Accumulation of ornithine decarboxylase–antizyme complex in HMO_A cells

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A new method was developed for the assay of ornithine decarboxylase (ODC)–antizyme complex, in which α -difluoromethylornithine (DFMO)-inactivated ODC was used to release active ODC competitively from the complex. ODC–antizyme complex was present in the extracts of hepatoma tissue-culture (HTC) cells and of ODC-stabilized variant HMO_A cells, in much larger amounts in the latter. Cellular amounts of the complex fluctuated after a change of medium in a similar manner in HTC and HMO_A cells, increasing during the period of ODC decay. After treatment with cycloheximide, the decay of ODC–antizyme complex in HMO_A cells was more rapid than the decay of free ODC, but it was much slower than the decay of free ODC or complexed ODC in HTC cells. Administration of putrescine caused a rapid increase in the amount of ODC–antizyme complex in both HTC and HMO_A cells, but nevertheless the decay of total ODC (free ODC plus ODC–antizyme complex) was more rapid with putrescine than with cycloheximide. These results suggested the possibility that ODC is degraded through complex-formation with antizyme. In contrast with complexed antizyme, free antizyme was not stabilized in HMO_A cells.

A variant clone of hepatoma tissue-culture (HTC) cells designated HMO_A has been isolated and characterized by Mamont *et al.* (1978). The HMO_A cells, which are resistant to α -methylornithine, have an elevated activity of ODC, with a concomitant increase in putrescine content (Mamont *et al.*, 1978). The half-life of ODC is much increased compared with that in HTC cells (McCann *et al.*, 1979; Pritchard *et al.*, 1982). The alteration in turnover rate seems to be specific to ODC, since it was not observed with total cellular proteins, S-adenosylmethionine decarboxylase or tyrosine aminotransferase (Pritchard *et al.*, 1982). Another difference between HTC and HMO_A cells is their sensitivity to putrescine: 10 μ M exogenous putrescine effected formation of inactive ODC–antizyme complex in the parent HTC cells, but not in HMO_A cells, and a much higher concentration of putrescine (10 mM) was required for the formation of the complex in HMO_A cells (McCann *et al.*, 1979). These facts suggested the possibility that the delay in ODC turnover in HMO_A cells may have resulted from deficiency of antizyme, a unique regulatory protein which is induced by diamines or polyamines and specifically binds to and inactivates ODC (Heller *et al.*, 1976; Canellakis *et al.*,

1979). In the present paper we determined the amount of inactive ODC–antizyme complex in HTC and HMO_A cells by a newly devised method with DFMO–ODC to examine this possibility. Our results showed that the inactive ODC complex accumulated in HMO_A cells under physiological conditions.

Experimental

Materials

DL-[1-¹⁴C]Ornithine (sp. radioactivity 59 Ci/mol) was obtained from New England Nuclear Corp. DFMO was kindly given by Dr. K. Samejima (Tokyo Biochemical Research Institute, Tokyo, Japan). Stocks of HTC and HMO_A cells were kindly provided by Dr. P. P. McCann of Merrell Research Center, Cincinnati, OH, USA.

Preparation of ODC, antizyme and DFMO–ODC

ODC was partially purified by DEAE-cellulose column chromatography from livers of rats injected intraperitoneally with thioacetamide as described previously (Kameji *et al.*, 1982). 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 previously

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

(Hayashi & Fujita, 1983). ODC inactivated by covalent binding of DFMO was prepared by incubating partially purified rat liver ODC (300 units/mg of protein) with DFMO (20 μ M) at 37° for 2 h and dialysed extensively against 25 mM-Tris/HCl, pH 7.4, containing 10 μ M-pyridoxal phosphate and 1 mM-dithiothreitol.

Assays

ODC and antizyme activities. ODC was assayed as described previously (Murakami *et al.*, 1981). Since NaCl inhibits ODC activity, control assay mixture was adjusted to contain the same salt concentration as in test assays. One unit of ODC activity is defined as the amount releasing 1 nmol of CO₂ from ornithine/h at 37°C. For antizyme assay a sample was added to an assay mixture for ODC containing a constant amount of partially purified ODC. The mixture was preincubated for more than 5 min at 0°C, and then the remaining ODC activity was assayed by addition of substrate. One unit of antizyme activity is defined as the amount inhibiting 1 unit of ODC activity.

Antigenic activity of ODC. Antibodies were produced in rabbits to homogeneous rat liver ODC (Kameji *et al.*, 1984) and partially purified by (NH₄)₂SO₄ fractionation (0–40% saturation); 1 mg of protein in this preparation caused precipitation of 0.63 μ g of rat liver ODC in the presence of bacterial protein A adsorbent. To assess the amount of ODC antigen in a cell extract, the Ab₅₀, defined as the amount of monospecific antibody required to inactivate 50% of the enzyme activity in that extract, was measured as described by Obenrader & Prouty (1977).

High-performance gel-filtration chromatography

Analysis of free antizyme. An 0.5 ml of sample was applied to a column (7.5 mm $\times$ 600 mm) of TSKgel G3000SW (Toyo Soda), which had been equilibrated with 25 mM-Tris/HCl buffer, pH 7.2, containing 0.01% Tween 80, 0.05 M-NaCl and 1 mM-dithiothreitol, and the column was eluted with the same buffer in 0.43 ml fractions. The flow rate was approx. 30 ml/h.

Analysis of ODC–antizyme complex. An 0.5 ml cell extract containing 1 M-NaCl was applied to a column and fractionated under the same conditions as for analysis of free antizyme, except that the concentration of NaCl was 1 M and 0.37 ml fractions were collected.

Separation of free and complexed antizyme by immunoprecipitation

Antizyme (17 units) was incubated for 5 min at 0°C in the presence or in the absence of native ODC (26 units) or DFMO–ODC (26 units) in a

mixture (0.45 ml) containing 55 μ M-pyridoxal phosphate, 6.9 mM-dithiothreitol, 55 mM-Tris/HCl and 0.0055% Tween 80, and then 0.1 ml of anti-ODC antibody (54 μ g of protein) was added. After an incubation for 1 h at 0°C, 0.1 ml of 10% (w/v) suspension of formalin-fixed *Staphylococcus aureus* cells in a buffer containing 50 mM-Tris/HCl, 0.15 M-NaCl, 5 mM-EDTA, 0.05% (v/v) Nonidet P-40 and 0.02% (w/v) NaN₃, pH 7.4, was added to each mixture, which was further incubated overnight at 0°C and then centrifuged at 3500g for 30 min at 4°C. The precipitate was washed with 0.3 ml of 40 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-dithiothreitol, 40 μ M-pyridoxal phosphate and 0.005% Tween 80. The combined supernatant was assayed for free antizyme activity. The washed precipitate was again resuspended by vortex-mixing in 0.2 ml of 25 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-dithiothreitol, 1 M NaCl and 0.01% Tween 80 and incubated at 0°C for 30 min. The suspension was then centrifuged as described above and the supernatant was assayed for the amount of antizyme that had been complexed with ODC.

Cell culture, ODC induction and preparation of cell extract

Cells were grown either as a suspension culture (only in the experiment shown in Fig. 1) in Swim's 77 medium (Gibco) containing 11.1 mM-glucose, 5.9 mM-NaHCO₃, 50 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, 2 mM-glutamine and 10% (v/v) newborn-calf serum (Gibco) or on 100 mm $\times$ 20 mm polystyrene Petri dishes (Corning) (all other experiments) in the same medium supplemented with 1 mM-pyruvate and 1.8 mM-CaCl₂. In suspension culture, ODC activity was induced by diluting cells with fresh medium to a density of 1.5×10^5 – 1.7×10^5 cells/ml. After incubation for the indicated time, samples containing approx. 3×10^6 cells were centrifuged at 1600g for 5 min at 4°C, washed three times with ice-cold phosphate-buffered saline (0.17 M-NaCl, 3.4 mM-KCl, 10 mM-Na₂HPO₄, 1.8 mM-KH₂PO₄, pH 7.2) and resuspended in 1 ml of 0.25 M-sucrose containing 1 mM-dithiothreitol. The cells were lysed by two cycles of freeze–thawing. The suspension of disrupted cells was centrifuged at 30000g for 40 min, and 0.4–0.8 ml of the supernatant was assayed for ODC activity. In monolayer culture ODC activity was induced either by simply replacing growth medium of a confluent monolayer of cells with fresh medium or by diluting the cells into fresh medium: confluent cells were shaken off a dish after trypsin treatment and plated (2×10^5 – 3×10^5 cells/ml in Swim's 77 growth medium containing glucose, NaHCO₃, Tricine, glutamine, pyruvate, CaCl₂ and newborn-calf serum; total volume 10 ml). After incubation for the indicated time, the

plate was rinsed three times with ice-cold phosphate-buffered saline, and 1 ml of 0.25 M-sucrose containing 1 mM-dithiothreitol was added to each plate. The cells were lysed by two cycles of freeze-thawing. The suspension of disrupted cells was transferred to a centrifuge tube with a rubber policeman and a Pasteur pipette, and centrifuged at 30000g for 40 min; 0.2–0.4 ml of the supernatant was assayed for ODC activity.

Results

Decay of ODC activity and ODC protein

Seely & Pegg (1983) have shown that their antibody produced in rabbits to homogeneous mouse kidney ODC could bind inactive ODC-antizyme complex in their radioimmunoassay, since an addition of antizyme to ODC *in vitro* did not affect the

amount of immunoreactive ODC protein detected. We first confirmed that our antibody to rat liver ODC also bound inactive ODC-antizyme complex prepared *in vitro* by showing that the antigenic activity, Ab₅₀, was identical for free ODC and inactive ODC-antizyme complex (result not shown). We then compared the time course for loss of ODC activity and ODC protein after a treatment with putrescine. As shown in Fig. 1, the decay of ODC protein was clearly slower than that of ODC activity in both HTC and HMO_A cells. This result suggested that ODC-antizyme complex may be stable and may accumulate in these hepatoma cells. Therefore, if any ODC-antizyme complex is formed under physiological conditions, it is expected to be detectable in these cells.

Determination of ODC-antizyme complex by DFMO-ODC

We have shown the presence of a macromolecular factor (antizyme inhibitor) in rat liver that inhibits the antizyme activity to ODC (Fujita *et al.*, 1982). Antizyme inhibitor re-activates ODC that has been inactivated by antizyme and therefore can be used to assay the amount of ODC-antizyme

Fig. 1. Decay of ODC activity and immunoreactive protein caused by putrescine in HTC cells and HMO_A cells. Cells grown to maximum density in suspension culture were diluted into fresh medium 16 h before addition of putrescine (10 mM). Extracts were prepared from samples of cells at the indicated times, and ODC activity and the immunoreactive ODC protein were assayed as described in the Experimental section.

Fig. 2. Re-activation of ODC-antizyme complex by DFMO-ODC

The indicated amounts of DFMO-ODC were added to a series of flasks containing free ODC (3.08 units) and ODC-antizyme complex (7.43 units), prepared by mixing partially purified preparation of ODC (10.42 units) and antizyme (7.34 units). After being left at 0°C for 30 min, the mixtures were assayed for ODC activity. Since about 4% of ODC was not inactivated by DFMO during the preparation of DFMO-ODC, this residual ODC activity was subtracted from the actual increase in ODC activity to obtain the experimental increase in ODC activity (●), and it was included in total ODC in calculating theoretical increase in ODC activity (○). Values shown in parentheses are percentages of theoretical.

complex. However, the content of antizyme inhibitor in rat liver was small, and its activity was rather labile. Therefore, we developed another method for the routine assay of the complex, in which DFMO-ODC was used instead of antizyme inhibitor to re-activate the complex. The equilibrium constant of the reaction between antizyme and ODC has been shown to be as high as $1.4 \times 10^{11} \text{ M}^{-1}$ (Kitani & Fujisawa, 1984b). If inactive DFMO-ODC competes with native ODC for antizyme, the amount of ODC-antizyme complex is expected to be determined by a competitive assay. Thus, when DFMO-ODC is added to a mix-

ture of free ODC and ODC-antizyme complex, it would replace some of ODC in the complex, and an increase in ODC activity would be observed, depending on the ratio of amount of DFMO-ODC to that of total ODC (sum of free ODC, complexed ODC and DFMO-ODC) and on the relative affinities of DFMO-ODC and native ODC for antizyme. Assuming that DFMO-ODC and native ODC have an equal affinity for antizyme and that all antizyme molecules exist as complexed form in the mixture, the theoretical increase in ODC activity would be given as follows:

$$\Delta \text{ODC} = \frac{(\text{DFMO-ODC}) \cdot (\text{complexed ODC})}{(\text{free ODC}) + (\text{complexed ODC}) + (\text{DFMO-ODC})} \quad (1)$$

Fig. 3. Disappearance of free antizyme after incubation with ODC or DFMO-ODC

Antizyme (68 units) was incubated at 0°C with or without ODC (104 units) or DFMO-ODC (104 units) for 30 min in 25 mM-Tris/HCl buffer, pH 7.2, containing 50 mM-NaCl, 1 mM-dithiothreitol and 0.01% Tween 80 in a final volume of 0.6 ml. A portion (0.5 ml) of the mixture was then applied to the column of TSKgel G3000SW (7.5 mm × 600 mm), and the column was eluted with the same buffer in 0.43 ml fractions. A 0.2 ml sample of each fraction was assayed for ODC activity in the presence of 13.0 units of partially purified ODC.

Fig. 4. Chromatography of NaCl-treated extract from HMO_A cells

A cell extract was prepared from HMO_A cells 20 h after cell dilution in the same manner as for ODC assay, except that sucrose and dithiothreitol were not added. Cell extract (0.5 ml) from six dishes were chromatographed on TSKgel G3000SW (7.5 mm × 600 mm) in the presence of 1 M-NaCl; 0.37 ml fractions were collected, and a 0.2 ml portion of each fraction was assayed for ODC activity in the presence of 12.0 units of partially purified ODC. Standard proteins (positions indicated by arrows) were eluted under the same conditions: 1, bovine serum albumin (M_r 67500); 2, ovalbumin (M_r 43000); 3, cytochrome c (M_r 12400).

Table 1. Co-precipitation of antizyme with DFMO-ODC-anti-ODC immunoprecipitation

Antizyme (17.0 units) was incubated alone, with ODC (26.0 units) or with DFMO-ODC (26.0 units) in the presence of antibody to ODC (54 µg of protein) and formalin-fixed *Staph. aureus* cells. After centrifugation, the supernatant was assayed for antizyme activity. Extract from the immunoprecipitate with 1 M-NaCl was also assayed for antizyme activity as described in Experimental section. Results are means ± S.E.M. for three experiments.

	Antizyme activity (units)	
	Supernatant after immunoprecipitation	NaCl extract from immunoprecipitate
Antizyme	16.0 ± 0.2	0.4 ± 0.2
ODC + antizyme	3.4 ± 0.4	7.5 ± 0.4
DFMO-ODC + antizyme	4.1 ± 0.2	6.4 ± 0.4

Table 2. ODC activity and amounts of ODC-antizyme complex in extracts of HTC and HMO_A cells

ODC activity was induced either by cell dilution or by change of medium of confluent monolayers of cells as described in the Experimental section. Cells were harvested 20h after cell dilution or 4h after change of medium. Cell extracts were prepared and assayed for ODC activity in the presence and in the absence of DFMO-ODC. The amount of ODC-antizyme complex was calculated as described in the text. Results are averages of seven experiments for HMO_A cells after change of medium, or four experiments for others. Ranges of values are in parentheses.

	20h after cell dilution		4h after change of medium	
	HTC cells	HMO _A cells	HTC cells	HMO _A cells
(A) Free ODC (units/mg of protein)	4.8 (4.0–5.9)	16.5 (13.1–19.0)	9.8 (4.0–16.8)	25.8 (8.9–40.8)
(B) Complexed ODC (units/mg of protein)	2.0 (1.8–2.2)	9.4 (6.8–12.7)	0.46 (0.04–1.05)	4.0 (2.2–6.9)
B/A	0.42 (0.38–0.48)	0.58 (0.41–0.84)	0.067 (0.002–0.15)	0.19 (0.07–0.37)

Fig. 2 shows both the theoretical and experimental increases in ODC activity obtained by adding increasing amounts of DFMO-ODC to mixtures containing fixed amounts of free ODC and ODC-antizyme complex in a constant final volume. The experimental curve is very close to the theoretical one, indicating that DFMO-ODC and native ODC have a nearly equal affinity for antizyme.

Kyriakidis *et al.* (1984) have reported the lack of interaction of antizyme with DFMO-ODC. Therefore, we further examined the affinity of DFMO-ODC for antizyme. Antizyme was incubated either alone or with about 1.5 equivalents of ODC or DFMO-ODC, and then remaining free antizyme was analysed by high-performance gel filtration. As shown in Fig. 3, almost all the free antizyme originally present disappeared when mixed with excess ODC, and about 86% of free antizyme disappeared when mixed with excess

DFMO-ODC. This result indicated that antizyme could interact with DFMO-ODC in almost identical manner as with native ODC. Next, antizyme was incubated either alone or with excess ODC or DFMO-ODC, and then treated with antibody to ODC and formalin-fixed *Staph. aureus* cells. The mixture was centrifuged and the supernatant was assayed for free antizyme activity. As shown in Table 1, 94% of antizyme activity originally present was recovered in the supernatant when antizyme alone had been incubated. However, only 20 or 24% of antizyme activity was recovered in the supernatant when it had been incubated with ODC or DFMO-ODC, respectively, and antizyme bound to immunoprecipitate could be partially (50–55%) recovered by extraction with 1 M-NaCl (Table 1). These results indicated that DFMO-ODC and native ODC have a nearly equal affinity for antizyme. Therefore, an approximate amount of ODC-antizyme complex can be calculated by eqn. (2), derived from eqn. (1):

$$\text{Complexed ODC} = \frac{(\text{DFMO-ODC}) + (\text{free ODC})}{(\text{DFMO-ODC}) - (\Delta\text{ODC})} \cdot (\Delta\text{ODC}) \quad (2)$$

Fig. 5. Changes in ODC activity and the amount of ODC-antizyme complex in HTC cells and HMO_A cells after change of medium

Cells were plated at about 10^6 per 100 mm dish and grown to an almost confluent state, and then the growth medium was replaced with fresh medium. Cell extracts were prepared at the indicated times after the change of medium, and ODC activity was determined in the presence and in the absence of DFMO-ODC, and amounts of ODC-antizyme complex were calculated as described in the text. Data were results of a single experiment. Similar results were obtained in one (HTC cells) or two (HMO_A cells) other experiments.

Presence of ODC-antizyme complex in extracts of HTC and HMO_A cells

ODC activity was induced either by diluting cells with fresh medium or by replacing growth medium as described in the Experimental section. Cells were harvested at 4h after the change of medium or 20h after the cell dilution, and cell extracts were assayed for ODC activity in the presence and in the absence of DFMO-ODC (50 units/assay mixture). The amounts of ODC-antizyme complex were calculated from the data by eqn. (2). When ODC was induced by cell dilution, fairly large amounts of ODC-antizyme complex were found in both HTC and HMO_A cells, as shown in Table 2. On the other hand, when ODC was induced by a change of medium of a confluent monolayer of cells, the amount of ODC-antizyme complex showed considerable variation from experiment to experiment, but it was always detected in HMO_A cells and almost always in HTC cells, too (Table 2). The average ratio of complexed ODC to free ODC was higher in HMO_A cells (Table 2).

We also determined ODC-antizyme complex by using antizyme inhibitor, and found that in an extract of HMO_A cells 20h after cell dilution the ratio of amounts of complexed to free ODC was 0.75:1. This was close to the value, 0.65:1, determined by using DFMO-ODC.

ODC-antizyme complex can be dissociated with high salt concentration and the components resolved on gel filtration (Heller *et al.*, 1976; McCann *et al.*, 1977). An extract from HMO_A cells was analysed by gel filtration. As shown in Fig. 4,

after almost all of ODC was eluted as a monomer (Kitani & Fujisawa, 1984a), antizyme activity was eluted from the column at a position approximately corresponding to a M_r of 26 700, which was close to the reported M_r of 26 500 of antizyme isolated from a variety of cells (Heller *et al.*, 1976). Approx. 57.4 units of ODC and 17.4 units of antizyme activity were recovered from original 49.0 units of ODC and 29.3 units of ODC-antizyme complex determined with DFMO-ODC. Assuming that the complex had been completely dissociated under those conditions, the recovery rates of ODC and antizyme activities were calculated to be 73% and 59%, respectively, whereas this gel filtration normally recovers approx. 85% of ODC and 70% of antizyme activities. These results therefore confirmed the presence of ODC-antizyme complex in the cell extract under physiological conditions and supported the validity of the DFMO-ODC method for assay of ODC-antizyme complex.

Changes in ODC activity and in the amount of ODC-antizyme complex in cells after a change of medium

Growth media of confluent monolayers of HTC cells and HMO_A cells were replaced with fresh media, and both the ODC activity and the amount of inactive ODC-antizyme complex in the cells were determined over 30h period. In HTC cells (Fig. 5a), the amount of ODC-antizyme complex was very small at the time of change of medium, but it began to increase after ODC activity had reached a peak at 4h, and the maximum amount of the complex was found at around 12h. In HMO_A cells (Fig. 5b), which had an elevated ODC acti-

Fig. 6. Changes in ODC activity, amount of ODC–antizyme complex and total ODC after treatment with cycloheximide or putrescine in HTC (a) and HMO_A (b) cells

Growth medium of confluent cells was changed with fresh medium 4 h before addition of cycloheximide (CH; 50 µg/ml) or putrescine (Put; 10 mM) at zero time. Cell extracts were prepared at the indicated times and assayed for ODC activity in the presence and in the absence of DFMO–ODC. The amounts of ODC–antizyme complex were calculated as described in the text. Data were the results of a single experiment. Similar results were obtained in three (HTC cells) or two (HMO_A cells) other experiments. Δ , Free ODC after cycloheximide; $\square$, complexed ODC after cycloheximide; $\circ$, total ODC after cycloheximide; $\blacktriangle$, free ODC after putrescine; $\blacksquare$, complexed ODC after putrescine; $\bullet$, total ODC after putrescine.

vity, a fairly large amount of ODC–antizyme complex was present even at the time of change of medium, and it increased after ODC activity had reached a peak at 4–6 h, exhibiting a broad and high peak at 8–18 h.

Changes in both ODC activity and the amount of ODC–antizyme complex were measured after addition of either cycloheximide or putrescine at around the peak of ODC activity. With a short time lag, ODC activity decayed more rapidly with putrescine than with cycloheximide in both HTC and HMO_A cells (Fig. 6). In HTC cells, the amount of complex was very low at zero time, and it began to increase immediately after the addition of putrescine. Nevertheless, except in the lag period the decay of total ODC (sum of free ODC and complexed ODC) was more rapid with putrescine than with cycloheximide (Fig. 6a). In HMO_A cells, the decay of ODC–antizyme complex was more rapid than the decay of ODC activity after treatment with cycloheximide, although it was much slower than the decay of ODC activity or complexed ODC in HTC cells. When HMO_A cells were treated with putrescine, ODC activity fell rapidly, with a concomitant increase in complexed ODC, and then complexed ODC began to decrease with time. The decay of total ODC was more rapid with putrescine than with cycloheximide. Furthermore, it

was noted that the decay of total ODC after treatment with putrescine was more rapid than the decay of complexed ODC after treatment with cycloheximide (Fig. 6b). These effects of putrescine were not observed when cycloheximide was added simultaneously (results not shown).

Half-life of antizyme in HTC cells and HMO_A cells

Antizyme was induced by putrescine, as described in the Experimental section. The activity of antizyme fell rapidly in both HTC and HMO_A cells after treatment with cycloheximide (Fig. 7a). For comparison, the rates of loss of ODC activity and ODC–antizyme complex were also determined in the cells cultured in the absence of putrescine 20 h after cell dilution (Fig. 7b). The half-life of free antizyme in HMO_A cells was much smaller than that of ODC–antizyme complex (complexed antizyme). On the other hand, the half-life of free antizyme in HTC cells was similar to that of complexed antizyme (Figs. 7a and 7b).

Discussion

Diamines and polyamines have been shown to cause a rapid decay of ODC activity and to induce a protein inhibitor termed ‘antizyme’ in several cell lines and rat tissues (Heller *et al.*, 1976; McCann *et*

Fig. 7. Decay of antizyme activity (free antizyme) and complexed antizyme in HTC and HMO_A cells
 (a) Cells were incubated for 8 h after cell dilution and then incubated for an additional 12 h in the presence of 10 mM-putrescine. Then cycloheximide (50 μ g/ml) was added. Cell extracts were prepared at the indicated times. Lysed cell suspensions from three dishes were combined before centrifugation, and 0.8 ml of the supernatant was assayed for inhibitory activity. Inhibitory activity was determined with three different amounts of ODC, and antizyme activity was calculated from the ordinate intercept of the line obtained by the method of least squares (Fujita *et al.*, 1984). Results of three (HTC cells) or four (HMO_A cells) separate experiments were combined. Initial antizyme activities (means $\pm$ S.E.M.) were 9.2 ± 1.6 units/mg of protein for HTC cells and 8.6 ± 1.8 units/mg of protein for HMO_A cells.
 (b) Cells were incubated for 20 h after dilution. Then cycloheximide (50 μ g/ml) was added. Cell extracts were prepared at the indicated times and assayed for ODC activity in the presence and in the absence of DFMO-ODC. Amounts of ODC-antizyme complex were calculated as described in the text. Results are means $\pm$ S.E.M. for three (HTC cells) or five (HMO_A cells) separate experiments. Initial ODC activities (means $\pm$ S.E.M.) were 4.4 ± 0.2 units/mg of protein for HTC cells and 21.2 ± 4.0 units/mg of protein for HMO_A cells. Initial amounts of ODC-antizyme complex were 1.9 ± 0.1 units/mg of protein for HTC cells and 9.6 ± 1.1 units/mg of protein for HMO_A cells.

al., 1977, 1979; Pösö *et al.*, 1978; Pegg *et al.*, 1978; Seely & Pegg, 1983). Antizyme interacts with ODC to form an inactive enzyme-antizyme complex (Heller *et al.*, 1976; McCann *et al.*, 1977). It has been supposed that the enhanced decrease in enzyme activity after polyamine treatment is due both to the inhibition of ODC synthesis and to the synthesis of antizyme, followed by the formation of inactive ODC-antizyme complex (Canellakis *et al.*, 1979; McCann *et al.*, 1979). We have shown that only a small amount of ODC-antizyme complex could be detected in 1,3-diaminopropane-treated rat liver, whereas ODC activity was decreased to an almost undetectable value (Fujita *et al.*, 1982), and we have subsequently obtained similar results also in cultured hepatocytes (K. Utsunomiya, R. Kanamoto & S. Hayashi, unpublished work). These results raised the possibility that ODC-antizyme complex may be degraded rapidly. On the other hand, McCann *et al.* (1979) have demonstrated the existence *in vivo* of ODC-antizyme complex in both HMO_A and HTC cells several hours after putrescine treatment. This suggested a stabilization of the complex in hepatoma cells. Our present result with antibody also suggested it (Fig. 1). In fact, inactive ODC-antizyme complex was found to be accumulated both in

HTC and in HMO_A cells not only after putrescine treatment but also under physiological conditions, indicating physiological involvement of antizyme in ODC regulation. It is not known exactly, however, what mechanism determines the cellular amount of ODC-antizyme complex under physiological conditions. Although a contribution of endogenous putrescine is suggested by the result in Fig. 5, it will be necessary to examine the correlation between fluctuations in cellular putrescine and ODC-antizyme complex to confirm this.

As shown in Fig. 6, the decay of total ODC was more rapid with putrescine than with cycloheximide in both HTC and HMO_A cells. This suggested the possibility that antizyme not only inactivates ODC but also accelerates its degradation. Seely & Pegg (1983) also suggested the same possibility from their observation that the decay of immunoreactive ODC protein in rat liver after treatment with 1,3-diaminopropane was faster than that after cycloheximide. If antizyme plays an essential role in ODC degradation, it must be supplied not only by synthesis *de novo* but also by some other mechanism, since rapid ODC decay occurs in the presence of cycloheximide that inhibits antizyme synthesis (Fong *et al.*, 1976). Recycling of antizyme could be speculated as being such a

Scheme 1.

mechanism (Scheme 1). According to this hypothesis, the long half-life of ODC activity in HMO_A cells could be due to delayed degradation of the ODC-antizyme complex. Moreover, we often observed a slow-down in the rate of ODC decay in HMO_A cells after treatment with cycloheximide (cf. Fig. 6b). In connection with this, it is noteworthy that the half-life of free antizyme was much shorter than that of complexed antizyme in HMO_A cells (Fig. 7). This great difference in half-life between free and complexed antizyme might cause a slow-down of the supply of antizyme and consequently a slow-down of ODC decay. In any case, more quantitative studies are required to provide a definite proof for the hypothesis that antizyme mediates degradation of ODC.

Finally, the above discussions were made with the assumption that putrescine inhibits ODC synthesis (McCann *et al.*, 1979). If that assumption is not correct, then ODC degradation caused by putrescine must take place more rapidly than it appears, and the role of the antizyme in ODC regulation would be more important.

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