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31. We thank J. Szejtli of Chinoin Pharmaceutical and Chemical Works, Budapest, Hungary, for methylated cyclodextrin; G. Reed of American Maize Product, Hammond, IN, for propylated cyclodextrins; Y. Kawano of Takeda Chemical Industries, Osaka, Japan, for synthesizing additional β-cyclodextrin tetradecasulfate; R. Levenson and G. Jackson for help with the chick embryo bioassays; and P. Breen and M. Brozna for secretarial assistance. Supported by USPHS grant R01-CA37395 (J.F.); the Preuss Foundation (J.F.); a grant to Harvard University from Takeda Chemical Industries; and by an interdisciplinary research grant from Mobil Foundation (P.B.W.).

18 July 1988; accepted 13 December 1988

Prevention of Rapid Intracellular Degradation of ODC by a Carboxyl-Terminal Truncation

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Ornithine decarboxylase (ODC) was converted from a protein with a short intracellular half-life in mammalian cells to a stable protein by truncating 37 residues at its carboxyl terminus. Cells expressing wild-type protein lost ODC activity with a half-life of approximately 1 hour. Cells expressing the truncated protein, however, retained full activity for at least 4 hours. Pulse-chase experiments in which immunoprecipitation and gel electrophoresis were used confirmed the stabilizing effect of the truncation. Thus, a carboxyl-terminal domain is responsible for the rapid intracellular degradation of murine ODC.

WE REPORT THAT TRUNCATION OF the carboxyl terminus of ornithine decarboxylase (ODC), a cytosolic enzyme with a short intracellular half-life in mammalian cells, converts it into a stable protein. Three lines of evidence suggested a role for the carboxyl terminus in the turnover of ODC. First, although ODC is rapidly degraded in mammalian cells (*1–8*), the equivalent enzyme is stable in trypanosomes (*9*). The mouse and parasite forms of ODC are highly homologous in structure and similar in enzymatic properties, but the former has a carboxyl-terminal portion not present in the latter (*9*). Second, previous studies on mouse ODC expressed in *Escherichia coli* showed that a truncated protein containing the first 423 amino acids of the 461 present in the native protein is enzymatically active (*10*). We thought the carboxyl terminus could mediate a biologically important nonenzymatic function because the sequence in this region is conserved among mammalian ODCs but dispensable for catalytic activity. Third, the "PEST" hypothesis postulates that stretches of amino acids comprised predominantly of proline (P), glutamatic acid (E), aspartic acid, serine (S), and threonine (T) are found in proteins that are rapidly degraded (*11–14*). Murine ODC has two regions that score high on the PEST test. Amino acids 423 through 449, which correspond to one of the PEST regions, lie within the carboxyl terminus of the murine enzyme (*11*).

ODC catalyzes the first committed step in polyamine synthesis, the decarboxylation of ornithine to putrescine (*15, 16*). Intracellular levels of ODC are rapidly modulated under different physiological conditions. Its rate of degradation has been examined by measuring the decay of enzymatic activity or immunoreactive protein after inhibition of protein synthesis by agents such as cycloheximide, and by pulse-chase labeling of ODC followed by immunoprecipitation or two-dimensional gel analysis (*1–8*). It appears that the decay in enzymatic activity is due to the instability of the protein itself rather than an inactivation process that preserves the gross

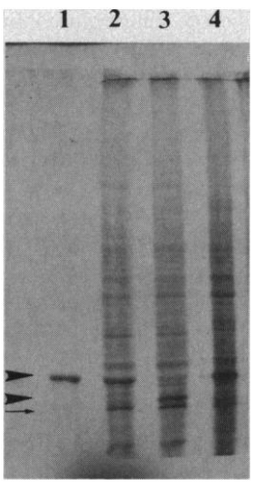

Fig. 1. Immunoprecipitation of ODC proteins expressed from pODg461 and pODg424. ODC⁻ C55.7 cells were cotransfected with pSV2neo (*28*) and either pODg461, a plasmid containing DNA coding for the full-length ODC, or pODg424, a plasmid containing DNA coding for the truncated ODC. After selection for G418 resistance, pooled transfectants were grown in the presence of 10 μ*M* α-difluoromethylornithine for 3 weeks to allow increased expression of the ODC protein (*5*). Cells were labeled for 10 min in methionine- and cysteine-free medium supplemented with 5% fetal calf serum and Tran[^{35}S]-Met (250 μCi/ml) (ICN). Lysates from transfectants carrying pODg461 and pODg424 contained 1.6×10^8 and 2.1×10^8 acid precipitable counts, respectively. These were immunoprecipitated with rabbit antibodies to murine ODC as described in (*5*) and were analyzed by 10% SDS-PAGE and autoradiography. Lanes 1 and lane 4, cytoplasmic lysate from D4.1 ODC overproducing cells (*29*), immunoprecipitated and unprecipitated, respectively; lane 2, immunoprecipitate of pODg461-transfected cells; lane 3, immunoprecipitate of pODg424-transfected cells. Arrowheads mark the positions of wild-type (top) or mutant (bottom) ODC; small arrow indicates the position of actin, whose mass is 42 kD.

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structural integrity of the protein but that abolishes activity (*5, 6*). These observations provided a basis for testing the hypothesis that the carboxyl terminus of mouse ODC is required for its rapid intracellular turnover.

Plasmids encoding a protein missing the last 37 residues at the carboxyl terminus, but otherwise identical to native ODC, were generated. To assure that the results would reflect the intended structure of the mutant protein rather than accidental consequences of the construction process or properties of the vectors, two distinct constructions designed to encode identical proteins were made. One used a mouse ODC recombinant genomic clone, and a second used a mouse ODC cDNA clone (*17*). Both contained a stop codon in place of amino acid codon 425. This site in the mouse protein is equivalent to the carboxyl terminus of the trypanosome protein. The recipient cells for the transfection of recombinant ODC genes were C55.7, a line of mutant Chinese hamster ovary cells devoid of endogenous ODC activity. These cells require putrescine in their medium and do not spontaneously acquire the ability to grow in the absence of exogenously supplied polyamines (*18, 19*).

When a recombinant gene (pODg461) encoding full-size wild-type ODC of 461 amino acids was transfected into the C55.7 cell line, transformants expressed ODC activity. Likewise, when a mutated derivative of the recombinant gene (pODg424) containing an opal (TGA) translation-termination codon at position 425 was transfected into C55.7 cells, transformants expressed ODC activity at levels comparable to those of wild-type transfectants. To establish that ODC proteins of the expected size were present in these two cell lines, we labeled transformants metabolically with ^{35}S-labeled amino acids. Both the wild-type and mutant ODC proteins were immunoprecipitated with an antibody directed against wild-type ODC protein and were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1). Cells transfected with pODg424, the mutated gene, expressed a protein that cross-reacted with antibody to ODC that was lower in molecular weight than wild-type ODC.

To determine the intracellular half-life of the wild-type or the truncated ODC, we treated transfected cells with cycloheximide, an inhibitor of protein synthesis, for various lengths of time. Extracts of these cells were prepared and assayed for ODC activity. Cells expressing the wild-type protein from pODg461 lost activity with a half-life of approximately 1 hour (Fig. 2). Cells expressing the truncated protein from pODg424, however, retained full activity in the presence of cycloheximide for at least 4 hours. Beyond this time, cycloheximide becomes toxic and cannot reliably be used to measure protein stability.

Analogous experiments were performed on C55.7 cells transfected with either the wild-type (pODc461) or mutant (pODc424) ODC cDNA genes. When transfectants expressing the wild-type ODC cDNA gene were treated with cycloheximide, ODC activity decayed to about one-tenth original values after 4 hours (Table 1). Transfectants expressing the mutated ODC cDNA gene retained full ODC activity throughout the 4-hour cycloheximide treatment, in agreement with the results obtained using the genomic transformants. These results indicate that a truncation in the carboxyl terminus of mammalian

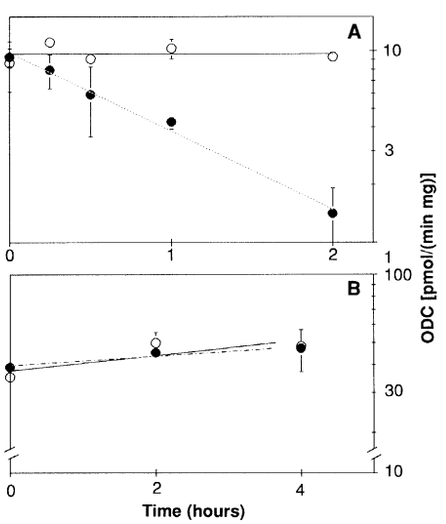

Fig. 2. Turnover of ODC activity expressed from pODg461 or pODg424. C55.7 cells carrying either the wild-type (**A**) or the mutant (**B**) ODC genes were plated at a density of 2×10^6 cells per 100-mm plate. One day later cells were treated with cycloheximide (100 μg/ml) for various times or were untreated. Cells were scraped into Dulbecco's phosphate-buffered saline, pelleted, and suspended in 200 μl of 10 m*M* potassium phosphate buffer, *p*H 7.5, containing 10 m*M* β-mercaptoethanol, and 0.1 m*M* EDTA. After centrifugation of sonicated extracts, 50 μl of each lysate was used in ODC reactions containing 285 μ*M* [1-^{14}C]ornithine (8.7 mCi/mmol), 1 m*M* dithiothreitol, and 100 μ*M* pyridoxal phosphate. ○, Untreated cells; ●, cycloheximide treated cells. Assays were done in triplicate; error bars are standard deviations.

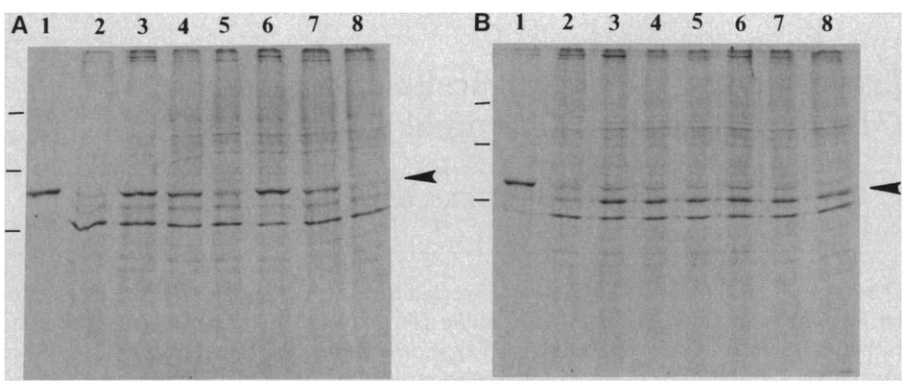

Fig. 3. Intracellular degradation of [^{35}S]methionine-labeled wild-type and truncated ODC. C55.7 cells carrying either the wild-type (**A**) or mutant (**B**) ODC genes were incubated for 2 hours in 3.5 ml of methionine-free medium supplemented with 5% fetal calf serum and [^{35}S]methionine (250 μCi/ml) (Amersham). After this, cells were incubated for 0, 2, or 4 hours in methionine-rich medium in the presence or absence of cycloheximide (100 μg/ml); 5×10^7 acid precipitable counts of each sample were immunoprecipitated and analyzed as in Fig. 1. Lane 1, ODC overproducing D4.1 cells. Lanes 3 to 5, cells incubated without cycloheximide. Lanes 6 to 8, cells incubated with cycloheximide. Lanes 3 and 6, 0-hour chase; lanes 4 and 7, 2-hour chase; and lanes 5 and 8, 4-hour chase. Lane 2, nonspecific precipitate with preimmune serum of the same lysate specifically precipitated in lane 3. Bars mark the positions of proteins whose masses are 97.4, 68, and 43 kD. Arrowheads mark the position of wild-type (A) or mutant (B) ODC.

Table 1. Activity of pODc461- and pODc424-encoded proteins expressed in C55.7 cells. Cells carrying either the mutant (pODc424) or the wild-type (pODc461) ODC genes were plated, treated, and analyzed for ODC activity as in Fig. 2. Activity is given in picomoles of CO_2 per minute per milligram of protein. Numbers in parentheses refer to standard deviations of triplicate determinations.

Time (min)	Activity [pmol/(min·mg)]			
	pODc461		pODc424	
	Untreated	Cycloheximide	Untreated	Cycloheximide
0	24.6 (0.7)	25.1 (0.9)	9.6 (2.7)	10.3 (1.2)
120	21.4 (1.2)	11.9 (4.2)	12.0 (2.8)	10.7 (0.7)
240	24.8 (1.1)	2.7 (0.2)	15.9 (0.1)	10.1 (0.7)

ODC stabilizes ODC activity in cycloheximide-treated cells.

The degradation of ODC is regulatable as well as rapid (*3, 4, 7*). When cells are treated with exogenous polyamines, ODC activity falls more rapidly than when cells are treated with an inhibitor of protein synthesis, such as cycloheximide (*7, 8, 20*). Cotreatment of cells with polyamines and cycloheximide has been found in some cases to diminish ODC activity less rapidly than treatment with polyamine alone (*8, 21–23*), suggesting that a labile protein participates in the degradation of ODC. Thus, the truncated form of ODC could possibly be stable only when cells are prevented from synthesizing proteins. To test this possibility, we labeled cells for 2 hours in the presence of [^{35}S]methionine (pulse) followed by incubation in unlabeled methionine-rich medium (chase) for 0, 2, or 4 hours in the presence or absence of cycloheximide. Analysis by SDS-PAGE of immunoprecipitates revealed that wild-type ODC was largely degraded over a 4-hour period in C55.7 cells carrying pODg461, whether or not cycloheximide was present (Fig. 3). The truncated protein expressed from pODg424, however, did not appear to diminish in the presence or absence of cycloheximide for a period of up to 4 hours. These results support the conclusion that truncation stabilizes ODC independent of protein synthesis. It appears also that under these experimental conditions, ODC turnover is not mediated by a short-lived protein.

Precedents exist for the conversion of short-lived to long-lived proteins. A decade ago, a long-lived form of ODC was shown to be present in a mutant rat cell line selected for resistance to an inhibitor of the enzyme (*24*). Available methods failed to reveal a structural alteration in the ODC produced by the mutant line (*25*), and it was therefore surmised that the mutation altered a cellular proteolytic degradation process, rather than the enzyme itself. Another labile enzyme, hydroxymethylglutamyl coenzyme A (CoA) reductase, has been mutagenized and thereby made more stable (*26*). The mutation that renders hydroxymethylglutamyl CoA reductase stable is a 331–amino acid deletion of a region in the amino terminus, which contains an extremely hydrophobic membrane-spanning domain; removal of that domain makes the protein stable and converts the protein from a membrane-associated to a cytoplasmic form. It is unlikely that a change of intracellular localization explains the results decribed here. Fractionation studies with cells that express the wild-type and truncated ODCs have indicated no significant change in the intracellular distribution of enzymatic activity and indicate that in both cases ODC is a cytoplasmic protein.

The structural characteristics of a protein that determine its rate of intracellular degradation are poorly understood. Several rules have been proposed that relate protein primary structure to stability (*11–14, 27*). The results presented here give significant support to the PEST hypothesis (*11*), and raise further questions relating to the necessity, sufficiency, and specificity of such sequences in rapid protein degradation. For example, it remains to be determined whether replacing the wild-type carboxyl-terminal domain with termini rich in amino acids other than those required by the PEST hypothesis would also promote rapid turnover of ODC. Furthermore, a second PEST sequence is retained in the truncated ODC protein whose role in protein degradation is undetermined. Elevation of intracellular polyamine levels by exogenously administered polyamines has been shown to accelerate the intracellular turnover of ODC (*7, 8, 20*). Although the truncated ODC is stable under the conditions of cell growth described here, the protein is labile in cells treated with polyamines. The second PEST sequence could play a role in this polyamine-dependent turnover of ODC.

One may consider two general ways in which the carboxyl-terminal domain of ODC could promote degradation. The domain may be engendering a destructive process, for example, by acting as an attractant or an activator of a protease. In contrast, it may act as a disrupter of a process that results in protein stabilization, for example, by preventing the folding of ODC into a tertiary or quaternary structure that is resistant to degradation. The two classes of models make distinct predictions. In the first case, one might expect that addition of the carboxyl-terminal domain of ODC to other proteins would destabilize them. In the second case, it is likely that a broad range of carboxyl-terminal structures would effectively interfere with ODC stability.

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17. The vector, pODc461, has ODC cDNA flanked on the 5′ by the SV40 early promoter and at the 3′ by the hepatitis B virus polyadenylation signal sequence. The vector was constructed in two steps. First, the large Cla I–Sst II fragment of the pFR400 plasmid [C. C. Simonsen and A. D. Levinson, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2495 (1983)], with the Sst II site blunt-ended by the use of T4 polymerase, was isolated and the Taq I–Pvu II fragment (1628 bp) of ODC cDNA [G. Gupta and P. Coffino, *J. Biol. Chem.* **260**, 2941 (1985)] was inserted, thereby producing a plasmid named pODM3. Next, a 514-nucleotide Sal I fragment containing the f1 ori was excised from pDM1 [D. A. Mead, E. S. Skorupa, B. Kemper, *Nucleic Acids Res.* **13**, 1103 (1985)] and cloned into the one Sal I site of pODM3 to facilitate the synthesis of single-stranded DNA. The resulting plasmid was named pODM3.3f1 or, alternately, pODc461. Mutagenesis of the 425 Phe codon of pODc461 into an amber (TAG) codon to create pODc424 was performed by the double-primer method [M. J. Zoller and M. Smith, *DNA* **3**, 479 (1984)] with pODM3.3f1 single-stranded DNA as a template. The mutagenic primer (AC CTC CGG CGG CTA GCC ATG GCT C, mutagenic mismatches underlined) spanned the 425 Phe codon of ODC and a second primer (CAC AAT GGG TCA CAA CAT AA) was complementary to the 3′ noncoding region of ODC cDNA. The mutation was confirmed by DNA sequencing. pODg461 contains the entire mouse ODC gene [M. Brabant, L. McConlogue, T. van Daalen Wetters, P. Coffino, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2200 (1988)] and approximately 7 kb of contiguous upstream DNA. pODg424 was made by inserting into the Nco I restriction site within ODC codons 422 to 424 a 150-bp Nco I fragment from the Rous sarcoma virus reverse transcriptase gene isolated from plasmid pSPGP-1 (obtained from T. Jacks, UCSF). The insertion converts the sequence form . . . CAG AGC CAT GGC TCC . . . to . . . CAG AGC CAT GGC TGA CCT . . . and thereby generates a TGA opal translation stop codon at position 425.
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30. Supported by NIH grants CA 29048, CA 47721, and CA 09043, and NSF grant DCB 8707375.

6 October 1988; accepted 11 January 1989