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# Ubiquitin dependent and independent protein degradation in the regulation of cellular polyamines

*Review Article*

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**Summary.** Protein degradation mediated by the ubiquitin/proteasome system is the major route for the degradation of cellular proteins. In this pathway the ubiquitination of the target proteins is manifested via the concerted action of several enzymes. The ubiquinated proteins are then recognized and degraded by the 26S proteasome. There are few reports of proteins degraded by the 26S protesome without ubiquitination, with ornithine decarboxylase being the most notable representative of this group. Interestingly, while the degradation of ODC is independent of ubiquitination, the degradation of other enzymes of the polyamine biosynthesis pathway is ubiquitin dependent. The present review describes the degradation of enzymes and regulators of the polyamine biosynthesis pathway.

**Keywords:** Ornithine decarboxylase – S-adenosylmethionine decarboxylase – Antizyme – Antizyme inhibitor – Protein degradation – Ubiquitin

**Abbreviations:** AdoMet, S-adenosylmethionine; AdometDC, S-adenosylmethionine decarboxylase; Az, Antizyme; AzI, Antizyme inhibitor; NQO1, NAD(P)H quinone oxidoreductase; ODC, ornithine decarboxylase; ORF, Open reading frame

## Introduction

Cellular proteins are in a dynamic state of synthesis and degradation. The degradation of the vast majority of cellular proteins is manifested by the ubiquitin-proteasome pathway (Hershko and Ciechanover, 1998). This multifactorial highly controlled system is involved in regulating numerous basic cellular processes such as disposal of defective proteins, regulation of the cell cycle, differentiation, apoptosis and signaling. Malfunction of components of the ubiquitin system contributes to the manifestation of a number of pathological situations. The central component of this proteolytic system is ubiquitin a 76 amino acids protein that is highly conserved from yeast to man. Degradation of proteins via the ubiquitin-mediated pathway involves the attachment of ubiquitin to lysine residues of the target protein, and degradation of the conjugated substrate by the 26S proteasome (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998).

There are a number of proteins that seem to be degraded by the proteasome in an ubiquitin independent manner. These includes Ornithine decaroxylase (ODC), the first enzyme in the polyamine biosynthesis pathway (Bercovich et al., 1989; Murakami et al., 1992; Rosenberg-Hasson et al., 1989), the cyclin dependent kinase inhibitor $p21^{Cip1}$ (Sheaff et al., 2000), the $\alpha$ subunit of the T cell antigen receptor (Yu et al., 1997), c-Jun (Jariel-Encontre et al., 1995), IκBα (Krappmann et al., 1996), and troponin (Benaroudj et al., 2001). In the case of some of these proteins the possibility that their degradation occurs in a ubiquitin-independent manner is based on the observations that mutant ubiquitin lacking all its lysines fails to stabilize them, that their lysineless variants are degraded in a rate indistinguishable from that of the wild-type proteins and that some of them are degraded in cells harboring a conditional allele of the ubiquitin activating enzyme E1. The fact that some of these proteins like $p21^{Cip1}$ are also degraded in a ubiquitin dependent manner, and their possible N-terminal ubiquitination (Ciechanover and Ben-Saadon, 2004) were used to question whether their degradation is truly ubiquitin-independent. ODC is distinct from the other proteins since ODC ubiquitinated forms

were never demonstrated and since an alternative mechanism for its presentation to the proteasome was provided. Interestingly, other enzymes of the polyamine biosynthesis pathway and regulators of the ODC degradation process are degraded in an ubiquitin-dependent manner.

## ODC degradation

ODC is the first rate-limiting enzyme in the biosynthesis of polyamines. ODC decarboxylates ornithine to putrescine. Next an aminopropyl group generated by the action of *S*-adenosylmethionine decarboxylase (AdoMetDC), EC 4.1.1.50) on *S*-adenosylmethionine (AdoMet), is attached to putrescine and spermidine by the action of spermidine and spermine synthase to form spermidine and spermine, respectively. Both ODC and SAMDC are characterized by rapid turnover rate.

### *ODC is degraded without ubiquitination*

Rapid degradation is a central element in regulating cellular ODC levels. ODC was the first protein demonstrated to be degraded without ubiquitination. Its degradation is ATP dependent, occurs in fraction-II of reticulocyte lysate that lacks ubiquitin and in cells harboring a thermosensitive allele of the ubiquitin activating enzyme, E1, even when grown at conditions that inactivate E1 and inhibit the degradation of substrates that are degraded in a ubiquitin dependent manner (Bercovich et al., 1989; Rosenberg-Hasson et al., 1989). In the case of ODC the requirement for ubiquitination as a mean of mediating the recognition of target proteins by the proteasome, is replaced by interaction with a polyamine induced protein termed antizyme (Az) (Murakami et al., 1992). Az has higher affinity to ODC monomer than these subunits display to each higher. ODC subunits that are in a constant state of association/dissociation can be therefore trapped by Az. Interaction with Az has two distinct but related outcomes; ODC is inactivated due to the formation of inactive ODC/Az heterodimer, and (2) the ODC/Az heterodimer is presented to the proteasome for degradation. Az stimulates ODC degradation by enhancing the interaction of ODC with the 26S proteasome rather than stimulating its degradation rate (Zhang et al., 2003). This may be an outcome of a conformational change imposed on ODC upon its interaction with antizyme resulting in the exposure of its C-terminal degradation signal that is recognized by the proteasome (Li and Coffino, 1993). Although it is not completely clear which proteasomal component recognize and interact with ODC, it seems that the ODC/Az complex and ubiquinated proteins are recognized by the same proteasomal component, as ubiquitinated proteins and polyubiquitin chains inhibit ODC degradation (Zhang et al., 2003). As in mammalian cells, also in yeast cells the degradation of yeast ODC is ubiquitin independent (Gandre and Kahana, 2002) and Az dependent (Palanimurugan et al., 2004).

### *Sequences that mediate ODC degradation*

Two segments of ODC are involved in regulating its degradation. The first is a C-terminal segment encompassing the last 37 amino acids that seems to serve as the proteasomal recognition signal (Ghoda et al., 1989; Rosenberg-Hasson et al., 1991; Zhang et al., 2003). The second, encompassing amino acids 117–140 serve as the Az binding site (Li and Coffino, 1992). Deletion of the Az binding region or mutations introduced into it stabilized the protein (Li and Coffino, 1992; Mamroud-Kidron et al., 1994a). Deletion of the C-terminal segment converts ODC into a stable protein (Ghoda et al., 1989; Rosenberg-Hasson et al., 1991). Interestingly, *Trypanosoma brucei* ODC naturally lacks this region and is a stable protein both in trypanosom cells and when expressed in mammalian cells (Ghoda et al., 1990). A chimerical trypanosom ODC that contains the C-terminus of mouse ODC is rapidly degraded in mammalian cells. Since trypanosome ODC does not bind mammalian Az (Li and Coffino, 1992), it should be assumed that the appended C-terminus of the mammalian enzyme is exposed within the context of the trypanosome enzyme. Like the trypanosome enzyme, ODC from *Leishmania donovani* is a stable protein whereas despite lacking the C-terminal destabilizing signal *Crithidia fasciculate* ODC is rapidly degraded both in the parasite and when expressed in mammalian cells (Persson et al., 2003).

Like trypanosome ODC also the yeast enzyme lacks the C-terminal destabilizing segment found in the mammalian enzyme. However, while being stable when expressed in mammalian cells, yeast ODC is rapidly degraded in yeast cells (Gandre and Kahana, 2002). An N-terminal segment that is unique to yeast ODC seems to mediate its degradation in yeast cells as its deletion stabilizes the enzyme (Gandre and Kahana, 2002). Interestingly, mammalian ODC with a mutated Az binding site is efficiently degraded in yeast cells while a mutant with a truncated C-terminus is stable (Gandre and Kahana, 2002; Mamroud-Kidron and Kahana, 1994; Mamroud-Kidron et al., 1994b). These results suggest that in yeast cells the C-terminal degradation signal of the mammalian

enzyme is exposed without requiring interaction with Az. The degradation of yeast and mammalian ODC differ also in their requirements for the proteolytic activities of the yeast proteasome (Mamroud-Kidron and Kahana, 1994; Mamroud-Kidron et al., 1994b).

### *ODC degradation by the 20S proteasome*

While the above described ubiquitin independent degradation of ODC is executed by the 26S proteasome, recently a novel pathway was described that degraded ODC via the action of the 20S proteasome (Asher et al., 2005; Kahana et al., 2005). Degradation via this pathway that is regulated by NAD(P)H quinone oxidoreductase (NQO1) does not require the C-terminal destabilizing segment and is independent of interaction with Az and ubiquitination. Transient ODC monomers seem to be the substrates of this system and binding to NQO1 protects them from degradation. Disruption of this interaction by dicoumarol promotes ODC degradation by the 20S proteasome. This pathway that was demonstrated to degrade ODC during oxidative stress does not seem to respond to the intracellular polyamine balance. This pathway seems to be a general poteolytic pathway that is responsible for default degradation of unfolded proteins before they assume their mature and protective conformation or before they enter into protective complexes.

## Antizyme synthesis and degradation

Az is a central regulator of the polyamine biosynthesis pathway that is conserved from the budding yeast saccharomyces cerevisiae to man. Az was originally described as an ODC inhibitory activity that is stimulated by polyamines (Heller et al., 1976). It was then demonstrated that this activity is actually a protein that binds to monomeric ODC subunits resulting in ODC inactivation followed by targeting the trapped ODC subunits to proteasomal degradation (Coffino, 2001; Hayashi et al., 1996). Az is a central component of an autoregulatory circuit that regulates cellular polyamine levels. Az synthesis is regulated by a polyamine stimulated ribosomal frameshifting (Matsufuji et al., 1995; Rom and Kahana, 1994). The complete Az protein is encoded by two different open reading frames (ORFs). Translation initiated at one of two possible initiation codons is terminated shortly thereafter at an in-frame termination codon. The synthesis of a complete Az protein requires that the scanning ribosomes will be subverted to the +1 reading frame. This shift in the translation reading frame is stimulated by polyamines and serves as a cellular polyamine sensing mechanism. Two segments of Az fulfill two different roles in mediating ODC degradation. The first is a C-terminal segment encompassing amino acids 104–183 that mediates the interaction with ODC resulting in its inactivation (Mamroud-Kidron et al., 1994a). The second, a more N-terminal segment containing amino acids 70–103 is required for targeting ODC to proteasomal degradation (Mamroud-Kidron et al., 1994a). It is possible that together with the C-terminal destabilizing signal of ODC and the N-terminal segment of Az form a more efficient proteosomal recognition signal.

While inhibition of ODC by Az is stoichiometric, Az stimulated ODC degradation appears to be catalytic (Mamroud-Kidron et al., 1994a). In an in vitro degradation assay, Az remains stable while stimulating ODC degradation supporting the notion that Az is recycled to support additional cycles of ODC degradation. Nevertheless, in cells Az is a rapidly degraded protein (Gandre et al., 2002). Whereas interaction with Az accelerates ODC degradation, this interaction does not affect the degradation rate of Az, suggesting that Az is not degraded together with ODC when presenting the latter to the proteasome (Gandre et al., 2002). Az degradation was inhibited in cells harboring a thermosensitive allele of the ubiquitin-activating enzyme E1 when the cells were grown at the non-permissive temperature. It was therefore concluded that Az is degraded in a ubiquitin-dependent manner (Gandre et al., 2002). Using mutant cell lines dependence on ubiquitination was also demonstrated for the degradation of yeast Az in yeast cells. Interestingly, this degradation of yeast Az is inhibited by polyamines (Palanimurugan et al., 2004).

## Degradation of antizyme-inhibitor (AzI)

Like Az, AzI was also described as an inhibitory activity capable of inhibiting Az activity (Fujita et al., 1982). Due to biochemical similarity to ODC, it was initially unclear whether AzI is distinct from ODC (Kitani and Fujisawa, 1989). This was solved when AzI was cloned demonstrating that it is highly homologous but distinct from ODC (Koguchi et al., 1997; Murakami et al., 1996). Despite its high similarity to ODC and its dimeric structure, AzI retains no ornithine decarboxylating activity (Murakami et al., 1996). The affinity of AzI for Az is greater than that of ODC (Fujita et al., 1982). It can therefore sequester Az and save ODC from degradation. Indeed, forced AzI expression was demonstrated to provide cells with growth advantage via neutralization of Az functions (Keren-Paz et al., 2006; Kim et al., 2006), although Az independent

mechanism was also suggested (Kim et al., 2006). Of the two segments that regulate ODC degradation, one, the Az binding segment of AzI functions even better than that of ODC. In contrast, the C-terminal segment of AzI differs from that of ODC. Despite this difference AzI is a rapidly degraded protein (Bercovich and Kahana, 2004). Furthermore, an AzI mutant lacking the C-terminal fragment is rapidly degraded, demonstrating that unlike ODC, AzI does not posses a C-terminal destabilizing signal that is recognized by the proteasome. Although AzI binds Az efficiently, this interaction is not required for AzI degradation. Furthermore, interaction with Az actually stabilized ODC (Bercovich and Kahana, 2004). This stabilization in complex with Az is a key element in defining AzI as an inhibitor of Az. Based on stabilization of AzI due to lesions in the ubiquitin system and on direct ubiquitination assays it was concluded that AzI is degraded in an ubiquitin-dependent manner. Furthermore, it was demonstrated that interaction with Az stabilized AzI by interfering with its ubiquitination (Bercovich and Kahana, 2004). Interestingly, Az also stabilized an AzI/ODC chimera in which the C-terminal segment of AzI was replaced with that of ODC. The inability of the C-terminal segment of ODC to confer ubiquitin independent/Az dependent degradation on AzI is interesting in light of the high affinity of AzI to Az and of previous observations demonstrating that the C-terminal 37 amino acids of mammalian ODC act as an autonomous degradation signal that confers rapid degradation when appended to stable proteins (Hoyt et al., 2005).

## Degradation of S-adenosylmethionine decarboxylase (AdoMetDC)

AdoMetDC that uses a covalently bound pyruvoyl as a cofactor catalayzes the decarboxylation of S-adenosylmethionine (AdoMet) provides an aminopropyl group for the synthesis of spermidine and spermine respectively. AdoMetDC is synthesized as a proenzyme that is autoclaved to form $\alpha$ and $\beta$ subunits with the former being pyruvoylated simultaneously with the cleavage at an internal serine residue (Stanley et al., 1989). Like ODC, AdoMetDC is a highly regulated enzyme that is characterized by a short intracellular half-life and inverse correlation to the intracellular concentration of polyamines (Pegg et al., 1987; Poso and Pegg, 1981). The regulatory similarity between ODC and AdoMetDC raised the possibility that Az also regulates the degradation of the latter. However, based on lack of co-immunoprecipitation it was concluded that Az does not regulate AdoMetDC degradation (Yerlikaya and Stanley, 2004). This conclusion was not supported by a functional experiment like co-transfection experiments monitoring for the ability of Az to stimulate AdoMetDC degradation. Interestingly, both ATP depletion and treatment with proteasome inhibitors stabilize the AdoMetDC protein but not the decay of its enzymatic activity. Since proteasome inhibitors did not affect AdoMetDC activity in vitro it was concluded that the activity decay is a result of inhibition of proteasomal functions (Yerlikaya and Stanley, 2004). This activity decay was correlated to increased concentration of the substrate AdoMet that mediates enzymes transamination. It was actually suggested that the substrate-mediated transamination stimulates AdoMetDC degradation. Demonstration of polyubiquitinated species in cells co-transfected with tagged AdoMetDC and ubiquitin suggested that the degradation of AdoMetDC is mediated by the ubiquitin dependent proteolytic system (Yerlikaya and Stanley, 2004).

## Degradation of spermidine/spermine $N^1$-acetyltransferase

Spermidine/spermine $N^1$-acetyltransferase (SSAT) is a rate-limiting enzyme in the catabolism of cellular polyamines. SSAT modifies spermidine and spermine to their $N^1$-acetylated derivatives that are either excreted or subjected to the action of polyamine oxidase that converts them to putrescine (Casero and Pegg, 1993; Seiler, 1987). SSAT is a highly regulated short-lived protein. The degradation of SSAT is mediated by the ubiquitin/proteasome system (Coleman and Pegg, 1997, 2001). Binding to polyamines and to polyamine analogues inhibits SSAT degradation probably by inducing conformational changes. SSAT degradation depends also on the carboxyl-terminal region of the protein, especially on two glutamic acid residues at positions 170 and 171. Although no single lysine of its 11 lysines is absolutely essential for SSAT's ubiquitination, lysine 87 seems to be a preferred ubiquitination site as its conversion to arginine significantly prolonged the half-life of the protein.

## Conclusions

ODC is unusual in being degraded by the 26S proteasome without requiring prior ubiquitination. The vast majority of cellular proteins that are degraded by the proteasome are marked for degradation by polyubiquitination. Although there are few cellular proteins that were demonstrated to be degraded in an ubiquitin-independent manner, they differ from ODC since most of them are also

degraded in an ubiquitin- dependent manner and since an alternative mechanism for their delivery to the proteasome was not demonstrated. Ubiquitination is manifested by the action of several enzymes and requires investment of energy. Targeting of ODC by interaction with Az seems to be simpler. The way ODC is targeted to degradation is special not only when compared to the degradation of totally unrelated cellular proteins, but it also differs from other enzymes and regulators in the polyamine synthesis and catabolic pathways that are degraded in a ubiquitin dependent manner. Of particular interest is the difference from the ubiquitin dependent degradation of AdoMetDC whose half-life like that of ODC, is inversely correlated to the intracellular level of spermidine and spermine (Autelli et al., 1991; Pegg et al., 1987; Poso and Pegg, 1981). Polyamines regulate degradation not only via induction of Az. For instance, binding of polyamines and polyamine analogues to SSAT, stabilize it by interfering with its ubiquitination. It will be of interest to determine whether in other cases components of the ubiquitin system respond to changes in the concentration of cellular polyamines.

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