Ornithine decarboxylase stability in HMOA and DH23b cells is not due to post-translational truncation of a C-terminal recognition site

John L. A. MITCHELL*, Chung-youl CHOE and Gary G. JUDD

Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, U.S.A.

The normally labile ornithine decarboxylase (ODC) becomes unusually stable when Cys-441 is replaced with Trp in the variant cell lines HMOA and DH23b. This stable ODC is also observed to have higher mobility on SDS/PAGE. Because previous studies have shown that ODC stability can be achieved when as few as five amino acid residues are removed from its C-terminus, it was suggested that the amino acid substitution in the variant ODC might alter its conformation sufficiently to promote a similar proteolytic loss of a C-terminal degradation signal, resulting in a stable yet active ODC. To examine this mechanism, amino acids

in the C-terminal regions of both wild-type and stable (Trp-441) ODC proteins were released, by means of carboxypeptidase-Y digestion, and identified by HPLC. The C-terminal ends were found to be the same, and are as predicted from the cDNA sequence. This study proves that stability of the Trp-441 form of ODC is not simply due to proteolytic removal of a C-terminal proteasome-targeting sequence, thereby implying that the stabilization of this mutant ODC form must result directly from a conformational change associated with the loss of Cys-441.

INTRODUCTION

Ornithine decarboxylase (ODC; EC 4.1.1.17) catalyses the initial and, arguably, rate-limiting step in biosynthesis of polyamines necessary for normal cell function (reviewed in [1]). To avoid potentially toxic over-accumulation of polyamines, this enzyme is rapidly inactivated in response to increased concentrations of free spermidine or spermine. These polyamines increase the rate of translation of mRNA for a regulatory protein, antizyme, which complexes with ODC monomers and targets this enzyme for rapid degradation by the 26 S proteasome (reviewed in [2]). Consequently, the half-life of ODC can range from 4 h or more, when cellular polyamine levels have been artificially lowered [3,4], to less than 20 min on addition of excess polyamines [3,5].

The signal for rapid degradation of ODC seems to involve its C-terminal end because truncation by as few as five amino acid residues results in a substantial increase in the stability of this protein [6–8], even in the presence of antizyme [9,10]. Li and Coffino [9] suggested a model, based on antibody recognition studies, in which antizyme binding serves to change the conformation of ODC such that its C-terminus becomes exposed, and presumably recognized by an as yet unidentified binding component of the proteasome.

A stable ODC mutant has been identified in HMOA cells, a variant of the HTC rat hepatoma cell line that has been selected for resistance to the competitive ODC inhibitor, α -methyl-ornithine [11]. Curiously, this ODC seems to have a normal affinity for antizyme although the complex is not subsequently recognized by proteasome [12,13]. Miyazaki et al. [13] traced this ODC stability to a mutation in the ODC gene causing a single amino acid substitution, Cys-441 $\rightarrow$ Trp. This same modification has now been observed in the stable ODC found in DH23b cells, which were selected for resistance to high levels of the irreversible inhibitor α -difluoromethylornithine [14,15]. Because the stabilizing alteration in ODC observed in these variants is relatively close (20 residues) to the C-terminal end of this protein, one

could speculate that the loss of this cysteine residue might in some way prevent the normal antizyme-induced exposure of this critical region. Paradoxically, modifications in amino acid sequence are generally expected to promote destabilization, especially when the change involves the loss of a cysteine that might be part of a conformation-stabilizing disulphide bond.

In view of this contradiction we have hypothesized that it might actually be the increased lability of the C-terminal region of ODC from HMOA and DH23b that results in the overall stabilization of the ODC protein. In this study we have examined the potential instability of the C-terminal end of ODC protein as a factor in determining the susceptibility of this protein to rapid degradation by the proteasome.

EXPERIMENTAL

Plasmids and protein expression

GST-ODC fusion protein (where GST is glutathione S-transferase) was purified, by using glutathione-Sepharose 4B, from *Escherichia coli* DH5 transformed with pGEX-ODC. This plasmid was produced by inserting the 2 kb *NcoI/EcoRI* ODC fragment (Cys-441) from *Rattus norvegicus* (provided by Dr. Harry vanSteeg [16]) into the *NcoI/EcoRI* sites of pGEX-3X (Pharmacia). GST-Z1AZ fusion protein was similarly prepared from bacteria transformed with the *NcoI* fragment of Z1AZ cut from pTV-Z1NN2 (provided by Dr. Shin-ichi Hayashi [17]) cloned into a pGEX-3X vector using a *Bam*-compatible *NcoI* linker.

For ODC expression *in vitro*, two different ODC cDNAs were used. First, pGEM-ODC was constructed by cloning the *HincII/EcoRI* rat ODC (Cys-441, as above) into a pGEM-3-Zf+ vector (Promega). Secondly, pBluescript II SK-ODC was produced by inserting the *BamHI* rat ODC fragment (both Cys-441 and Trp-441 forms) from pSVL-ODC (provided by Dr. Riccardo Autelli [18]) into the *BamHI* site of pBluescript II SK vector (Stratagene).

Abbreviations used: GST, glutathione S-transferase; ODC, ornithine decarboxylase; OPA, o-phthalaldehyde.

* To whom correspondence should be addressed.

For antizyme expression *in vitro*, pBluescript II SK-AZ was constructed by cutting the Z1 antizyme sequence from the pTV-Z1NN2 plasmid (as above) with *Nco*I and inserting it into the *Bam*HI site of pBluescript II SK with a linker.

Purified DH23b ODC was prepared as described previously [19].

Transcription and translation *in vitro*

pGEM-ODC was linearized by *Eco*RI or *Bst*XI and transcribed with T3 RNA polymerase (Promega) to yield truncated and intact ODC mRNA respectively. These RNAs were translated into truncated and intact ODC proteins by using L-[³⁵S]methionine (Amersham) and rabbit reticulocyte lysate (Promega). pBluescript II SK-ODC was linearized by *Hind*III for transcription and translation *in vitro* as with pGEM-ODC. Translation products were separated by SDS/PAGE and blotted to nitrocellulose and detected by either autoradiography or immunological localization. Affinity-purified polyclonal rabbit antibody specific for antizyme was prepared as described previously by using an antizyme fusion protein (pGEX-AZ) expressed in bacteria [20].

Analysis of C-terminal amino acids

ODC protein samples were denatured by treatment with 0.1 M sodium bicarbonate, pH 7.8, containing 4.5 mM dithiothreitol and 1% (w/v) SDS. Iodoacetic acid (250 μ M; Sigma) was added with incubation in the dark at 22 °C for 30 min. Excess reagents were then removed by extraction with trichloroacetic acid. The reduced, carboxymethylated ODC was digested by carboxypeptidase-Y (an exopeptidase with specificity for all free C-terminal residues; Sigma) using a carboxypeptidase-to-ODC ratio of 1:100 (w/w), at 25 °C in the presence of 0.1% SDS.

Aliquots (20 μ l) of the digestion reaction were mixed with 5 μ l of a 2% SDS solution in 0.4 M sodium borate containing 5 μ l of *o*-phthalaldehyde (OPA) derivatizing solution. After incubation at 22 °C for 1 min, the reaction was stopped by addition of 10 μ l of 0.1 M sodium acetate, pH 7.2, and a 25 μ l aliquot was injected on to the reverse-phase HPLC column. The OPA derivatizing solution was made by dissolving 50 mg of OPA (Fisher) into 1.25 ml of methanol, followed by the addition of 50 μ l of 2-mercaptoethanol and 11.2 ml of 0.4 M sodium borate, pH 9.5. The mixture was flushed with N₂ and stored in the dark until use.

HPLC analysis of released amino acids was performed with a Waters Nova-Pak C₁₈ reverse-phase column (4 μ m particle size, 60 Å pore, 3.9 mm $\times$ 150 mm). A tetrahydrofuran/methanol solvent gradient [solvent A, tetrahydrofuran/methanol/0.1 M sodium acetate, pH 7.2 (5:95:900, by vol.); solvent B, methanol] was used for the separation of amino acids with a flow rate of 2.2 ml/min. The OPA-derivatized amino acids were monitored with a Waters 470 fluorescence detector set for excitation at 335 nm and emission at 455 nm.

RESULTS AND DISCUSSION

The involvement of antizyme in ODC protein degradation can be reproduced in a heterologous test system *in vitro*. Rabbit reticulocyte lysate used for mRNA translation *in vitro* also degrades ODC in an ATP- and antizyme-dependent manner. Murakami et al. [21] and Mamroud-Kidron et al. [22] have shown that such lysates contain both the necessary 26 S proteasome and a small amount of antizyme. As shown in Figure 1, wild-type ODC synthesized *in vitro* is degraded by components of the reticulocyte

Figure 1 Degradation of ³⁵S-labelled ODC forms *in vitro*

Wild-type (wt; Cys-441), truncated and mutant (Trp-441) ³⁵S-labelled ODC were synthesized *in vitro* as described in the Experimental section. Samples (approx. 2 units) of active ODC protein were added to 16 μ l of rabbit reticulocyte lysate (Promega) in the presence of the prescribed ATP-generating system necessary for translation, but without added mRNA or amino acids. As indicated, the degradation reaction was run in the absence and presence of 4 units of purified GST-AZ fusion protein. The mixtures were incubated at 30 °C and samples removed for analysis by SDS/PAGE and autoradiography at 0, 30 and 60 min. Activity assays and unit (U) values are as described by Mitchell et al. [20].

lysate mixture, and the addition of purified fusion protein containing the effective C-terminal half of antizyme greatly enhances this reaction. In contrast, ODC protein synthesized without its last 39 amino acid residues does not degrade noticeably in this system, even in the presence of added antizyme. This observation is consistent with the stability of such truncated ODC forms noted in intact cells [6]. In that case, however, it was unclear whether such truncation actually interfered with the rapid antizyme-induced ODC degradation, and later evidence suggests that the ODC C-terminus is essential for all ODC degradation [8,10,22,23]. Like truncated ODC, the mutant ODC form found in HMOA and DH23b cells (Cys-441 $\rightarrow$ Trp) is also stable *in vitro* in this proteolytic system, even in the presence of added antizyme (Figure 1).

We have previously reported that it is the ODC monomer and not the active, intact dimer that complexes readily with antizyme [24]. Therefore it is not surprising that the presence of antizyme during the emergence of the nascent ODC polypeptide chain in translation promotes efficient formation of an ODC-antizyme complex, and consequently extremely rapid ODC degradation ($t_{1/2}$ < 5 min) [5,25]. Because of this, the erroneous appearance is given that antizyme has a strong inhibitory effect on ODC mRNA translation itself.

If, as many believe, antizyme acts as a chaperone to facilitate immediate degradation of newly formed ODC, then how might this process be altered by the single amino acid change noted in the mutated ODC isolated from HMOA and DH23b cells? One possibility is that the change induces improper folding in the C-terminal region, allowing this sensitive end of the ODC protein to be exposed, perhaps even in the absence of antizyme. As previously noted, this exposure might promote recognition by the 26 S proteasome with subsequent ODC degradation, but this is not observed in the mutated ODC form. Alternatively, abnormal exposure of this critical C-terminal ODC end in this fashion might specifically allow proteolytic cleavage of the exposed peptide. This would result in a C-terminal truncation that would leave active, stable ODC protein, exactly as observed.

The possibility of a truncation event that occurs immediately on completion of ODC synthesis is supported by the observation that the Trp-441 form of ODC synthesized *in vitro* runs slightly

Figure 2 Comparison of the mobility of different ODC forms with SDS/PAGE

ODC protein purified from DH23b cells was compared with wild-type (wt) and Trp-441 ODC protein synthesized *in vitro*, by using SDS/PAGE analysis. The proteins were blotted to nitrocellulose and ODC was detected with affinity-purified polyclonal ODC antibody. Bands were revealed by Amplified alkaline phosphatase goat anti-rabbit immuno-blot assay kit (Bio-Rad).

faster on SDS/PAGE than similarly made wild-type ODC (Figure 2). This apparent difference in size was also noted by Miyazaki et al. [13], with several different replacements for Cys-441 in the ODC protein, and Autelli et al. [18]. Further, this apparent size difference is also noted in the stable mutant ODC form that has been isolated and purified from DH23b cells (Figure 2, lane 3).

If the above hypothesis is correct and replacement of Cys-441 confers a structural change resulting in post-translational truncation of several C-terminal amino acids, this would explain the stability seen in the mutant ODC form and would also support the current belief that antizyme destabilizes ODC by exposing this region to recognition by the proteasome. We tested this hypothesis directly by analysing the C-terminal amino acids present in pure Trp-441 ODC protein isolated from DH23b cells. This protein preparation does run slightly faster than wild-type ODC on SDS/PAGE (Figure 2) and it is resistant to degradation, so according to the model it should have C-terminal amino acids different from those encoded by the cDNA sequence.

In the study shown in Figure 3 we have used carboxypeptidase-Y to release amino acids from the C-terminal ends of denatured Cys-441 (wild-type) and Trp-441 (mutant) ODC species. This exoprotease is known to remove C-terminal residues with varying facility, depending on exactly which amino acids are involved. From the predicted sequence of wild-type cDNA we expected the appearance of free Val, Asn, Ile, Ser and Ala respectively. As shown in Figure 3(A), the last three (Val, Asn and Ile) all seemed to be released at about the same time with very little evidence of Ser, or any other amino acids, even after 80 min. Because carboxypeptidase-Y is most effective when the penultimate and/or terminal residues have aromatic or large aliphatic side chains, it is not surprising that all of the last three amino acids were released at about the same time, with essentially no serine evolution. As shown in Figure 3(B), the release of amino acids from stable ODC purified from DH23b cells showed the exact same pattern. Because this combination of amino acids is not predicted to be in close proximity anywhere else in this half of the ODC protein, we must conclude that the C-terminal end of the stable ODC protein has not been truncated.

Although the stable form of ODC does contain the C-terminal degradation target, it is still possible that this recognition signal is not exposed sufficiently to be recognized by the proteasome components. Li and Coffino [9] suggested that antizyme functions by altering the conformation of ODC to increase the exposure of this C-terminal target sequence. Because carboxypeptidase-Y generally only recognizes exposed C-terminal amino acids, as in the denatured substrates used in the above experiments, we speculated that there might be a differential sensitivity of wild-type and stable ODC molecules to this protease. Unfortunately, carboxypeptidase-Y was found to be completely ineffective at cleaving non-denatured ODC (either wild-type or stable mutant)

Figure 3 Comparison of C-terminal amino acids of wild-type and stable (Trp-441) ODC protein

(A) GST-ODC (Cys-441; wild-type sequence) was prepared as described in the Experimental section, and approx. 150 μ g was digested with carboxypeptidase-Y to reveal only the C-terminal amino acids. At the indicated intervals, samples were removed and analysed with reverse-phase HPLC. Only the indicated amino acids were detected to be free in quantities above background (●, Val; ■, Asn; ▲, Ile). (B) Stable ODC protein containing Trp-441 was purified from DH23b as described previously [19]. Samples (50 μ g) were digested with carboxypeptidase-Y as above and the resulting data are presented as in (A).

even in the presence of GST-antizyme (results not shown). Thus if a structural change in ODC is induced by antizyme binding, it does not expose the protein C-terminal end sufficiently for recognition by carboxypeptidase-Y.

In these experiments we have clearly demonstrated that the C-terminal destabilization region of ODC remains intact in the stable, Trp-441 form of the protein found in HMOA and DH23b cells. If it is true that the ODC C-terminal amino acids constitute a destabilization domain, it is now necessary to discover the reason that antizyme fails to expose this critical region of the mutant form of ODC, as this seems to be the key to understanding normal antizyme-induced ODC lability.

This work was supported by Research Grant GM33841 from the National Institutes of Health.

REFERENCES

- 1 Marton, L. J. and Pegg, A. E. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35**, 55–91
- 2 Hayashi, S. I. and Murakami, Y. (1995) *Biochem. J.* **306**, 1–10
- 3 Mitchell, J. L. A., Mahan, D. W., McCann, P. P. and Qasba, P. (1985) *Biochim. Biophys. Acta* **840**, 309–316
- 4 Glass, J. R. and Gerner, E. W. (1986) *Biochem. J.* **236**, 351–357
- 5 Murakami, Y., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1994) *Biochem. J.* **304**, 183–187
- 6 Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D. and Coffino, P. (1989) *Science* **243**, 1493–1495
- 7 Rosenberg-Hasson, Y., Bercovich, Z. and Kahana, C. (1991) *Eur. J. Biochem.* **196**, 647–651
- 8 Ghoda, L., Sidney, D., Macrae, M. and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 2178–2185
- 9 Li, X. and Coffino, P. (1993) *Mol. Cell. Biol.* **13**, 2377–2383

- 10 Tamori, A., Nishiguchi, S., Kuroki, T., Koh, N., Kobayashi, K., Yano, Y. and Otani, S. (1995) *Cancer Res.* **55**, 3500–3503
- 11 Mamont, P. S., Duchesne, M., Grove, J. and Tardif, C. (1978) *Exp. Cell Res.* **115**, 387–393
- 12 Murakami, Y., Fujita, K., Kameji, T. and Hayashi, S. (1985) *Biochem. J.* **225**, 689–697
- 13 Miyazaki, Y., Matsufuji, S., Murakami, Y. and Hayashi, S. (1993) *Eur. J. Biochem.* **214**, 837–844
- 14 Mitchell, J. L. A., Diveley, Jr., R. R., Bareyal-Leyser, A. and Mitchell, J. L. (1992) *Biochim. Biophys. Acta Mol. Cell Res.* **1136**, 136–142
- 15 Mitchell, J. L. A., Choe, C. Y., Judd, G. G., Daghfal, D. J., Kurzeja, R. J. and Leyser, A. (1996) *Biochem. J.* **317**, 811–816
- 16 Van Steeg, H., Van Oostrom, C. T. M., Van Kranen, H. J. and Van Kreijl, C. F. (1988) *Nucleic Acids Res.* **16**, 8173–8174
- 17 Miyazaki, Y., Matsufuji, S. and Hayashi, S. (1992) *Gene* **113**, 191–197
- 18 Autelli, R., Persson, L. and Baccino, F. M. (1995) *Biochem. J.* **312**, 13–16
- 19 Mitchell, J. L. A., Hoff, J. A. and Bareyal-Leyser, A. (1991) *Arch. Biochem. Biophys.* **290**, 143–152
- 20 Mitchell, J. L. A., Judd, G. G., Bareyal-Leyser, A. and Ling, S. Y. (1994) *Biochem. J.* **299**, 19–22
- 21 Murakami, Y., Matsufuji, S., Tanaka, K., Ichihara, A. and Hayashi, S. (1993) *Biochem. J.* **295**, 305–308
- 22 Mamroud-Kidron, E., Omeritsicovich, M., Bercovich, Z., Tobias, K. E., Rom, E. and Kahana, C. (1994) *Eur. J. Biochem.* **226**, 547–554
- 23 Ghoda, L., Phillips, M. A., Bass, K. E., Wang, C. C. and Coffino, P. (1990) *J. Biol. Chem.* **265**, 11823–11826
- 24 Mitchell, J. L. A. and Chen, H. J. (1990) *Biochim. Biophys. Acta* **1037**, 115–121
- 25 Li, X. and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 3556–3562