The antizymes constitute a conserved gene family with at least three mammalian orthologs. As described previously, in a degradation system utilizing rabbit reticulocyte lysate, antizyme 1 (AZ1) accelerates proteasomal ornithine decarboxylase (ODC) degradation, but antizyme 2 (AZ2) does not. To examine the relationship between antizyme structure and function, we further characterized the properties of AZ1 and AZ2 and protein chimeras composed of elements of the two. AZ1 binds to ODC with about a 3-fold higher potency than AZ2, but this cannot account for their distinct degradative activities. The dissimilar degradative capacity of AZ1 and AZ2 is also observed using purified proteasomes. A series of reciprocal AZ1/AZ2 chimeras was used to determine the sequence elements needed to direct ODC degradation. An element contained within amino acids 130–145 of AZ1 is essential for this function. Constructs in which amino acids 130–145 were exchanged between the antizymes confirmed the critical nature of this region. Within this region, amino acids 131 and 145 proved responsible for the functional difference between the two forms of AZ.

The bulk of cellular proteins are long-lived, but some are synthesized, function briefly, and are then destroyed. Labile proteins control an extensive range of biological processes (1). These processes include cell cycle control, transcription, apoptosis, antigen processing, biological clock control, signal transduction, differentiation, and surface-receptor desensitization. Many of these processes have in common the need for rapid or transient responses. The major protease that destroys naturally labile proteins, as well as those that are mutant or misfolded, is the proteasome (2). This structurally complex and highly conserved molecular machine contains an interior nanochamber where specific catalytic sites perform proteolysis. Some form of traffic control is needed to accurately direct appropriate substrates to the proteasome interior for destruction, while excluding other proteins. For many well-characterized labile proteins, post-translational modification with polyubiquitin chains serves as the marker directing degradation (3). In a few cases, proteasomal degradation takes place without the participation of ubiquitin. A well-characterized example is the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) (4–6).

ODC is a proteasome substrate with a basal half-life in cells of less than an hour. Its degradation is more rapid still in cells with superabundant polyamines. Polyamine excess induces the protein antizyme 1 (AZ1), which binds tightly to ODC, forming a heterodimer. In association with AZ1, ODC becomes a very efficient substrate of the proteasome, with a half-life of a few minutes (7–9). AZ1 was shown recently to belong to a conserved gene family, with at least three members in the vertebrate lineage (10). AZ1 and antizyme 2 (AZ2) are found in all tissues, with AZ1 transcripts generally present in 10- to 20-fold greater amounts than AZ2 (11). AZ3 is found only in the testes and is expressed solely during postmeiotic stages of spermatogenesis (12).

AZ1 takes part in polyamine homeostasis. By binding to ODC, it disrupts the active form of the enzyme, a homodimer, and then causes its destruction (13). AZ2 also binds and inhibits, but whether it has degradative capacity is unclear. Our previous studies using an in vitro system of proteasomal degradation (derived from rabbit reticulocyte lysates) demonstrated that, in this context, AZ1 directs degradation, but AZ2 does not (14). However, when mammalian ODC and AZ1 or AZ2 were co-expressed in insect cells using baculovirus-derived vectors, both AZs greatly reduced the steady state levels of ODC, a result consistent with accelerated degradation (14). In the present study, we demonstrate that AZ1 and AZ2 indeed differ in their ODC degradative activity when made and tested in vitro or in bacteria and that this functional difference depends on two amino acids.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—All DNA manipulations used standard molecular methods. Constructions that utilized PCR steps were verified by sequencing; constructions that relied on restriction-ligation utilized fully sequenced constituents. The ODC gene was mouse (*Mus musculus*), AZ1 was rat (*Rattus norvegicus*), and AZ2 was human (*Homo sapiens*). Site-directed mutagenesis was carried out using the megaprimer method (15).

**Plasmids for ODC, AZ1, and AZ2 Bacterial Expression**—The *Escherichia coli* expression vector pQE30 (Qiagen) was used for expression of recombinant ODC, AZ1, and AZ2 proteins, each with a His6 sequence positioned near the N terminus to facilitate purification by metal-ion chromatography. The AZ1 construct was designed such that the N-terminal sequence Met-Arg-Gly-Ser-His6-Gly-Ser-Ala-Cys replaced the initial methionine of the native protein sequence; AZ2 was similarly constructed but with Met-Arg-Gly-Ser-His6-Gly-Ser. In vectors used for bacterial expression of the AZs, deletion of a single T nucleotide was used to align native ORFs 1 and 2 (16). To facilitate purification and other protein manipulations outside the scope of this paper, the initial methionine of the native sequence of ODC was replaced with a sequence containing a His6 tag, tobacco etch virus (TEV) protease cleavage site and FLAG tag: Met-Arg-Gly-Ser-His6-Gly-Ser-Glu-Asn-Leu-Tyr-Phe-Gln-Gly (TEV site)-Gly-Ser-Met-(Asp-Tyr-Lys-Asp-Asp-Asp-Lys (FLAG epitope)).

**In Vitro Transcription and Translation**—All plasmids or PCR products used for *in vitro* transcription/translation contained a T7 promoter positioned upstream of the ORF. The plasmids used for transcription/
ODC Degradation in Reticulocyte Lysate and AZ Binding to ODC—AZ-stimulated ODC degradation was assayed as previously described (14). Briefly, $^{35}$S-labeled ODC was produced by coupled in vitro transcription/translation in a rabbit reticulocyte lysate system (TNT, Promega). AZ1 and AZ2 were similarly produced. The reticulocyte lysates used for synthesis of ODC and the AZs also provided the proteasomes required for degradation. Lysates containing ODC and AZs were incubated at 37°C for 1 h in the presence of ATP and an ATP-regenerating system (30 mM Tris pH 7.5, 5 mM MgCl$_2$, 2 mM dithiothreitol, 1 mM ATP, 10 mM creatine phosphate, 1.6 mg/ml creatine phosphokinase). The reaction was stopped by addition of SDS-PAGE loading buffer. The fraction of ODC remaining undegraded was determined by SDS-PAGE and quantitation of the $^{35}$S-labeled autolysate. The relative stoichiometry of the labeled proteins was determined in parallel enzymatic inhibition and stimulation of ODC activity in the absence of template (7, 17). In contrast, AZ2 did not augment ODC degradation when added at similar concentrations. At the highest concentration of AZ2 tested in the inhibition assay (Fig. 1B), ODC was more than half saturated by AZ2. The avidity of AZ1 for ODC was about 3-fold greater than that of AZ2: a 3-fold higher concentration of AZ2 compared with AZ1 was required to produce 50% inhibition of ODC activity (Fig. 1B). However, at amounts of the two AZs that were equipotent in binding, AZ1 caused degradation, but AZ2 did not. Therefore, a difference in the extent of their association cannot explain the difference in degradative activity.

The Distinct Activities of AZ1 and AZ2 Are Maintained in a Degradation System Composed of Purified Components—The reticulocyte is a highly specialized end-stage tissue that may not reflect the characteristics of ODC degradation in other cells. To determine whether the complex cell extracts used for degradation contain an activity that can differentially inhibit AZ2-mediated ODC targeting (or enhance that of AZ1), the activities of AZ1 and AZ2 were compared using purified components. Purified $^{35}$S-methionine-labeled ODC and recombinant His$_6$-AZ1 or His$_6$-AZ2 were incubated with purified proteasomes and Mg$^{2+}$-ATP. His$_6$-AZ1 caused proteolysis of about 1/3 of the $^{35}$S-methionine-ODC substrate in 1 h (Fig. 2). His$_6$-AZ2, in contrast, produced little detectable degradation compared with a control incubation without AZ. The distinction between the degradative activities of the recombinant AZs therefore is preserved in a system containing only ODC and the proteasome as additional macromolecular components.

RESULTS

Dissimilar Degradative Capacity of AZ1 and AZ2 Does Not Result from Dissimilar Avidity for ODC—We have shown previously using an in vitro degradation system derived from rabbit reticulocytes that AZ1 accelerates degradation of ODC but AZ2 does not (14). In these experiments, the rabbit reticulocyte extract used for in vitro translation provides the AZs and ODC, and is also a rich source of proteasomes, the protein machine that degrades ODC. Titration experiments comparing AZ1 and AZ2 in this in vitro model have shown the former to be at least 16 times more potent than the latter in directing degradation. However, no measurements were made in this system to determine whether the two AZs used to test degradation activity were both binding to ODC. If the avidity of AZ2 for ODC is markedly inferior to that of AZ1, a failure of AZ2 to bind ODC under the experimental conditions used could trivially account for their distinct degradative capacities. To address this question, we measured in parallel two activities of both AZ1 and AZ2: the capacity to accelerate ODC degradation and ODC binding. Binding to ODC was determined by measuring ODC enzymatic activity. This surrogate measure of binding is available because AZ binding converts the ODC homodimer into an enzymatically inactive AZ:ODC heterodimer. The reduction in activity therefore reflects the extent of ODC occupancy by antizyme.

$^{35}$S-methionine-labeled ODC, AZ1, and AZ2 were individually generated by coupled in vitro transcription and translation, and the relative protein amounts were determined by SDS-PAGE and autoradiography. Various amounts of AZ1 and AZ2 were compared in both an ODC degradation assay and ODC activity inhibition assay, using in each case a fixed quantity of ODC (Fig. 1). The effect of different stoichiometric ratios of AZ:ODC was assessed. The lowest amount of AZ1 tested caused the degradation of about half of the ODC substrate (Fig. 1A). This amount of AZ1 represents a stoichiometric ratio of 0.3:1 (AZ1:ODC). Greater amounts of AZ1, up to 20-fold more (equivalent to a stoichiometric ratio of about 6:1 AZ1:ODC) similarly resulted in degradation of about half of the substrate. The capacity of sub-stoichiometric amounts of AZ1 to produce significant degradation is consistent with the catalytic role of AZ1, in which one molecule can direct the degradation of multiple molecules of ODC (7, 17). In contrast, AZ2 did not augment ODC degradation when added at similar concentrations. At the highest concentration of AZ2 tested in the inhibition assay (Fig. 1B), ODC was more than half saturated by AZ2. The avidity of AZ1 for ODC was about 3-fold greater than that of AZ2: a 3-fold higher concentration of AZ2 compared with AZ1 was required to produce 50% inhibition of ODC activity (Fig. 1B). However, at amounts of the two AZs that were equipotent in binding, AZ1 caused degradation, but AZ2 did not. Therefore, a difference in the extent of their association cannot explain the difference in degradative activity.

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Antizyme Structures Specifying ODC Degradation

A, dependence of ODC degradation on AZ1 and AZ2. The degradative effects of various amounts of AZ1 or AZ2 were tested in a reticulocyte lysate-based degradation reaction. The molar ratio of [35S]methionine-labeled AZ1 or AZ2 to [35S]methionine-labeled ODC was in the range of 0.3:1 to 6:1. Top panel, AZ1; Bottom panel, AZ2. Band intensities were quantitated, and the data were plotted as the molar ratio of AZ:ODC versus percent AZ-stimulated degradation. [AZ1], AZ1; [AZ2], AZ2. B, dependence of ODC association on AZ1 and AZ2 concentration. AZs were prepared as in A, and the capacity of AZ1 or AZ2 to inhibit ODC enzymatic activity was measured.

Degradative Activities of Chimeras Indicate the Importance of Amino Acids 130–145—The region consisting of amino acids 70–120 of AZ1 was previously identified by deletion analysis as important for mediating ODC degradation (18, 19). Deletions, however, can disturb folding of the residual protein, thus propagating functional changes outside of the region that has been deleted. To test the function of this region in the context of an intact AZ, we constructed the chimeric protein N-AZ1121–AZ2129-C, composed of the N-terminal residues 1–121 of AZ1 followed by residues from 122 to the C terminus of AZ2, but found that this did not efficiently mediate ODC degradation (results not shown). This may be because some crucial region in AZ1 is absent in the chimera. To help localize the region or regions responsible for the functional difference between the two AZs, we made a systematic series of reciprocal AZ1/AZ2 chimeras and measured the capacity of each chimera to direct ODC degradation. The strong homology of the second ORF of the two AZs, the ORF expressed as the result of polyamine-stimulated frame-shifting (10), makes it possible to unambiguously identify residues that can be used as homologous crossover points in constructing such paired chimeras. One chimera protein series consisted of an N-terminal portion originating in AZ1 up to residue n, followed by a crossover to the AZ2 sequence at residue n + 1 (Fig. 3A). For example, one such protein was N-AZ1129–AZ2130-C. A second parallel chimera protein series was similarly constructed, but with the N-terminal region originating in AZ2 rather than AZ1, e.g. N-AZ2129–AZ1130-C. These chimera pairs had junction sites at residues 121, 129, 138, 145, 157, or 172. In the simplest and most readily analyzed outcome, a single small linear region would be found to account for the greater part of the functional difference. This proved true; the only region of functional importance was the region between AZ1 residues 121 and 145 (Fig. 3B). The chimeras with breakpoints intermediate between 121 and 145, at 129 or 138, had intermediate activity, and the reciprocal pairs with breakpoints at 129 or 138 within the critical region had approximately additive activities.

Amino Acids 130–145 of AZ1 Are Primarily Responsible for the Distinct ODC Degradative Activity of AZ1 and AZ2—To determine whether AZ1 amino acids 130–145 are responsible for the distinct degradative activities of the two proteins, we carried out reciprocal swaps of this sequence. We concentrated on this region because the properties of the single-breakpoint chimeras suggested that amino acids 130–145 capture most of the functional difference between AZ1 and AZ2. When amino acids 130–145 of AZ2 were used to replace the corresponding region of AZ1 (forming the double chimera AZ1-2-1), AZ1 losses the ability to target ODC degradation (Fig. 4A), although it retains high ODC binding potency (Fig. 4B). Conversely, the reciprocal molecule in which the corresponding amino acids of AZ1 are implanted within the body of AZ2 (double chimera AZ2-1-2) exhibits 65% of the degradative ability of AZ1, despite having a somewhat reduced ability to bind ODC compared with AZ1.

The rank order of avidity for ODC was AZ1-2-1 > AZ1 > AZ2 > AZ2-1-2 (Fig. 4B). Chimera AZ1-2-1 showed higher ODC binding potency than AZ2-1-2, although it lacks the capacity to degrade ODC. In contrast, chimera AZ2-1-2 showed a relatively low avidity for ODC, but it can mediate ODC degradation efficiently. These results imply that amino acids 130–145 of AZ1 are crucial for degradation but not for ODC association.

Amino Acids 131 and 145 Account for the Functional Difference between AZ1 and AZ2—To determine which specific residues among amino acids 130–145 of AZ1 were important for degradation, we mutated all of those that differed between AZ1 and AZ2. There are 10 such amino acids. All of these in AZ1 were altered to correspond to the sequence of AZ2; amino acids were changed individually (131, 143, 145), or in clusters of two or three (134–135, 136–138, and 140–141). The degradative activity of each mutant was compared with that of wild-type AZ1 (Fig. 5). Only two of the amino acid exchanges reduced activity by 20% of more, Arg131Asp and Ala145Asp, each of

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
which reduced activity about 2-fold. The Arg\textsuperscript{131}Asp, Ala\textsuperscript{145}Asp double mutation was then made and tested; its residual activity was 6% that of wild-type AZ1.

**DISCUSSION**

The chief findings of this paper are as follows. When rabbit reticulocyte lysate is used as the environment of proteolysis, AZ1 can direct ODC degradation but AZ2 cannot. The difference in degradative function is intrinsic to the AZs, because it is also observed in a purified system in which proteasomes, ODC, and AZ are the only proteins present. A greater avidity of AZ2 for ODC does not explain their disparate function. Analyses of chimeras between AZ1 and AZ2 and of regional reciprocal swaps demonstrate that most of the functional difference is conferred by amino acids 130–145 of AZ1. Site-directed mutagenesis of Arg\textsuperscript{131} and Ala\textsuperscript{145} of AZ1 shows that these two amino acids fully account for the difference in functional properties of the two AZs.

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**FIG. 3.** Degradative activity of AZ1/AZ2 and AZ2/AZ1 chimeric proteins. A, schematic representation of AZ chimeras. Junction sites of crossover between the AZ sequences were at residues 121, 129, 138, 145, 157, or 172. The solid bar represents protein sequences corresponding to AZ1, the speckled bar sequences corresponding to AZ2. B, ODC degradation stimulation by chimeras. The AZs were in vitro-translated in \(^{35}\text{S}\)methionine-labeled form and adjusted to identical levels. After incubation with each AZ, the \(^{35}\text{S}\)methionine-labeled ODC remaining undegraded was determined. The data are plotted for both chimera series: one with AZ1 sequence at the N-terminal end ( ), the second with the AZ2 sequence at the N-terminal end ( ). The horizontal axis represents the crossover point of each chimera, and the vertical axis represents the extent of ODC degradation, normalized to 0% for no AZ and 100% for full-length AZ1.

**FIG. 4.** Amino acids 130–145 of AZ1 determine degradative activity. A, degradative activity of AZ1, AZ2, and AZs with reciprocal exchanges of amino acids 130–145. The percent degradation of ODC was quantitated and expressed normalized to 100% for AZ1-stimulated degradation. B, ODC binding capacity of AZ1, AZ2, and AZs with reciprocal exchanges of amino acids 130–145. A, AZ1; B, AZ2; C, AZ 1-2-1; D, AZ 2-1-2.

**FIG. 5.** Sequence comparison of amino acids 111–150 of AZ1 and AZ2 and effect of amino acid mutation. Upper, AZ1 and AZ2 residues 111–150 aligned. Among amino acids 130–145, 10 residues are marked that differ between AZ1 and AZ2 sequences. E designates residues predicted to be involved in \(\beta\)-sheet formation. Lower, effect on degradative activity of mutating amino acids of AZ1 to the corresponding amino acids of AZ2 individually or in clusters. Recombinant \(^{35}\text{S}\)methionine-labeled ODC (~50 nM) was incubated with wild-type AZ1 or AZs with the indicated mutations. AZs were individually produced by in vitro translation in rabbit reticulocyte lysate, which provided proteasomes as well. The extent of degradation was determined by measuring acid-insoluble radioactivity, normalized to 100% degradation for wild-type AZ1.
The strategy employed here started with the finding that two proteins of similar structure have dissimilar properties: one can and the other cannot direct degradation of ODC \textit{in vitro}. This suggested that chimeras and regional and local exchanges of sequence information between the two forms of AZ could be used to identify the responsible residues. As anticipated from their similarity of structure, these hybrid proteins retained the capacity to bind ODC, eliminating this potentially confounding factor in the analysis of the data. The overall approach was validated by the readily interpreted unambiguous results. Chimeras had transitions of activity that depended smoothly on breakpoint location. Reciprocal chimeras had reciprocal activities, thus implicating a single small region as critical. Exchange of this region between the two parent proteins inverted their functional properties. The partial activity of chimeras with breakpoints between residues 130–145 suggested that more than one residue within this region contributes to the functional difference. Exhaustive amino acid exchanges within this region identified two amino acids as critical to function.

The limitations as well as benefits of this approach merit consideration. We began with the agnostic stance that nothing is known about the sequence elements responsible for the difference of degradative function, and asked a very specific question: using \textit{in vitro} degradation by the proteasome, where do the responsible sequence elements lie? In fact, previous work based on other methods had told us much about the functional domains of AZ1. The C-terminal AZ1 half molecule (amino acids 120–227) is sufficient for ODC binding, and additional sequence information within amino acids 70–119 is required to direct ODC degradation (18, 19). Furthermore, deletion of amino acids 113–118 also destroys the degradative capacity of AZ1 (18). There is no contradiction between these data and our present results identifying residues 131 and 145 as critical. We have answered a single but significant question, the identity of the amino acids responsible for the different \textit{in vitro} degradative properties of the two AZs. The use of reciprocal chimeras and replacements rather than truncations and deletions is less likely to result in artifactual misidentification of functional elements, because removing sequence elements can disturb folding into a native conformation. The further question of whether and under what conditions AZ2 can mediate ODC degradation remains to be resolved.

ODC is degraded by the proteasome in either the presence or absence of AZ (9). The process is accelerated by but not dependent on AZ. The C terminus of ODC has an essential role in its degradation (20). It bears residues necessary for degradation, preeminently Cys\textsuperscript{414} and the five terminal residues. AZ1 exposes the ODC C terminus. Antibodies directed at the ODC C terminus gain greater access to the molecule in the presence of AZ1, and such antibodies impair proteasomal degradation (21). We have not tested whether AZ2, like AZ1, alters access to the ODC C terminus. Our previous studies (18) and those of others (19) have shown that a portion of AZ1 consisting of approximately the C-terminal half of that molecule (amino acids 120–227) binds to ODC and can disrupt ODC enzymatic activity. Like intact AZ1, the AZ1 C-terminal half molecule presumably displaces the ODC homodimer, replacing it with an ODC:AZ heterodimer. Like the AZ1 C-terminal half molecule, AZ2 must inactivate ODC by disrupting the homodimer, and like the AZ1 half molecule, cannot direct degradation. This reinforces the conclusion, based previously on AZ1 deletions and truncations, that disrupting the ODC homodimer is not sufficient for enhancing degradation.

It is possible that AZ1 functions in degradation merely by mobilizing the ODC C terminus. Alternatively, it may do this, but perform some additional function that promotes degradation. Amino acids 131 and 145 may be critical contact residues in one or both of these roles, interacting with ODC or the proteasome. The crystal structure of truncated (22) or full-length ODC containing all 461 amino acids (23) shows little electron density corresponding to amino acids C-terminal to residue 420, implying that the most distal part of ODC is disordered. It remains to be determined whether this region is more or less flexible when ODC is complexed with AZ1.

\textbf{Acknowledgment—}We thank Mingsheng Zhang of this laboratory for providing purified rat proteasomes.

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Structural Elements of Antizymes 1 and 2 Are Required for Proteasomal Degradation of Ornithine Decarboxylase
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J. Biol. Chem. 2002, 277:45957-45961.
doi: 10.1074/jbc.M206799200 originally published online September 30, 2002

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