Involvement of the proteasome and antizyme in ornithine decarboxylase degradation by a reticulocyte lysate

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Ornithine decarboxylase (ODC) degradation in a freshly prepared reticulocyte lysate was examined. Immunodepletion of proteasomes from the reticulocyte lysate resulted in almost complete loss of ODC degradation. In contrast with the previously reported degradation in extracts of hepatoma tissue-culture (HTC) and Chinese-hamster ovary (CHO) cells or that by the purified 26 S proteasome, efficient degradation of ODC was observed in the lysate without exogenous antizyme, an ODC protein inhibitor induced by polyamines, owing to the presence of a significant amount of antizyme in the lysate. The degradation

of ODC in the lysate was strongly suppressed on inactivation of antizyme in the lysate with antizyme inhibitor, a protein which binds to the antizyme and releases ODC from the ODC–antizyme complex. Thus the main pathway for ODC degradation in a reticulocyte lysate was essentially the same as that characterized previously in extracts of HTC and CHO cells, namely an ATP- and antizyme-dependent 26 S proteasome-catalysed pathway that is presumed to be responsible for ODC degradation in whole cells.

INTRODUCTION

A reticulocyte lysate has often been used for *in vitro* analysis of the degradation of various cellular proteins synthesized *in vitro* by transcription/translation of their cDNAs in the presence of [³⁵S]methionine [1–13]. Its not known, however, whether protein degradation in a reticulocyte lysate is identical with that in intact cells [14]. This system has been used to study the degradation of ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis with a short half-life; Bercovich et al. [2] have found that ODC synthesized *in vitro* was degraded in an ATP-dependent, but ubiquitin-independent, manner in a reticulocyte lysate. However, in contrast with ODC synthesized *in vitro*, the enzyme isolated from mammalian cells was stable in the lysate [2]. We also observed slow degradation of ODC in a CHO-cell extract on incubation with a reticulocyte lysate in the presence of ATP [15]. We recently developed an *in vitro* ODC degradation system in which isolated ODC was degraded as it is *in vivo*, its degradation depending on both ATP and antizyme [15,16], an ODC-inhibitory protein induced by polyamines [17]. We also showed that, in this system, the 26 S proteasome was responsible for ODC degradation [16]. The role of antizyme in ODC degradation was also confirmed directly in cultured cells by forced expression of the antizyme gene [18]. The apparent difference between ODC degradations in cell extracts and a reticulocyte lysate suggested that the proteolytic mechanisms in the two are different. Alternatively, the difference may be due to post-translational modification of ODC [3,19]. Possibly the proteasome can degrade newly synthesized ODC (unassembled monomer) efficiently, but not mature ODC (dimer) in the absence of antizyme. In the present study we examined ODC degradation in a freshly prepared reticulocyte lysate, finding that the degradation was essentially the same as that in cell extracts, being catalysed by an ATP- and antizyme-dependent 26 S proteasome pathway.

MATERIALS AND METHODS

Materials

Plasmid pOD20.7 containing mouse ODC cDNA was a gift from Dr. P. Coffino (University of California, San Francisco, CA, U.S.A.) and pODC24 derived from pOD20.7 was kindly provided by Dr. K. Igarashi (Chiba University, Chiba, Japan). [³⁵S]Methionine (1000 Ci/mmol) was purchased from Amersham International. A rabbit reticulocyte lysate and nuclease-treated lysate were prepared by the methods of Pelham and Jackson [20]. Rat reticulocyte lysates were prepared similarly from Wistar rats (200–250 g). Anti-ODC [21], anti-antizyme [22] and anti-proteasome [23] antibodies were prepared as described previously. Recombinant antizyme was produced in *Escherichia coli* and purified by immunoaffinity chromatography as described previously [22]. Antizyme inhibitor was prepared from rat liver as described previously [24] and concentrated by ultrafiltration (MINICENT 30, Toyo Soda, Tokyo, Japan). Purified 20 S and 26 S proteasomes were prepared as described previously [25].

Preparation of ODC synthesized *in vitro* and metabolically labelled with ³⁵S

ODC mRNA was prepared *in vitro* as described by Ito et al. [26] and translated at 30 °C for 90 min in a total volume of 15 µl of solution containing 0.6 pmol of the mRNA, 25 µCi of [³⁵S]methionine and 10.5 µl of ribonuclease-treated rabbit reticulocyte-lysate cocktail. For measurement of the activity of ODC, the labelled methionine was replaced by 20 µM unlabelled methionine, and then ODC activity was measured by incubation of the reaction mixture in a total volume of 0.125 ml [21]. Metabolically labelled ODC was isolated from ODC-overproducing FM3A cells (EXOD-1) [27], which were kindly provided by Dr. K. Igarashi (Chiba University) and was purified by immunoaffinity chromatography as described previously [21].

ODC degradation assay

Purified ^{35}S -labelled ODC (59 units) or $1.75\ \mu\text{l}$ of a solution of [^{35}S]ODC synthesized *in vitro* as described above was incubated in a total volume of $100\ \mu\text{l}$ of degradation assay mixture containing 40 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine, 5 μg of creatine kinase (Sigma), 0.1 mM cycloheximide and $25\ \mu\text{l}$ of rabbit reticulocyte lysate or $60\ \mu\text{l}$ of rat reticulocyte lysate. When ODC synthesized *in vitro* was used as a substrate, remaining ODC was immunoprecipitated and analysed by SDS/PAGE as described previously [16], and the ODC band was quantified with an image analyser (FUJIX, BAS 2000). When purified ODC isolated from cells was used as a substrate, its degradation was also monitored by release of trichloroacetic acid-soluble radioactive materials.

Immunoprecipitation experiments

A reticulocyte lysate was incubated with the indicated amounts of anti-proteasome IgG or control IgG in a total volume of $80\ \mu\text{l}$ at 4°C for 2 h, and then centrifuged at $14000\ g$ for 30 min, and the supernatant was used for proteolytic assay of purified ^{35}S -labelled ODC. When the reticulocyte lysate was used for proteolytic assay of ODC synthesized *in vitro*, it was treated with control or anti-proteasome IgG by an alternative method to avoid interference from remaining antibody on subsequent immunoprecipitation of ODC. Rat anti-proteasome IgG (0.34 mg) or control IgG (0.34 mg) was incubated with $200\ \mu\text{l}$ of a 10% suspension of formalin-fixed *Staphylococcus aureus* Cowan 1 cells (Pansorbin, Calbiochem) at room temperature for 15 min, and then centrifuged at $12000\ g$ for 1 min. The precipitate was washed twice with $0.5\ \text{ml}$ of washing buffer [50 mM Tris/HCl (pH 7.4)/0.15 M NaCl/5 mM EDTA/0.05% (v/v) Nonidet P40/0.02% (w/v)/ NaN_3] and once with 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). The antibody-bound Pansorbin was suspended in PBS. Suspensions containing the indicated amounts of IgG were re-centrifuged at $12000\ g$ for 1 min, and the pellets were suspended in $80\ \mu\text{l}$ of reticulocyte lysate. After incubation at room temperature for 15 min, the precipitates were removed by centrifugation at $14000\ g$ for 10 min and the supernatants were used for proteolytic assay of synthesized ODC. This method was also used for preparation of anti-antizyme-antibody-treated reticulocyte lysates after adding anti-antizyme antibody instead of anti-proteasome antibody; $80\ \mu\text{l}$ of reticulocyte lysate was treated with the indicated amounts of anti-antizyme antibody.

RESULTS AND DISCUSSION

Mouse ODC purified from cultured cells and ODC synthesized *in vitro* were each incubated with a rabbit reticulocyte lysate in the presence of ATP. As shown in Figure 1, in contrast with a previous report [2], purified ODC was degraded at a rate comparable with that of ODC synthesized *in vitro*. We have found that immunoprecipitation of the proteasome from extracts of HTC or CHO cells caused almost complete loss of ATP- and antizyme-dependent degradation of ODC [16]. We therefore examined the effect of immunoprecipitation of the proteasome from a reticulocyte lysate on degradation of ODC. Since anti-proteasome antibody raised in rabbits did not precipitate the proteasome from a rabbit reticulocyte lysate, we used a rat reticulocyte lysate in this experiment. As Figure 2 shows, the degradation of each type of ODC was suppressed completely by immunoprecipitation of the proteasome from the reticulocyte

lysate. This result indicates that the proteasome is necessary for ODC degradation in a reticulocyte lysate.

We next examined the possible participation of antizyme in the degradation of ODC in the reticulocyte lysate. A reticulocyte lysate was pre-incubated with anti-antizyme-antibody-bound Pansorbin and then the Pansorbin was removed by centrifugation as described in the Materials and methods section. Thus the reticulocyte lysate added to the ODC degradation mixture as a protease source did not contain antizyme or anti-antizyme antibody. Treatment of the reticulocyte lysate with anti-antizyme antibody clearly suppressed the degradation of purified ODC, but not that of ODC translated *in vitro* (Figure 2). This suggested that ODC degradation in the reticulocyte lysate was antizyme-dependent. The apparent absence of effect on degradation of ODC translated *in vitro* may be explained by contamination of the preparation with antizyme, since this ODC was synthesized in a reticulocyte lysate and was not treated with anti-antizyme antibody before use as a substrate. To examine this possibility,

Figure 1 Degradation of ODC in a reticulocyte lysate

ODC synthesized *in vitro* (a) or purified isolated ODC (b) was incubated with a reticulocyte lysate. At the indicated times, labelled ODC was precipitated with anti-ODC IgG and analysed by SDS/PAGE. Percentage degradations, determined with an image analyser, are shown at the bottom of each lane.

Figure 2 Effect of immunoprecipitation of the proteasome on degradation of ODC in a reticulocyte lysate

A rat reticulocyte lysate was treated with control (○), anti-proteasome (●) or anti-antizyme (△) IgG as described in the Materials and methods section, and the degradation activities of the supernatants were assayed by using [^{35}S]ODC synthesized *in vitro* (a) or purified exogenous [^{35}S]ODC (b). Values are shown as percentages of the activity measured with no added IgG, which was 47.9% degradation/h for ODC synthesized *in vitro* and 43.3% degradation/h for purified [^{35}S]ODC.

Table 1 Effect of antizyme inhibitor on the activity of ODC synthesized *in vitro*

The activity of ODC synthesized *in vitro* was assayed in the presence or absence of excess antizyme inhibitor (3.4 units) [24]. Results are averages for triplicate determinations.

	ODC activity		ODC-antizyme complex (b-a) (units)	Antizyme/ODC ratio (b-a)/ (b)
	- Antizyme inhibitor (a) (units)	+ Antizyme inhibitor* (b) (units)		
Reticulocyte lysate				
Before incubation	0.005	0.025	0.02	0.8
After incubation with ODC mRNA	0.249	0.600	0.35	0.59

* Activity was completely lost on heating at 80 °C for 5 min.

Table 2 Inhibitory effect of antizyme inhibitor on ODC degradation in a reticulocyte lysate and its restoration by antizyme

ODC synthesized *in vitro* or purified ODC was incubated with a reticulocyte lysate at 37 °C for 70 min. Antizyme inhibitor (6.8 units) and/or antizyme (156 units) was added to the degradation assay mixture as indicated (total volume 100 µl, as described in the Materials and methods section).

Addition	Degradation of ODC (%)	
	ODC synthesized <i>in vitro</i>	Purified ODC
None	53.8	39.3
Antizyme inhibitor	18.7	7.2
Antizyme	61.2	57.4
Antizyme inhibitor + antizyme	54.4	39.4

we determined the amounts of ODC synthesized *in vitro* and complexed with antizyme. For this we measured ODC activity in the presence or absence of excess antizyme inhibitor, which specifically binds to antizyme with high affinity [28] and thus can remove antizyme from the ODC-antizyme complex [24,29], releasing active ODC. As shown in Table 1, about 60% of the ODC synthesized *in vitro* was bound with endogenous antizyme in a reticulocyte lysate. At this antizyme/ODC ratio (0.6), ODC degradation by the 26 S proteasome was close to the maximum level observed with a saturating concentration of antizyme (results not shown). Therefore we used the antizyme inhibitor to inactivate endogenous antizyme in both substrate and protease preparations. As shown in Table 2, the antizyme inhibitor markedly suppressed the degradation of both ODC synthesized *in vitro* and ODC purified from cultured cells, and the suppression was reversed by an excess amount of antizyme. These results indicate that antizyme is involved in ODC degradation in the reticulocyte lysate.

Removal of Mg²⁺ from the assay mixture by treatment with EDTA completely inhibited the degradation of ODC (results not shown). This result, together with a previous observation that ATP is essential for ODC degradation in a reticulocyte lysate [2], indicated the involvement of ATP hydrolysis in ODC degradation. This was also consistent with our previous findings that both ATP and Mg²⁺ were necessary for antizyme-dependent ODC degradation by the 26 S proteasome and that non-hydrolysable ATP analogues had no effect [16]. Thus the main ODC degradation pathway operating in a freshly prepared

reticulocyte lysate was essentially the same as that in extracts of HTC and CHO cells, namely the ATP- and antizyme-dependent and 26 S-proteasome-catalysed pathway that is presumed to be responsible for ODC degradation in whole cells.

The discrepancy with the previous observation [2] that ODC synthesized *in vitro* was degraded in a reticulocyte lysate, but that ODC isolated from mammalian cells was stable in the lysate [2], appears to be due to a difference in the antizyme/ODC ratios. A small amount of endogenous antizyme would be enough for degradation of a small amount of endogenously synthesized ODC, but not for that of a much larger amount of purified ODC. In the present study we used relatively small amounts of purified ODC with high specific radioactivity.

The antizyme inhibitor did not completely inhibit degradation of either purified ODC or ODC synthesized *in vitro* (Table 2). This finding may be partly attributable to basal degradation of ODC. We previously suggested the existence of basal degradation of ODC in HTC cells and mouse kidney from the finding that the intercept of the regression line between the ODC decay rate (ordinate) and relative content of antizyme (abscissa) was not zero; the degradation rate in the absence of antizyme was approx. 10% of the maximal rate stimulated by antizyme [30,31]. Ghoda et al. [32] postulated, from their results with engineered ODC protein, that constitutive degradation of ODC and polyamine-mediated regulation require distinct domains of mouse ODC. It is difficult, however, to determine the basal degradation of ODC directly, since the existence of antizyme *in vivo* cannot be ruled out. From this point of view, it is interesting to identify the enzyme that catalyses the basal ODC degradation *in vitro*. Either treatment of a reticulocyte lysate with excess anti-proteasome antibody (Figure 2) or depletion of Mg²⁺ (results not shown) caused complete loss of the degradation activity, suggesting that basal degradation of ODC is also catalysed by the proteasome in an energy-dependent manner. Conceivably, the 26 S proteasome recognizes the same domain of ODC in the presence or absence of antizyme, and antizyme accelerates degradation of ODC by exposing this domain and thus increasing the chance of its binding to the proteinase.

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