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Post-translational arginylation of ornithine decarboxylase from rat hepatocytes

Jürgen KOPITZ, Brigitte RIST and Peter BOHLEY*
Physiologisch-chemisches Institut, Universität Tübingen, Hoppe-Seyler-Strasse 4, D7400 Tübingen, Federal Republic of Germany

Ornithine decarboxylase (ODC) was purified 6500-fold from NMRI mouse kidneys under conditions designed to inhibit degradation by proteinases. The enzyme was homogeneous by SDS/polyacrylamide-gel electrophoresis, and the specific activity was among the highest reported. The yield was 70%. A monoclonal antibody against this preparation was generated and used in studies to investigate the half-life of ODC in cultured rat hepatocytes labelled with [^{35}S]methionine. This value was 39 ± 4 min and was unchanged when either NH_4Cl (as a lysosomotropic agent) or leupeptin (as a lysosomal proteinase inhibitor) was added to the culture medium. Thus the intracellular turnover of ODC in cultured hepatocytes occurs mainly in extra-lysosomal compartments. Arginylation of rat ODC was investigated *in vitro* by incubation with L-[^{3}H]arginyl-tRNA, and the incorporation of the label was compared with that of total cytosolic proteins. Arginylated ODC had a specific radioactivity 8600 times that of the bulk of cytosolic protein. Edman degradation of this ODC showed that the post-translational arginylation occurred only at the α-amino end of the enzyme. The inhibitor of arginyl-tRNA:protein arginyltransferase (EC 2.3.2.8), L-glutamyl-L-valyl-L-phenylalanine, increased the half-life of ODC in cultured hepatocytes from 39 min to more than 90 min. The possible significance of the preferential post-translational arginylation of ornithine decarboxylase to its rapid turnover is discussed.

INTRODUCTION

Cytosol proteins in rat hepatocytes and in many other cells display a wide range in half-lives from a few minutes to many weeks, and it is helpful to define three main groups with respect to this turnover: first very-fast-turnover proteins with half-lives of less than 1 h, secondly fast-turnover proteins with half-lives between 1 and 24 h, and thirdly slow-turnover proteins with half-lives of more than 24 h.

As far as we know, only a few individual proteins belong to the first group of very-fast-turnover proteins [1–3], but the rate-limiting enzyme in the synthesis of polyamines, ornithine decarboxylase (ODC, EC 4.1.1.17), shows a half-life between 10 and 40 min in the cells of many higher organisms [3–6]. The structural features for such a rapid turnover of a molecule are still largely unknown, and this might be one of the reasons for the existence of so many hypotheses, very much differing, in this field (for some recent reviews see [1–3,7–11]).

The unusually short half-life of ODC in most animal cells enables rapid changes in the enzyme content and therefore in polyamine synthesis, which is important for the regulation of growth. It seems possible that an increase in the stability of ODC is a common correlate in neoplastic transformation of cells [12].

For all these reasons, ODC is a substrate of special interest for the investigation of intracellular proteolysis. In the present paper we report the isolation of the enzyme and the generation of a monoclonal antibody against it. We used this antibody to determine the half-life of ODC in the absence and in the presence of lysosomotropic agents, in order to decide whether the very fast degradation of ODC occurs in lysosomes. We found that ODC must be degraded mainly by a non-lysosomal pathway.

Recently, a new mechanism for rapid non-lysosomal degradation of proteins has been described [11] by which a protein is designated to fast degradation by its *N*-terminal amino acid. According to this '*N*-end-rule' [11], especially an *N*-terminal arginine residue should have an extremely destabilizing effect on cytosolic proteins. On the other hand, it was shown that for certain proteins the ATP-dependent non-lysosomal degradation was also dependent on tRNA [13]. For these reasons it seemed possible that a post-translational arginylation of very-short-lived cytosolic proteins by the enzyme arginyl-tRNA:protein arginyltransferase (arginyltransferase, EC 2.3.2.8) might be one of the first steps of rapid degradation of these proteins. Arginyltransferase is a cytosolic enzyme which catalyses the post-translational arginylation of proteins by transferring arginine from arginyl-tRNA to the α-amino-terminal amino acid of a substrate protein [13–15]. We investigated the post-translational arginylation of ODC by this cytosolic enzyme arginyltransferase, and we present evidence from Edman degradation that this arginylation of ODC occurs only at the α-amino-terminal end.

Arginylated ODC had a specific radioactivity 8600 times that of the bulk of cytosolic proteins. If post-translational arginylation is a first step in degradation, the inhibition of arginyltransferase should also diminish the degradation of those proteins which can be marked for more rapid turnover by this post-translational arginylation. The peptide L-glutamyl-L-valyl-L-phenylalanine (Glu-Val-Phe) is an effective inhibitor of arginyltransferase [14]. We found a remarkable prolongation of the half-life of ODC, from 39 min to more than 90 min, after treatment of cultured hepatocytes with 1 mM-Glu-Val-Phe.

EXPERIMENTAL

Materials

Animals. Hepatocytes for primary cultures were isolated from livers of male Sprague–Dawley rats (body wt. 250 g). For isolation of rat liver cytosol used for measuring the arginylation of ODC, male Sprague–Dawley rats (body wt. 250 g) were given an

Abbreviation used: ODC, L-ornithine decarboxylase.

* To whom correspondence and reprint requests should be addressed.

intraperitoneal injection of thioacetamide (37.5 mg in 0.5 ml of 0.9% NaCl) 18 h before being killed and their livers excised [16].

Chemicals, biochemicals and media for cell culture. L-[^{35}S]Methionine (1228 Ci/mmol), L-[1-^{14}C]ornithine hydrochloride (61 mCi/mmol), L-[5(n)-^{3}H]arginine monohydrochloride (15 Ci/mmol), [^{14}C]methylated proteins and Amplify were purchased from Amersham (Braunschweig, Germany). Williams E Medium was from Flow Laboratories (Bonn, Germany). Glu-Val-Phe was obtained from Bachem (Bubendorf, Switzerland). All other chemicals and biochemicals were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Sigma (St. Louis, MO, U.S.A.).

Methods

Protein determination. Protein was determined by the method of Lowry *et al.* [17].

ODC assay. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine [18]. One unit of ODC activity catalysed the decarboxylation of 1 μmol of ornithine/min at 37 °C.

Purification of ODC and antibody production. ODC was purified from the kidneys of testosterone-treated male NMRI mice as described elsewhere [10,19]. Purification steps were affinity chromatography on pyridoxamine–Sepharose, ion-exchange chromatography on Mono Q and affinity chromatography on heparin–Sepharose. The highly purified ODC was used to generate monoclonal antibodies to ODC [10].

Primary cultures of hepatocytes. Male Sprague–Dawley rats were kept for more than 6 days in a 12 h-dark/12 h-light cycle on the standardized diet of ALMA (Botzenhardt, Kempten, Germany) and water *ad libitum*. Liver parenchymal cells were isolated by a collagenase perfusion method [20]. The isolated cells were allowed to attach to collagen-coated Petri dishes in Williams E Medium [21] with 10% (v/v) calf serum, 1 μM-insulin and 0.1 μM-dexamethasone for 2 h, and the medium was changed afterwards to the same medium without serum.

Half-life of ODC in hepatocytes. After 24 h in the Williams E/insulin/dexamethasone medium, the cells were incubated with Williams E/10 mM-asparagine medium for 3 h to induce ODC activity [22]. For radioactive labelling, the medium was then changed to Williams E/10 mM-asparagine/[^{35}S]methionine (500 μCi/dish) for 1 h. After washing the dishes for 3 × 10 min with 1 mM-L-methionine in Williams E, the cells were incubated with Williams E medium. Incubations were stopped by harvesting the cells after 0, 20, 40, 60, 80, 100 and 120 min. The cells were washed three times with ice-cold Williams E medium, rapidly harvested from the monolayers with a rubber policeman and homogenized in a Potter micro-homogenizer (80 μm clearance, 10 strokes up and down) with 400 μl of homogenization buffer (see above), and the cytosol was isolated by centrifugation for 30 min at 430000 $g_{av.}$ in a Beckman TL-100 ultracentrifuge. Immunotest tubes were prepared by coating with 1 μg of anti-ODC in 500 μl of 200 mM-Na_2CO_3 buffer, pH 10.6, for 12 h, blocking with 4 ml of 99% PBS (120 mM-NaCl/12 mM-Na_2HPO_4/1.5 mM-KH_2PO_4)/1% BSA and washing twice with 99.5% PBS/0.5% Tween 20. The supernatant fractions (300 μl) were shaken in these test tubes for 12 h at 4 °C. Then the tubes were washed three times with PBS/Tween (see above), and the protein bound to the test tubes was solubilized by 0.2 M-NaOH, mixed with Aqualuma plus and counted for radioactivity in a liquid-scintillation counter with automatic d.p.m. calculation.

Effects of NH_4Cl and leupeptin on the half-life of ODC. The half-life of ODC in hepatocytes with blocked lysosomal pathway for protein degradation was measured as described above, but 20 mM-NH_4Cl or 2 mM-leupeptin was included in the incubation medium after labelling and washing the cells.

Arginylation of ODC. The liver of a thioacetamide-treated rat was homogenized in 50 mM-Tris/HCl (pH 7.8)/250 mM-sucrose/30 mM-KCl/5 mM-$MgCl_2$/5 mM-dithiothreitol. Cytosol was isolated by centrifugation for 1 h at 100000 $g_{av.}$, and the buffer was changed to 20 mM-Tris/HCl (pH 7.8)/5 mM-dithiothreitol by ultrafiltration. Samples (3 ml) of this cytosol were incubated with [^{3}H]arginyl-tRNA (230 kBq in 200 μl) for 15 min at 37 °C (for preparation of [^{3}H]arginyl-tRNA, see [23]), and then 30 μl of an aminopeptidase-inhibitor mixture (1 mM-bestatin, 10 mM-leucinol, 10 mM-L-3-amino-1-chloro-5-methylhexan-2-one) was added.

To measure the extent of ODC arginylation under these conditions, 3 ml of the [^{3}H]arginyl-tRNA-treated cytosol was transferred to immunotest tubes (coated with anti-ODC as described above). After shaking the tubes for 12 h at 4 °C, the supernatant fractions were removed, the tubes were washed three times with PBS/Tween and the protein bound to the test tubes was solubilized with 0.2 M-NaOH, mixed with Aqualuma plus and counted for radioactivity in a liquid-scintillation counter.

To measure the extent of arginylation of all cytosolic proteins, they were precipitated from 100 μl portions of cytosol after [^{3}H]arginyl-tRNA treatment with 100 μl of 10% trichloroacetic acid, collected by centrifugation at 13000 $g_{av.}$ for 10 min and solubilized again in 100 μl of 0.2 M-NaOH. This precipitation and solubilization was repeated twice to remove [^{3}H]arginine bound to tRNA. Finally, the solubilized proteins were mixed with Aqualuma plus and counted for radioactivity in a liquid-scintillation counter.

SDS/polyacrylamide-gel electrophoresis and fluorography. [^{3}H]Arginylated ODC, isolated by using anti-ODC-coated immunotubes as described above, was subjected to SDS/polyacrylamide-gel electrophoresis [stacking gel: 125 mM-Tris/HCl (pH 6.8)/0.1% SDS, 6% acrylamide, 3% cross-linker; running gel: 375 mM-Tris/HCl (pH 8.8)/0.1% SDS, linear 12–15% T-acrylamide gradient with 3% cross-linker]. Fluorography was carried out with the fluorographic reagent Amplify. [^{14}C]Methylated proteins were used as M_r markers.

Edman degradation of arginylated ODC. [^{3}H]Arginylated ODC bound to the immunotest tubes as described above was solubilized in 0.2 M-NaOH and was subjected to one single step of Edman degradation as described by Wittmann-Liebold *et al.* [24]. The release of radioactivity from the protein was used for calculation of the amount of arginine bound to the *N*-terminus of ODC.

Effect of Glu-Val-Phe on the half-life of ODC. The half-life of ODC was measured as described above, but 1 mM-Glu-Val-Phe was included in the incubation medium.

RESULTS

Purification of ODC

The purification of ODC from kidneys of testosterone-treated NMRI mice is summarized in Table 1. Specific activity in cytosol was higher than in other ODC purifications from mouse kidney. Overall fractionation of a total of 53.6 g of kidney tissue yielded 271 μg of enzyme protein with a specific activity of 50.6 units/mg of protein. This represented a relative purification of 6570-fold

Table 1. Purification of ODC from testosterone-treated NMRI mice [10]

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Relative activity
Cytosol	2530	19.6	0.0077	100	1
Pyridoxamine–Sepharose	18.3	18.4	1.01	94.2	131
Mono Q	2.78	14.4	5.19	73.7	674
Heparin–Sepharose	0.271	13.7	50.6	70.1	6570

and a recovery of 70.1% of the cytosol activity. The specific activity of the purified enzyme corresponded to the highest values described previously, and the recovery was far higher than in other procedures. The enzyme gave a single band ($M_r \approx 55000$) when tested by SDS/polyacrylamide-gel electrophoresis on a PhastSystem [10].

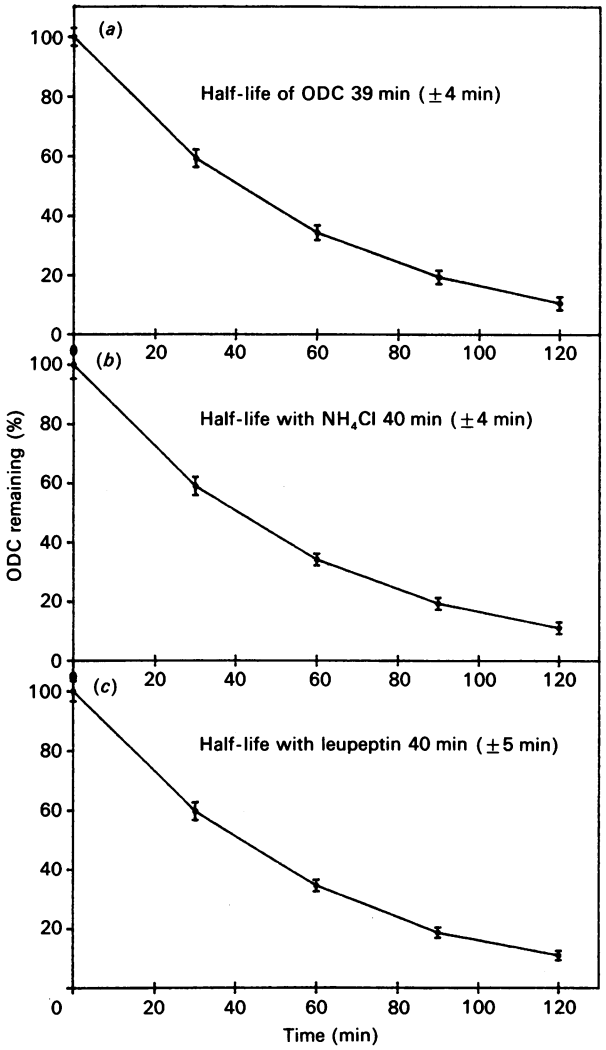

Fig. 1. Half-life of ODC in hepatocytes

ODC in cultured hepatocytes was induced with 10 mM-asparagine. Intracellular protein was pulse-labelled by incubating the cells with L-[^{35}S]methionine for 1 h. The half-life of ODC was determined by measuring the decrease in immunoreactive ^{35}S-labelled ODC with anti-ODC-coated immunotubes after pulse-labelling (*a*). To measure the effect of inhibitors of lysosomal function on the degradation of ODC, the cells were incubated with a 20 mM-NH_4Cl (*b*) or 2 mM-leupeptin (*c*) after pulse-labelling, and the half life of ODC under these conditions was determined as described above.

Monoclonal antibody

The immunoreactivity of the monoclonal antibody generated with the highly purified ODC as antigen was checked in immunotest tubes coated with anti-ODC as described above. The antibody showed an excellent immunoreactivity with native ODC from different sources (kidneys of non-treated and testosterone-treated mice, non-treated and thioacetamide-treated rats) and with DL-α-difluoromethyl[3,4-^{3}H]ornithine-labelled ODC [10].

Half-life of ODC in hepatocytes

To determine this, induction with asparagine was necessary. Without induction the amount of immunoreactive ODC was too low to be detected in the ODC radioimmunoassay. Cellular protein of the hepatocytes was labelled with [^{35}S]methionine in a pulse–chase procedure which allowed us to measure the half-life of ODC with the use of the monoclonal antibody in a radioimmunoassay. The time curve of the degradation of [^{35}S]methionine-labelled ODC is given in Fig. 1. With the assumption that the degradation of ODC is a first-order process, the half-life of ODC was calculated from the curve in Fig. 1 to be 39 min.

Intracellular location of ODC degradation

In order to check whether the lysosomal pathway of protein degradation is responsible for the very fast degradation of ODC in intact cells, this pathway was blocked by leupeptin or NH_4Cl. Neither leupeptin (an inhibitor of lysosomal cysteine proteinases) nor the lysosomotropic amine NH_4Cl had any influence on the very fast degradation of ODC (Fig. 1). This indicates that a non-lysosomal pathway for protein degradation must be responsible for the very fast turnover of ODC in hepatocytes.

Arginylation of ODC

All immunotest tubes used for measuring the extent of ODC arginylation contained 59 mg of cytosolic protein. By measuring the ODC activity in the cytosol used in this experiment, the total ODC activity per test tube was determined to be 2.24 munits of ODC. From the highest specific ODC activity described [25], the amount of ODC in each tube was calculated to be 44.9 ng of ODC protein, which is 0.000076% of the total cytosol protein. After the arginylation with [^{3}H]arginyl-tRNA, 3.34×10^6 d.p.m. were bound to the total cell protein, of which 2.22×10^4 d.p.m. was bound to the anti-ODC-coated tubes and which were therefore supposed to be due to ODC arginylation. Evidence that only post-translationally [^{3}H]arginylated ODC contributes to the radioactivity bound to the anti-ODC coated immunotubes is given in Fig. 2. This means that 0.66% of the total arginylation of cytosolic proteins can be attributed to ODC arginylation. Considering the extremely low amount of active ODC present in

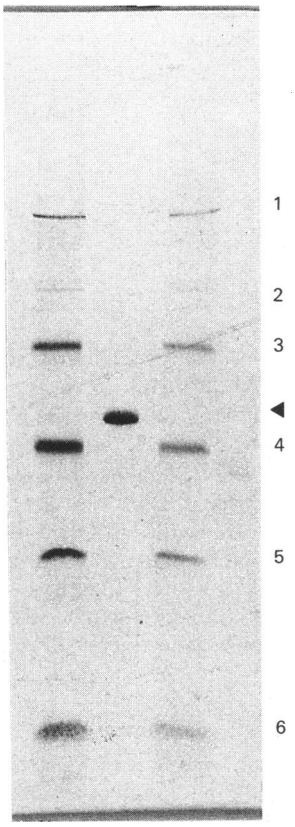

Fig. 2. Fluorography of [^{3}H]arginylated ODC after SDS/polyacrylamide-gel electrophoresis

[^{3}H]Arginylated ODC was isolated with anti-ODC-coated immunotubes and subjected to SDS/polyacrylamide-gel electrophoresis. [^{14}C]Methylated proteins were used as M_r markers: 1, myosin (~200000); 2, phosphorylase *b* (~97400); 3, BSA (~69000); 4, ovalbumin (~46000); 5, carbonic anhydrase (~30000); 6, lysozyme (~14300). Radioactively labelled proteins were then detected by fluorography. [^{3}H]Arginylated ODC is indicated by ◀.

cytosol, the share of ODC in total arginylation is about 8600 times the proportion of ODC in the total cytosol protein.

The M_r of an ODC monomer is about 55000 [25], which means that 44.9 ng of ODC is equivalent to 0.816 pmol. Therefore, arginylation of all ODC monomers with [^{3}H]arginine (sp. radioactivity 15 nCi/pmol) would result in 2.69×10^5 d.p.m. bound to ODC. Actually 2.22×10^4 d.p.m., corresponding to 8.2% of the maximum possible radioactivity, was found in ODC at the end of the incubation with [^{3}H]arginyl-tRNA. The results, which were taken as a basis of the calculations described above, are summarized in Table 2.

Table 2. Post-translational arginylation *in vitro* of ODC and the total cytosol protein

Cytosol from rat livers was treated with [^{3}H]arginyl-tRNA and transferred to immunotest tubes coated with anti-ODC (see the text). Each test tube contained 59 mg of total cytosol protein and 44.9 ng of ODC protein (2.24 munits of ODC activity). The control experiment was done with 20 mM-Tris/HCl, pH 7.8, and 5 mM-dithiothreitol instead of cytosol. Results are means ± S.D. for five observations.

	Experimental (d.p.m.)	Control (d.p.m.)
[^{3}H]Arginine in total cytosol protein	3.34×10^6 ($\pm 1.2 \times 10^4$)	1090 (±121)
[^{3}H]Arginine in ODC	2.22×10^4 (±594)	112 (±10)

N-Terminus of post-translationally arginylated ODC

Some 88.9% of the radioactivity bound to ODC after arginylation with [^{3}H]arginyl-tRNA was released from the protein by one single step of Edman degradation. Therefore it is evident that post-translational arginylation of ODC occurs at the *N*-terminus of the protein.

Post-translational arginylation and degradation of ODC

In order to check whether post-translational arginylation of ODC might have an effect on the degradation of this protein in intact cells, we investigated the effect of Glu-Val-Phe, an inhibitor of arginyltransferase [14], on the half-life of ODC in hepatocytes. As shown in Table 3, inhibition of arginyltransferase caused a significant ($P < 0.001$) prolongation of the half-life of ODC in cultured hepatocytes. The half-life of ODC was more than doubled by treating the cells with 1 mM-Glu-Val-Phe.

DISCUSSION

Our purification scheme results in an apparently homogeneous preparation of ODC with a specific activity which is among the highest reported and an overall yield of 70%, which is substantially higher than the yields obtained by previous investigators [25–27]. These results are achieved by the use of

Table 3. Effect of inhibition of arginyltransferase on the half-life of ODC

ODC in cultured hepatocytes was induced with 10 mM-asparagine. Intracellular protein was pulse-labelled by incubating the cells with [^{35}S]methionine. For measuring the effect of an arginyltransferase inhibitor, the cells were incubated without and with 1 mM-Glu-Val-Phe after pulse-labelling. The remaining amount of immunoreactive ^{35}S-labelled ODC after the pulse-labelling was measured with anti-ODC coated immunotubes as described in the text. Results are means ± S.D. for three observations.

Incubation time (min)	^{35}S-labelled immunoreactive ODC remaining after incubation (%)	
	Without Glu-Val-Phe	With 1 mM-Glu-Val-Phe
0	100.0 (±0.3)	100.0 (±1.9)
30	59.0 (±2.1)	74.5 (±3.4)
60	34.4 (±1.4)	58.4 (±0.3)
90	19.7 (±1.5)	46.5 (±1.5)
120	12.7 (±0.4)	36.1 (±0.7)

conditions designed to inhibit degradation of the very labile ODC by proteinases from the very beginning of purification and by starting with the affinity-chromatography step ([10]; J. Kopitz, G. Adam & P. Bohley, unpublished work). It was already known that a monoclonal antibody against mouse kidney ODC also interacts with the enzyme from other mouse, rat and hamster tissues [28], and we also found such an interaction of our monoclonal antibody from mouse kidney with ODC from rat liver. Therefore it was possible to use this antibody in studies to investigate the degradation of ODC in cultured rat hepatocytes labelled with [^{35}S]methionine. The half-life of about 40 min was unchanged when either the lysosomotropic agent NH_4Cl or the proteinase inhibitor leupeptin was added to the culture medium. This means that the intracellular degradation of ODC in cultured hepatocytes occurs mainly in extralysosomal compartments (cf. [29]), which is in agreement with other studies on the autophagic sequestration of cytosolic proteins with widely differing turnover rates in hepatocytes. In those studies ODC showed the same sequestration rate as tyrosine aminotransferase, tryptophan 2,3-dioxygenase, serine dehydratase, glucokinase, aldolase and lactate dehydrogenase (J. Kopitz, G. Kisen, P. Gordon, P. Bohley & P. Seglen, unpublished work).

The initial events in degradation of ODC and the cellular compartments and proteinases involved in this process are unknown, though several mechanisms have been proposed. The primary steps in ODC degradation may involve reactions with antizyme [30–32] and/or reversible inactivation by the formation of mixed disulphides [33,34]. It has also been suggested that antizyme recycles during the degradation of ODC in a manner similar to the ubiquitin-dependent proteolysis in reticulocytes [30,31]. Some reports have indicated that ubiquitin is not involved in ODC degradation [12,29], but others have suggested that it is [35].

By removal of 37 amino acids at its *C*-terminus, ODC was converted into a stable protein which is labile only in cells treated with polyamines [36], and one may consider different ways in which this *C*-terminal part of ODC could promote degradation: by acting as activator of cellular proteinases or by preventing the folding into a structure that is resistant to degradation [36]. However, some proteins are more rapidly degraded after *C*-terminal truncation [37].

Post-translation *N*-terminal arginylation of cytosolic proteins [10,13–15,23,35,38] introduces one of the degradation signals for a short-lived protein according to the '*N*-end-rule' [11,39–41], and has been found to occur especially with more hydrophobic cytosolic proteins from hepatocytes [23]. Many fast-turnover proteins bind preferentially to hydrophobic surfaces [1–3], and in this respect ODC bound more strongly to octyl-Sepharose and to phenyl-Sepharose compared with any other cytosol protein so far reported [42]. The arginylation of ODC with labelled arginyl-tRNA in cytosol was therefore investigated, and we found that the arginylated ODC had a specific radioactivity more than 8600 times that of the bulk of cytosolic protein. The very rapid degradation of ODC after the arginylation could explain the finding that only 8% of all ODC molecules contained labelled arginine residues. There are different highly active exopeptidases in the cytosol, and it seems worthwhile to investigate the possible role of cytosolic aminopeptidases [43] in the removal of the *N*-terminal arginine as a new way for regulation of intracellular proteolysis.

The cytosolic enzyme arginyltransferase [14,15] catalyses the specific addition of one arginine molecule to the *N*-terminus of proteins or peptides [14,15], and we also showed here that nearly 90% of the [^{3}H]arginine bound to ODC after the arginylation can be released from ODC by one single step of Edman degradation. After an *N*-terminal processing [43] of ODC by cytosolic aminopeptidases [44], this post-translational arginylation of ODC might be one of the initial events in the degradation of this very-fast-turnover protein in cytosol.

The importance of arginyltransferase for intracellular proteolysis of ODC in living cells is demonstrated by our experiments with cultured hepatocytes and the arginyltransferase inhibitor Glu-Val-Phe, which prolonged the half-life of ODC from 39 min to more than 90 min.

Further work is needed to clarify the possible role of cellular proteinases, especially of the multi-catalytic proteinase [45–47], and of ubiquitin [11,39–41], in the degradation of post-translationally arginylated ODC.

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