# Feedback repression of ornithine decarboxylase synthesis mediated by antizyme

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The induction of antizyme by spermidine and the resulting enhancement of ornithine decarboxylase (ODC) degradation have been well studied; however, little is known about the mechanism whereby elevated spermidine levels decrease synthesis of the polyamine biosynthetic enzyme. To evaluate the relative contribution of inhibited synthesis, as distinct from enhanced degradation of ODC, spermidine levels were manipulated in a variant cell line that overproduces a stable form of ODC. Spermidine did not selectively inhibit ODC synthesis in these variant cells, supporting the concept that spermidine diminishes ODC synthesis in normal cells owing to enhanced degradation of the protein in the presence of elevated antizyme levels. This model was further investigated *in vitro* by use of rabbit reticulocyte lysate, which catalyses simultaneous ODC mRNA translation and antizyme-stimulated degradation of ODC protein. Antizyme strongly repressed the incorporation of labelled amino acids into normal rat ODC. Unexpectedly it also diminished the apparent translation of ODC mRNA species coding for enzyme forms that are not destabilized by the post-translational addition of antizyme. The effect of antizyme on ODC translation was not observed in wheatgerm extract, in which there is no antizyme-induced degradation. Further, deletion of a short segment of antizyme necessary for the destabilization of ODC (amino acid residues 113–118) resulted in a form that bound ODC but did not diminish its apparent translation. These results suggest that the co-translational addition of antizyme to ODC results in a complex that is different from, and innately less stable than, that formed when antizyme is added post-translationally.

## INTRODUCTION

The polyamines putrescine, spermidine and spermine are essential to normal mammalian cell physiology, yet potentially cytotoxic when present at elevated concentrations. To avoid excessive polyamine synthesis, the initial enzyme in their biosynthetic pathway, ornithine decarboxylase (ODC; EC 4.1.1.17), is tightly feedback-regulated at the post-translational level, and perhaps also during translation (reviewed recently in [1–3]). Post-translationally, elevated levels of free cellular spermidine stimulate the synthesis of a regulatory protein, antizyme, which specifically and avidly complexes with ODC and promotes its rapid degradation by the 26 S proteasome [2,3]. Recent studies by Coffino and co-workers [4–6] suggest that in forming this complex with antizyme a critical C-terminal degradation target is exposed on the ODC protein, and this domain, combined with a region on the N-terminal portion of antizyme, serves to mark ODC for rapid degradation.

Elevated levels of cellular spermidine not only enhance ODC degradation but they also seem to inhibit ODC synthesis [7–11]. This control might involve structural components of the long, GC-rich 5′ untranslated region of the ODC message, as recently reviewed by Pegg et al. [1]. Others argue that the observed spermidine feedback on ODC synthesis relies only on the coding regions of the mRNA and is not due to an alteration in translation velocity; rather it reflects an enhanced rate of degradation of ODC protein when synthesized in the presence of antizyme [12–14]. Mitchell and Chen [15] demonstrated that the ability of antizyme to complex with ODC was greatly facilitated by modifications in the native ODC structure such as the dissociation of the homodimer into monomeric units. It is therefore reasonable to assume that antizyme would preferentially bind partly folded ODC monomers during translation, resulting in an even shorter half-life than observed when antizyme is added to extant, native ODC protein. By using a cell line in which they could force the over-expression of antizyme, Murakami et al. [14] demonstrated that the cellular half-life of the ODC–antizyme complex is less than 5 min. This accelerated turnover of ODC is consistent with a model in which spermidine suppresses the incorporation of labelled amino acids into ODC protein by increasing the rate of degradation of ODC protein synthesized in the presence of antizyme.

If the effect of spermidine on ODC synthesis is due to this increased rate of ODC degradation, one would predict that this result should not be observed under conditions where ODC has been modified to prevent antizyme-induced degradation. In the present study we have tested this hypothesis by using two stable ODC forms, one truncated to delete a portion of the C-terminal ODC destabilization domain [4,16–18] and the other containing a single amino acid substitution of Trp for Cys-441 [19–21]. Contrary to expectations, accumulation of these notably stable ODC forms was affected by the co-translational presence of antizyme, and paradoxically, this effect depended on enhanced turnover of these mutant ODC forms. The results suggest that, by binding to the N-terminal end of the nascent ODC polypeptide, antizyme exposes additional protease-sensitive sites on ODC that are not revealed when antizyme binds to ODC that has already achieved its native structure.

## EXPERIMENTAL

### Cell culture

ODC-stable DH23b cells [21] were selected from rat hepatomal (HTC) cells as described previously [22] and cultures maintained in monolayer and suspension cultures in Swim's 77 medium containing 10 % (v/v) calf serum and 10 mM difluoromethyl-

Abbreviations used: BMV, brome mosaic virus; CAT, chloramphenicol *O*-acetyltransferase; ODC, ornithine decarboxylase; wt, wild-type.

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ornithine (Eflornithine; kindly provided by Marion Merrell Dow Research Institute).

### ODC immunoprecipitation

Preparation of monospecific ODC antibody and immunoprecipitation techniques were as described previously [22].

### Preparation of GST–antizyme fusion protein

Truncated cDNA coding for rat liver antizyme was obtained in a prokaryotic expression plasmid pTV-Z1NN2 from Dr. S. Matsufuji and Dr. S. Hayashi [23]. The plasmid was cut with *Nco*I, and the fragment coding for antizyme was ligated into the *Nco*I site of a pGEX-3X (Pharmacia) vector with a *Bam*HI-compatible *Nco*I linker. Subsequent purification of the fusion protein, activity assays and unit values were as previously described [24]; 4 units of this protein were sufficient to induce degradation of wild-type (wt) ODC in reticulocyte lysates [25].

### Site-directed mutagenesis of Z1-antizyme

Amino acid residues 113–118 (based on wt antizyme from rat containing 227 residues) were deleted to form Z1-AZΔ113–118 by site-directed mutagenesis with the Altered Sites *in vitro* mutagenesis system (Promega). Mutated Z1-antizyme in the pALTER-1 vector was verified by DNA sequencing.

### mRNA preparations and translation *in vitro*

Wild-type rat liver ODC (Cys-441) and the stable mutant form isolated from HMOA cells (Trp-441 ODC) were prepared from pSVL-ODC plasmids provided by Dr. Riccardo Autelli [20]. Both the Cys-441 and Trp-441 ODC forms were removed from the respective pSVL-ODC vectors with *Bam*HI and inserted into the *Bam*HI site of pBluescript II SK vector (Stratagene). These were linearized for transcription with *Hin*dIII. To produce truncated ODC (407 amino acids; ODC-N407) lacking the C-terminal destabilization domain, the Cys-441 ODC was also cut by *Sna*BI. All three forms of ODC cDNA were transcribed by T3 RNA polymerase.

Z1-antizyme and Z1-AZΔ113–118 in pALTER-AZ were linearized by *Afl*III and transcribed by T3 RNA polymerase (Promega) to produce intact and deleted antizyme mRNA.

Control and standard mRNA species were obtained as follows: luciferase mRNA was purchased from Promega. Rabbit globin mRNA containing both the $\alpha$-chain (600 bases) and $\beta$-chain (650 bases) was purchased from Gibco/BRL. pCDNA3/CAT vector (Invitrogen) containing an 787 bp fragment coding for chloramphenicol *O*-acetyltransferase (CAT) was linearized with *Bam*HI and transcribed with T7 RNA polymerase. Brome mosaic virus (BMV) RNA1 cDNA on the plasmid pB1TP3 [26] was kindly provided by Dr. J. Bujarski. This was linearized with *Sma*I and transcribed by T7 RNA polymerase. The amounts and quality of mRNA species transcribed *in vitro* were evaluated by electrophoresis on 1% (w/v) agarose gels and comparison of ethidium bromide-stained bands with appropriate standards.

Translation of the mRNA species transcribed *in vitro* was by use of rabbit reticulocyte lysate (Promega) and wheatgerm extract (Promega) translation systems as indicated, using L-[$^{35}$S]methionine (Amersham) under the conditions recommended by the manufacturer for a 25 $\mu$l reaction volume. Translation products were separated by SDS/PAGE, blotted to nitrocellulose and detected by autoradiography. The autoradiographs were subsequently scanned and digitized, and quantified by the use of Image Calc, a shareware program created by C. H. A. van de Lest.

## RESULTS

### Effect of spermidine on ODC synthesis in intact cells

The addition of polyamines to cells in culture induces a marked decrease in [$^{35}$S]methionine incorporation into ODC. To distinguish any direct effect of the polyamines on ODC translation itself from enhanced degradation due to the co-translational presence of polyamine-induced antizyme, we have explored this phenomenon in cells containing stable ODC forms. The replacement of Cys-441 with Trp in the ODC isolated from HMOA cells has been shown to result in a protein that is relatively stable ($t_{\frac{1}{2}}$ of approx. 10 h) [27] even though antizyme seems to complex normally with this mutant ODC [28,29]. Unfortunately both unstable wt and mutant ODC are expressed simultaneously in this line (as shown by Autelli et al. [20]), making this a poor test system in which to analyse label incorporation into the stable ODC band separated on SDS/PAGE gels. Recently we demonstrated that this same stable ODC mutation is also overexpressed in the difluoromethylornithine-resistant variant DH23b, but in this cell the level of unstable ODC expressed is relatively small [21]. The effect of elevated intracellular spermidine on ODC synthesis can therefore be evaluated in cultures of these cells exposed to low levels of added spermidine, as summarized in Table 1. After 1 h of spermidine exposure, which raised the intracellular levels approx. 3-fold to 15–20 nmol/mg of protein, the cells were exposed to [$^{35}$S]methionine for 30 min, and labelled ODC was measured as a fraction of total protein synthesis. Previous studies had shown that higher levels or longer polyamine exposures could induce even larger intracellular spermidine increases, but these greatly inhibited total protein synthesis as well [30]. Under the conditions described in Table 1 there were no specific differences noted in the relative amounts of ODC protein synthesized even though there was a 3-fold increase in the intracellular level of spermidine.

The inability of spermidine to preferentially decrease the synthesis of stable ODC in DH23b cells tends to support the model that enhanced ODC degradation is necessary for the apparent effect of elevated spermidine levels on ODC synthesis in normal cells. It should be noted, however, that DH23b cells also lack feedback inhibition of polyamine transport [30]. This

**Table 1 ODC protein synthesis is not selectively inhibited by exogenous spermidine in DH23b cells**

Spermidine (60 $\mu$M) and aminoguanidine (1 mM) were added to cultures of DH23b cells growing in the presence of 10 mM difluoromethylornithine and incubation was continued for 1 h. The cells were then washed and resuspended at a concentration of $5.0 \times 10^6$ cells/ml in methionine-free medium and labelled for 20 min with 250 $\mu$Ci/ml [$^{35}$S]methionine. After multiple washes with ice-cold isotonic PBS the cells were homogenized by sonication, and samples were immunoprecipitated with mono-specific ODC antibody and counted in a scintillation counter. Samples of the crude cell extracts were also precipitated with 10% (w/v) TCA and counted. The [$^{35}$S]methionine incorporated into ODC protein is reported below as a percentage of total TCA-precipitable counts. The results are means ± S.D. for three distinct experiments comparing spermidine-treated cells with controls; figures in parentheses are the number of replicates.

| | ODC activity (% of control) | |
|---|---|---|
| Experiment no. | Control | With 60 $\mu$M spermidine |
| 1 | 1.0 ± 0.08 (4) | 1.18 ± 0.12 (6) |
| 2 | 0.65 ± 0.14 (8) | 0.52 ± 0.10 (8) |
| 3 | 0.63 ± 0.01 (8) | 0.59 ± 0.11 (8) |

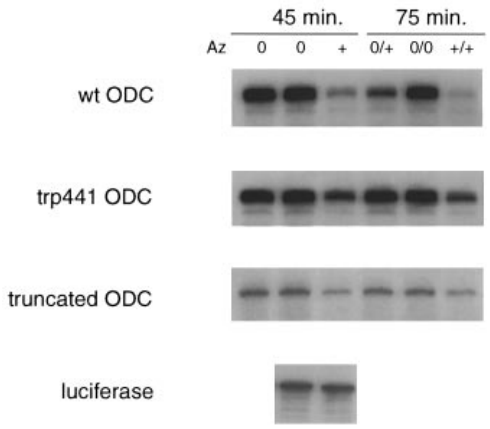


**Figure 1 Demonstration *in vitro* of the effect of GST–antizyme (Az) on the translation and stability of ODC**

mRNA species transcribed *in vitro* (0.1–0.2 $\mu$g) for wt ODC (Cys-441), Trp-441 ODC, the truncated ODC (ODC-N407) and luciferase were translated in rabbit reticulocyte lysate for 45 min in the presence (+) or absence (0) of an excess of purified GST–antizyme protein. At this time, samples were withdrawn for evaluation of $^{35}$S-labelled proteins by SDS/PAGE and, as indicated, GST–antizyme was added to some of the reactions that did not have this addition during translation (0/+). The reactions were incubated for an additional 30 min and samples again withdrawn for analysis to determine the extent of protein degradation that had occurred in the period after the translation reaction had ceased.

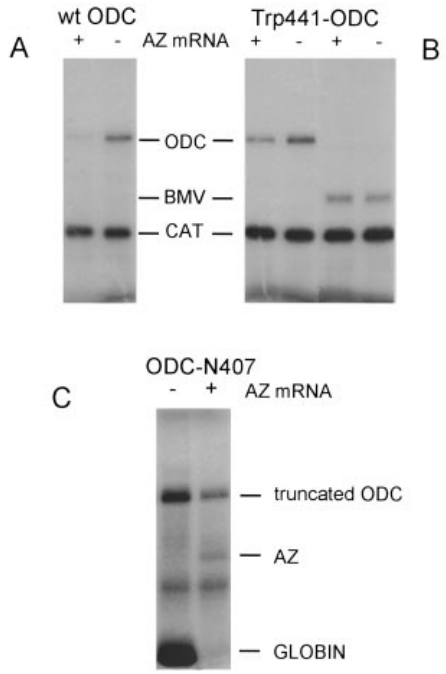


**Figure 2 Effect of Z1-antizyme (AZ) translated *in vitro* on the incorporation of [$^{35}$S]methionine into ODC**

(**A**) Translation was initiated in 25 $\mu$l of rabbit reticulocyte lysate reaction mixtures by using either Z1-antizyme mRNA (first lane; +) or globin mRNA as a control (second lane; −). After 5 min an additional 25 $\mu$l of lysate was added to each reaction along with mRNA species for both wt ODC and CAT, and translation was continued for an additional 45 min. The labelled products were analysed by SDS/PAGE and autoradiography. (**B**) mRNA species transcribed *in vitro* for Trp-441 ODC and CAT were co-translated for 30 min in the presence of mRNA for either Z1-antizyme or globin, in the first and second lanes respectively. The reactions shown in the third and fourth lanes reflect 30 min co-translations of mRNA species for BMV and CAT in the presence or absence respectively of Z1-antizyme mRNA. (**C**) mRNA species for Z1-antizyme (+) or the control, globin (−), were translated separately for 5 min as in (**A**). The reaction mixtures were then added in equal volumes to translation mixtures containing the truncated ODC (ODC-N407) mRNA, and the reactions were continued for an additional 45 min. A longer autoradiographic exposure was used to show the presence of the faint antizyme band in the second lane of (**C**).

deficiency has been traced to the fact that very high accumulations of the inactive, stable ODC tend to bind up any available antizyme, thereby preventing its normal function in transporter regulation [21,24,30]. It is possible that antizyme is also a necessary intermediate in the polyamine-induced depression of ODC synthesis. This was examined in the studies *in vitro* described below.

### Effect of antizyme protein on ODC synthesis and degradation *in vitro*

In the following experiments the effect of antizyme on ODC mRNA translation *in vitro* was evaluated with the rabbit reticulocyte lysate system. This lysate has also been shown to be a good cellular model for studies of antizyme-stimulated ODC degradation [31–33] and is therefore ideal for these investigations of co- and post-translational actions of antizyme on ODC protein.

The effect of GST–antizyme, a functional antizyme fusion protein constructed from Z1-antizyme [23,24], on [$^{35}$S]methionine incorporation into wt ODC in a 45 min translation *in vitro* is shown in Figure 1. Under these conditions antizyme inhibited ODC production by almost 60 % compared with the duplicate controls without antizyme. After 45 min, when the translation reaction had ceased, antizyme was added to one of the control reactions and all lysates were incubated for an additional 30 min and sampled again. As expected, a portion (almost 35 %) of the newly synthesized ODC was degraded during this 30 min post-translational exposure to antizyme. Clearly such antizyme-induced degradation during translation is a major contributor to the relatively poor incorporation of label into wt ODC in the presence of antizyme.

Contrary to expectations, however, this same level of GST–antizyme also adversely affected the apparent translation of Trp-441 ODC, the stable ODC form found in HMOA and DH23b cells. The co-translational presence of antizyme decreased the level of label incorporated into Trp-441 ODC by approx. 23 %. In a parallel reaction, co-translational antizyme also decreased the incorporation of label into a stable truncated form of ODC by 41 %. In this case, the wt ODC cDNA was cut with *Sna*BI to produce a truncated ODC mRNA that translates to an ODC lacking 54 C-terminal residues, which are a necessary part of the destabilization domain. Consistently with previous studies [25], neither the Trp-441 ODC nor the truncated ODC (ODC-N407) was destabilized by the post-translational addition of antizyme. Also as expected, antizyme did not seem to affect the translation of a control mRNA, luciferase.

### Co-translation of antizyme and ODC

To eliminate problems associated with purification and maintenance of active antizyme, and to minimize the possibility that non-specific inhibitory factors might be carried over in the GST–antizyme protein preparations, the effect of antizyme on ODC translation was further tested by using Z1-antizyme synthesized *in vitro*. In the studies shown in Figure 2, Z1-

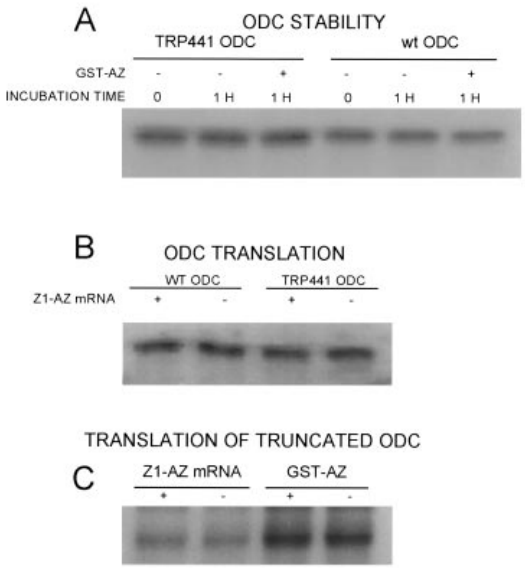


**Figure 3 Effects of antizyme on ODC synthesis and stability in the wheatgerm extract translational system**

(**A**) Wild-type ODC and Trp-441 ODC mRNA species were translated with the wheatgerm translational system and samples were withdrawn for analysis by SDS/PAGE. GST–antizyme (GST–AZ; 10 units) was then added to portions of each of these translation reactions and incubation continued for a further 1 h, at which time the reactions with or without antizyme were again sampled for analysis of the label remaining in the ODC band. (**B**) Wild-type ODC and Trp-441 ODC mRNA species were each co-translated with either Z1-antizyme (Z1–AZ) or globin mRNA as described in Figure 2(A), except that this was translated with the wheatgerm extract system. (**C**) The first two lanes show the product of truncated ODC (ODC-N407) mRNA that was co-translated with either Z1-antizyme or globin mRNA in wheatgerm extract as in (**B**). ODC-N407 mRNA translation was performed in the presence or absence of an excess of GST-antizyme (20 units), and the resulting ODC bands are shown in the third and fourth lanes respectively.

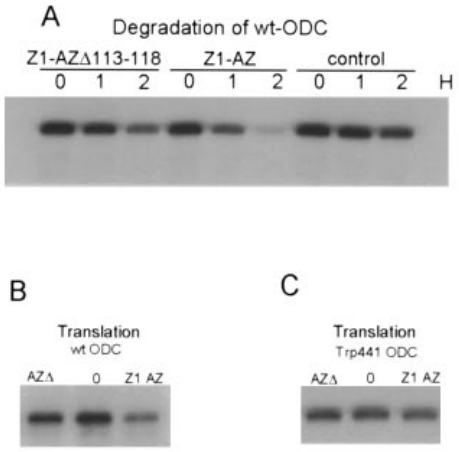


**Figure 4 Comparison of the effects of Z1-antizyme and Z1-AZ*Δ*113–118 on the translation and stability of ODC in rabbit reticulocyte lysates**

(**A**) Z1-antizyme (Z1-AZ) , Z1-AZΔ113–118 and wt ODC were made separately by translation *in vitro* in rabbit reticulocyte lysates. Equal amounts of the antizyme proteins (approx. 0.1 ng) were added to [$^{35}$S]ODC and the degradation of this protein in reticulocyte lysates with ATP was followed for 2 h. (**B**, **C**) Reticulocyte lysate translation mix was incubated for 5 min either alone (0) or with mRNA for Z1-antizyme or Z1-AZΔ113–118 (AZΔ). These reactions were subsequently mixed in equal volumes with fresh reticulocyte lysate containing either wt ODC mRNA (**B**) or Trp-441 ODC mRNA (**C**), and co-translation was continued for an additional 45 min.

antizyme mRNA was translated in a reticulocyte lysate translation mix for 5 min before ODC and control mRNA species were added. Such pre- and co-translation of antizyme mRNA had a marked effect on the apparent translation of wt ODC without affecting translation of a control mRNA, CAT (Figure 2A).

Consistently with the studies presented in Figure 1, both Trp-441 and ODC-N407 (truncated) were also inhibited in their apparent translation (49 % and 84 % respectively) by the presence of antizyme mRNA, and thus antizyme, in the translation reaction (Figures 2B and 2C). This affect of antizyme on the incorporation of label into even the stable ODC forms seems to be specific because in all cases the translations of control mRNA species (CAT and BMV) were not similarly decreased by the presence of antizyme mRNA. Further, it did not seem that the translation of antizyme mRNA caused a significant depletion of translational system components because the amount of antizyme synthesized was small relative to either the control or ODC mRNA. It should be noted that, with only two methionine residues per Z1-antizyme, detection of the antizyme band generally required extended autoradiograph exposures (compare Figure 2C with Figures 2A and 2B). To minimize possible non-specific mRNA effects, equivalent amounts of globin mRNA were added to control (no Z1-antizyme mRNA) reactions. As shown in Figure 2(C), the amount of label incorporated into globin protein greatly exceeded that into antizyme, yet the Z1-antizyme mRNA caused repression of label incorporation into ODC.

## Translation with wheatgerm extract

The above observations, that even 'stable' ODC forms show antizyme-induced repression of translation, suggest that this inhibition might be independent of antizyme-induced ODC degradation. To examine this unexpected possibility further, we tested for this effect in another translation system *in vitro*, wheatgerm extract. As shown in Figure 3(A), this system differs from reticulocyte lysate in that it does not support the antizyme-induced proteolytic degradation of even wt ODC. After a 1 h incubation in the presence or absence of added GST–antizyme protein, there was no difference in labelled ODC protein levels from those in unincubated controls. In this system, where rapid ODC degradation was not observed, translation of wt ODC mRNA was not affected by the co-translation of antizyme mRNA (Figure 3B) even though this level of Z1-antizyme mRNA was sufficient to suppress ODC accumulation by over 60 % in rabbit reticulocyte lysate. The presence of antizyme mRNA (Figures 3B and 3C) or GST–antizyme during translation in wheatgerm extract also did not diminish translation of 'stable' ODC forms, Trp-441 and ODC-N407. The lack of response to antizyme in the wheatgerm system supports the original model that the antizyme effect on apparent translation depends upon ODC degradation. This seems, however, to be in striking contrast with the observed effects of antizyme on ODC translation of the 'stable' ODC forms in reticulocyte lysates.

## Studies with antizyme from which the ODC-destabilization domain has been deleted

Because of the apparent paradox that degradation of even 'stable' ODC is required for the observed effect of antizyme on ODC translation, the following studies were performed to test whether it is antizyme's destabilizing activity specifically that is necessary for this effect. The C-terminal half of antizyme is

capable of binding and inactivating ODC, but stimulated degradation of ODC protein requires a region in the N-terminal half of this regulatory protein [5,34]. In particular, Ichiba et al. [34] have extrapolated that residues 113–118 might be most critical in the ODC destabilization reaction.

Using site-directed mutagenesis we deleted this suspected destabilization domain from Z1-antizyme (Z1-AZΔ113–118) to create an antizyme form that in theory should bind ODC efficiently but not stimulate its degradation. Translation of this construct *in vitro* produced a protein, of approximately the same molecular mass as Z1-antizyme, that cross-reacted with antizyme antibody and inactivated ODC (results not shown). Equal amounts of Z1-antizyme or Z1-AZΔ113–118 synthesized *in vitro* were examined for their ability to stimulate ODC degradation in reticulocyte lysates. As shown in Figure 4(A), the deletion mutant did stimulate ODC degradation slightly with respect to controls without antizyme, but this antizyme form was not nearly as effective as the unmutated Z1-antizyme.

These two antizyme forms were then tested for their action on the translation *in vitro* of wt and Trp-441 ODCs in rabbit reticulocyte lysate, as depicted in Figure 4 (B and C). The Z1-AZΔ113–118 construct was found to be much less effective than the parental Z1-antizyme in decreasing the production of both wt ODC (14 % compared with 49 % decrease) and stable ODC (4 % compared with 18 % decrease), again confirming that it is the degradation function of antizyme that is required for this co-translational effect.

## DISCUSSION

The opposing models to explain the spermidine-induced suppression of incorporation of labelled methionine into ODC protein are (1) a direct effect of the polyamine on the structure of the 5′ untranslated region of ODC mRNA resulting in inefficient translation [1], and (2) the accelerated degradation of newly synthesized ODC in the presence of spermidine-stimulated antizyme [12–14]. The original intent of this study was to use a variant line of HTC cells (DH23b) that overproduces a stable ODC form so that we could distinguish between these modes of action. To this end, spermidine levels inside intact DH23b cells were rapidly elevated 3-fold and the production of ODC protein was monitored. The lack of any immediate effect on ODC synthesis was a clear rejection of the first model in favour of the second.

To examine the validity of the second model, involving antizyme-mediated accelerated degradation of newly synthesized ODC, we used a system *in vitro* in which ODC translation and degradation proceed simultaneously. This model predicts that the incorporation of labelled methionine into wt ODC protein should be greatly decreased by the presence of antizyme, whereas this regulatory factor should have no effect on the incorporation of label into modified ODC forms, such as Trp-441 or C-terminal-truncated ODC, as these are not degraded rapidly even in the presence of antizyme [4,16–21]. As expected, the synthesis of wt ODC was markedly affected by antizyme. Surprisingly, synthesis of both the modified ODC forms was also suppressed somewhat by antizyme, even though the post-translational addition of antizyme did not stimulate their degradation. Further, this response was independent of whether the effective protein was added as a purified GST–antizyme construct or as Z1 antizyme synthesized *in vitro*.

The observation that antizyme represses the synthesis of even stable ODC *in vitro* seems incongruous with the results of Table 1 showing that spermidine addition did not affect the incorporation of label into ODC in DH23b cells. As previously noted, however, the very large (more than 1000-fold) excess of ODC in this variant cell line effectively titrates the relatively small amount of antizyme produced in response to elevated intracellular spermidine [21]. Thus polyamine transport, which is normally repressed by antizyme, is also not turned off when spermidine levels are elevated in these DH23b cells [24,30].

The inhibitory effect of antizyme on label incorporation into the apparently stable ODC forms would suggest either a direct effect of antizyme on the translational efficiency of ODC mRNA or some hitherto unknown mechanism of antizyme-induced ODC degradation. The first possibility is inconsistent with the observation that antizyme failed to have any effect on ODC mRNA translation in the wheatgerm extract system, in which there is no degradation of ODC. This conclusion is supported by the results of Murakami et al. [14], in which they observed that antizyme does not directly alter the translation of ODC mRNA *in vitro*. To specifically test the possibility that ODC turnover is necessary for the antizyme effect on translation of the modified ODC forms, we produced an antizyme, Z1-AZΔ113–118, construct that bound and inactivated ODC but had a greatly diminished capacity to stimulate ODC degradation. This modified antizyme form was much less effective than intact antizyme at suppressing the incorporation of label into ODC protein in the reticulocyte lysate translation/degradation system. These results indicate that rapid ODC turnover is essential for the ability of antizyme to suppress label incorporation into ODC, even in the enzyme forms that are not destabilized by post-translational addition of this same antizyme.

Murakami et al. [14] demonstrated that ODC synthesized in the presence of antizyme was degraded at an accelerated rate, and they suggested that this enhanced degradation resulted from the facility with which antizyme complexed with newly synthesized ODC monomers. The current studies clearly point to a different explanation for this accelerated degradation, namely that the complex formed by the co-translational presence of antizyme must be distinct from the complex achieved when antizyme is added to pre-existing ODC. This distinction is evidenced by the fact that antizyme present during the synthesis of Trp-441 ODC or the C-terminal-truncated form induces degradation of both forms, whereas antizyme complexes made post-translationally with these enzyme forms are not subject to rapid degradation.

Li and Coffino [4] suggested that the post-translational attachment of antizyme to ODC caused a conformational change that made a destabilization domain on the C-terminus of ODC more accessible. This identified degradation target is either not present or not revealed by the addition of antizyme to the ODC-N407 or Trp-441 mutant forms respectively. Does the destabilization of these forms when translated in the presence of antizyme imply the exposure of perhaps another ODC destabilization domain that would promote degradation by the 26 S proteasome? Li et al. [6,35] have shown that grafting the N-terminal portion of antizyme to heterologous proteins containing known destabilization domains can induce such lability. A potential candidate for this second destabilization region might be the protease-sensitive loop in eukaryotic ODC at residues 160–170, identified by Osterman et al. [36]. Interestingly, this region is quite close to the antizyme-binding domain (residues 117–140) identified by Li and Coffino [37].

Alternatively, co-translational engagement of antizyme might interfere with the actions of chaperones in folding this protein. Normal folding of cell proteins is thought to involve co-translational attachment of members of the Hsp70 family of chaperones to early hydrophobic regions, with subsequent folding being facilitated by a large oligomeric complex such as the

chaperonins [38,39]. Conceivably, bound antizyme would inhibit one or both of these actions, resulting in unfolded, and therefore unstable, ODC protein.

Antizyme was initially identified for its ability to inactivate ODC and promote its degradation [2,3,40,41]. Recently it has been discovered that this regulatory protein is also a necessary intermediate in the control of polyamine transport activity [24,42]. Now we have suggested yet another role for this interesting protein: it seems to be able to inhibit the normal folding of ODC, and by this mechanism is at least partly responsible for the decrease in ODC production in cells replete with spermidine.

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