Existence of antizyme and ornithine decarboxylase-antizyme complex in RK13 kidney cells

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It has been reported that 'antizyme', a protein inhibitor of ornithine decarboxylase (ODC) induced by its product, is not found in rat or mouse kidney. We determined whether antizyme was present in rabbit kidney cells (RK13) in culture. Antizyme could be induced in these cells by putrescine treatment, a substantial portion being in the particulate fraction in contrast with hepatic antizyme. Furthermore, ODC-antizyme complex was present even in untreated cells. Pretreatment of cells with putrescine increased the relative amount of ODC-antizyme complex and accelerated decay of ODC. These results support the ubiquitous existence of antizyme and its role in ODC degradation.

Ornithine decarboxylase *Antizyme* *(Kidney cell)* *Enzyme-inhibitor complex*

1. INTRODUCTION

ODC, a key enzyme in biosynthesis of polyamines, has an extremely short half-life in eukaryotic cells [1]. Exogenously added polyamines cause a rapid decrease of ODC activity and induce a protein inhibitor 'antizyme' which specifically binds to ODC and inhibits its activity [2]. A possible role of antizyme has been assumed to be either acceleration of ODC degradation or storage of available ODC for subsequent cellular use [2]. The former possibility was supported by the accelerated decay of immunoreactive ODC protein in rat liver after treatment with 1,3-diaminopropane, an analog of putrescine [3]. We have found in HTC cells a good correlation between the reciprocal of the half-life of ODC in the presence of cycloheximide and the relative amount of antizyme, suggesting an essential role of antizyme in degradation of ODC in vivo [4]. If this is the case, antizyme should be found in all tissues or cells containing rapidly turning-over ODC. Antizyme, however, has not been found in rat or mouse kidney even after treatment with diamines, despite the short half-life of renal ODC [5,7]. The difficulty in finding antizyme in the kidney may have been due, in part, to rapid excretion of polyamines from kidney cells [5,7,8]. Here, we investigated whether antizyme is present in kidney cells in culture, where a high concentration of extracellular putrescine can be maintained.

Abbreviation: ODC, ornithine decarboxylase (EC 4.1.1.17)

2. MATERIALS AND METHODS

DL-[1-^{14}C]Ornithine was obtained from New England Nuclear. Stocks of rabbit kidney cells (RK13 [9]) were kindly provided by Dr Matsunaga, National Institute for Health, Tokyo. ODC was induced in rat liver and partially purified by DEAE-cellulose column chromatography as described [10]. Cells were grown as monolayers in Swimm's 77 medium (Gibco) on 100 × 20 mm petri dishes (Corning) under the same conditions as described for HTC cells [11]. Cell lysates were prepared in 0.25 M sucrose or 0.155 M KCl, each containing 1 mM dithiothreitol, by 3 cycles of freeze-thawing and centrifuged at 30000 × g for 40 min. The

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supernatant was used for assay of ODC or antizyme activity. The precipitates were homogenized with 1 M NaCl containing 0.01% Tween 80, 10 mM mercaptoethanol and 25 mM Tris-HCl, pH 7.4, and centrifuged at 30000 × *g* for 40 min. The supernatant was dialysed for 3 h in the same buffer without NaCl, and assayed for inhibitory activity against ODC. ODC and antizyme activities were assayed as in [11]. One unit of ODC activity is defined as the amount releasing 1 nmol CO_2 from ornithine per 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 in [11], in which α-difluoromethylornithine-inactivated ODC was used to release active ODC competitively from the complex. The amount of ODC protein was determined by immunoprecipitation according to [12]. Protein was determined by the method of Lowry et al. [13]. Antizyme and ODC-antizyme complex were analysed by high-performance gel chromatography as described [11].

3. RESULTS AND DISCUSSION

3.1. *Induction of antizyme by putrescine*

Putrescine (10 mM) was added to confluent cells containing low ODC activity, at 19 h after change of growth medium. Cells were harvested 6 h later and the extracts prepared with isotonic sucrose or KCl solution. The extracts inhibited constant amounts of ODC activity irrespective of the amount of enzyme activity present (fig.1a). Such a stoichiometric inhibitory action is characteristic of antizyme [14]. It was noted, however, that the inhibitory activity of the KCl extract was about twice as high as that of the sucrose extract. This was in contrast to hepatic antizyme, which could be extracted to the same extent with either KCl or sucrose (not shown). Some antizyme activity could be further extracted with 1 M NaCl from the particulate fraction, especially from that left after sucrose extraction (fig.1b). The sum of inhibitory activities in soluble and particulate fractions was about the same between sucrose and KCl extractions. Sucrose extraction was used throughout the following experiments.

When putrescine was added at around the peak of ODC activity, 4 h after change of medium of confluent cells, the inhibitory activity appeared 2 h later, reached a peak at around 6 h and remained high for at least 29 h. A concentrated extract was prepared from cells harvested 15 h after addition of putrescine and analysed by gel filtration. As shown in fig.2a, the inhibitory activity was eluted from the column at a position approximately corresponding to an M_r of 27000, which is close to the reported M_r of 26500 of antizyme from a variety of cells [2]. These results indicated that the inhibitory activity was attributable to antizyme. The

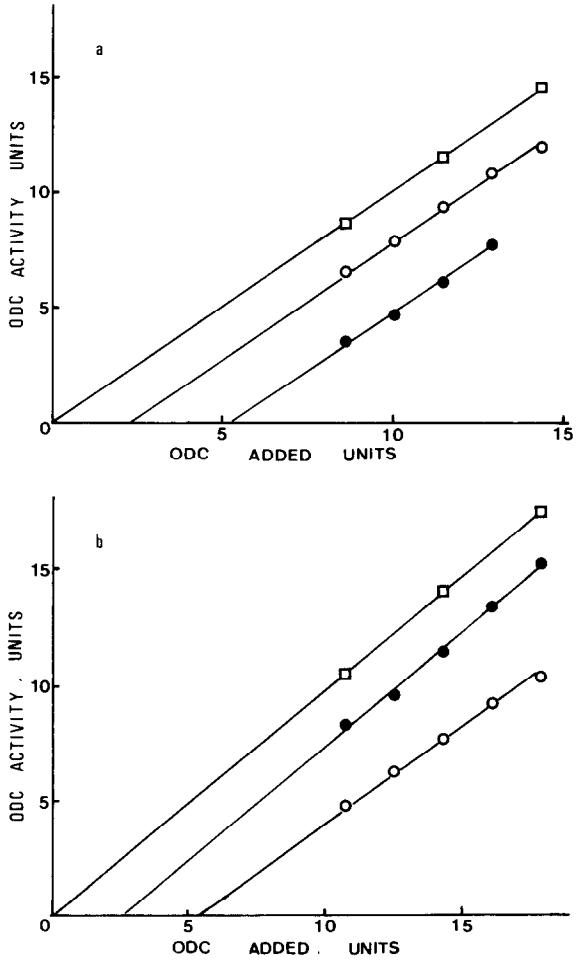

Fig.1. Inhibitory activity of extracts from putrescine-treated RK13 cells against various amounts of ODC. (a) ○, 0.25 M sucrose extract (4.1 × 10^6 cells); ●, 0.155 M KCl extract (4.8 × 10^6 cells); □, control. (b) ○, 1 M NaCl extract from 0.25 M sucrose precipitate (8.4 × 10^6 cells); ●, 1 M NaCl extract from 0.155 M KCl precipitate (9.2 × 10^6 cells); □, control.

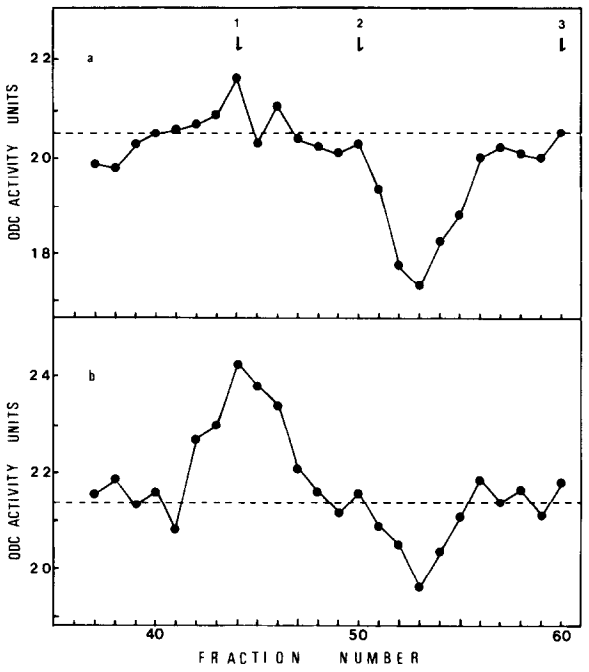

Fig.2. Gel filtration analysis of extracts from RK13 cells. (a) Extract from putrescine-treated RK13 cells (3.6 dishes) containing 25.6 units ODC-inhibitory activity was chromatographed on TSK gel G3000SW in the presence of 1 M NaCl, and an 0.2 ml aliquot of each fraction assayed for ODC activity in the presence of 20.5 units partially purified ODC. Collectively, 23.1 units antizyme activity was recovered. Standard proteins were eluted under the same conditions. (1) Bovine serum albumin (M_r 67500); (2) ovalbumin (M_r 43000); (3) cytochrome *c* (M_r 12400). (b) Extract from untreated RK13 cells (5 dishes) containing 14.9 units ODC activity was chromatographed as in (a). An 0.2 ml aliquot of each fraction was assayed for ODC activity in the presence of 21.3 units partially purified ODC. Collectively, 11.1 units antizyme activity and 21.5 units ODC activity were recovered.

appearance of antizyme was prevented by simultaneous addition of cycloheximide (not shown).

3.2. *Existence of antizyme in untreated RK13 cells*

We determined whether antizyme is present under physiological conditions, namely without exogenous polyamines. At high salt concentrations, ODC-antizyme complex can be dissociated and the components can be resolved on gel filtration [2]. An extract was prepared from RK13 cells 19 h after change of medium. This extract contained some ODC activity and no free antizyme activity. On gel filtration analysis, however, some antizyme activity was eluted from the column at a position approximately corresponding to an M_r of 27000 (fig.2b). The amount of ODC activity recovered from the column substantially exceeded that applied to the column. These results indicated the presence of ODC-antizyme complex in RK13 cells under physiological conditions.

3.3. *Effect of putrescine on decays of ODC activity and immunoreactive protein*

RK13 cells containing high ODC activity were preincubated with putrescine (10 mM) for 40 min, and then cycloheximide (50 μg/ml) was added to inhibit both ODC and antizyme syntheses. The pretreatment with putrescine increased the ODC-antizyme complex/ODC ratio 1.5-fold and accelerated the decay of ODC protein 2-fold ($t_{1/2}$: from 112 to 56 min) in parallel with the activity ($t_{1/2}$: from 111 to 63 min). These results suggested that antizyme was involved in degradation of ODC in RK13 cells.

The present results support the ubiquitous existence of antizyme and its significant role in ODC degradation. Previous difficulties in observing antizyme activity in mouse or rat kidney [5,6] may have been due, in part, to the large antizyme-binding capacity of particulate fraction in kidney cells under low ionic strength condition, suppressing overshoot of free antizyme in cytosol.

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