 mg of protein) without any preincubation. ODC activity assayed in the presence of the extracts was corrected for endogenous ODC activity of the extracts (approx. 0.1 unit) and plotted against the activity of partially purified ODC added. Values are means $\pm$ s.d. for four extracts. Broken line indicates ODC activity without any inhibition. The procedure for partial purification of ODC was described in the Experimental section.

as that of antizyme, which is 25000 (Heller *et al.*, 1976; Fujita *et al.*, 1983).

Kinetic properties of the inhibitor

As shown in Fig. 3(a), the partially purified inhibitor caused an inhibition of almost the same amount of ODC activity irrespective of the amounts of the enzyme in the reaction mixture within the range tested. The inhibition against the smallest amount of ODC seems to be less than with others. Such non-linearity of the inhibition was usually observed in the titration experiments of ODC with antizyme in a range beyond 75% inhibition (Fong *et al.*, 1976). Fig. 3(b) shows that the enzymic reaction of ODC proceeded linearly with time for at least 60 min either in the presence or in the absence of the inhibitor, indicating that the inhibition was time-independent. The results show that the inhibitor, like antizyme (Fong *et al.*, 1976;

Fig. 2. Gel filtrations of antizyme (a) and the inhibitor (b)

Antizyme and the inhibitor were each partially purified by DEAE-cellulose chromatography as described in the Experimental section. Then 2 ml of antizyme or the inhibitor was applied to a column (1.1 cm $\times$ 73 cm) of Sephadex G-75 equilibrated with buffer A, and eluted with buffer A at a flow rate of 20 ml/h. Recovery of the antizyme activity in the gel filtration was 65%. Standard proteins used were ovalbumin (Ov, M_r 43000), chymotrypsinogen (Ch, M_r 25000) and ribonuclease A (Rn, M_r 13700).

Heller *et al.*, 1976), is not an enzyme attacking ODC, but a protein that stoichiometrically binds to ODC to inactivate it.

Formation of ODC-inhibitor complex

Affinity binding to a column of ODC-anti-ODC-Sepharose 4B was studied to examine whether the inhibitor, like antizyme, forms a complex with ODC. Beforehand, we confirmed that authentic antizyme was adsorbed to the ODC-anti-ODC-Sepharose 4B column and eluted with buffer containing 1M-NaCl (results not shown). As shown in Table 1, although the cytoplasmic inhibitor was not adsorbed to the anti-ODC-Sepharose 4B column, it was adsorbed to

Fig. 3. Kinetic properties of the inhibitor

(a) Inhibitor activity against various amounts of ODC. Different amounts of ODC were assayed in the presence (●) or in the absence (○) of a fixed amount of the inhibitor without any preincubation. (b) Effect of the inhibitor on the rate of the catalytic reaction of ODC. ODC was assayed in the presence (●) or in the absence (○) of the inhibitor for the indicated periods. Values are means $\pm$ s.d. for three determinations. Procedures for partial purification of ODC and the inhibitor and for assay of ODC activity are described in the Experimental section.

ODC-anti-ODC-Sepharose 4B and eluted with buffer B containing 1 M-NaCl. This result indicates that the inhibitor forms a complex with ODC, and

Table 1. Adsorption of the inhibitor to ODC-anti-ODC-Sepharose 4B

In Expt. 1 the inhibitor was applied to a column of anti-ODC-Sepharose 4B. The column was washed with buffer B containing 0.2 M-NaCl. Effluent was dialysed against buffer B and assayed for the inhibitor activity. In Expt. 2, the inhibitor was filtered through a column of ODC-anti-ODC-Sepharose 4B three times at a flow rate of 4 ml/h. The column was washed with buffer B containing 0.2 M-NaCl and 0.1% Brij-35. The adsorbed inhibitor was eluted with buffer B containing 1 M-NaCl and 0.1% Brij-35 and assayed for the inhibitor activity. Procedures for partial purification of the inhibitor and preparation of two kinds of affinity gel are described in the Experimental section. One unit of the inhibitor activity was defined as the amount inhibiting one unit of ODC activity.

Expt. no.	Inhibitor activity (units)		
	Applied	Non-adsorbed	Eluted
1	3.66	3.63	
2	15.4	3.8	11.9

Table 2. Suppression of the inhibitor activity by antizyme inhibitor and heat treatment

ODC was assayed in the presence of the indicated effectors without any preincubation; 7.1 units of antizyme inhibitor were used where indicated. Heat treatment was at 55°C for 30 min. Procedures for partial purification of ODC, antizyme, the inhibitor and antizyme inhibitor are described in the Experimental section. Values are means $\pm$ s.d. for three determinations.

Addition	ODC activity (units)
None	4.54 $\pm$ 0.07
Inhibitor	2.16 $\pm$ 0.03
Inhibitor + antizyme inhibitor	4.53 $\pm$ 0.11
Inhibitor after heat treatment	4.52 $\pm$ 0.00
Antizyme	2.84 $\pm$ 0.03
Antizyme + antizyme inhibitor	4.45 $\pm$ 0.04

dissociates from it in the presence of 1 M-NaCl, as antizyme does.

Inactivation by antizyme inhibitor

As described by Fujita *et al.* (1982), antizyme inhibitor does not affect the activity of free ODC, but re-activates ODC that had been inactivated by antizyme. Table 2 shows the effects of antizyme inhibitor on the activity of the partially purified inhibitor and on that of antizyme. The antizyme inhibitor reversed the inhibition of ODC activity caused by the inhibitor, as well as that caused by antizyme. Table 2 also shows that the inhibitor was labile to heat treatment at 55°C.

Table 3. *Presence of ODC-antizyme complex in liver of starved rats*

Rats (body wt. 240–250 g) were killed at 12 : 10 h after starvation for 23 h. The extracts were prepared as described in the Experimental section and dialysed against buffer B containing 0.1% Tween 80. ODC activity in the presence or in the absence of antizyme inhibitor was measured by duplicate assay as described in the Experimental section, except that the concentration of pyridoxal phosphate in the reaction mixture was 0.5 mM. All the differences in duplicate assays were less than 2% of mean values. The values were corrected for ODC inhibition of non-antizyme type (Fig. 1). The amount of ODC-antizyme complex was determined as the increase in ODC activity by addition of antizyme inhibitor.

Liver extract	ODC activity (munits/mg of protein)		Amount of ODC-antizyme complex (munits/mg of protein) (b-a)
	In the absence of antizyme inhibitor (a)	In the presence of antizyme inhibitor (b)	
Rat 1	34.7	81.1	46.4
Rat 2	32.1	78.5	46.4
Rat 3	28.1	88.8	60.7
Rat 4	23.2	66.5	43.3

These results together clearly identified the inhibitor as ODC antizyme.

Activities of antizyme and ODC in the livers of starved rats

Since antizyme acts irrespective of the amounts of ODC, the amounts of free antizyme in liver cytosols of starved rats were estimated to be 99 ± 10 munits/mg of protein (mean $\pm$ S.D.) from the ordinate intercepts in Fig. 1. The value is 8–10% of the antizyme activity maximally induced by putrescine (Heller *et al.*, 1976) and roughly corresponds to the basal hepatic ODC activity of rats fed *ad libitum* (Noguchi *et al.*, 1979). ODC activity in the same extracts was extremely low (3.1 ± 0.3 munits/mg of protein), about 0.2% of the enzyme activity induced by feeding (Noguchi *et al.*, 1979).

Presence of ODC-antizyme complex in the livers of starved rats

Since the presence of antizyme suggests the formation of inactive ODC-antizyme complex, we measured the amount of the complex in the liver cytosols of starved rats by using antizyme inhibitor, which can re-activate ODC of the inactive complex (Fujita *et al.*, 1982). As shown in Table 3, small but significant amounts of ODC-antizyme complex were detected in such extracts.

Discussion

This study firmly demonstrated the presence of a small amount of antizyme and ODC-antizyme complex in liver cytosols of starved rats. Great care should be taken before identifying an ODC inhibitor as antizyme, since ODC activity could be affected by various macromolecular inhibitors, such as proteinases, phosphatases and ODC-in-

activating factor (Icekson & Kaye, 1976), as well as by several low-molecular-weight compounds such as NaCl and KCl (Obenrader & Prouty, 1977), ornithine and arginine, which may be easily converted into ornithine in liver extracts (Hayashi & Kameji, 1983). In the present study the identification was based on the following evidence. (1) The inhibitor is a heat-labile macromolecule of a molecular weight similar to that of antizyme (25000). (2) It acts in a time-independent and stoichiometric manner. (3) It forms a complex with ODC and dissociates from it in the presence of 1 M-NaCl. (4) Its action on ODC can be reversed by antizyme inhibitor.

It has been reported that a small amount of inactive antizyme was bound to subcellular particles of uninduced H-35 cells and rat liver (Heller *et al.*, 1977) and HTC and HMO_A cells (McCann *et al.*, 1979) and that active antizyme could be released from the particles *in vitro* by treatment with putrescine. Clarification of the physiological function of the bound antizyme awaits future studies, including its exact identification.

Antizyme is known to be degraded rapidly *in vivo* with a half-life of 24 min (Heller *et al.*, 1976). Therefore its presence in the liver of starved rats suggests that synthesis of antizyme *de novo* takes place in such liver, where intracellular putrescine concentration is about one-fifth of that of fed rats (Kameji *et al.*, 1979).

Our previous results (Fujita *et al.*, 1982) showed that a much smaller amount of ODC-antizyme complex than expected was present in liver cytosols of rats treated with an exogenous diamine. This suggests that ODC-antizyme is extremely unstable *in vivo*. The present study revealed the existence of ODC-antizyme complex in the liver of starved rats (about one-fifth of that found in the livers of the diamine-treated rats). This suggests

that ODC synthesis also takes place at a significant rate under starved conditions, even when hardly any ODC activity is detected there.

The presence of antizyme and ODC-antizyme complex in the liver of starved rats without any treatment with exogenous diamines or polyamines strongly suggests that antizyme is a physiological factor involved in the regulation of ODC activity even at low intracellular putrescine concentration. Future studies are needed to clarify the exact role and action mechanism of antizyme in the process of ODC regulation.

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