Biochem. J. (1991) 277, 671–675 (Printed in Great Britain)

Identification of residues in ornithine decarboxylase essential for enzymic activity and for rapid protein turnover

Li LU*, Bruce A. STANLEY* and Anthony E. PEGG†

* Department of Cellular and Molecular Physiology and † Department of Pharmacology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

The importance of certain amino acid residues in mammalian ornithine decarboxylase activity and degradation was studied by site-specific mutagenesis. Changes were made to the mouse ornithine decarboxylase cDNA in a plasmid containing a T7 RNA polymerase promoter. The plasmid was then used for the synthesis of RNA, which was translated in a reticulocyte lysate system. The activity of the ornithine decarboxylase formed and the stability of the protein to degradation in a reticulocyte lysate system were determined. Changes of lysine-169 or of histidine-197 to alanine completely abolished enzyme activity, indicating that these residues are essential for enzyme activity. The removal of the *C*-terminal 36 residues, the mutation of lysine-349 to alanine, of lysine-298 to alanine or the double change of serine-303 and glutamic acid-308 to alanine residues still resulted in an active enzyme. The last-mentioned finding indicates that the phosphorylation of serine-303 does not play an essential role in the catalytic activity of ornithine decarboxylase. The control ornithine decarboxylase protein was degraded rapidly in a reticulocyte lysate provided that ATP was added. The truncated protein missing the 36 residues from the *C*-terminus was much more stable in this system, and the protein containing the double change of serine-303 and glutamic acid-308 to alanine residues was slightly more stable than control ornithine decarboxylase protein. These results indicate that the altered residues may play a role in interaction with factors responsible for the rapid turnover of ornithine decarboxylase.

INTRODUCTION

Ornithine decarboxylase (ODC), which catalyses the conversion of ornithine into putrescine, is an essential enzyme in the synthesis of polyamines in mammalian cells. ODC activity is necessary for cell growth unless exogenous polyamines are provided, and many studies have shown that its activity can fluctuate very rapidly in response to a wide variety of growth-promoting stimuli (Jänne *et al.*, 1978; Russell, 1980; Heby, 1981; Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1988). ODC has a very short half-life, and this may be a critical factor in the regulation of its activity (Jänne *et al.*, 1978; Pegg & McCann, 1982; Pegg, 1986). Despite intensive investigation of the control of ODC concentrations, very little is known of the structural features of the protein needed for ODC activity and degradation. Rechsteiner and colleagues have proposed that PEST regions are involved in the degradation of rapidly turning-over proteins, and ODC contains two such regions located at residues 298–333 and 423–449 (Rogers *et al.*, 1986; Rechsteiner, 1987).

In the present studies, we have altered several residues in the mouse ODC sequence by site-specific mutagenesis and have examined the activity of the altered enzyme. These experiments were carried out by producing the mutations of the cDNA inserted into a plasmid containing a T7 promoter site. The plasmid was then used for the synthesis of RNA by T7 RNA polymerase and the RNA was translated in a reticulocyte lysate. The synthesis of ODC protein was monitored by using the incorporation of [^{35}S]methionine and the activity of the synthesized ODC was measured by determining the release of $^{14}CO_2$ from [1-^{14}C]ornithine. The effect of changes in the ODC sequence on the degradation of the protein was studied by measuring the rate of breakdown of the ^{35}S-labelled protein in a reticulocyte lysate.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized in the Macromolecular Core Facility, Hershey Medical Center, by using a Milligen 7500 DNA synthesizer or were purchased from Operon Technologies, San Pablo, CA, U.S.A. A plasmid derived from pmODC-1, which contains the mouse ODC cDNA sequence, was generously given by Dr. D. Nathans, Johns Hopkins University School of Medicine. [α-[^{35}S]Thio]dATP, L-[^{35}S]methionine and L-[1-^{14}C]ornithine were purchased from Du Pont–New England Nuclear, Boston, MA, U.S.A. pGEM3zf(−), helper bacteriophage M13K07 and RNasin were purchased from Promega, Madison, WI, U.S.A. Rabbit reticulocyte translation system was purchased from BRL Laboratories, Gaithersburg, MD, U.S.A. T4 DNA ligase was purchased from New England Biolabs, Beverly, MA, U.S.A. Sequenase 2.0 was obtained from United States Biochemical, Cleveland, OH, U.S.A. Other biochemical reagents were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Sigma Chemical Co., St. Louis, MO, U.S.A., and Pharmacia–LKB Biotechnology, Piscataway, NJ, U.S.A. Antiserum against ODC was raised as described by Seely *et al.* (1982).

Site-directed mutagenesis of residues in mouse ODC

A 1690 bp *Eco*RI–*Bam*HI fragment containing the mouse ODC cDNA was subcloned from a plasmid derived from pmODC-1 (Kahana & Nathans, 1985) into pGEM3Zf(−) to produce pGEM-ODC. Oligonucleotide-directed mutagenesis was carried out by a modification of the method described by Kunkel (1985). Single-stranded DNA containing uracil was prepared from pGEM-ODC grown in *Escherichia coli* strain CJ236 in the presence of M13K07 helper bacteriophage and purified on 1% NuSieve GTG Agarose gels (FMC Products,

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

† To whom correspondence should be addressed.

Rockland, ME, U.S.A.). The orientation of the f1 origin of replication in the pGEM-ODC was such that the single-stranded DNA produced contained the DNA equivalent to sense mRNA, so the following antisense oligodeoxynucleotides were synthesized for use as mutagenic primers to produce the indicated amino acid or base changes (mismatches are underlined): lysine-169→alanine, 5′-GGCACCAAACGCAACAC-3′; histidine-197→alanine, 5′-GCCCACAGCGAAGCTC-3′; lysine-298→alanine, 5′-GGGCTGCTCCGCCCACACG-3′; lysine-349→alanine, 5′-GGATGAGTAATACGCCTCG-3′; serine-303→alanine, glutamic acid-308→alanine, 5′-CATTTGACGCGTCTTCATCGTCGGCGCCGGGCTGC-3′. The mutagenic primers were phosphorylated, annealed to the uracil-containing single-stranded DNA in a ratio of 25:1 (primer/template), and a complementary strand was synthesized and ligated by using Sequenase and T4 DNA ligase. The double-stranded DNA formed was introduced into *E. coli* JM109 cells by electroporation (Dower *et al.*, 1988). Ampicillin-resistant colonies were picked, grown up overnight and used for isolation of small amounts of DNA (Holmes & Quigley, 1981). This double-stranded DNA was then sequenced (Toneguzzo *et al.*, 1988) by using Sequenase and a suitable primer to identify plasmids containing the desired mutation. (The nucleotide changes in the serine-303→alanine, glutamic acid-308→alanine mutant introduces a *Mlu*I site into the plasmid. For this mutation, an initial screen before sequencing the DNA was carried out by testing candidate plasmid DNAs for the presence of this site.)

The truncated ODC mutant (ODC-425) was obtained by cutting the plasmid with *Nco*I and *Xba*I and inserting an adapter sequence made by annealing the oligodeoxynucleotides 5′-CATGGCGCCTAGTGACGT-3′ and 5′-CTAGACGTCACTAGGCGC-3′. The insertion was completed by ligation of the gel-purified fragment of pGEM-ODC with the adapter by using T4 DNA ligase. The ligated DNA was introduced into *E. coli* JM109 by electroporation. Ampicillin-resistant colonies were picked and grown up overnight as for other mutants, and those plasmids that contained a unique *Nar*I site introduced by the adapter sequence were selected. The insertion of the correct change in the DNA sequence was then checked by DNA sequencing.

Large-scale DNA preparations of DNA from the control pGEM-ODC plasmid and the desired mutants were obtained from 500 ml cultures using the alkaline lysis method followed by centrifugation in CsCl (Birnboim & Doly, 1979).

Transcription and translation of RNA from the mutants of ODC

Portions (50 μg) of purified DNA from pGEM-ODC and mutants from it were linearized with *Xba*I, and RNA was synthesized by using T7 RNA polymerase (Krieg & Melton, 1984; Stanley *et al.*, 1989). This RNA was used for the synthesis of ODC protein in translation assays *in vitro*. The RNA was heated for 10 min at 65 °C and then cooled on ice immediately before use. Translation was carried out in a total volume of 30 μl containing 20 μl of an assay mix solution and 10 μl of a nuclease-treated rabbit reticulocyte lysate (BRL Laboratories). According to the supplier's information, this lysate contained 3.5 mM-$MgCl_2$, 0.05 mM-EDTA, 25 mM-KCl, 70 mM-NaCl, 0.5 mM-dithiothreitol, 25 μM-haemin, 0.5 μg of creatine kinase, 1 mM-$CaCl_2$ and 2 mM-EGTA. The assay mix solution contained about 1 μg of RNA (see below), 180 mM-potassium acetate, 1.5 mM-magnesium acetate, 37.5 mM-Hepes, pH 7.2, 60 mM-KCl, 15 mM-phosphocreatine, 75 μM each amino acid except methionine, 2.25 μM-[^{35}S]methionine (about 60 μCi) and 25 units of RNasin. After incubation for 90 min at 30 °C, the samples were separated by PAGE under denaturing conditions, and the labelled bands corresponding to ODC were detected by fluorography and quantified by scanning the films with a laser densitometer as described previously (Stanley *et al.*, 1989). The amount of RNA added from the various ODC mutants was adjusted to get approximately the same level of ODC synthesis in all cases. Precipitation of the translation products with an antiserum specific for ODC was carried out as described by Pegg *et al.* (1988).

The concentration of univalent cations and Mg^{2+} added in the translation assay mix was based on that found to give an optimal rate of ODC synthesis when mRNA from mouse kidney was used (Kameji & Pegg, 1987). The synthetic mRNA used in the present experiments contains only 52 nucleotide residues of the 313-nucleotide-residue 5′ non-translated leader sequence of mouse ODC and translated much more efficiently than complete ODC mRNA. This is consistent with published reports that ODC mRNA translates very poorly and that this may be caused by the secondary structure and a small open reading frame in the 5′ leader sequence (Pegg, 1986; Coffino, 1988; Wen *et al.*, 1989; Ito *et al.*, 1990; Manzella & Blackshear, 1990).

Assay of ODC activity

When the activity of ODC and mutants derived from it was measured, the labelled methionine for translation *in vitro* was replaced by 20 μM unlabelled methionine. The products from 30 μl translation reactions *in vitro* were assayed for ODC activity by incubation in a total volume of 0.25 ml containing 40 μM-pyridoxal 5′-phosphate, 2.5 mM-dithiothreitol, 50 mM-Tris/HCl and 19.1 μM-[1-^{14}C]ornithine (0.25 μCi). The production of $^{14}CO_2$ was determined as previously described (Seely *et al.*, 1982).

ODC degradation assays

The ^{35}S-labelled ODC synthesized as described above was used as a substrate for degradation by incubating 5 μl portions of the synthesis mix with a reticulocyte lysate in a total volume of 0.3 ml at 37 °C. This contained 40 mM-Tris/HCl, pH 7.5, 5 mM-$MgCl_2$, 2.0 mM-dithiothreitol, 0.5 mM-ATP, 10 mM-phosphocreatine, 0.05 mg of creatine kinase/ml, 0.1 mM-cycloheximide and 75 μl of reticulocyte lysate. This lysate was prepared from rabbits treated with phenylhydrazine by a modification of the method of Pelham & Jackson (1976). Tubes containing 6.5 ml of blood were centrifuged at 500 *g* for 5 min at 0 °C. The pellets were then resuspended in 19.5 ml of wash buffer (130 mM-NaCl, 5 mM-KCl, 7.6 mM-$MgCl_2$) and re-centrifuged as described above. The pellets were then washed four more times by resuspension in 7.8 ml of the wash buffer followed by centrifugation. The final cell pellets (each having a total volume of about 2.4 ml) were lysed by the addition of 2.4 ml of ice-cold sterile water, and the tubes were kept on ice for 5 min and then centrifuged at 15000 *g* for 8 min at 0 °C. The supernatants were removed and stored frozen in 1 ml portions at −70 °C until used.

In experiments testing an energy requirement for ODC degradation, ATP, phosphocreatine and creatine kinase were omitted, and 2-deoxyglucose (20 mM) and hexokinase (10 μg/ml) were added. In order to monitor ODC degradation, 90 μl samples were removed at various times and analysed for the presence of ODC as described above.

RESULTS

Oligodeoxynucleotide-directed mutagenesis was used to change the ODC sequence. Residues lysine-169, histidine-197, lysine-298 and lysine-349 were each changed separately into an alanine residue, and a double mutation where residues serine-303 and glutamic acid-308 were changed into alanine residues was

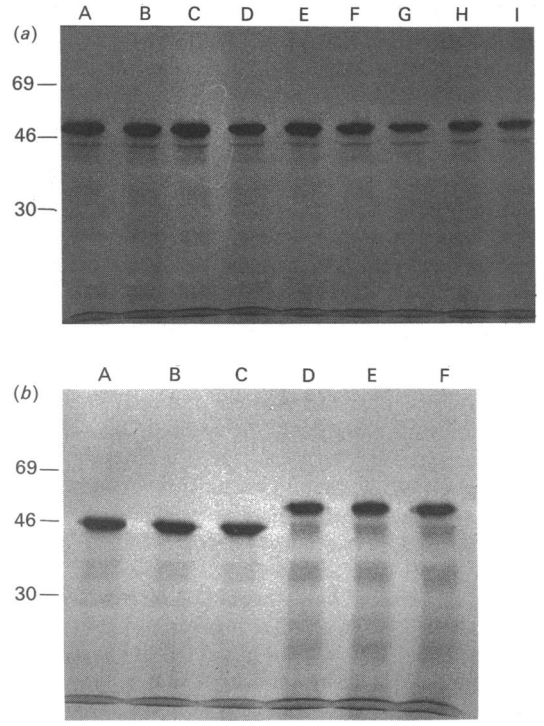

Fig. 1. Translation *in vitro* of RNA prepared from pGEM-ODC and mutants in reticulocyte lysate

Triplicate samples of RNA were prepared, translated and analysed by PAGE as described in the Materials and methods section. (*a*) Lysine-349→alanine mutant (lanes A–C), the serine-303→alanine, glutamic acid-308→alanine mutant (lanes D–F) and the control non-mutant (lanes G–I). (*b*) ODC-425 (lanes A–C) and the lysine-298→alanine mutant (lanes D–F). The positions of M_r markers ($\times 10^{-3}$) are indicated.

Table 1. Effect of changes in amino acid sequence on activity of ODC

The results are expressed as the percentage of the control ODC activity. The activity measurements were compared on the basis of the activity produced in a reticulocyte lysate by the translation of samples of RNA that in parallel experiments gave an equal synthesis of ^{35}S-labelled ODC. Results are means ± S.E.M. (n = 4–6).

Change in sequence	ODC activity (% of control)
None	100
Histidine-197→alanine	< 1
Lysine-169→alanine	< 1
Lysine-298→alanine	33 ± 5
Lysine-349→alanine	93 ± 35
Serine-303→alanine, glutamic acid-308→alanine	123 ± 23
ODC-425 (phenylalanine 425→alanine, proline-426→termination)*	102 ± 29

* This ODC contains only 13 methionine residues compared with 15 in the control but the results were not corrected for this difference.

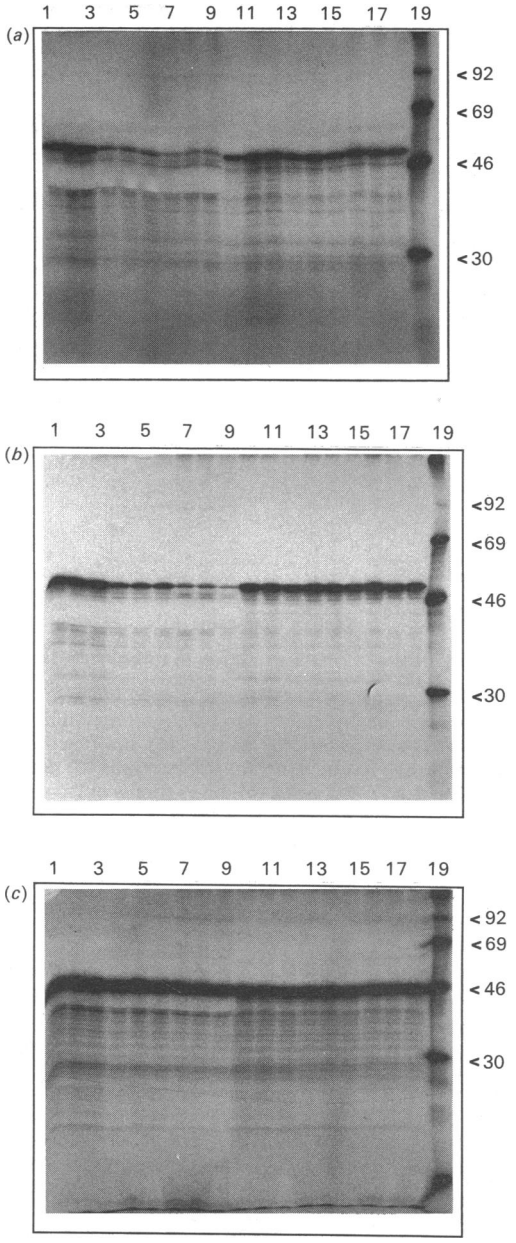

Fig. 2. Degradation of ODC, ODC-425 and ODC mutant (serine-303→alanine, glutamic acid-308→alanine) in reticulocyte lysates

Labelled ODC was synthesized and then incubated in the reticulocyte lysate degradation system in the presence or in the absence of ATP as described in the Materials and methods section. Samples were removed at 0, 1 or 3 h and analysed in triplicate by PAGE as shown. Results are shown for degradation of control ODC (*a*), ODC (serine-303→alanine, glutamic acid-308→alanine) (*b*) and ODC-425 (*c*). In each panel the lanes on the left-hand side (lanes 1–9) show results in the presence of ATP and the lanes on the right-hand side (lanes 10–18) show results in the absence of ATP. Incubation was for 0 h (lanes 1–3 and 10–12), 1 h (lanes 4–6 and 13–15) and 3 h (lanes 7–9 and 16–18). Lane 19 shows M_r markers.

also made. A truncated ODC (ODC-425) was produced by cutting the pGEM-ODC with *Nco*I and *Xba*I and inserting a short linker sequence containing a termination codon after the sequence corresponding to amino acid 425. The plasmids containing each of these cDNAs were used for the synthesis of RNA. The translation of these RNAs in a reticulocyte lysate in the presence of [^{35}S]methionine gave rise to a labelled protein of the expected size (Fig. 1). All of the radioactivity in the bands at about M_r 47000 (ODC-425) and 51000 (control ODC and the other mutants) was precipitable by a monospecific antiserum raised to purified mouse ODC (results not shown). The activity of the ODC encoded by these RNA preparations was determined

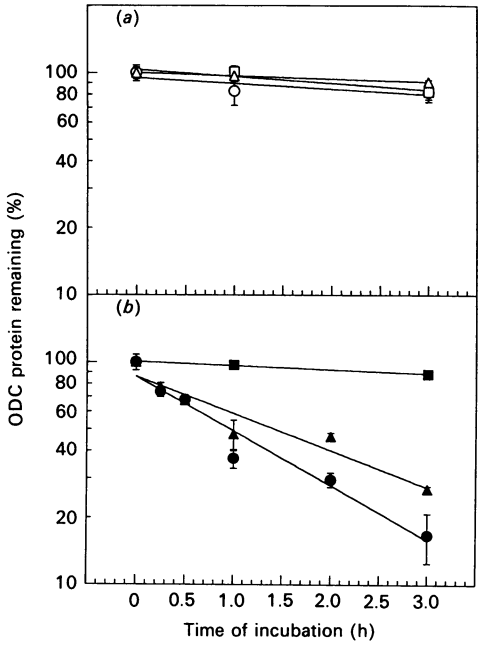

Fig. 3. Quantification of ODC degradation in reticulocyte lysates

Synthesis and degradation of ODC was carried out as indicated in Fig. 2 legend and the amount of the band corresponding to ODC was determined densitometrically and plotted as a function of time of incubation in the lysate. Panel (*a*) shows results for degradation in the absence of ATP and panel (*b*) shows results in the presence of ATP. Results, which are pooled from three experiments, are shown for control ODC (○ and ●), ODC-425 (□ and ■) and ODC mutant (serine-303→alanine, glutamic acid-308→alanine) (△ and ▲).

by measuring ODC activity produced in the reticulocyte lysate and comparing it with the activity found when RNA from the control pGEM-ODC was used. Differences in the amount of protein synthesized (which were minor) were corrected by normalizing the activity measurements to the amount of protein synthesized as determined by densitometric scanning of the fluorograms of translation products. The results shown in Table 1 indicate that changes of lysine-169 or histidine-197 to alanine completely abolished ODC activity. In contrast, the mutation of lysine-349 to alanine had no effect on activity and changing lysine-298 to alanine reduced activity to 33% of control. The change of serine-303 and glutamic acid-308 to alanine residues and the truncation of ODC by deletion of 36 residues from the *C*-terminus had no effect on activity.

The degradation of ODC was studied by using a reticulocyte lysate system as reported by Bercovich *et al.* (1989). The ^{35}S-labelled ODC produced by translation of the RNA in the presence of [^{35}S]methionine was incubated with reticulocyte lysates in the presence or in the absence of an ATP-generating system (Figs. 2 and 3). The control ODC protein was degraded rapidly in this system provided that ATP was present. However, the truncated ODC-425 was much more stable and its degradation was not affected by ATP. The ODC in which serine-303 and glutamic acid-308 were mutated was degraded in an ATP-dependent reaction, but more slowly than the control ODC (Figs. 2 and 3).

DISCUSSION

ODC is present in mammalian cells at very low abundance. The difficulty in obtaining significant amounts of the enzyme protein has prevented detailed studies of the structure and function of the enzyme protein. Before the present study, the only amino acid residue present in ODC known to be essential for activity was glycine-381 in the hamster sequence, which was converted into aspartic acid in the inactive CHO-cell mutant ODC studied by Pilz *et al.* (1990). The system described here, in which mutations are produced in the cDNA and the effects on enzyme activity are determined by measuring the ODC activity formed in a reticulocyte lysate translation system supplemented with RNA made from this template, provides a rapid and convenient method to identify key residues. The more interesting mutants can easily be subcloned into a bacterial expression system and used for the production of larger amounts of the enzyme protein that can then be used for more detailed characterization. Our finding that sufficient ODC can be synthesized in a reticulocyte lysate for the convenient assay of ODC activity is in agreement with the work of Glass *et al.* (1987).

cDNAs corresponding to ODC have been isolated from a variety of organisms, including yeast, trypanosomes, humans and rodents (Fonzi & Sypherd, 1987; Phillips *et al.*, 1987; Hickok *et al.*, 1987; Gupta & Coffino, 1985; Kahana & Nathans, 1985; Wen *et al.*, 1989). Residues histidine-197 and lysine-169, which we have found to be essential for enzyme activity, are conserved in all the known ODC sequences and are contained in regions of the protein that show a high level of similarity. Either of these residues could be responsible for the donation of the proton needed for the decarboxylase action. At present, the lysine residue that forms the Schiff base with pyridoxal 5′-phosphate is not known. Our results rule out the lysine residues at positions 298 and 349, since changes to alanine at these sites did not greatly reduce the activity, but lysine-169 is a possible site for the interaction with pyridoxal 5′-phosphate. The activity of the ODC with the lysine-298 to alanine change does not support the suggestion (Pegg, 1986) that this lysine residue may be the site of interaction with the enzyme-activated irreversible inhibitor α-difluoromethylornithine. The truncation of ODC at position 426 had no effect on the activity, in agreement with a report that ODC missing the *C*-terminal 37 residues was enzymically active when expressed in *E. coli* (Macrae & Coffino, 1987).

The serine residue at position 303 is a probable site of phosphorylation by casein kinase II, and it has been suggested that such phosphorylation might have a role in ODC activity and turnover (Donato *et al.*, 1986; Lougovoi & Kyriakidis, 1989; Meggio *et al.*, 1987; Flamigni *et al.*, 1990; Haddox *et al.*, 1990). Our results indicate clearly that phosphorylation at this site is not needed for ODC activity. The reduced rate of degradation of the ODC in which the serine-303 and glutamic acid-308 were changed to alanine residues suggests that this region may play some role in the degradation of ODC, but it remains to be determined whether phosphorylation is involved. The increased stability of ODC after changes of these two residues, which make up an important part of the 'PEST motif', is consistent with the possible role of the internal PEST sequence in the process (Rogers *et al.*, 1986; Rechsteiner, 1987).

The major effect in diminishing protein breakdown of the truncation of ODC, which removes all of the critical regions of the PEST sequence located at positions 423–449, is consistent with the putative role of this sequence in protein degradation. Ghoda *et al.* (1989, 1990) have reported that truncation of mouse ODC by removal of 37 residues at the *C*-terminus renders the protein stable when expressed in CHO cells. Our results confirm their finding, and show that the physiological factors controlling degradation of ODC can be studied in a cell-free reticulocyte lysate system since this system retains the ability to distinguish between forms of the enzyme that differ in their stability *in vivo*. The use of labelled ODC synthesized *in vitro* from RNA transcribed from plasmids containing appropriate changes in the

ODC cDNA sequence should be very useful in the investigation of the key residues in the deleted ODC that are responsible for the rapid turnover. The plasmid construct containing the cDNA for ODC-425 contains three unique sites for cutting by restriction enzymes (*Nco*I, *Nar*I and *Xba*I), which can be used to insert oligodeoxynucleotides to extend the truncated ODC with any desired sequence.

Our results and those reported by Rosenberg-Hasson *et al.* (1989) and Bercovich *et al.* (1989), who have also noted the rapid degradation of ODC in reticulocyte lysates, indicate clearly that this degradation is an ATP-dependent process, but the other factors controlling ODC degradation are still poorly understood. Ubiquitin does not appear to be involved in the turnover (Glass & Gerner, 1987; Rosenberg-Hasson *et al.*, 1989; Bercovich *et al.*, 1989). It has been suggested that a putrescine-inducible protein termed ODC antizyme may stimulate the degradation by binding to ODC and that the antizyme–ODC complex may be the substrate for degradation (Seely & Pegg, 1983; Murakami & Hayashi, 1985; Murakami *et al.*, 1989). The extent to which antizyme is present in reticulocyte lysates is unclear, but this concept could be tested by addition of additional antizyme. It has also been suggested that the post-translational addition of arginine to the *N*-terminus of ODC may increase its breakdown perhaps by introducing a degradation signal in accordance with the '*N*-end-rule' (Kopitz *et al.*, 1990; Gonda *et al.*, 1989).

This research was supported in part by Grant CA-18138 from the National Cancer Institute. Oligodeoxynucleotide synthesis was carried out with equipment purchased with the aid of a National Science Foundation Biological Facility Center Grant DIR 8804758.

REFERENCES

Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. & Kahana, C. (1989) J. Biol. Chem. **264**, 15949–15952
Birnboim, H. C. & Doly, J. (1979) Nucleic Acids Res. **7**, 1513–1523
Coffino, P. (1988) Gene **69**, 365–368
Donato, N. J., Ware, C. F. & Byus, C. V. (1986) Biochim. Biophys. Acta **884**, 370–382
Dower, W. J., Miller, J. F. & Ragsdale, C. W. (1988) Nucleic Acids Res. **16**, 6127–6145
Flamigni, F., Marmiroli, S., Meggio, F., Guanrieri, C. & Pinna, L. A. (1990) Biochim. Biophys. Acta **1052**, 345–347
Fonzi, W. A. & Sypherd, P. S. (1987) J. Biol. Chem. **262**, 10127–10133
Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D. & Coffino, P. (1989) Science **243**, 1493–1495
Ghoda, L., Phillips, M. A., Bass, K. E., Wang, C. C. & Coffino, P. (1990) J. Biol. Chem. **265**, 11823–11826
Glass, J. R. & Gerner, E. W. (1987) J. Cell. Physiol. **130**, 133–141
Glass, J. R., MacKrell, M., Duffy, J. J. & Gerner, E. W. (1987) Biochem. J. **245**, 127–132
Gonda, D. K., Bachmair, A., Wünning, I., Tobias, J. W., Lane, W. S. & Varshavsky, A. (1989) J. Biol. Chem. **264**, 16700–16712
Gupta, M. & Coffino, P. (1985) J. Biol. Chem. **260**, 2941–2944
Haddox, M. K., Rose, K. M. & Worth, L. L. (1990) J. Cell. Biochem. Suppl. **14C**, 88
Heby, O. (1981) Differentiation **19**, 1–20
Hickok, N. J., Seppänen, P. J., Gunsalus, G. & Jänne, O. A. (1987) DNA **6**, 179–187
Holmes, D. S. & Quigley, M. (1981) Anal. Biochem. **114**, 193–197
Ito, K., Kashiwagi, K., Watanabe, S., Kameji, T., Hayashi, S. & Igarashi, K. (1990) J. Biol. Chem. **265**, 13036–13041
Jänne, J., Pösö, H. & Raina, A. (1978) Biochim. Biophys. Acta **473**, 241–293
Kahana, C. & Nathans, D. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1673–1677
Kameji, T. & Pegg, A. E. (1987) J. Biol. Chem. **262**, 2427–2430
Kopitz, J., Rist, B. & Bohley, P. (1990) Biochem. J. **267**, 343–348
Krieg, P. A. & Melton, D. A. (1984) Nucleic Acids Res. 7057–7070
Kunkel, T. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
Lougovoi, C. P. & Kyriakidis, D. A. (1989) Biochim. Biophys. Acta **996**, 70–75
Macrae, M. & Coffino, P. (1987) Mol. Cell. Biol. **7**, 564–567
Manzella, J. M. & Blackshear, P. J. (1990) J. Biol. Chem. **265**, 11817–11822
Meggio, F., Flamigni, F., Guarnieri, C. & Pinna, L. A. (1987) Biochim. Biophys. Acta **929**, 114–116
Murakami, Y. & Hayashi, S. (1985) Biochem. J. **226**, 893–896
Murakami, Y., Nishiyama, M. & Hayashi, S. (1989) Eur. J. Biochem. **180**, 181–184
Pegg, A. E. (1986) Biochem. J. **234**, 249–262
Pegg, A. E. (1988) Cancer Res. **48**, 759–774
Pegg, A. E. & McCann, P. P. (1982) Am. J. Physiol. **243**, C212–C221
Pegg, A. E., Madhubala, R., Kameji, T. & Bergeron, R. J. (1988) J. Biol. Chem. **263**, 11008–11014
Pelham, H. R. B. & Jackson, R. J. (1976) Eur. J. Biochem. **67**, 247–256
Phillips, M. A., Coffino, P. & Wang, C. C. (1987) J. Biol. Chem. **262**, 8721–8727
Pilz, R. B., Steglich, C. & Scheffler, I. E. (1990) J. Biol. Chem. **265**, 8880–8886
Rechsteiner, M. (1987) Adv. Enzyme Regul. **27**, 135–151
Rogers, S., Wells, S. & Rechsteiner, M. (1986) Science **234**, 364–368
Rosenberg-Hasson, Y., Bercovich, Z., Ciechanover, A. & Kahana, C. (1989) Eur. J. Biochem. **185**, 469–474
Russell, D. H. (1980) Pharmacology **20**, 117–129
Seely, J. E. & Pegg, A. E. (1983) Biochem. J. **216**, 701–707
Seely, J. E., Pösö, H. & Pegg, A. E. (1982) Biochemistry **21**, 3394–3399
Stanley, B. A., Pegg, A. E. & Holm, I. (1989) J. Biol. Chem. **264**, 21073–21079
Tabor, C. W. & Tabor, H. (1984) Annu. Rev. Biochem. **53**, 749–790
Toneguzzo, F., Glynn, S., Levi, E., Mjolsness, S. & Hayday, A. (1988) Biotechniques **6**, 460–469
Wen, L., Huang, J. K. & Blackshear, P. J. (1989) J. Biol. Chem. **264**, 9016–9021

Received 3 December 1990/24 January 1991; accepted 5 February 1991