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Antizyme, a protein induced by polyamines, accelerates the degradation of ornithine decarboxylase in Chinese-hamster ovary-cell extracts

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Ornithine decarboxylase (ODC), the key regulatory enzyme for polyamine biosynthesis, is known to have a short intracellular half-life, and antizyme, an ODC-binding protein induced by polyamines, has been suggested to be involved in the process of ODC degradation. In the present study we demonstrated that antizyme markedly accelerated ATP-dependent degradation of ODC *in vitro* in an extract from ODC-overproducing Chinese-hamster ovary cells.

INTRODUCTION

Mammalian ornithine decarboxylase (ODC) is the first and key enzyme for synthesis of polyamines, which are essential in cellular growth and differentiation processes [1]. The enzyme turns over rapidly and its degradation rate is accelerated by polyamines [2–8]. We have suggested from several lines of indirect evidence that the polyamine effect is mediated by an ODC-inhibitory protein 'antizyme' [5,9,10], which is known to be induced by polyamines and to inhibit the enzyme activity by forming a complex with it [11,12]. Thus formation of a complex with antizyme would render ODC highly susceptible to a specific proteolytic system. Recently we transfected HTC cells with antizyme cDNA driven by a glucocorticoid-inducible promoter and demonstrated that dexamethasone induced antizyme in these cells and elicited a marked acceleration of ODC decay in the absence of exogenous polyamines, indicating that antizyme indeed stimulates ODC degradation in cells [13]. In the present study we examined ODC degradation in extracts from ODC-overproducing Chinese-hamster ovary (CHO) cells and demonstrated that antizyme markedly stimulated ATP-dependent degradation of ODC in this cell-free system. This is the first demonstration *in vitro* of degradation of native ODC with a short half-life comparable with that observed *in vivo*.

EXPERIMENTAL

Materials

A mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine (Expre^{35}S^{35}S) was purchased from du Pont–New England Nuclear. L-[^{14}C]Ornithine was from ICN Biomedicals Inc. Rabbit anti-ODC antibody was prepared as described previously [14]. Recombinant antizyme was produced in *Escherichia coli* and purified by immunoaffinity chromatography as described previously [15]. Briefly, antizyme in an extract of *Escherichia coli* (~ 0.8 g of protein) was applied to a monoclonal anti-antizyme antibody (HZ-3H1)–AffiGel 10 column (1 ml) and the column was washed with 25 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 2 M-NaCl. Antizyme was then eluted with 6.5 ml of 0.2 M-glycine buffer, pH 2.3, and the eluate was immediately neutralized with 2 M-Tris base, dialysed against 25 mM-Tris, pH 7.8, containing 1 mM-dithiothreitol (DTT), and applied to a DEAE-cellulofine column (0.2 ml). Antizyme was eluted with 25 mM-Tris/HCl, pH 7.5, containing 0.5 M-NaCl, 0.01% Tween 80 and 1 mM-DTT. Rabbit anti-antizyme antibody was prepared as described previously [15]. Reticulocyte lysate was prepared as described previously [16].

Cell culture

α-Difluoromethylornithine (DFMO)-resistant CHO cell line (DF3) was kindly supplied by Dr. I. E. Scheffler (University of California, San Diego, CA, U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 2% (v/v) fetal-calf serum, 4% newborn-calf serum, non-essential amino acid mixture (GIBCO) and neomycin sulphate (100 mg/l).

ODC induction, metabolic labelling and preparation of cell extract

ODC activity was induced in DF3 cells by replacing growth medium with fresh medium, which was methionine- and cysteine-free Dulbecco's modified Eagle's medium supplemented with 2% fetal-calf serum, 4% newborn calf serum, non-essential amino acid mixture and neomycin sulphate. After incubation for 2.5 h, a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine (see above) was added at 80 μCi/ml and cells were further incubated for 1 h. Then the dishes were washed twice with ice-cold phosphate-buffered saline (8.26 mM-sodium/potassium phosphate/0.171 M-NaCl, pH 7.2) and, after addition of 0.05 ml of phosphate-buffered saline containing 20 mM-methionine/100 mm-diameter dish, cells were collected in Eppendorf tubes and disrupted by three cycles of freeze–thawing. The homogenates were centrifuged at 12000 rev./min ($r_{av.}$ 6 cm) for 15 min and the supernatants were used for degradation experiments.

ODC-degradation assay

Assay mixture contained cell extract (50–100 μg of protein), 750 nmol of Tris/HCl, pH 9.0 or 7.5, 250 nmol of $MgCl_2$ and ATP-regenerating system (50 nmol of ATP, 5 μg of creatine kinase and 500 nmol of phosphocreatine). The total volume was 25 μl or 50 μl. The cell extract, containing ^{35}S-labelled ODC as substrate for degradation, was prepared as described above. In experiments testing an energy requirement for ODC degradation, the ATP-regenerating system was omitted and 500 nmol of glucose and 5 μg of hexokinase were added unless otherwise mentioned. After various periods of incubation at 37 °C, labelled ODC was precipitated with anti-ODC immunoglobulin after

Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; DTT, dithiothreitol.

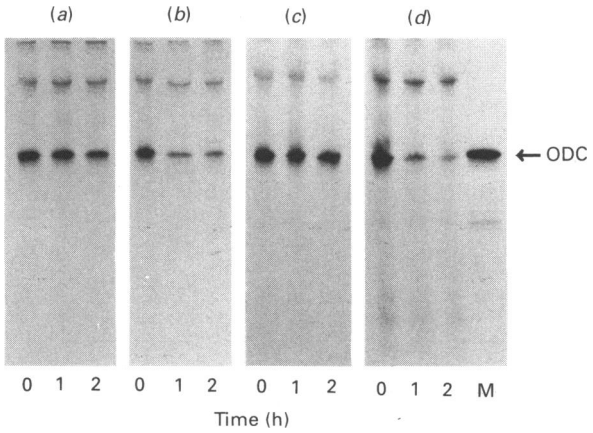

Fig. 1. Degradation of ODC in the presence or absence of reticulocyte lysate

Cell extracts which contained labelled ODC as a substrate were incubated with or without reticulocyte lysate (20 μl) in the degradation mixture in a total volume of 50 μl. When reticulocyte lysate was omitted, 1.8-times as much cell extract was added. At indicated times, labelled ODC was precipitated with anti-ODC immunoglobulin and analysed by SDS/PAGE. (*a*) Reticulocyte lysate + ATP; (*b*) reticulocyte lysate + ATP + 23.5 units of antizyme; (*c*) reticulocyte lysate + 23.5 units of antizyme; (*d*) ATP and 23.5 units of antizyme. Lane M was ^{35}S-labelled ODC marker. Details are given in the Experimental section.

dilution with 0.5 ml of ice-cold phosphate-buffered saline containing 20 mM-methionine and analysed by SDS/PAGE as described previously [8]. After fluorography the ODC band was quantified by measuring its radioactivity after cutting from the gels.

Assays of ODC and antizyme activities

The activities of ODC and antizyme were measured as described previously [17]. One unit of ODC is defined as the amount releasing 1 nmol of CO_2/h at 37 °C and 1 unit of antizyme as the amount which inhibits 1 unit of ODC. When the activity of ODC in cell extract containing labelled methionine and cysteine was assayed, radioactivity trapped on KOH-impregnated filter paper was determined in the presence and absence of L-[^{14}C]ornithine, and the activity was obtained by difference.

RESULTS AND DISCUSSION

For the development of a cell-free protein-degradation system the nature of the substrate protein should be an important factor. To avoid possible 'artifactual' modification during purification or labelling with ^{125}I of ODC we used metabolically labelled ODC in a crude cell extract as the substrate. We prepared extracts from cells of DF3, a DFMO-resistant CHO cell line, since they produced ODC to high activity in the absence of DFMO, the half-life of ODC was short and the ODC has been shown to be normal by several criteria [18,19].

We first examined ODC degradation in a reticulocyte-lysate system, since Bercovich *et al.* [20] recently reported that ODC synthesized *in vitro* was efficiently degraded in reticulocyte lysates via an ATP-dependent and ubiquitin-independent proteolytic pathway.

The labelled ODC in the cell extract described above was incubated together with a reticulocyte lysate in the degradation assay mixture of pH 9.0 described in the Experimental section. ODC was degraded only slightly, even in the presence of ATP, as reported by Loetscher *et al.* [21] (Fig. 1*a*), whereas the addition of antizyme markedly stimulated the degradation of ODC (Fig. 1*b*). Antizyme-stimulated ODC degradation was not observed in the absence of ATP (Fig. 1*c*). Thus ODC in cell extracts was degraded in this system in an ATP- and antizyme-dependent manner. Interestingly, the degradation of ODC was observed in the absence of reticulocyte lysate as well (Fig. 1*d*). The reticulocyte lysate appeared not to affect the ODC degradation at all in this system (Fig. 1*b* versus Fig. 1*d*). In the following experiments, therefore, we omitted reticulocyte lysate from the assay mixture for ODC degradation. ODC degradation in cell extract was also ATP-dependent (results not shown). Since it exhibited a broad pH optimum around 7.5 (results not shown), Tris/HCl buffer of pH 7.5 was used in the following experiments.

We reported previously that the ODC decay rate in cultured cells and animal tissues correlated well with antizyme/ODC ratio in the cells [9,17]. Then we examined whether ODC degradation

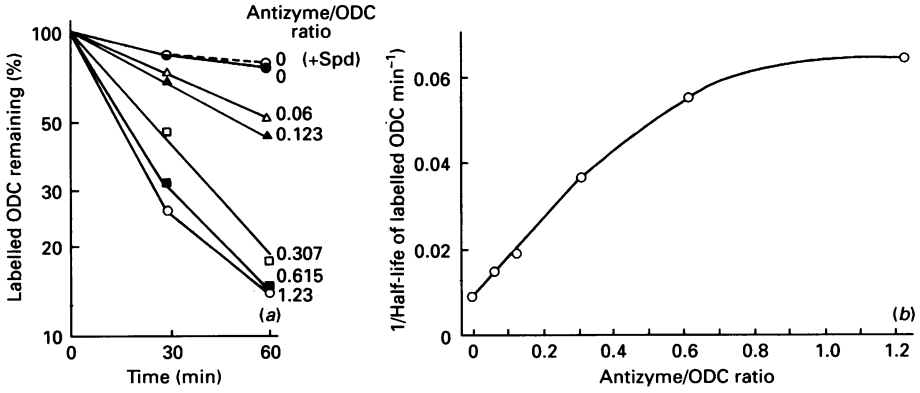

Fig. 2. Dose-dependent response of ODC degradation *in vitro* to antizyme in cell extracts

Extracts of DF3 cells (17 μl) containing metabolically labelled ODC were incubated for the indicated period with various amounts of antizyme or spermidine (1 mM) in the degradation assay mixture of a total volume of 25 μl. Labelled ODC that remained in the mixture was immunoprecipitated and analysed by SDS/PAGE. After fluorography the ODC band was cut out from the gel and its radioactivity determined with a scintillation counter. The antizyme/ODC ratio was calculated by using initial ODC activity (0.96 unit/μl) in cell extracts before incubation, which was measured as described in the Experimental section. (*a*) Time courses of ODC degradation in the presence of various amounts of antizyme or 1 mM-spermidine (+Spd). The results were expressed as the percentage of the initial amount of ODC protein (13000 c.p.m. of ^{35}S), which was determined at zero time. (*b*) Dose–response curve for antizyme. The decreases in labelled ODC during 30 min incubation were plotted as a function of the antizyme/ODC ratio. Data were obtained from (*a*).

Table 1. Decrease of ODC activity in the cell extract after incubation with ATP and antizyme

The tubes containing the assay mixture for ODC degradation (creatine kinase and phosphocreatine were added to all tubes except tube no. 1) were incubated at 37 °C for 30 min and then excess antizyme antiserum was added to all tubes except tube no. 6, to which it was added before the incubation. Tubes were kept on ice for about 30 min and ODC activity was determined after the addition of pyridoxal phosphate and L-[^{14}C]ornithine. The results are expressed as the percentage of the initial ODC activity (10.6 units), which was the activity of a duplicate of tube no. 1 without the incubation for ODC degradation.

Tube no.	Additions to degradation assay mixture...	ATP	Antizyme	Antizyme/ODC ratio	Antiserum	ODC activity (% of initial activity)
1		−	−	0	−	113
2		−	+	1.9	−	79
3		+	−	0	−	75
4		+	+	1.9	−	7
5		+	+	0.5	−	7
6		+	+	1.9	+	80

correlates with the antizyme/ODC ratio in the present '*in vitro*' degradation system. ODC degradation in the cell extracts, which appeared to proceed with first-order kinetics, was progressively accelerated by increasing amounts of antizyme (Fig. 2*a*). The rate appeared to reach a maximum at an antizyme/ODC ratio of about 1 (Fig. 2*b*). This suggested that ODC bound with antizyme was selectively degraded by a proteinase. Since small amounts of antizyme stimulated the degradation of large amounts of ODC, antizyme was likely to be freed to bind to another ODC molecule, as had been suggested in whole cells [5,9]. It should be noted that slow degradation of ODC was observed in the absence of exogenous antizyme. This could have been due, at least in part, to stimulation by endogenous antizyme. DF3-cell extracts prepared under our experimental conditions usually contained a small amount of antizyme, the antizyme/ODC ratio being less than 0.1. In addition, this could have been caused by constitutive degradation, as suggested in whole cells [9].

Spermidine, a strong inducer of antizyme in cells [22], did not affect the rate of ODC degradation *in vitro* (Fig. 2*a*), indicating that polyamines do not act by themselves to accelerate ODC degradation. This result was consistent with previous observations in living cells, namely that new protein synthesis, but not continued presence of exogenous polyamines, was necessary for the acceleration of ODC degradation [9].

Finally, we confirmed that ODC activity in cell extract was also decreased dramatically by incubation with both ATP and antizyme (Table 1). The decrease in activity was strongly, but not completely, inhibited by an excess amount of antibody to antizyme. Because the action of the antibody was incomplete and some degradation was observed even in the absence of antizyme, the degradation of ODC must have continued slowly during ODC assay (60 min) in the presence of excess antizyme antibody. This may have caused the larger decrease in activity (Table 1) than in the amount of labelled ODC protein (Figs. 1 and 2).

The present study clearly demonstrated that ODC degradation is stimulated by antizyme. Further studies are needed to elucidate the precise mechanism of antizyme action. On the other hand, important structural domains responsible for the rapid degradation of ODC and its regulation by polyamines have been examined extensively [23–26] since the 'PEST' hypothesis was proposed [27]. It is also important to determine whether the rapid ODC degradation is due to the presence of a specific degradation system for ODC or due to the high susceptibility of ODC protein to a common proteolytic machinery. The present '*in vitro*' system should be useful for these studies, since it is presumably responsible for physiological ODC degradation within whole cells as judged from the following evidence: first, mature ODC isolated from cells is degraded with short half-lives comparable with that observed for whole cells [19]; secondly, ODC degradation *in vitro* is, like ODC decay in cells, ATP-dependent [28–31]; and thirdly, the ODC degradation *in vitro*, like that in whole cells [9,13], is markedly accelerated by antizyme.

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