

Antizyme, a mediator of ubiquitin-independent proteasomal degradation

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Abstract — Ornithine decarboxylase (ODC) is among the small set of proteasome substrates that is not ubiquitinated. It is instead degraded in conjunction with the protein antizyme (AZ). ODC and AZ are participants in a regulatory circuit that restricts pools of polyamines, the downstream products of ODC enzymatic activity. Functional studies using directed mutagenesis have identified regions of ODC and AZ required for the process of ODC degradation. Within ODC, there is a region that is required for AZ binding which lies on the surface of an alpha-beta barrel forming one domain of the ODC monomer. A carboxy-terminal ODC domain is needed for both AZ-dependent and AZ-independent degradation. Within AZ, the carboxy-terminal half molecule is sufficient for binding to ODC, but an additional domain found within the AZ amino terminus must be present for stimulation of ODC degradation by the proteasome. Recently, the AZs have been found to consist of an ancient gene family. Within vertebrate species, multiple isoforms are found, with distinct functions that remain to be sorted out. Although AZ homologs have been found in some yeast species, homology searches have failed to identify an AZ homolog in *Saccharomyces cerevisiae*. Nevertheless, the close parallel between polyamine-induced ODC degradation in *S. cerevisiae* and in animal cells suggests that this organism will also be found to harbor an AZ-like protein. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Saccharomyces cerevisiae / antizyme / ubiquitin / proteasomal degradation

1. Antizyme, a mediator of proteasomal degradation

Proteasomes degrade ubiquitinated proteins. This general rule must recognize exceptional cases, for there is a small set of proteins that are proteasome substrates, but use other signals to mark themselves for degradation [1]. The best characterized of these is ornithine decarboxylase (ODC). When ODC binds to a second protein called antizyme (AZ), it becomes a good substrate for the proteasome. AZ is (usually) spared destruction; a single AZ molecule can therefore catalyze multiple rounds of ODC degradation. Both in vitro [2, 3] and in vivo [4] studies have shown that ubiquitin takes no part in this process. In this brief review, I give special attention to the work of this laboratory. A fuller presentation of this subject, to which other laboratories have made prominent contributions, can be found in recent reviews [5–8].

ODC and AZ are participants in a form of feedback regulation that constrains a metabolic pathway dedicated to polyamine biosynthesis. The polyamines are small abundant poly-cations that are essential for life [9]. ODC initiates the synthesis of polyamines. When these rise, AZ is induced by a remarkable mechanism: translational frameshifting [10]. The AZ mRNA contains two overlap-

ping open reading frames. The second of these encodes most of the protein, but lacks an initiation codon. Translation initiates in reading frame 1, but must shift to reading frame 2 for production of functionally active AZ. Polyamines increase the efficiency of AZ mRNA frameshifting just before translation would otherwise terminate in reading frame 1. The rise of AZ impairs polyamine synthesis by destroying ODC. AZ also inhibits polyamine uptake by cells. Together these mechanisms stabilize cellular polyamine levels.

Mutagenesis and structural studies have begun to reveal the elements of ODC and AZ that are responsible for their interaction and for ODC degradation. The 461 amino acid ODC polypeptide subunit must form a homodimer to become enzymatically active. In contrast to its weak self-association, the ODC monomer has very high affinity for AZ. AZ therefore stoichiometrically creates ODC:AZ heterodimers, which lack enzymatic activity, and catalytically directs ODC degradation by the proteasome [11].

ODC in the African parasite *Trypanosoma brucei* is structurally similar to vertebrate ODC, but cannot interact with AZ and is not a proteasome substrate. Using chimeras between mouse ODC and *T. brucei* ODC, the region critical for association with AZ was shown to include residues 117–140 [12]. This region, close to the ODC homodimer interface, encompasses a large electropositive patch which lies on the surface of the alpha-beta barrel

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forming one domain of the mouse ODC monomer structure [13]. Association with AZ makes the carboxy terminus of ODC more accessible to antibody, suggesting that a conformational change is propagated to that part of the molecule [14]. Conversely, truncating as few as five amino acids from the carboxy terminus of ODC makes it refractory to proteasomal degradation [15, 16]. These observations imply that the last amino acids of ODC have a critical role in proteasomal degradation.

Within AZ, approximately half the molecule, the carboxy-terminal part of the 227 amino acid protein, is sufficient for binding to ODC and for exposure of the ODC carboxy terminus. However, the AZ carboxy-terminal half does not accelerate ODC degradation by the proteasome [17]. Therefore, the amino-terminal half of AZ is contributing something critical to the process, a contribution that may consist of proteasome recognition or activation. A function for the amino-terminal half of AZ independent of ODC binding can be affirmed by grafting it to ODC as a fusion protein. In this form, AZ also enhances the efficiency of proteasomal degradation, and also requires the presence of the ODC C terminus for function [18]. Taken together, these results indicate that the amino terminus of AZ augments a signal carried by the ODC carboxy terminus.

2. A family of antizymes

Recently, the antizymes (AZs) have been shown to consist of an ancient gene family [19, 20]. Representatives with conserved structural, functional and regulatory features are present from fungi to mammals. Within vertebrate species, multiple isoforms are found; humans have at least four. AZs appear to share the following features: 1) structural homology, strongest in the carboxy-terminal half; 2) association with ODC, which reduces its enzymatic activity and may reduce its abundance; 3) polyamine-stimulated translational frameshifting that depends on conserved motifs near the site of frameshifting. These shared characteristics are uniformly suited for preventing excessive changes in polyamine pools. Why then are there conserved isoforms within a species, how are these distributed among tissues, and what biological needs do they individually fulfill? Only very partial answers are known at present. Let us consider AZs 1 through 4. The AZ we have been discussing above, which we will now term AZ1, is the first described. It has a wide tissue distribution. AZ2 is similar in tissue distribution to AZ1, but is less abundant. AZ3 has a very narrow distribution: it is found only in male germ cells in a post-meiotic stage of their differentiation to mature sperm [21, 22]. AZ4 is presently known only as an EST. AZ1 and AZ2 both bind to ODC, but seemingly differ in their capacity to direct proteasomal degradation [23]. Using an *in vitro* assay in which a cell extract provides the source of

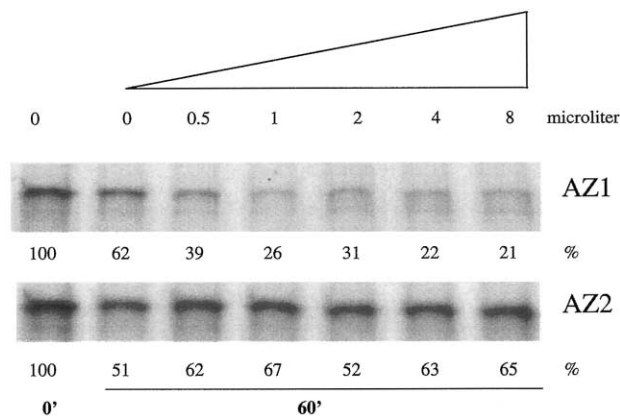


Figure 1. *In vitro* degradation of ODC directed by AZ1 or by AZ2. ^{35}S -methionine-labeled ODC produced by *in vitro* translation was incubated with 0–8 μL of extracts in which AZ1 or AZ2 had been similarly translated, as indicated. After mixing and preincubation of ODC and the AZ on ice and addition of an ATP generating system, samples were immediately analyzed (left lanes) or incubated at 37 °C for 60 min before analysis by SDS-PAGE and autoradiography. The amount of ODC that remained undegraded is shown below each lane as a percentage of that present in the sample not subject to 60 min of incubation at 37 °C. Relative intensity of labeling, normalized to the respective methionine content of each protein, was used to estimate relative protein stoichiometry. At the highest concentration of each AZ used in the experiment shown, the AZs and ODC were initially present at approximately equimolar concentrations.

proteasomes, AZ2 elicited no degradation of ODC, whereas a 16-fold lower concentration of AZ1 produced significant degradation (*figure 1*). However, when ODC was co-expressed with AZ1 or AZ2 in insect cells using baculovirus-based vectors, the presence of either of these AZs lead to marked reduction of the ODC level. Neither system is very physiologic: AZ expression is massive in the insect cells, not low-level as *in vivo*, and *in vitro* systems may lack (or contain) components that alter processes that are normally rate-determining in cells. Conditional expression of AZs at normal levels under conditions not dependent on polyamines should answer the question more reliably. AZ3 binds to and inactivates ODC [21], but no further information on its biochemical properties has as yet emerged.

Gene disruption in mice has shown that AZ1 is not essential, although perinatal mortality is high for pups with a homozygous deficiency (S. Matsufuji and T. Noda, personal communication). Cultured cells derived from these animals do not degrade ODC in response to polyamines. This seems to support a special role for AZ1 in degradation, but may instead simply reflect the greater abundance of AZ1 mRNA compared to AZ2 mRNA in normal cells. Should AZ2 prove ineffective as a catalyst of

ODC degradation, its function as a stoichiometric inhibitor suggests an interesting possibility: AZ2 could form an inactive complex with ODC, one that could later release active enzyme. The very narrow realm of AZ3 expression implies a special function in sperm development, but one that remains to be tested in genetically manipulated animals. Interestingly, transgenic mice that express ODC at very high levels in the testes are defective in the late stages of sperm production [24]. It is likely that AZ3 evolved to provide a specialized and temporally restricted control of polyamine production in the sperm cell lineage.

The inferior degradative power in vitro of AZ2 compared to AZ1 points to an experimental strategy for associating this function with specific structural elements of the more proficient AZ1: make chimeras with a portion of each AZ and test their capacity to cause ODC degradation. The experiments described above, consisting of hacking off bits of AZ1 from each end, have provided some information that localizes functions. However, more revealing data may come from examining pairs of reciprocal AZ1/AZ2 chimeras with identically placed break-points. Preliminary results have supported the utility of this approach. These findings imply that residues functionally important for degradation lie beyond the region previously fingered by truncation analysis.

3. Yeast ODC degradation

In the budding yeast *Saccharomyces cerevisiae*, polyamines control ODC very much as they do in animal cells [25, 26]. Addition of polyamines to the medium reduces the steady-state level of ODC activity and protein more than ten-fold within 4 h. Accelerated degradation is responsible for this change [27]. For newly synthesized ODC, polyamines reduce the half-life from 3 h to approximately 10 min. Interestingly, degradation of bulk ODC pools is also accelerated by polyamines, but the absolute rate of turnover is slower, changing from a half-life of 5 h in untreated cells to 1 h in treated cells. This implies that the newly synthesized protein undergoes a maturation process that makes it more resistant to both basal and induced degradation. Mutations that alter proteasome catalytic residues impair polyamine-induced degradation. Despite the kinetic and phenomenological similarity of this form of regulation in animal cells and *S. cerevisiae*, no direct evidence for an AZ homolog has been found in this yeast species. In contrast, sophisticated database searches reveal that the genome of another yeast, *Schizosaccharomyces pombe*, encodes an AZ [19, 20]. AZ functions in *S. pombe* much as in animal cells. It is possible that an AZ homolog is also present in *S. cerevisiae*, but is so greatly diverged as to escape detection by database searches based on homology. Alternatively, a paralogous protein may have undertaken an AZ-like role in this yeast. The protein synthesis inhibitor cycloheximide prevents polyamine-

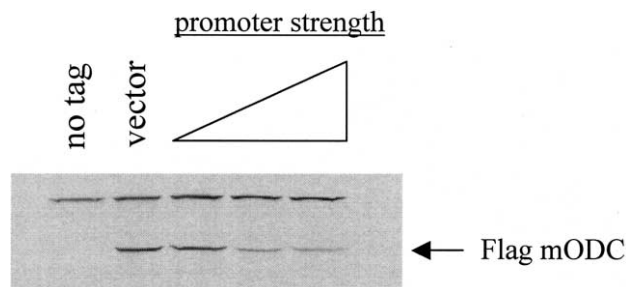


Figure 2. Effect of AZ expression on mouse ODC protein in yeast cells. Yeast cells carrying a vector expressing FLAG epitope-tagged mouse ODC from a weak constitutive promoter were transformed with vectors carrying promoters of increasing strength expressing AZ1, or an empty vector. The rat AZ1 ORF was engineered to bypass the frameshifting requirement. Heterologous expression of N-terminal FLAG epitope-tagged or untagged mouse ODC in a Δ spe1 yeast strain [27] was accomplished using the p424CYC1 vector [34]. An in-frame full-length AZ1 coding region was expressed in yeast transformants using the p426 vector series [34] containing promoters from ADH, TEF, and GPD. By varying the choice of promoter, AZ1 was expressed at different levels. The empty vector control ('vector') indicates cells transformed with the Flag-ODC vector and an empty p426GPD vector. The 'no tag' control was transformed with a mouse ODC expression vector lacking the FLAG epitope tag. Protein extracts were prepared from yeast transformants grown to mid-exponential growth phase in synthetic defined media lacking appropriate supplements, and epitope-tagged ODC was detected by immunoblotting using anti-Flag antibodies (Sigma Chemical Co.). A high molecular mass non-specific protein detected with the anti-Flag antibody serves as a loading control.

induced ODC degradation in *S. cerevisiae*; this finding implies that production of some protein, presumably one with AZ-like properties, must be required.

The difficulty in identifying an AZ in *S. cerevisiae* creates an impediment to mechanistic studies of yeast ODC degradation. While search for this is in progress, a parallel approach has been undertaken, one that utilizes yeast as a genetically tractable model organism for examining the proteolytic events associated with the interaction of mammalian AZ and ODC. This approach was suggested by the observation that *S. cerevisiae* proteasomes degrade mouse ODC in vitro, and do so in accordance with the same constraints as mammalian proteasomes: both AZ and the ODC carboxy terminus promote degradation [28]. We have now examined this question in vivo, by expressing mouse ODC together with rat AZ in *S. cerevisiae*. An AZ-dependent reduction in ODC was observed (figure 2), and this required that the ODC carboxy terminus be present. This observation opens the possibility of using genetic means to ascertain components that are involved in AZ-dependent ODC degradation. This approach may identify not only intrinsic pro-

teasomal proteins, but also other gene products involved in ODC degradation.

4. Why use such unusual means for regulating an enzyme?

Allosteric regulation is the near-universal means by which metabolic end products of a pathway regulate the enzymes responsible for flux through that pathway. The combination of frameshifting and proteolysis offers a unique solution to this control problem. Why do things this way? One way to address this question is to break the engineering problem into two parts: 1) there must be a way to sense the polyamine level; 2) there must be a way to control pools by changing synthesis, catabolism or transport, or more than one of these.

The first problem is made a little more complex because polyamines are largely bound to polynucleotides in cells [29], more strongly to RNA than to DNA. Therefore, only a small fraction of bulk pools can be sampled, those that are free or in rapid equilibrium with weak binding sites. The affinity of polyamines for RNA may make it a good sensor. (Whether polyamine-induced AZ frameshifting is mediated by direct association of polyamines with AZ mRNA, with another RNA or with something else is not known.) Finding a solution to the sensing problem relatively early in evolution may have locked in this choice, regardless of whether it provides an optimal or unique means for polyamine sensing. At the least, frameshifting, the solution used, provides a fast response to changes in polyamines, because it bypasses the need to adjust mRNA level.

Fast response is also an important attribute of a labile protein. As R. Schimke noted decades ago, when the rate of synthesis of a protein changes, the time required to attain a new steady-state depends on the half-life of the protein [30]. Three enzymes are key in determining polyamine levels: ODC, S-adenosylmethionine decarboxylase and spermidine/spermine acetyltransferase. All have short half-lives. Rapid and modulated proteolysis, the solution to the second engineering problem, assures a rapid response.

AZ targets not only ODC but also polyamine transport [31, 32], by mechanisms that are not yet understood. AZ can therefore act as the coordinator of a polyamine-specific local network with at least two effector modes, synthesis and transport. Beyond the need to maintain polyamines within acceptable limits, their supply also must be adjusted in response to cellular needs. Polyamine synthesis is for example up-regulated by mitogens. AZ has been found to have an important role in the growth of prostate cells [33] suggesting that AZ may participate in coordinating polyamines with cell growth signals.

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