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Degradation of Ornithine Decarboxylase: Exposure of the C-Terminal Target by a Polyamine-Inducible Inhibitory Protein

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Polyamine-mediated degradation of vertebrate ornithine decarboxylase (ODC) is associated with the production of antizyme, a reversible tightly binding protein inhibitor of ODC activity. The interaction of antizyme with a binding element near the N terminus of ODC is essential but not sufficient for regulation of the enzyme by polyamines (X. Li and P. Coffino, Mol. Cell. Biol. 12:3556–2562, 1992). We now show that a second element present at the C terminus is required for the degradation process. Antizyme caused a conformational change in ODC, which made the C terminus of ODC more accessible. Blocking the C terminus with antibody prevented degradation. Tethering the C terminus by creating a circularly permuted, enzymatically active form of ODC prevented antizyme-mediated degradation. These data elucidate a form of feedback regulation whereby excess polyamines induce destruction of ODC, the enzyme that initiates their biosynthesis.

Proteins that control important cellular processes are commonly short lived (30). This requires a means of recognizing them and tagging them for destruction. A few protein pairs have been identified, such that the first binds to and apparently targets the second for degradation, e.g., the human papillomavirus oncoprotein E6 and the tumor suppressor p53 (5, 33, 39). How these interactions direct proteolysis has not been determined. We (18) and others (4, 7, 15, 25, 26) have shown that ornithine decarboxylase (ODC), a short-lived enzyme, and antizyme, a tightly binding protein inhibitor of ODC, represent such a pair: antizyme accelerates the intracellular destruction of ODC. This interaction subserves a form of feedback regulation: ODC initiates the synthesis of polyamines, excess polyamines induce the production of antizyme (7, 15), and antizyme both inhibits ODC activity and leads to its destruction (26). Evidence for this chain of events comes from correlations among cellular polyamine pool size, antizyme level, and the rate of ODC turnover (25), and, more recently, the observation that forced expression of cloned antizyme accelerates the turnover of ODC (27). Conversely, if ODC is structurally altered so as to disrupt the site of antizyme binding, the ability of antizyme to inhibit enzymatic activity in vitro is abolished and the enzyme is no longer regulated by polyamines in vivo (18). Although it has been maintained that intracellular polyamines decrease ODC by reducing the translation of its mRNA, we have considered and negated these claims (9, 18, 38).

Mouse ODC is a homodimer of subunits that each contains 461 amino acids. Turnover can proceed along two different pathways, constitutive and polyamine dependent. The C terminus is both necessary and sufficient to make an ODC protein constitutively unstable. However, the region required for antizyme binding, amino acids 117 to 140, is necessary but not sufficient for regulated degradation. This is made evident by the properties of recombinant chimeras that bind to and respond to antizyme in vitro but do not regulate. An example is a chimera having an N terminus consisting of the first 315 amino acids of mouse ODC followed by the complementary region of trypanosome ODC (18). This chimera binds to and is inhibited by antizyme in vitro but is unresponsive to polyamines in vivo. Several questions arise naturally from this observation. What structural elements of ODC outside the N terminus are required for antizyme-mediated degradation? Are the constitutive and regulated pathways discrete or convergent? How does the binding of antizyme provide a signal for proteolysis? We found that two elements of ODC are required for antizyme-directed degradation: an N-terminal sequence for antizyme binding and a C-terminal sequence for proteolysis. Binding of antizyme exposes the C terminus of ODC, making the protein more accessible to proteolysis.

MATERIALS AND METHODS

Construction of recombinant DNAs encoding chimeric ODCs. Chimeric proteins and the corresponding DNAs that encode them are named by specifying the site(s) of junction between mouse and trypanosome ODC sequences; e.g., M110T145M contains sequentially amino acids 1 to 110 of the mouse, 111 to 145 of the trypanosome, and 146 to 461 of the mouse. Because mouse and trypanosome ODCs are 69% identical within their core region of homology, with no gaps required for alignment (29), the numbering system of the mouse can be applied to both proteins without ambiguity. Because regions of identity are extensive, the numeric specification of junction sites within such regions is necessarily arbitrary. All chimeric junctions were chosen to precisely maintain mouse-trypanosome homology. The construction was done by conventional methods (18), using restriction-ligation, polymerase chain reaction (PCR), or overlap extension PCR (16). In some cases, restriction sites were created by site-directed mutagenesis without altering amino acid coding. The structures of all constructs were verified by restriction mapping or sequence analysis. Details of construction methods will be provided on request. Construct M285T376M was made by removing a *Hpa*I-*Pvu*II

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DNA fragment encoding a portion of trypanosome ODC from pODTbM (11) and replacing it with an equivalent DNA fragment encoding mouse ODC.

In vitro translation. Plasmid DNA was used as a template to amplify chimeric ODC cDNA by PCR with a primer containing a T7 RNA polymerase recognition site as the 5′ oligonucleotide, and the amplified DNA was transcribed into cRNA with T7 RNA polymerase (9, 18). PCR was also used to amplify antizyme cDNA, with the rat Z1 cDNA clone (21, 24) as a template. A T7 RNA polymerase recognition sequence and ATG codon were introduced into a 5′ oligonucleotide primer identical to the first 18 nucleotides of Z1 cDNA. ODC or antizyme cRNA was translated with rabbit reticulocyte lysate in vitro as described previously (9, 18).

Circularly permuted mouse ODC. Two copies of the ODC coding region were placed in a head-to-tail configuration. The TAG stop codon of the 5′ cDNA copy was converted to CAT (encoding His) by site-directed mutagenesis and abutted directly in frame with the methionine initiating codon of the 3′ cDNA copy (We thank S. Tsirka for making this construction). PCR was used to produce a template for transcription of a cRNA specifying the permuted ODC. The 5′ oligonucleotide used for PCR contained a T7 RNA polymerase recognition site followed by ATG and then by 13 nucleotides identical to those beginning at codon 308 of mouse ODC. The 3′ primer was the complement of codons 304 to 307 of ODC, followed by repeated in-frame TAG stop codons. The PCR product was transcribed and translated as above.

In vivo regulation by polyamines and stability. Chimeric constructs were cloned into a eukaryotic expression vector by using the simian virus 40 early promoter (10). These were cotransfected with plasmid pMC1neo (37), which confers resistance to the antibiotic G418, into ODC-deficient mutant CHO cells (36), and transformants were selected with G418. Pools of 100 or more stably transformed clones were plated (approximately 10^6 cells per plate) and incubated at 37°C overnight. Putrescine was added to the cell culture to a final concentration of 0.5 mM. Cell lysates were prepared at various times after treatment and assayed for ODC activity as described previously (10). To test the sensitivity of ODC extracted from cells, we mixed the lysates with different amounts of antizyme bound to Pansorbin as described previously (18) and incubated them on ice for 5 min. We then assayed the mixture for ODC activity. To determine intracellular ODC stability, we treated the cells with 100 μg of cycloheximide per ml for 4 h to inhibit protein synthesis and then prepared cell lysates for the ODC assay.

In vitro degradation of ODC. Mouse ODC and antizyme were produced by in vitro translation in a rabbit reticulocyte lysate as described above. A 10-μl volume of translation lysate containing [^{35}S]methionine-labeled mouse ODC was mixed with 2 μl of lysate used to translate unlabeled antizyme, and the mixture was incubated for 5 min on ice. A 12-μl volume containing an ATP-regenerating system (40 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 40 mg of creatine phosphokinase per ml) was added to the mixture and incubated at 37°C for 1 h. The ODC remaining undegraded was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. To determine the degradation of monomer induced by NaCl, in vitro-translated mouse ODC was either preincubated with antizyme as above or mock treated and then mixed with NaCl to a final NaCl concentration of 0.125, 0.25, or 0.5 M. The ATP-regenerating system was added, and incubation was continued at 37°C for 1 h. The amount of ODC remaining undegraded was assessed by SDS-PAGE. To determine the effect of C-specific antibody on the degradation of ODC, we added 1 μl of the antibody solution to the mixture after preincubating ODC with antizyme. Degradation was carried out at 37°C for 1 h, and the residual ODC was determined by SDS-PAGE.

Preparation of C-specific antibodies. To obtain specific antibodies against the mouse C terminus, we made a fusion protein of glutathione-S-transferase and mouse ODC amino acids 376 to 461 by cloning a DNA fragment encoding that region of mouse ODC into the *Bam*HI-*Eco*RI fragment of the pGEX-3 plasmid. The fusion protein was prepared and purified as described previously (34). The fusion protein was used to affinity purify antibody (35) from polyclonal rabbit antiserum against mouse ODC; a control antibody was similarly prepared from the serum of an unimmunized rabbit.

Dot blot assay. To examine the conformational change of ODC induced by antizyme binding, we preincubated in vitro-translated [^{35}S]methionine-labeled ODC with antizyme as described above. The mixture was then spotted onto nitrocellulose paper for dot blot immunoassay. The paper was treated with C-specific antibody and developed to visualize bound antibody (2). The dots were photographed, and their relative intensity was measured with a Zeineh soft-laser scanning densitometer (Biomed Instruments). To determine the effect of NaCl on immunoreaction of ODC with the tail-specific antibodies, we subjected NaCl-treated ODC to the same blot assay.

RESULTS

Interaction with antizyme is necessary but not sufficient for regulation. Two chimeric ODCs, M285T and M314T, contain, respectively, the N-terminal 285 and 314 amino acids of mouse ODC followed by the complementary part of trypanosome (*Trypanosoma brucei*) ODC. (Mouse and trypanosome ODCs are highly homologous [29], and chimeras generally retain enzymatic activity.) Both constructs have a functional antizyme-binding site, as demonstrated by in vitro assays of binding and inhibition of enzymatic activity (18). However, neither is negatively regulated by polyamines when expressed in vivo. This was shown by using ODC-deficient mutant CHO cells (36) stably transformed with constructs that encode enzymatically active ODCs (9, 18). Cells expressing M285T (the structure depicted in Fig. 1, top) showed no decrease of activity when treated with putrescine to augment polyamine pools (Fig. 1A); in fact, activity even increased. In sharp contrast, ODC activity falls to low levels within hours in response to putrescine in stable transfectants expressing wild-type ODC (9, 18).

The failure of chimeric M285T ODC to respond like wild-type ODC is not due to an in vivo modification that prevents it from interacting with antizyme: cell extracts from CHO cells expressing M285T ODC were challenged with recombinant antizyme, and the activity, like that of M285T ODC made by in vitro translation (18), was inhibited (data not shown). The properties of chimeras containing a functional antizyme-binding site but deficient in regulated degradation suggested a specific hypothesis: antizyme binding initiates a series of events that result in proteolysis of intact mouse ODC, but these chimeras lack a domain needed for completion of the process. A plausible candidate for the missing element was the C-terminal sequence of mouse ODC, which is required for basal degradation (10). Therefore, we restored this element by appending amino acids 376 to 461 to M285T in place of the corresponding region of

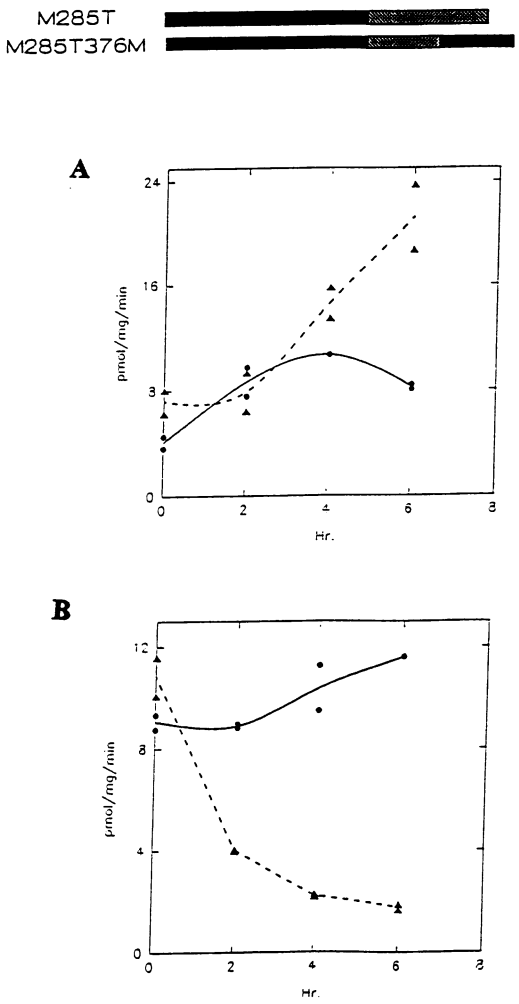

FIG. 1. In vivo regulation of M285T and M285T376M chimeras. Amino acids contributed by trypanosome or mouse ODC are indicated in the block diagram by cross-hatched and solid areas, respectively. ODC⁻ cells transformed with chimeric M285T (A) or M285T376M (B) were treated with 0.5 mM putrescine (▲) or were untreated (●) for 0, 2, 4, or 6 h. Duplicate cell lysates were prepared and assayed for ODC activity (9).

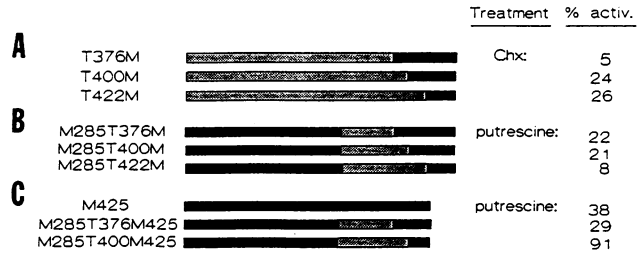

FIG. 2. Structure, stability, and regulation of chimeric ODCs. Amino acids contributed by trypanosome or mouse ODC are indicated in block diagrams as in Fig. 1. ODC⁻ CHO cells stably expressing chimeric ODCs were treated with cycloheximide (Chx) or putrescine, as indicated. After 4 h of treatment, ODC activity in the cell extracts was demonstrated and reported as a percentage of that present in untreated control cells. (A) Trypanosome ODC fused to the mouse ODC C terminus at residue 376, 400, or 422. (B) A series of chimeric proteins similar to those in panel A but containing amino acids 1 to 285 of mouse ODC, the region required for antizyme binding. (C) C-truncated forms of mouse ODC, divested of 37 terminal amino acids, and chimeras with amino acids 286 to 375 or 286 to 400 of mouse ODC replaced by the equivalent trypanosome regions.

trypanosome ODC, to form M285T376M (Fig. 1, top). Unlike M285T, this chimeric ODC containing both the antizyme-binding site and C terminus of mouse ODC showed reduced activity in response to polyamines (Fig. 1B). Amino acids 376 to 461 thus contain a region which complements the amino terminus; together, they confer the negative regulation observed with wild-type ODC.

Identification of the elements needed to complement antizyme binding. Next, we mapped more precisely the region needed to complement antizyme binding. We also sought to determine the relationship of this region to the C-terminal element, contained within amino acids 376 to 461, shown previously to be needed for basal degradation: are they the same or different, or do they overlap? First, we determined with greater precision the region required for basal degradation, i.e., the region which functions without the cooperation of antizyme binding. We reduced the size of the mouse 376-to-461 region, which was previously tested in the T376M chimera (9), by making two additional chimeric proteins of trypanosome ODC (N terminus) and mouse ODC (C terminus), one with the junction at 400 (T400M) and the other with the junction at 422 (T422M). These structures are depicted in Fig. 2A. We expressed all three proteins in ODC⁻ CHO cells and determined their constitutive stabilities by measuring the decline in ODC activity 4 h after treating the cells with cycloheximide to inhibit protein synthesis. The activity of T376M declined with a half-life of about 1 h (Fig. 2A); T400M and T422M were somewhat less labile, and their activity declined with a half-life of about 2 h. We conclude that 422 to 461 is the region both necessary (9) and sufficient for basal degradation.

To map the extent of the C terminus needed for complementation with antizyme, we made parallel constructs containing amino acids 376 to 461, 400 to 461, and 422 to 461 of mouse ODC, now coupled to the chimera M285T containing the antizyme-binding region (Fig. 2B). Each construct, M285T376M, M285T400M, and M285T422M, was expressed in CHO cells, and regulation was examined by measuring the extent of the change in ODC activity 4 h after elevating cellular polyamine levels; this was done by treating the cells with putrescine in the absence of cycloheximide. All three were negatively regulated. We conclude that the basal degradation element can complement antizyme binding to provide regulation.

However, M425, mouse ODC lacking the last 37 amino acids, has been shown to respond to polyamines in vivo (9). This result seems to conflict with our conclusion and suggests that another element may be able to provide complementation. If so, it should be present within amino acids 314 to 424, because chimeric M314T ODC which lacks this region is not regulated. We used the same strategy as above to identify the presumed alternative complementing element (Fig. 2C). First, amino acids 285 to 376 in M425, the C-truncated mouse ODC, were replaced by the equivalent region of trypanosome ODC. This construct displayed regulation (Fig. 2C). However, when we replaced a larger region, amino acids 285 to 400, with trypanosome-derived amino acids, regulation was lost (Fig. 2C). Therefore, mouse amino acids 376 to 424 can function as an alternate to the 422-to-461 basal degradation element; either can collaborate

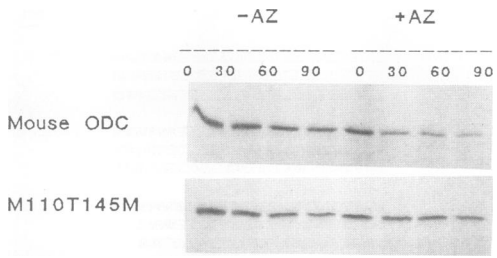

FIG. 3. In vitro degradation of mouse or M110T145M ODC: dependence on antizyme (AZ). [^{35}S]methionine-labeled mouse and M110T145M ODC produced by in vitro transcription and translation were incubated with or without antizyme. Degradation was allowed to proceed for the indicated time (minutes) at 37°C, and the ODC remaining intact was analyzed by SDS-PAGE.

with the antizyme-binding region to confer polyamine-dependent regulation. Because the 376-to-421 and 422-to-461 regions so defined are contiguous, we treat them as integral in the following experiments.

Antizyme binding can drive in vitro degradation. More detailed examination of the mechanism of antizyme-promoted degradation required an in vitro system. We used a rabbit reticulocyte extract (1, 32), with [^{35}S]methionine-labeled ODC produced by in vitro translation as the target for degradation. During a 90-min incubation, mouse ODC was only slightly degraded in the absence of antizyme; degradation was stimulated by the addition of antizyme (Fig. 3). The process required the presence of ATP (results not shown). A similar result was recently reported by Murakami et al. (28). The chimera M110T145M, without a functional antizyme-binding region (18), was, as expected, unresponsive to antizyme in this assay (Fig. 3). We conclude that antizyme-mediated degradation can be reproduced in vitro and requires the same binding site needed for in vivo regulation. Degradation is thus resolved into two steps. First, ODC and antizyme interact to form a catalytically inactive complex. Second, ODC is degraded by cellular components in an ATP-dependent process.

Antizyme promotes the degradation of ODC by exposing its C terminus. It is plausible that proteolysis requires access to the C-terminal element of ODC, the region we have shown to provide functional complementation to the antizyme-binding site. This suggests that interaction of ODC with antizyme makes the C terminus of ODC more accessible. To test this, we used an antibody directed against the C terminus (amino acids 376 to 461) of ODC. If antizyme binding exposes the C terminus of mouse ODC, reaction with this antibody should be promoted. In vitro-translated mouse ODC was used as the target. The inhibitory activity of the antizyme preparation was titrated: 10 μl of a reticulocyte lysate used for in vitro translation of antizyme sufficed to produce 90% inhibition of activity (Fig. 4). An identical titration was then carried out, and the resulting ODC-antizyme complexes were spotted on nitrocellulose. These were subjected to immuno-dot-blot assay to measure the binding of antibody to the C terminus of ODC. The immunoreaction between ODC and antibody became more pronounced with increasing amounts of antizyme (Fig. 4); densitometry showed that the amount of immunoreaction product increased more than 5-fold with the largest amount of antizyme used (the range was 2.5- to 5.9-fold in independent experiments). This result could have been an artifact caused by antizyme's altering the extent to which ODC associates with nitrocellulose. We

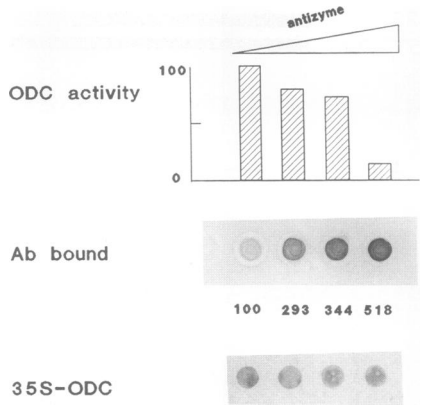

FIG. 4. Effect of antizyme on the reaction of mouse ODC with antibody against the ODC C terminus. Mouse ODC (10 μl of in vitro translation reaction product) and increasing amounts of antizyme (0, 0.5, 2.0, and 10 μl of in vitro translation reaction product) were mixed. ODC activities (histogram) of the resulting complexes are shown as a percentage of that present without antizyme. Identical complexes were dot blotted to a nitrocellulose filter and immunoreacted with antibody (Ab) against the C terminus of ODC. Bound antibody was determined. Immunoblots are shown, along with densitometric determinations of the intensity of the immunoreaction product (the control with no antizyme was arbitrarily set equal to 100). The reaction and blotting were carried out as above, but with [^{35}S]-methionine-labeled ODC, and the association of ODC with the filter was determined by autoradiography.

excluded this by repeating the experiment with [^{35}S]methionine-labeled mouse ODC spotted on nitrocellulose paper after being mixed with antizyme. Autoradiography showed that ODC associated with the filter matrix to the same extent regardless of the amount of antizyme used (Fig. 4). These results are consistent with the conclusion that antizyme acts on ODC to expose the C terminus.

We next tested the effect of the C-specific antibody on antizyme-promoted degradation of ODC by adding it directly to the in vitro degradation system. The antizyme-induced degradation of mouse ODC was blocked by the C-specific antibody but not by control antibody (Fig. 5). In this assay, the C-specific antibody did not alter the binding of antizyme to ODC (data not shown). We conclude that the C terminus is made accessible by antizyme and that C-specific antibody can block access of the terminus to degradative processes.

Dissociation of ODC dimer to monomer does not cause degradation. ODC is catalytically active as a dimer but exists as a monomer form in the complex of ODC and antizyme (17, 23). Does antizyme expose the C terminus merely by dissociating dimer to monomer, or is more extensive confor-

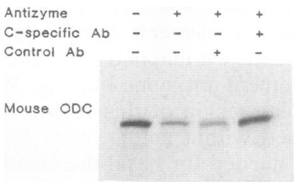

FIG. 5. Effect of C-specific antibody on in vitro degradation. The same antibody preparation as in Fig. 4 was incubated with the ODC-antizyme complex. Degradation was initiated by addition of ATP. Preincubation was carried out with a control antibody (Ab) preparation, with no antibody, or without antizyme, as indicated above the lanes.

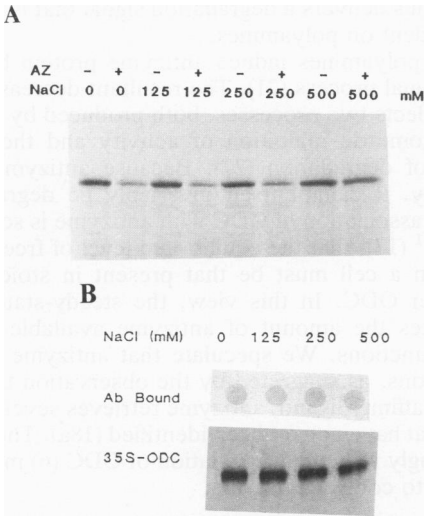

FIG. 6. Effect of dissociating ODC with NaCl. Different concentrations of NaCl (0, 125, 250, and 500 mM) were added to mouse ODC produced by in vitro transcription and translation. The effect on in vitro degradation (A), immunoreaction with the C-specific antibodies (Ab) (B; upper row), and coprecipitation with antizyme (B; lower row) was determined as in reference 18. AZ, antizyme.

mational change involved? To test whether monomer formation is adequate to make ODC a substrate of degradation, we used NaCl to alter the equilibrium between monomers and dimers. Monomer formation is favored by an NaCl concentration of 0.25 M or greater (17, 23). We asked whether this salt concentration can promote degradation of ODC as antizyme does. We found that ODC activity was reduced to 66, 26, and 10% of control values by, respectively, 125, 250, and 500 mM NaCl, an observation consistent with dissociation to monomer. However, salt did not promote in vitro degradation of ODC (Fig. 6A). To exclude the possibility that salt itself inhibited degradation, we tested whether 250 mM NaCl had any effect on in vitro degradation stimulated by antizyme. It did not, although a higher concentration, 500 mM NaCl, did somewhat inhibit proteolysis (Fig. 6A). We also used the same dot blot assay described above to examine the effect of NaCl on the exposure of the C terminus of ODC. Concentrations up to 0.5 M had no stimulatory effect on binding of C-specific antibody to ODC (Fig. 6B, upper row). No obvious effect on ODC-antizyme complex formation, determined by coprecipitation, was found at salt concentrations up to 0.5 M (Fig. 6B, lower row). Therefore, antizyme-induced degradation of ODC is not simply the result of converting ODC to a monomeric form.

Tethering the C terminus prevents degradation. If antizyme mobilizes the C terminus to permit degradation, constraining the terminus should suppress degradation. To examine this, we constructed a circularly permuted form of ODC in which the C terminus is tied to the N terminus, presumably making it less mobile. This construct is identical in primary structure to wild-type ODC, except that its amino terminus begins at natural amino acid 308 (preceded by the methionine necessary to initiate translation), the stop codon following the natural terminal amino acid 461 is mutated to a histidine codon, and the natural amino terminus, from amino acids 1 to 307, then follows, forming the C terminus of the novel protein. The C and N termini are thus connected through a histidine bridge, and the peptide chain is broken between 307

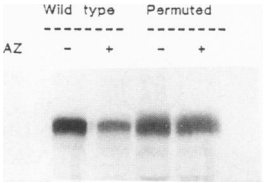

FIG. 7. In vitro degradation of [^{35}S]methionine-labeled wild-type or circularly permuted ODCs in the presence or absence of antizyme (AZ). The conditions of the experiment were as in Fig. 3, with incubation carried out for 60 min.

and 308. Following the lead of workers who have successfully rearranged the topology of other enzymes (3, 19), we expected that the altered ODC might be enzymatically active because (i) amino acids 307 to 308 are likely to lie within a surface loop that is not conserved among eukaryotic ODCs (29), so that chain scission need not greatly disturb folding or function and (ii) the C-terminal 37 amino acids of wild-type ODC are not required for catalytic activity (20), suggesting that covalently approximating the N and C termini might not perturb conformation of the catalytic center. Consistent with these expectations, circularly permuted ODC produced by in vitro translation is enzymatically active. Its specific activity was calculated as the ratio of catalytic activity to ODC synthesized. Under assay conditions not specifically optimized for either enzyme, the activity of permuted ODC measured 37% that of wild-type ODC.

The catalytic activities of wild-type and circularly permuted ODC, produced by in vitro translation, responded to antizyme. Both were almost fully inhibited (more than 10-fold) by antizyme, and submaximally effective amounts of antizyme inhibited both to a similar extent (the antizyme present in 0.5, 2, and 10 μl of a reticulocyte lysate used for its in vitro translation caused, respectively, 34, 38, and 91% inhibition of wild-type ODC and 32, 63, and 89% inhibition of circularly permuted ODC). The effect of antizyme on in vitro degradation was next examined. As described above, antizyme promoted the degradation of wild-type ODC. However, it had no effect on proteolysis of circularly permuted ODC (Fig. 7). Circular permutation thus prevents degradation. It probably does so by reducing the antizyme-induced mobilization of the C terminus, although we have not directly demonstrated this. (Experiments to determine whether antizyme can increase the immunoreactivity of circularly permuted ODC with C-specific antibody gave equivocal results.) It is additionally or alternatively possible that proteolysis requires a C terminus that is actually topologically or chemically terminal, rather than one made internal, as in the permuted form of ODC. This would be so if, for example, the recognition or precessive processing of the terminus required an unblocked carboxyl group as well as a particular primary structure.

DISCUSSION

We can distinguish two successive stages in antizyme-directed degradation (Fig. 8). The first requires only the association of antizyme and ODC. This results in catalytic inactivation of ODC and exposure of the C terminus, demonstrated here by increased access to antibody specific for the 376-to-461 region. The change in conformation induced by antizyme is not, however, sufficiently global to produce a readily detectable change in the rate or pattern of fragmentation produced by V8 protease (results not shown). The

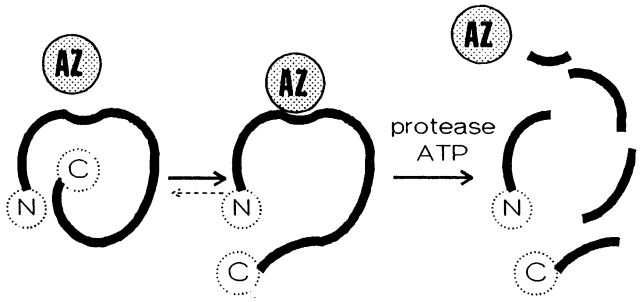

FIG. 8. A model of antizyme-promoted degradation. ODC and antizyme (AZ) associate in the first step, stabilizing an enzymatically inactive conformation with the C terminus of ODC exposed. Proteolysis occurs in the second, ATP-dependent step.

second stage encompasses proteolytic degradation per se and can be recreated in vitro by using extracts from rabbit reticulocytes (see above and reference 28). In this system, degradation requires antizyme and ATP and, within ODC, an antizyme-binding site and a C-specific element. Degradation is inhibited by antibody to the C terminus. Antizyme dissociates ODC dimer to monomer, but such dissociation, when induced by salt (17), is insufficient to expose the C terminus or enhance degradation by the in vitro system. The function of ATP in this system is not yet known. ATP may be required by a chaperone-associated ATPase (8) or may act as a component of an ATP-dependent protease (14).

Ubiquitin conjugation plays a well-documented role in protein degradation, but there is evidence in vivo and in vitro that ODC is not a substrate of the ubiquitin pathway (12, 32). Ubiquitin associates covalently with its target; antizyme associates tightly but noncovalently. Both pathways need ATP. They differ in target specificity; several endogenous targets of the ubiquitin pathway have been found, including cyclin B (13), but the only known target for antizyme is ODC. Both systems exemplify the utilization of a second protein to deliver a degradation signal to a protein target. Mouse ODC contains two PEST (rich in proline, glutamate, serine, and threonine) regions (31). The internal PEST region is dispensable for degradation; the potential importance of the C-terminal PEST region is suggested by the stabilizing effect of deletion mutations (9). It is of interest that antizyme itself contains a PEST region (24).

Degradation of ODC within cells takes place under two conditions, one polyamine dependent and driven by antizyme, and the second constitutive. C-terminal sequences unite these processes. For regulated degradation, these must cooperate with an antizyme-binding element located near the N terminus. Cooperation can be provided by the 376-to-461 C-terminal region or (see above and reference 9) the 376-to-424 amino acid subregion. Kinetic and thermodynamic barriers to the proper folding of ODC are seemingly negligible. The in vitro translation product is enzymatically active; the purified protein efficiently regains activity on dilution from chemical denaturants (37a). As we have shown here, even a radical mutation of topology is consistent with correct folding of the active site. Despite an equilibrium that favors tertiary and quaternary integrity of the active site, the C terminus of ODC is sufficiently available to allow constitutive intracellular degradation to occur with a half-life of 1 h or less (9). We propose that antizyme, by altering the conformation of ODC, raises the C terminus from an occasionally available ground state to one more uniformly accessible and thus delivers a degradation signal that is conditionally dependent on polyamines.

Cellular polyamines induce antizyme protein by a post-transcriptional process (21). The resultant decrease in ODC activity reflects two processes, both produced by antizyme: the stoichiometric inhibition of activity and the catalytic induction of degradation (27). Because antizyme can act catalytically, it cannot itself invariably be degraded with ODC. The association of ODC with antizyme is so tight, $1.4 \times 10^{11}\ M^{-1}$ (17), that the equilibrium level of free antizyme available in a cell must be that present in stoichiometric excess over ODC. In this view, the steady-state level of ODC titrates the amount of antizyme available for other potential functions. We speculate that antizyme has alternate functions, as suggested by the observation that, when used as an affinity ligand, antizyme retrieves several cellular proteins that have not yet been identified (18a). The complex and seemingly redundant regulation of ODC (6) may thus in turn serve to control antizyme.

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