

Role of antizyme in degradation of ornithine decarboxylase in HTC cells

Yasuko MURAKAMI and Shin-ichi HAYASHI

Department of Nutrition, The Jikei University School of Medicine, Minato-ku, Tokyo 105, Japan

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A good correlation was observed between the reciprocal of the half-life of ornithine decarboxylase (ODC) activity in the presence of cycloheximide and the relative amount of ODC–antizyme complex to total ODC (free ODC plus complexed ODC) activity in HTC cells examined at various times after cell dilution or change of medium. Pretreatment of cells with putrescine increased the relative amount of ODC–antizyme complex and decreased the half-life of ODC decay. These results suggested that antizyme plays a key role in ODC degradation.

ODC is a rate-limiting enzyme in biosynthesis of polyamines in mammalian tissues (Tabor & Tabor, 1976). Exogenously added polyamines cause a rapid decrease in ODC activity (Jänne & Hölttä, 1974; Fong *et al.*, 1976; Pegg *et al.*, 1978; McCann *et al.*, 1979). It has been suggested that these amines act both by blocking ODC synthesis and by inducing a protein inhibitor, ‘antizyme’, which binds to ODC and inhibits its activity (Heller *et al.*, 1976; McCann *et al.*, 1979). In a previous paper we reported that exogenous putrescine transiently increased the cellular amount of ODC–antizyme complex in hepatoma tissue-culture (HTC) cells and ODC-stabilized variant HMO_A cells, and nevertheless the decay of total ODC (free ODC plus complexed ODC) activity was more rapid with putrescine than with cycloheximide, and suggested the possibility that ODC degradation is accelerated by complex-formation with antizyme (Murakami *et al.*, 1985). It is conceivable that ODC degradation under physiological conditions without exogenous putrescine is also mediated by antizyme, since both HTC and HMO_A cells contained substantial amounts of ODC–antizyme complex under these conditions, especially during the period of ODC decay (Murakami *et al.*, 1985).

In the present paper we show that the reciprocal of the half-life of ODC decay in HTC cells correlates with the relative amount of ODC–antizyme complex, suggesting a key role of antizyme in degradation of ODC.

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

Materials and methods

Materials

DL-[1-¹⁴C]Ornithine (sp. radioactivity (59 Ci/mol) was obtained from New England Nuclear Corp. Stocks of HTC cells were kindly provided by Dr. P. P. McCann of Merrell Research Center, Cincinnati, OH, U.S.A.

Cell culture and ODC induction

Cells were grown on 100mm × 20mm polystyrene Petri dishes (Corning), and ODC activity was induced either by diluting the cells into fresh medium or by simply replacing growth medium of a confluent monolayer of cells as described previously (Murakami *et al.*, 1985).

Assays

Cell extracts were prepared and assayed for ODC and antizyme activities as described previously (Murakami *et al.*, 1985). One unit of ODC activity is defined as the amount releasing 1 nmol of CO₂ from ornithine/h at 37°C. One unit of antizyme activity is defined as the amount inhibiting one unit of ODC activity. The amount of ODC–antizyme complex was determined by the competitive assay method described previously, in which α-difluoromethylornithine-inactivated ODC was used to release active ODC competitively from the complex (Murakami *et al.*, 1985). One unit of amount of ODC–antizyme complex is defined as the amount consisting of one unit each of ODC activity and antizyme activity.

Results and discussion

Relation between half-life of ODC activity and the relative amount of ODC-antizyme complex in total ODC activity

HTC cells were cultured for 2–20 h after cell dilution into fresh medium or for 2–5 h after change of medium at confluency, and then cycloheximide was added to determine the decay rate of ODC activity. Half-lives of ODC activity were plotted against the ratios of ODC-antizyme complex to total ODC determined at the time of addition of cycloheximide. The half-life of ODC activity decreased as the ratio of ODC-antizyme complex to total ODC activity increased. When the reciprocal of half-life of ODC activity was plotted against the relative amount of ODC-antizyme complex, a positive correlation was observed between them, as shown in Fig. 1. The intercept of the regression line on the ordinate was not zero. This indicated the existence of basal degradation of ODC in the absence of antizyme. A possibility remained, however, that the basal degradation is mediated by membrane-bound antizyme. Heller *et al.* (1977) and McCann *et al.* (1979) have shown the presence of membrane-bound antizyme in uninduced cells. The half-life of

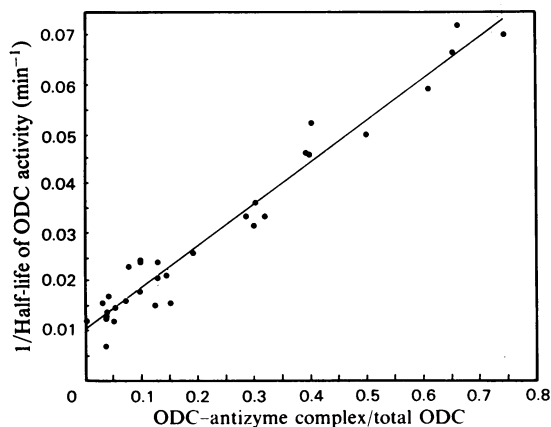


Fig. 1. Correlation between reciprocal of half-life of ODC activity and relative amount of ODC-antizyme complex in total ODC activity

HTC cells were cultured for 2–20 h after cell dilution into fresh medium or for 2–5 h after change of medium at confluency, and then cycloheximide was added (50 µg/ml). Cells were harvested at 0, 20, 40, 60 and 80 min or 0, 15 and 30 min in duplicate after the addition of cycloheximide. Cell extracts were prepared and assayed for ODC activity and the amount of ODC-antizyme complex. The half-life of ODC activity was calculated from the slope of the line, which was determined by linear regression analysis.

ODC activity in basal degradation was about 100 min, as obtained from the intercept. On the other hand, the shortest half-life of ODC activity in HTC cells was about 15 min. Thus the decay of ODC appeared to be accelerated up to 7-fold by increase in antizyme. In HTC cells the half-life of total ODC (free ODC plus complexed ODC) was about the same as that of free ODC under various conditions (Murakami *et al.*, 1985). Therefore it can be said that the half-life of decay of total ODC activity also correlates with the ratio of ODC-antizyme complex to total ODC activity.

Effect of pretreatment with putrescine on decay of ODC activity in HTC cells

The about result, together with previously reported results (Murakami *et al.*, 1985), suggested that antizyme accelerates the degradation of ODC both in the presence and in the absence of exogenous putrescine. Then we examined the effect of pretreatment with putrescine on decay of ODC activity. Cells containing high ODC activity were preincubated with putrescine for 40 min, and then cycloheximide was added to inhibit both ODC and antizyme synthesis. The pretreatment with putrescine accelerated the decay of ODC activity after addition of cycloheximide (Fig. 2). A similar effect was observed when putrescine was washed out before addition of cycloheximide (Fig. 2b). When putrescine was added simultaneously with cycloheximide, such an accelerative effect was not observed, in agreement with previous results (Fig. 2a) (Pösö *et al.*, 1978; Seely & Pegg, 1983). These facts suggested that ODC decay in the presence of cycloheximide was accelerated by antizyme which had been synthesized during 40 min preincubation with putrescine. Furthermore, the pretreatment with putrescine accelerated the decay of ODC protein, measured by using a monospecific anti-ODC antibody (Kameji *et al.*, 1984), in parallel with the activity (results not shown). Some discrepancy was noted, however, between the results in Fig. 1 and those in Fig. 2: we repeatedly observed that pretreatment of cells with putrescine caused more decrease of half-life than predicted from an increase in the ODC-antizyme complex:ODC ratio. The reason for this is not clear at present. One possibility is that putrescine has an additional effect not explicable by the change in antizyme. Determination of the amount of antizyme protein present is required to examine this possibility.

Thus ODC decay was more rapid in cells with a higher ratio of complexed antizyme activity to total ODC activity (Figs. 1 and 2). It should be noted that ODC decay was measured in the presence of cycloheximide, where antizyme synthesis *de novo* was inhibited (Fong *et al.*, 1976), and that all the

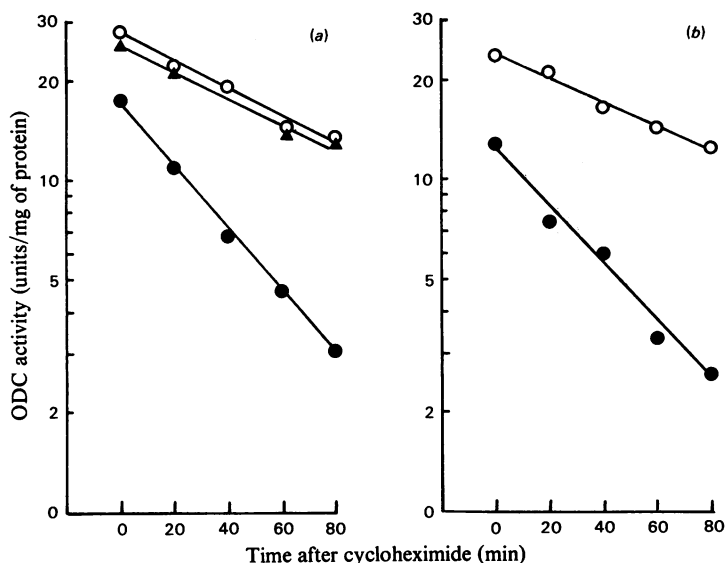


Fig. 2. Effect of preincubation with putrescine on decay of ODC activity

(a) In order to induce ODC activity, HTC cells were incubated for 3 h after cell dilution. Then putrescine (10 mM) in phosphate-buffered saline (0.17 M-NaCl, 3.4 mM-KCl, 10 mM- Na_2HPO_4 , 1.8 mM- KH_2PO_4 , pH 7.2) was added to one-third of the plates and incubated for a further 40 min to induce antizyme. Phosphate-buffered saline alone was added to the remaining plates. Cycloheximide (50 $\mu\text{g}/\text{ml}$) was then added to the putrescine-treated group (●) and to half of the phosphate-buffered-saline group (○), and cycloheximide plus putrescine were added to the rest of the phosphate-buffered-saline group (▲). Cells were harvested at the indicated times after cycloheximide. (b) HTC cells were incubated for 3 h after cell dilution, and preincubated in the presence (●) or in the absence (○) of putrescine for 40 min as described in (a). Then the monolayers of cells were rinsed three times with cold phosphate-buffered saline, and replaced with fresh growth medium containing cycloheximide (50 $\mu\text{g}/\text{ml}$). Pretreatment with putrescine increased the ratio of ODC-antizyme complex to total ODC by 2.1-fold (a) or 2.3-fold (b). Similar results were obtained in one (b) or three (a) more experiments.

performed antizyme existed as complexed form under such conditions. These results therefore suggested that antizyme mediates ODC degradation by recycling, as speculated in our previous paper (Murakami *et al.*, 1985), and that the relative amount of total reusable antizyme determines the rate of decay of total ODC.

Although putrescine clearly accelerated ODC degradation, it is not known whether this is the sole mechanism for the rapid loss of ODC activity caused by putrescine or whether inhibition of ODC synthesis is also involved. Persson *et al.* (1984) have reported that the rapid losses of renal ODC activity and protein after administration of 1,3-diaminopropane appeared to be caused both by an increase in the rate of the enzyme degradation and by a decrease in the rate of general protein synthesis. When putrescine was added to HTC and HMO_A cells, ODC-antizyme complex immediately increased, but decay of total ODC activity was accelerated after a certain lag period as compared with decay in the presence of cycloheximide (Murakami *et al.*, 1985). The appearance of the lag period was especially remarkable when putrescine

was added to cells during the phase of rapid ODC increase. This suggested the possibility that ODC synthesis continued after addition of putrescine. We therefore examined this possibility further. If ODC synthesis continues in the presence of putrescine, appearance of free antizyme activity will be decreased by neutralization with ODC. Then, when ODC synthesis is decreased by actinomycin D, free antizyme activity would be increased, since antizyme synthesis is not inhibited by actinomycin D (Fong *et al.*, 1976). In fact we observed significant increases of free antizyme activity by actinomycin D (Table 1). This indicated indirectly that ODC synthesis more or less continued in the presence of putrescine. More direct studies are required to elucidate the quantitative effect of putrescine on ODC synthesis.

It is conceivable that regulation of ODC decay by antizyme participates to amplify both the rapid increase and subsequent rapid decrease of ODC activity in cells after inducing stimuli. The relative amount of ODC-antizyme complex becomes smaller early after stimulation of ODC synthesis, resulting in a decreased decay rate of ODC and

Table 1. *Effect of actinomycin D on induction of antizyme activity upon addition of putrescine*

Confluent HTC cells were diluted with fresh growth medium with or without actinomycin D (1 µg/ml) and/or putrescine (10mM) and incubated for 3.7h to induce ODC or antizyme. Cell extracts were prepared and assayed for ODC or antizyme activity as described in the Materials and methods section. Results are means ± s.d. for four cultures. Results of Student's *t* test: *a* versus *b*, *P* < 0.005; *c* versus *d*, *P* < 0.005. Similar results were obtained in three more experiments. Abbreviation: ND, not detectable.

Additions	Free ODC activity (units/mg of protein)	Free antizyme activity (units/mg of protein)
None	6.2 ± 0.3 ^a	ND
Actinomycin D	2.8 ± 0.2 ^b	ND
Putrescine	ND	6.8 ± 0.4 ^c
Putrescine + actinomycin D	ND	10.1 ± 0.4 ^d

therefore in a further increase of ODC activity. On the other hand, it becomes larger after the peak of ODC activity, resulting in an accelerated decay rate of ODC and therefore in a further decrease of ODC activity.

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