

# Ubiquitin-independent Mechanisms of Mouse Ornithine Decarboxylase Degradation Are Conserved between Mammalian and Fungal Cells\*<sup>§</sup>

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The polyamine biosynthetic enzyme ornithine decarboxylase (ODC) is degraded by the 26 S proteasome via a ubiquitin-independent pathway in mammalian cells. Its degradation is greatly accelerated by association with the polyamine-induced regulatory protein antizyme 1 (AZ1). Mouse ODC (mODC) that is expressed in the yeast *Saccharomyces cerevisiae* is also rapidly degraded by the proteasome of that organism. We have now carried out *in vivo* and *in vitro* studies to determine whether *S. cerevisiae* proteasomes recognize mODC degradation signals. Mutations of mODC that stabilized the protein in animal cells also did so in the fungus. Moreover, the mODC degradation signal was able to destabilize a GFP or Ura3 reporter in GFP-mODC and Ura3-mODC fusion proteins. Co-expression of AZ1 accelerated mODC degradation 2–3-fold in yeast cells. The degradation of both mODC and the endogenous yeast ODC (yODC) was unaffected in *S. cerevisiae* mutants with various defects in ubiquitin metabolism, and ubiquitinated forms of mODC were not detected in yeast cells. In addition, recombinant mODC was degraded in an ATP-dependent manner by affinity-purified yeast 26 S proteasomes in the absence of ubiquitin. Degradation by purified yeast proteasomes was sensitive to mutations that stabilized mODC *in vivo*, but was not accelerated by recombinant AZ1. These studies demonstrate that cell constituents required for mODC degradation are conserved between animals and fungi, and that both mammalian and fungal ODC are subject to proteasome-mediated proteolysis by ubiquitin-independent mechanisms.

The 26 S proteasome is the major neutral protease of the cytoplasmic and nuclear compartments of eukaryotic cells. It is a multisubunit, ATP-dependent, protease composed of at least two subcomplexes: a barrel-shaped 20 S core particle that sequesters the proteolytic active sites from the intracellular environment, and a 19 S regulatory particle that contains activities required for recognition, unfolding, and translocation of substrates to the interior of the core particle (1). Its substrate

proteins include major regulators of cell growth and differentiation, and also proteins that fail to fold into a native conformation. The proteasome needs therefore to recognize appropriate substrates accurately to support proper timing of cellular events and to eliminate proteins that have failed to follow normal folding pathways. Many of these short-lived proteins are targeted to the proteasome by the conjugation of ubiquitin, an evolutionarily conserved 76-amino acid globular protein (2). The efficient recognition of ubiquitinated substrates by the proteasome requires, minimally, the formation of a tetraubiquitin chain on the substrate protein (3). The polyubiquitin signal is in turn recognized by a receptor site that includes the Rpt5/S6' ATPase subunit of the regulatory particle (4). Additionally, the proteasome has the capacity to recognize a class of substrates that do not require ubiquitin modification for their regulated degradation (5). These include the cyclin-dependent kinase inhibitor p21 and ornithine decarboxylase (ODC),<sup>1</sup> which catalyzes the initial step in polyamine biosynthesis.

In most eukaryotic cells, ODC is subject to regulation by spermidine and spermine, the end products of the polyamine biosynthetic pathway. These act not through allosteric feedback regulation, the usual mechanism, but by changing the abundance of the ODC protein (6). This form of feedback regulation is accomplished by an autoregulatory circuit composed of ODC, polyamines, and the regulatory protein antizyme 1 (AZ1) (7). Excess polyamines induce a +1 translational frameshift of the AZ1 mRNA that is required to align a short upstream open reading frame (ORF) with a downstream ORF encoding the functional protein (8). AZ1 binds the ODC monomer, dissociating the enzymatically active ODC homodimer and thereby inhibiting its activity (9). AZ1 binding exposes a COOH-terminal degradation signal in the ODC protein that leads to an increased rate of degradation of ODC by the proteasome (10). The AZ1-dependent degradation of ODC by the proteasome is remarkable in that it occurs in mammalian cells independently of the ubiquitinylation of ODC. Both *in vivo* data (11, 12) and *in vitro* data (13, 14) support this conclusion.

The ODC-AZ regulatory circuit has been best characterized in mammalian cells, but functional AZ homologs have also been described in a number of other organisms, including the nematode *Caenorhabditis elegans* (15), the fly *Drosophila melanogaster* (16), several filamentous fungi, and the fission yeast *Schizosaccharomyces pombe* (15, 17). However, no AZ homolog is evident in the genome sequence of the budding yeast *Saccharomyces cerevisiae* (18). In this organism, polyamine regu-

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains Fig. 1.

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<sup>1</sup> The abbreviations used are: ODC, ornithine decarboxylase; AZ1, antizyme 1; GFP, *Aequorea victoria* green fluorescent protein; HA, hemagglutinin; mODC, *Mus musculus* ornithine decarboxylase; ORF, open reading frame; yODC, *Saccharomyces cerevisiae* ornithine decarboxylase; E1, ubiquitin-activating enzyme; Ub, ubiquitin.

lation of ODC resembles that of mammalian cells in that excess polyamines increase the rate of ODC degradation (19). Polyamine regulation in *S. cerevisiae* also involves *de novo* protein synthesis (20), a requirement of an AZ-like regulatory mechanism, but not of polyubiquitinylation. Both yeast and mouse ODC are rapidly degraded by the 26 S proteasome in *S. cerevisiae* (19, 21).

Proteasome structure is highly conserved between mammals and fungi, and so too is the general use of ubiquitin modification as a marker for recognition of substrates. We ask here whether, and to what extent, mammalian and yeast proteasomes also share the capacity to recognize and respond to the structural hallmarks that are used in mammalian cells for the constitutive and regulated degradation of ODC. We show here that for both yeast and mouse ODC, degradation in yeast cells does not depend on ubiquitin. We found that recognition of mouse ODC requires similar structural and functional elements, regardless of the source of the proteasome, implying that ubiquitin-independent substrates, like those that depend on ubiquitin, must utilize a conserved discriminatory capacity of the proteasome.

#### EXPERIMENTAL PROCEDURES

**Yeast Strains**—Yeast strains were maintained and manipulated using standard protocols (22). For analysis of <sup>FLAG</sup>mODC and yODC<sup>FLAG</sup> turnover (reported in Figs. 1, 3, and 4), the appropriate expression vectors were transformed into strain Y13 (*MAT $\alpha$  his3 leu2 trp1 ura3 spe1 $\Delta$ ::HisG spe2 $\Delta$ ::LEU2*), a polyamine auxotroph (19). Growth in defined medium of polyamine auxotrophic strains was maintained by addition of 0.1  $\mu$ M spermidine, a concentration insufficient for repression of endogenous yODC; the level of spermidine in the medium was increased to 1 mM to induce repression of yODC. Vectors for the expression of GFP or Ura3 fusion proteins (Fig. 2) were transformed into strain MHY501 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 trp1-1 ura3-52*) (23) or the proteasome mutant WCG4-11/22a (*MAT $\alpha$  his3-11,15 leu2-3,112 ura3 pre1-1 pre2-2*) and its congenic wild type strain WCG4a (*MAT $\alpha$  his3-11,15 leu2-3,112 ura3*) (24). An *rpn4 $\Delta$*  mutant, MHY74 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 trp1-1 ura3-52 rpn4 $\Delta$ ::kanMX4*) was created by replacement of the entire *RPN4* ORF of strain MHY501 with a *kanMX4* cassette from pRS400 generated by PCR as described (25). To examine the effects of ubiquitin metabolism on ODC turnover (Fig. 6), <sup>FLAG</sup>mODC, yODC<sup>FLAG</sup>, or Leu- $\beta$ -galactosidase vectors were transformed into MHY1409 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 trp1-1 ura3-52 gal2 uba1-2 (mTn URA3)*) (26), MHY623 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 trp1-1 ura3-52 doa4- $\Delta$ 1::LEU2*) (27), or MHY501. Proteasomes were affinity-purified from strain RJD1144 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 trp1 $\Delta$ 63 ura3-52 PRE1<sup>FH</sup>::YIplac211 (URA3)*) (28).

**Plasmid Construction**—All plasmid constructions utilized standard molecular biology techniques (29), and the identities of DNA fragments generated by PCR were verified by sequencing. The sequences of primers used for PCR constructions are available upon request. FLAG epitope tags were added to the termini of mODC (30), mODC<sup>C441A</sup> (31), and yODC (32) by PCR using primers that also appended flanking restriction sites. The PCR products were digested with the appropriate restriction endonucleases and ligated into the similarly digested expression vectors of Mumberg *et al.* (33). These vectors include promoters of various strengths for heterologous expression in *S. cerevisiae*. We designed a similar vector containing the yODC (*SPE1*) promoter by PCR amplification of a region containing the 574 nucleotides upstream of the yODC ORF in the *SPE1* gene while appending 5' *SacI* and 3' *XbaI* sites. The *ADH* promoter was removed from the p414ADH vector (33) by digestion with *SacI* and *XbaI*, and replaced with the similarly digested PCR product containing the *SPE1* promoter by ligation. The resulting *TRP1*-marked *CEN/ARS* vector was designated p414SPE1. The COOH-terminal FLAG-tagged yODC ORF was cloned as a *BamHI-XhoI* PCR product into p414SPE1 to create p414SPE1-yODCF. To generate COOH-terminal truncations of mODC, the mODC ORF was amplified using a common sense primer complementary to the NH<sub>2</sub> terminus of mODC (including the FLAG epitope tag and flanking restriction sites) and antisense primers that introduce stop codons at Phe<sup>425</sup> (mODC $\Delta$ 425–461) or Ala<sup>457</sup> (mODC $\Delta$ 457–461). The AZI1AT ORF was cloned from pGEM4Z/ $\Delta$ T (8) as a *HindIII-EcoRI* fragment into similarly digested *URA3*-marked Mumberg vectors.

GFP-mODC fusions were constructed by splice overlap extension PCR (SOE-PCR) using a pGFPuv vector (BD Sciences Clontech) as a template. A PCR fragment containing the COOH-terminal half of the GFP ORF was generated by a sense primer overlapping the *XhoI* site within the GFPuv coding region, and an antisense primer complementary to the COOH terminus of GFPuv and amino acids 425–461 of mODC. A second PCR fragment was generated by amplification of the COOH terminus of the mODC or mODC<sup>C441A</sup> coding region using a sense primer complementary to the antisense primer used to generate the GFP fragment and an antisense primer that appended an *EcoRI* site following the stop codon of the mODC ORF. Following SOE-PCR, the resulting DNA fragments were digested with *XhoI* and *EcoRI*, and ligated into a similarly digested pGFPuv vector from which the fragment containing the COOH terminus of GFPuv had been removed. The ORFs encoding GFPuv and GFP-mODC fusion proteins were subcloned from the pGFPuv-based vectors into yeast expression vectors as *XbaI-EcoRI* fragments.

Ura3-mODC fusions were constructed similarly to the GFP-mODC fusions by SOE-PCR. The coding region of *URA3* was amplified from the pRS306 vector (34) using a sense primer that appended an NH<sub>2</sub>-terminal FLAG epitope tag and a flanking *BamHI* site, and an antisense primer complementary to the COOH terminus of Ura3 and amino acids 425–461 of mODC. A DNA fragment containing the COOH terminus of mODC was generated using a sense primer complementary to the antisense primer used to amplify the *URA3* coding region and an antisense primer that appended an *EcoRI* site following the stop codon of the mODC ORF. Following SOE-PCR, the DNA fragment encoding Ura3-mODC was digested with *BamHI* and *EcoRI* and ligated into similarly digested yeast expression vectors.

**Metabolic Labeling, Immunoprecipitations, and Immunoblotting**—Pulse-chase analysis was carried out similarly to the protocol described by Suzuki and Varshavsky (35). 10-ml cultures of yeast transformants were grown to midexponential growth phase ( $A_{600}$  0.5–1). Cells were harvested by centrifugation (3 min, 2000  $\times$  g), and washed twice in 1 ml of SD medium lacking methionine (SD –Met). The cells were resuspended in 0.4 ml of SD –Met and labeled for 5 min at 30 °C with 200  $\mu$ Ci of a L-[<sup>35</sup>S]methionine and L-cysteine mixture (Expres<sup>35</sup>S<sup>35</sup>S protein labeling mixture, PerkinElmer Life Sciences). To terminate incorporation of radiolabel and initiate a chase, cells were collected by brief centrifugation in a microcentrifuge, and resuspended in 0.4 ml of SD medium containing 10 mM methionine, 1 mM cysteine, and 0.5 mg/ml cycloheximide. Incubation was continued at 30 °C. At each time point 100  $\mu$ l of cells were removed and transferred to a 2-ml screw-top microcentrifuge tube containing 700  $\mu$ l of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and 500  $\mu$ l of 0.5-mm glass beads. Cells were lysed by 4 pulses of 30 s duration in a Mini-beadbeater (Biospec Products), with cooling on ice between pulses. Lysates were cleared by centrifugation (13,000  $\times$  g, 10 min at 4 °C), and the amount of trichloroacetic acid-insoluble radioactivity was determined. The volumes of lysates were adjusted to contain equivalent amounts of trichloroacetic acid-insoluble radioactivity for immunoprecipitations.

For immunoprecipitations of FLAG-tagged proteins, normalized volumes of lysates were incubated for 2 h at 4 °C with 20  $\mu$ l of a 50% slurry of anti-FLAG M2 affinity gel (Sigma) in lysis buffer. Immunoprecipitations of Leu- $\beta$ -galactosidase or GFP were carried out by incubation of lysates with 1  $\mu$ l of anti- $\beta$ -galactosidase antibody (Promega) or anti-GFP antibody (BD Sciences Clontech) for 2 h at 4 °C, followed by the addition of 20  $\mu$ l of a 50% slurry of protein G-Sepharose (Amersham Biosciences) and a further 1-h incubation at 4 °C. Immunoprecipitates were collected by brief centrifugation (2000  $\times$  g, 20 s), and washed 4 times with 1 ml of lysis buffer containing 0.1% SDS. The immunoprecipitates were resuspended in SDS sample buffer (29), heated to 100 °C for 5 min, and fractionated by SDS-PAGE on 10% (<sup>FLAG</sup>mODC, yODC-<sup>FLAG</sup>, <sup>FLAG</sup>Ura3 fusions, or GFP fusions) or 7.5% ( $\beta$ -galactosidase) polyacrylamide gels. Radiolabeled proteins were visualized by autoradiography, and quantified using Scion Image software (Scion Corp.).

For immunoblot analysis 5-ml cultures of yeast cells were grown to midexponential growth phase in SD medium lacking the appropriate nutritional supplements. Cells were collected by centrifugation (3 min, 2000  $\times$  g), and washed in 5 ml of water, followed by resuspension in 200  $\mu$ l of phosphate-buffered saline + 1% Triton X-100. The cell suspension was added to 200  $\mu$ l of glass beads and lysed by 3 pulses of 20 s duration in a Mini-beadbeater with intermittent cooling on ice. Cell debris was removed by centrifugation (13,000  $\times$  g for 5 min at 4 °C), and volumes of lysates containing 20  $\mu$ g of total protein were fractionated by SDS-10% PAGE. Fractionated proteins were transferred to nitrocellulose filters, and developed using the Amersham ECL detection kit and proto-

col. Filters were blocked in Tris-buffered saline + 1% Triton X-100 (TBS-T) including 5% powdered low-fat milk and 5% bovine serum albumin, and washed 3 times for 5 min with TBS-T. FLAG-tagged proteins were detected with mouse anti-FLAG M2 antibody (Sigma, 1:2000 dilution) and sheep anti-mouse Ig-horseradish peroxidase conjugates (Amersham Biosciences, 1:15,000 dilution).

For detection of ubiquitin conjugates, cells were grown and harvested as described above, but lysis and immunoprecipitations were carried out in buffers containing 50 mM *N*-ethylmaleimide. Hemagglutinin-ubiquitin conjugates were detected, following fractionation by SDS-PAGE and transfer to nitrocellulose, by incubation with anti-HA-horseradish peroxidase conjugates (Santa Cruz Biotechnology, 1:1000 dilution).

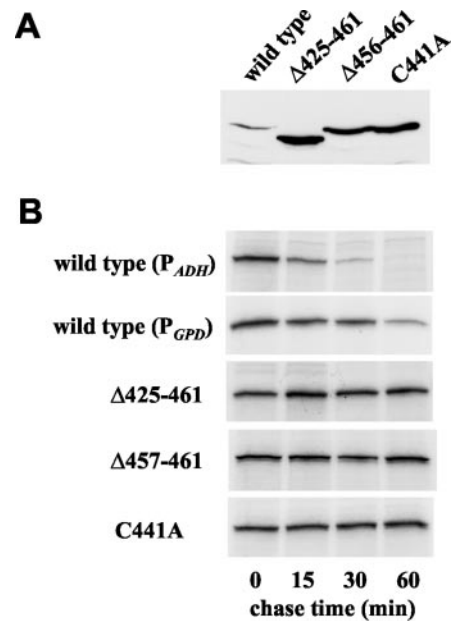
**Proteasome Purification**—Proteasomes were affinity purified from RJD1144 cells as described (28). Normally, this purification protocol involves separating tagged proteasome complexes from extracts using anti-FLAG antibody conjugated to agarose beads followed by elution of the complexes with FLAG peptide. We have found that the FLAG peptide causes activation of the normally gated 20 S core particle that results in a reproducible ATP-independent degradation of mODC.<sup>2</sup> To avoid exposure to the FLAG peptide, we used proteasome complexes still associated with the antibody-agarose, a form active for ATP-dependent proteolysis of mODC. Briefly, cells were grown to early stationary phase ( $A_{600}$  2–2.5) in 2 liters of SD medium and harvested by centrifugation ( $2000 \times g$ , 20 min). The cells were pooled and washed in 100 ml of ice-cold water. The cell pellet was resuspended in an equal volume of buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 10% glycerol) containing 5 mM ATP and drop frozen in liquid N<sub>2</sub> in a mortar resting in a bed of dry ice. The cells were ground with a pestle for 30 min with periodic additions of liquid N<sub>2</sub> to keep the material frozen. The ground cells were thawed and most cell debris was removed from the lysate by centrifugation ( $4,000 \times g$ , 10 min), followed by a second centrifugation ( $30,000 \times g$ , 20 min) to remove remaining debris. 10 ml of the cleared lysate ( $\sim 200 \mu\text{g}$  of protein) was incubated with 300  $\mu\text{l}$  of anti-FLAG M2 affinity gel for 90 min at 4 °C on a rocking platform. The agarose beads were collected by centrifugation ( $2000 \times g$ , 10 min), transferred to a microcentrifuge tube, and washed with 10 ml of buffer A containing 2 mM ATP and 0.2% Triton X-100, followed by 2 washes with 1 ml of buffer A with 2 mM ATP. The proteasome-agarose beads were resuspended in an equal volume of buffer A with 2 mM ATP and used for degradation assays. Total protein bound to the anti-FLAG agarose was estimated by elution in 1 M NaCl followed by protein determination of the resulting eluate.

**In Vitro Degradation Assays**—The degradation of mODC by purified proteasomes was followed by a released radiolabel assay. Recombinant radiolabeled mODC was produced in *Escherichia coli* as described elsewhere.<sup>3</sup> Degradation assays were performed in 20- $\mu\text{l}$  reaction volumes at 37 °C and contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM ATP, 10 mM KCl, 10% glycerol, an ATP regenerating system (2 mM dithiothreitol, 10 mM creatine phosphate, 1.6 mg/ml creatine kinase), 2 mg/ml bovine serum albumin and proteasomes. Reactions were initiated by addition of substrate and quenched by addition of 140  $\mu\text{l}$  of 20% (w/v) trichloroacetic acid. The trichloroacetic acid-insoluble material was removed by centrifugation ( $14,000 \times g$ , 30 min) at 4 °C, and 150  $\mu\text{l}$  of the supernatant was removed and released counts were measured in a liquid scintillation counter. Percentage degradation of radiolabeled mODC was determined as the released counts/min minus background counts/min divided by total input counts/min. Total input counts were determined by substituting water for trichloroacetic acid in the reactions. Background counts/min were determined in reactions that excluded proteasomes, and were typically  $\sim 1\%$  of total input counts.

**Fluorescence Measurements**—The fluorescence of GFPuv and GFP-mODC proteins in yeast transformants was measured in arbitrary units with a TD-700 laboratory fluorometer (Turner Designs) using a 394-nm excitation filter and a 510-nm emission filter. Fluorescence measurements were made in cultures at exponential growth phase ( $A_{600}$  0.5–1), and background autofluorescence was measured in an empty vector transformant and subtracted from raw fluorescence measurements.

## RESULTS

**Carboxyl-terminal Recognition Elements of Mouse ODC Are Recognized in Yeast**—To facilitate studies of mODC degradation in *S. cerevisiae*, we appended a single copy of the FLAG



**FIG. 1. COOH-terminal mODC mutants are stable in yeast cells.** *A*, immunoblot analysis with anti-FLAG antibody (Sigma) of *spe1Δspe2Δ* cells transformed with p414ADH vectors expressing wild type<sup>FLAG</sup>mODC or COOH-terminal mutants. *B*, pulse-chase analysis of<sup>FLAG</sup>mODC in *spe1Δspe2Δ* transformants. Wild type<sup>FLAG</sup>mODC was expressed from the *ADH* (*P<sub>ADH</sub>*) or *GPD* (*P<sub>GPD</sub>*) promoters in p414 vectors. COOH-terminal mutants were all expressed from the *ADH* promoter of p414ADH. Epitope-tagged mODCs were immunoprecipitated with anti-FLAG antibody.

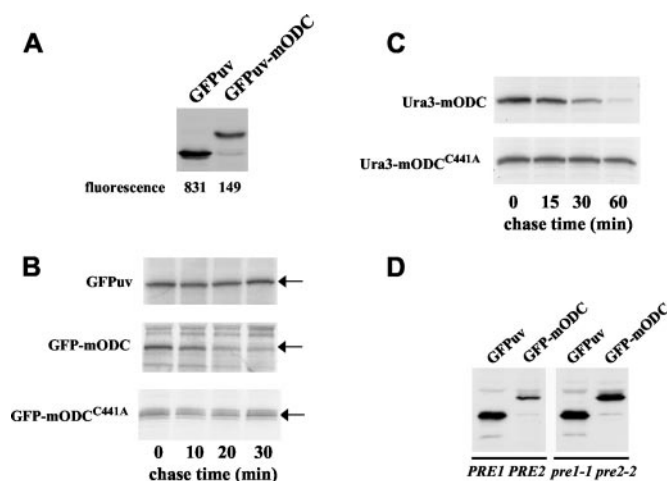
epitope tag to the NH<sub>2</sub> terminus of mouse ODC (<sup>FLAG</sup>mODC). The tagged mODC was expressed from yeast centromeric expression vectors (33) in a *spe1Δspe2Δ* mutant. This mutant lacks enzymes that catalyze key steps in polyamine biosynthesis in *S. cerevisiae*, and cannot accumulate spermidine, the effector of polyamine regulation in this organism. Polyamine pools are thus accessible to experimental control in this genetic background.

Mouse ODC expressed in yeast cells is rapidly degraded by the 26 S proteasome (21). We compared, in yeast transformants, the degradation of FLAG-tagged wild type mODC expressed from either the yeast *ADH* promoter or the stronger *GPD* promoter. We found that wild type<sup>FLAG</sup>mODC expressed from the *ADH* promoter was easily detectable by immunoblotting (Fig. 1*A*) and rapidly degraded in *spe1Δspe2Δ* cells with a half-life of  $\sim 10$  min, as measured by pulse-chase analysis (Fig. 1*B*). The expression of<sup>FLAG</sup>mODC protein from a vector containing the more active *GPD* promoter led to a 3-fold stabilization of<sup>FLAG</sup>mODC in those transformants (half-life =  $\sim 30$  min, Fig. 1*B*), suggesting that overexpression interferes with normal degradation of mODC as it does with turnover of endogenous yODC (19, 36, 37).

We used vectors containing the *ADH* promoter to compare turnover of wild type and mutant forms of mODC in yeast cells. The mODC mutants analyzed included truncated forms lacking the COOH-terminal 5 or 37 amino acids of the mODC, and full-length mODC containing a Cys<sup>441</sup> to Ala mutation. The COOH-terminal 37 amino acids of mODC (residues 425–461) contains a signal required for its rapid degradation in animal cells or *in vitro* (10, 38), and both Cys<sup>441</sup> and the 5 terminal residues in this region are critical for its function (31, 39). Truncation of 5 or 37 residues from the COOH terminus, or the mutation of Cys<sup>441</sup> to Ala, led to greater expression of these proteins than of the wild type<sup>FLAG</sup>mODC in exponentially growing cultures (Fig. 1*A*), and significant stabilization of these proteins throughout a 1-h chase period following metabolic

<sup>2</sup> M. A. Hoyt and P. Coffino, unpublished results.

<sup>3</sup> M. Zhang, C. M. Pickart, and P. Coffino, *EMBO J.*, in press.



**FIG. 2. GFP- and Ura3-mODC fusion proteins are unstable in yeast cells.** *A*, immunoblot analysis with anti-GFP antibody of MHY501 transformed with p416ADH vectors expressing wild type GFPuv or a GFPuv fusion to amino acids 425–461 of mODC. The GFP fluorescence of transformants expressing the corresponding proteins is indicated in arbitrary fluorescence units. *B*, pulse-chase analysis of GFPuv and GFP-mODC fusion protein turnover in yeast transformants. GFPuv and GFP-mODC fusions were immunoprecipitated with anti-GFP antibody and protein G-Sepharose. Arrows indicate the position of the appropriate proteins. *C*, pulse-chase analysis of <sup>FLAG</sup>Ura3-mODC and <sup>FLAG</sup>Ura3-mODC<sup>C441A</sup> fusion protein turnover in yeast transformants. <sup>FLAG</sup>Ura3-mODC fusions were immunoprecipitated with anti-FLAG M2 affinity gel. *D*, immunoblot analysis with anti-GFP antibody of a *pre1-1 pre2-2* mutant (WCG4-11/22a) or a congenic *PRE1 PRE2* wild type strain (WCG4a) transformed with GFPuv and GFPuv-mODC expression vectors. Extracts from mutant and wild type cells were prepared and analyzed in parallel and immunoblots were identically exposed.

labeling (Fig. 1*B*). Thus, COOH-terminal mutations that alter mODC degradation in animal cells similarly affect this process in yeast cells.

*The Carboxyl Terminus of Mouse ODC Is a Portable Proteasome Recognition Element in Yeast*—Because mODC is unstable in yeast cells, and truncation of its COOH terminus leads to stabilization, we asked whether this region of mODC conferred rapid turnover when attached to either a heterologous or endogenous protein expressed in *S. cerevisiae*. The fusion of the COOH-terminal 37 amino acids of mODC to the COOH terminus of *Trypanosoma brucei* ODC or GFP leads to the rapid turnover of these otherwise stable proteins in mammalian cells (40, 41). We attached the COOH-terminal 37 amino acids of mODC (425–461) to the COOH terminus of either GFP from *Aequorea victoria*, or orotidine-5'-monophosphate decarboxylase encoded by the *URA3* gene of *S. cerevisiae*. We constitutively expressed the GFP-mODC fusion protein and unmodified GFP in yeast transformants from the *ADH1* promoter. Immunoblot analysis with an anti-GFP antibody (Fig. 2*A*) showed that the steady-state level of GFP-mODC was greatly reduced compared with that of unmodified GFP in the yeast transformants. GFP fluorescence was similarly reduced in cells expressing the GFP-mODC fusion (Fig. 2*A*).

We measured the degradation of GFP and the GFP-mODC fusion in yeast cells directly by pulse-chase analysis. Whereas unmodified GFP was stable throughout the 30-min chase period, the GFP-mODC fusion was degraded with a half-life of ~10 min (Fig. 2*B*), confirming that the COOH-terminal 37 amino acids of mODC behaved similarly as a degradation signal in fungal and mammalian cells. We conclude that the decreased expression of GFP, to which the COOH-terminal portion of mODC has been appended, was caused by its increased rate of degradation. We found similar effects when we attached these mODC sequences to the COOH terminus of the

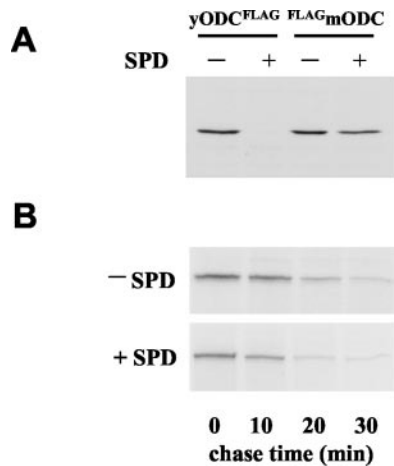
*S. cerevisiae URA3* ORF, which encodes a normally stable protein (Fig. 2*C*). Thus, the mODC COOH terminus appears capable of acting in *cis* to impart instability to a number of unrelated proteins in yeast cells.

Whereas appending the COOH terminus of mODC could import a *cis*-acting degradation signal to GFP (or Ura3) functionally akin to that present in native mODC, it is possible that the attachment of these sequences could result in some other effect, such as misfolding, which could result in accelerated degradation. To rule this out, we introduced a C441A mutation in the GFP- and Ura3-mODC fusion proteins. Pulse-chase analysis demonstrated that the C441A mutation led to the stabilization of both fusion proteins (Fig. 2, *B* and *C*). This mutation should prevent degradation effects specific to the mODC COOH terminus, but not more general effects, such as perturbation of structural integrity, which could lead to rapid degradation of the fusion protein. Additionally, the GFP-mODC fusion protein was fluorescent (Fig. 2*A*), and the Ura3-mODC fusion protein supported the growth of a *ura3* mutant (data not shown), indicating that the mODC extensions did not perturb the normal functions of these proteins. We therefore conclude that the destabilization of the fusion proteins is because of the presence of the mODC COOH terminus specifically, rather than misfolding or other artifactual characteristics of the fusion protein.

To confirm that the reduction in steady-state levels of the fusion proteins was because of increased degradation by the 26 S proteasome, we examined expression of the GFP-mODC fusion protein in a *pre1-1 pre2-2* mutant. This mutant carries mutations in the  $\beta_4$  and  $\beta_5$  subunits of the 20 S core particle and shows marked defects in the degradation of proteasome substrates (24). The GFP-mODC fusion protein accumulated to a significantly higher steady-state level in the *pre1-1 pre2-2* mutant when compared with the congenic wild type strain, whereas the abundance of the GFP control was unchanged (Fig. 2*D*). In summary, these results demonstrate that, as in mammalian cells, the COOH terminus of mODC is sufficient to confer proteasome-mediated proteolysis on an otherwise stable protein in *S. cerevisiae*, and that Cys<sup>441</sup> is critical for this function.

*Effects of Polyamine Pool Repletion and Antizyme on Turnover of Mouse and Yeast ODC*—In mammalian cells, increases in cellular polyamine pools leads to the rapid degradation of mODC, a process mediated by *de novo* synthesis of the regulatory protein AZ1 in response to polyamines. In *S. cerevisiae* a similar mechanism apparently functions in the regulation of endogenous yODC (20), although no AZ1 homolog has yet been identified in this organism (18). We asked whether this *S. cerevisiae* polyamine-responsive regulatory factor acted on mODC that was expressed in yeast cells, and if heterologous expression of a mammalian AZ1 further accelerated mODC degradation in these cells. We compared the effect of polyamine administration on the expression of <sup>FLAG</sup>mODC and COOH-terminal tagged yODC<sup>FLAG</sup>, also expressed from a centromeric vector in the *spe1Δspe2Δ* mutant (NH<sub>2</sub>-terminal epitope tags interfere with expression and regulation of yODC 42).<sup>4</sup> The *spe1Δspe2Δ* mutant, by precluding polyamine biosynthesis, allows manipulation of cellular polyamine pools by addition of exogenous spermidine. The addition of excess exogenous spermidine had no effect on the steady-state level of <sup>FLAG</sup>mODC protein (Fig. 3*A*) or its activity (data not shown). Measurements of <sup>FLAG</sup>mODC turnover by pulse-chase analysis in the presence or absence of exogenous spermidine confirmed that polyamines did not accelerate the degradation of <sup>FLAG</sup>mODC in

<sup>4</sup> F. Merz and P. Coffino, unpublished results.

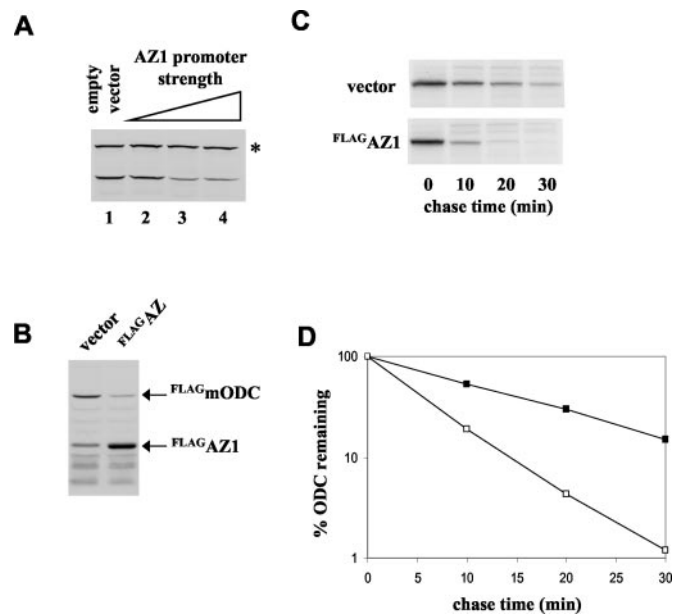


**FIG. 3. Polyamines do not regulate mODC stability in yeast cells.** *A*, immunoblot analysis with anti-FLAG antibody of *spe1Δspe2Δ* cells transformed with p414ADH vectors expressing wild type <sup>FLAG</sup>mODC or yODC<sup>FLAG</sup>. Transformants were maintained in medium with 0.1 μM spermidine (–SPD) or treated with 1.0 mM spermidine (+SPD). *B*, pulse-chase analysis of <sup>FLAG</sup>mODC turnover in *spe1Δspe2Δ* transformants. Cultures were supplemented with spermidine as described in *A*, or left untreated, and <sup>FLAG</sup>mODC was immunoprecipitated with anti-FLAG affinity gel.

yeast cells (Fig. 3*B*). In contrast, excess polyamine administration greatly reduced the abundance of yODC<sup>FLAG</sup> (Fig. 3*A*), indicating that the yODC regulatory factor was indeed induced, and that the epitope tag had no effect on polyamine regulation of yODC. We conclude that the endogenous yODC regulatory system does not act on mODC.

We next determined the effect of expression of AZ1 on the degradation of <sup>FLAG</sup>mODC in yeast cells. For these studies, we used a rat AZ1 cDNA in which the two partial ORFs comprising full-length AZ1 had been aligned by a single base deletion (8). This AZ1 ORF (AZ1ΔT) obviated the polyamine-induced frameshifting otherwise required for the translation of AZ1. In initial experiments we expressed the AZ1ΔT ORF from multicopy vectors with promoters of various strengths (33) in yeast transformants also expressing <sup>FLAG</sup>mODC from a multicopy vector (Fig. 4*A*). The steady-state levels of <sup>FLAG</sup>mODC in these transformants exhibited a modest decrease in response to expression of AZ1 from vectors with increasing promoter strength. This decrease was presumably because of increased levels of AZ1 expression, an assumption corroborated by the almost complete inhibition of ODC enzymatic activity in the transformants also expressing AZ1 from stronger promoters (data not shown). To confirm AZ1 expression directly, we appended a single copy of the FLAG epitope to the NH<sub>2</sub> terminus of AZ1ΔT, and expressed this ORF (<sup>FLAG</sup>AZ1ΔT) and <sup>FLAG</sup>mODC from centromeric yeast expression vectors. We compared the steady-state level expression of <sup>FLAG</sup>mODC from the *ADH* promoter in yeast transformants also carrying <sup>FLAG</sup>AZ1ΔT expressed from the stronger *GPD* promoter, or an empty vector. In agreement with the previous result, expression of <sup>FLAG</sup>AZ1 reduced the steady-state level of <sup>FLAG</sup>mODC compared with the empty vector control (Fig. 4*B*).

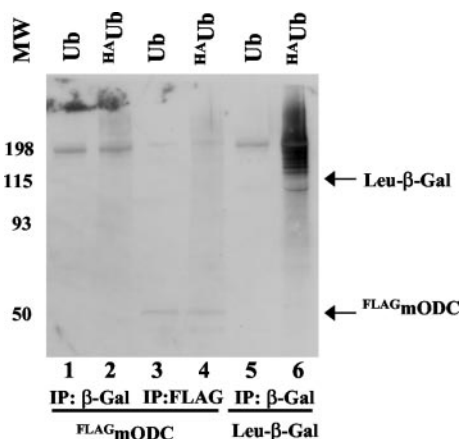
To verify that the decreased levels of <sup>FLAG</sup>mODC in transformants in which AZ1 was also expressed were because of accelerated degradation, we measured the effects of AZ1 co-expression on <sup>FLAG</sup>mODC turnover by pulse-chase analysis. The results showed that the half-life of <sup>FLAG</sup>mODC decreased from ~10 min in a transformant carrying an empty vector control (Fig. 4*C*, top panel) to ~4 min in transformants co-expressing <sup>FLAG</sup>AZ1 (Fig. 4, *C*, bottom panel, and *D*). We conclude that AZ1 expression causes a modest, but reproducible



**FIG. 4. AZ1 accelerates mODC degradation in yeast cells.** *A*, immunoblot analysis with anti-FLAG antibody of *spe1Δspe2Δ* cells transformed with a p424ADH vector expressing wild type <sup>FLAG</sup>mODC, and an empty vector (p426GPD, lane 1) or untagged AZ1 expressed from p426ADH (lane 2), p426TEF (lane 3), or p426GPD (lane 4) expression vectors. A nonspecific, cross-reacting protein (\*) is shown as a loading control. *B*, immunoblot analysis with anti-FLAG antibody of *spe1Δspe2Δ* cells transformed with a p414ADH vector expressing wild type <sup>FLAG</sup>mODC and an empty vector (p416GPD) or <sup>FLAG</sup>AZ1 expressed from p416GPD. *C*, pulse-chase analysis using anti-FLAG affinity gel for immunoprecipitation of labeled <sup>FLAG</sup>mODC from the transformants described in *B*. *D*, quantitation of <sup>FLAG</sup>mODC turnover data shown in *C*. Transformants co-expressed with <sup>FLAG</sup>mODC and <sup>FLAG</sup>AZ1 (open squares) or an empty vector control (filled squares).

acceleration of mODC turnover in yeast cells. In contrast with the effect of AZ1 on mODC in mammalian cells, in which a small amount of AZ1 relative to mODC produces a large effect on turnover, an excess of AZ1 leads to a modest increase in turnover of mODC in *S. cerevisiae*.

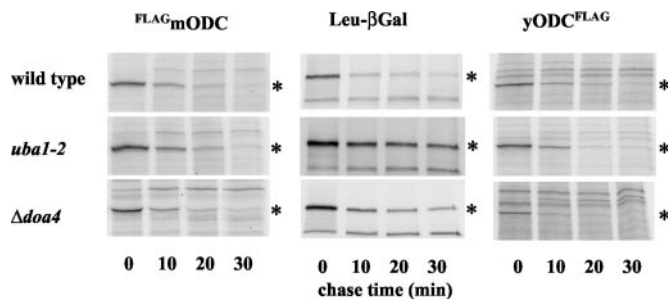
*Turnover of Mouse and Yeast ODC Is Independent of Ubiquitin*—In both cultured mammalian cells (12), and *in vitro* (14), AZ1-dependent degradation of mODC by the proteasome is independent of the ubiquitin system. To determine whether ubiquitinated forms of <sup>FLAG</sup>mODC were present in yeast cells, <sup>FLAG</sup>mODC was immunoprecipitated from yeast transformants expressing either ubiquitin (Ub) or a hemagglutinin epitope-tagged form of ubiquitin (<sup>HA</sup>Ub). As a positive control, transformants expressing Leu-β-galactosidase and Ub or <sup>HA</sup>Ub were similarly analyzed in parallel. Leu-β-galactosidase is a substrate of the N-end rule-mediated ubiquitinylation pathway, and is rapidly degraded in yeast cells because of the presence of a destabilizing Leu residue at its NH<sub>2</sub> terminus (43). We expressed both <sup>FLAG</sup>mODC and Leu-β-galactosidase from vectors bearing the *ADH* promoter. To further enhance the detection of transient polyubiquitinated forms, we utilized a proteasome inhibitor and carried out expression in a strain deleted for *RPN4*, a transcriptional activator of genes encoding proteasomal subunits. We confirmed a previous report (44) that cells with an *rpn4Δ* allele, unlike cells with wild type *RPN4*, are sensitive to the effects of proteasome inhibitors (data not shown). Proteasome function in these transformants was inhibited by treatment with 100 μM MG132, a peptide aldehyde inhibitor of the proteasome (45). In cells expressing both Leu-β-galactosidase and <sup>HA</sup>Ub, <sup>HA</sup>Ub-Leu-β-galactosidase conjugates were detectable with anti-HA antibodies following immunoprecipitation with anti-β-galactosidase antibody (Fig.



**FIG. 5. Ub-mODC conjugates are not detectable in yeast cells.** Vectors (Yep96 or Yep112 (59)) bearing Ub or <sup>HA</sup>Ub expressed from the Cu<sup>2+</sup>-inducible *CUP1* promoter were co-transformed into MHY74 *rpn4Δ* mutant cells with either p415ADH vectors expressing FLAG-mODC (lanes 1–4) or Leu-β-galactosidase (lanes 5 and 6). Following induction with 100 μM CuSO<sub>4</sub> and inhibition of proteasome-specific proteolysis with 100 μM MG132, cell extracts were immunoprecipitated (IP) with the indicated antibody, fractionated by SDS-7.5% PAGE, transferred to nitrocellulose, and probed with anti-HA-horseradish peroxidase conjugates. The positions of FLAG-mODC and Leu-β-galactosidase following fractionation by SDS-7.5% PAGE are indicated.

5, lane 6). Ub conjugates were not detectable with anti-HA antibody following immunoprecipitation if an untagged form of Ub was co-expressed with Leu-β-galactosidase (lane 5). In contrast, following co-expression of FLAG-mODC with <sup>HA</sup>Ub, no <sup>HA</sup>Ub conjugates were detectable in association with mODC (lane 4). Some very high molecular weight material stained faintly with the anti-HA antibody in the transformant expressing FLAG-mODC and <sup>HA</sup>Ub following immunoprecipitation with anti-FLAG antibody (lane 4), but similar staining was seen when the anti-β-galactosidase antibody was used (as a control for nonspecific immunoprecipitation) in a transformant expressing FLAG-mODC and <sup>HA</sup>Ub (lane 2), providing evidence that this material was not specifically associated with FLAG-mODC. A similar experiment was performed in an *RPN4* wild type strain without use of a proteasome inhibitor and yielded similar results (data not shown). In summary, ubiquitinated forms of FLAG-mODC were not detectable in yeast transformants under conditions that readily supported detection of conjugated forms of a control protein.

Ubiquitin conjugates to a particular protein may be difficult to detect because of their low abundance, transience, or the action of deubiquitinating enzymes before or after cell lysis. To verify the inference that ubiquitin was not involved in proteasomal degradation of FLAG-mODC, we examined the degradation of this protein in *S. cerevisiae* mutants affecting ubiquitin metabolism. The *uba1-2* mutant carries an insertion of a mini-transposon 84 bp upstream of the translation start site of the *UBA1* gene (26). The *UBA1* gene encodes the ubiquitin-activating enzyme, E1, and is essential for viability of *S. cerevisiae* (46). The *uba1-2* allele reduces wild type E1 function, and causes defects in a variety of ubiquitin-dependent processes (26). The *DOA4* gene encodes a deubiquitinating enzyme that interacts with the 26 S proteasome and acts late in the proteolytic pathway (27, 47). Doa4 is involved in the recycling of ubiquitin from proteolytic substrates, and in *doa4Δ* mutants cellular ubiquitin levels are severely depleted (48). We compared the degradation of FLAG-mODC and Leu-β-galactosidase in *uba1-2* and *doa4Δ* mutants, and a congenic wild type strain. In both mutants, pulse-chase analysis demonstrated that the ubiquitin-dependent substrate, Leu-β-galactosidase, was stabilized (Fig. 6, middle panels). However, there was no



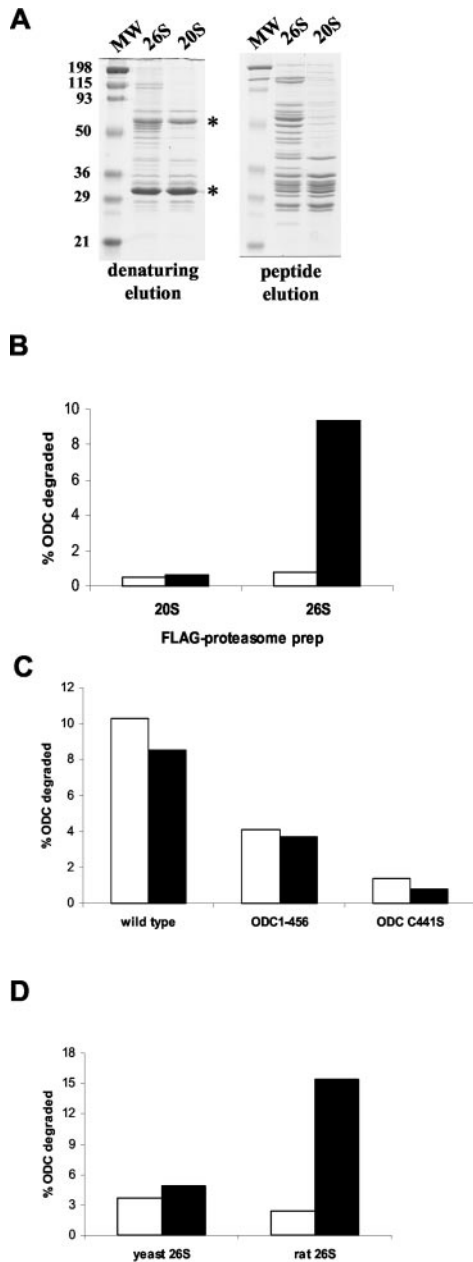
**FIG. 6. Mouse ODC degradation is not impaired in yeast mutants defective for ubiquitin metabolism.** Pulse-chase analysis of FLAG-mODC, yODC<sup>FLAG</sup>, and Leu-β-galactosidase expressed in MHY501 (wild type), MHY1409 (*uba1-2*), or MHY623 (*doa4Δ*) transformants. FLAG-tagged mODC and yODC were expressed from p415ADH and p415SPE1 vectors, respectively, and immunoprecipitated with anti-FLAG affinity gel. Leu-β-galactosidase was expressed from p415ADH, and immunoprecipitated with anti-β-galactosidase antibody. Transformants expressing yODC<sup>FLAG</sup> were treated with 1 mM spermidine. The positions of the relevant band in each panel are marked (\*).

apparent effect on the degradation of FLAG-mODC in these mutants compared with wild type (Fig. 6, left panels).

Using the criteria described above for FLAG-mODC, we asked whether ubiquitin plays a role in the degradation of yODC<sup>FLAG</sup> in response to excess polyamines. We measured the half-life of yODC<sup>FLAG</sup> in *uba1-2* and *doa4Δ* mutants and the congenic wild type strain by pulse-chase analysis following treatment with excess spermidine (Fig. 6, right panels). This analysis indicated that yODC<sup>FLAG</sup> was degraded with similar kinetics in wild type and mutant strains. We also found that polyamine regulation of endogenous yODC activity in these mutants was indistinguishable from that of the congenic wild type strain (data not shown). These results suggest that both the constitutive rapid degradation of mODC and the polyamine-induced degradation of endogenous yODC occur independently of ubiquitinylation in *S. cerevisiae*.

**In Vitro Degradation by Yeast Proteasomes Reproduces Salient Characteristics of in Vivo Degradation**—Both the AZ1-dependent (14) and AZ1-independent<sup>3</sup> degradation of mODC can be reconstituted *in vitro* with 26 S proteasomes from mammalian sources. We used affinity-purified yeast proteasomes to determine whether ubiquitin-independent mODC degradation could be replicated in a yeast *in vitro* system. Yeast proteasomes were isolated from a strain carrying a COOH-terminal FLAG-His<sub>6</sub> tagged form of the Pre1 β subunit of the 20 S core particle. We prepared agarose beads loaded with either the 26 S holoenzyme or 20 S core particle of the proteasome. We verified the composition of the immunopurified complexes by SDS-PAGE of a portion of the preparations eluted under denaturing conditions or following elution with FLAG peptide (Fig. 7A). The proteins visualized following Coomassie staining of these gels showed the expected patterns for 20 S and 26 S proteasomes (28, 49).

Using the immunoaffinity purified yeast proteasomes and recombinant mODC produced in *E. coli* as substrate, we verified that degradation of mODC was linear with time and increasing proteasome concentrations, and inhibited >90% by epoxomicin (Supplemental Fig. 1). Epoxomicin is a specific inhibitor of the proteasome that covalently binds to its catalytic sites in the 20 S core particle (50). As expected for a substrate of the 26 S proteasome, mODC degradation was ATP-dependent, and required association of the 19 S regulatory particle with the 20 S core (Fig. 7B). We compared the effects of mODC mutations that limit its degradation in cells on degradation *in vitro* using either yeast or rat proteasomes (Fig. 7C). Mutations that truncated the last 5 amino acids of the mODC COOH terminus or altered Cys<sup>441</sup> had similar inhibitory effects on



**FIG. 7. Mouse ODC is degraded by purified yeast proteasomes.** A, SDS-PAGE of proteasome preparations eluted from anti-FLAG affinity gel by boiling in SDS loading buffer (denaturing elution) or by incubation with 100  $\mu$ g/ml FLAG peptide (peptide elution). MW, molecular weight markers (kDa); Ig H and L chain eluted by buffer. B, effects of ATP and proteasome composition on mODC degradation in a purified degradation system. Degradation assays were carried out using equivalent amounts (100 nM) of the proteasome holoenzyme (26 S), containing both the 20 S core particle and 19 S regulator, or the 20 S core particle alone (20S). Reactions were carried out at 37  $^{\circ}$ C for 30 min in the presence of 1 mM ATP (filled bars), or following ATP depletion by washing proteasomes affixed to the affinity matrix in reaction buffer lacking ATP (open bars). C, effects of ODC COOH-terminal mutants on the degradation of mODC by yeast 26 S (open bars) or rat liver 26 S (filled bars) proteasomes in an *in vitro* degradation system. Degradation assays were carried out as in B using radiolabeled wild type mODC (wild type), or mODC lacking its 5 COOH-terminal amino acids (ODC1-456), or containing the C441S mutation (ODC C441S). D, effects of AZ1 on the *in vitro* degradation of 50 nM mODC by yeast 26 S or rat liver 26 S proteasomes in the absence (open bars) or presence (filled bars) of 400 nM AZ1.

degradation by proteasomes from either source.

Surprisingly, the addition of AZ1, even in large stoichiometric excess, had no stimulatory effect on mODC degradation by

yeast 26 S proteasomes (Fig. 7D). Under similar assay conditions, AZ1 stimulated the degradation of mODC by rat liver proteasomes  $\sim$ 6.5-fold (Fig. 7D). Both yeast and rat proteasomes showed similar degradative activity toward mODC in the absence of AZ1, but AZ1 significantly stimulated only degradation by the mammalian protease. We conclude that mODC degradation by the yeast 26 S proteasome can be reconstituted *in vitro*, in the absence of the ubiquitinylation of mODC. However, degradation in the purified system differed from both *in vivo* degradation in yeast cells and *in vitro* degradation using mammalian-derived proteasomes in that AZ1 had no stimulatory effect on mODC degradation.

#### DISCUSSION

For the majority of labile proteins, post-translational modification with polyubiquitin constitutes the necessary and sufficient marker for proteasomal recognition. The addition of polyubiquitin chains depends on a complex series of enzymes (2). The task of substrate identification and marking is thus removed from the proteasome itself and devolves instead on a series of ubiquitin activators and transferases. Such delegation of executive authority seems to limit the proteasome to a straightforward binary decision: if and only if a protein bears a polyubiquitin marker of the requisite size, it is to be recognized, unfolded, inserted into a hollow catalytic chamber, and there hydrolyzed to peptides.

This view of proteasome function likely understates its discriminatory capacity. Both prokaryotes and eukaryotes contain compartmentalized proteases that accomplish substrate recognition by association with regulatory complexes. The prokaryotic *E. coli* Clp/Hsl proteolytic complexes are composed of two stacked hexameric rings of a protease subunit flanked on one or both sides by hexamers of a regulatory ATPase subunit. These ATPases each recognize distinct and limited sets of substrate proteins (51). The greater structural complexity of the eukaryotic proteasome 19 S regulator suggests that it is capable of more elaborate forms of substrate recognition and processing. The 19 S complex includes two subassemblies. The base is juxtaposed to the ends of the 20 S core complex and contains six ATPase proteins plus two additional proteins. The lid, hinged to the base and positioned distally, contains 12–14 other proteins. The mammalian proteasome 19 S regulatory complex, in addition to making a binary decision based on the presence or absence of a ubiquitin chain, has the capacity to edit polyubiquitin chains, perhaps performing thereby a proofreading function (52). The proteasome also has direct interactions with the enzymes that carry out late steps in the process of adding ubiquitin chains (28, 53). Additionally, the proteasome has the capacity to recognize a class of substrates that do not require ubiquitin modification for their regulated degradation. This class of substrates includes the cyclin-dependent kinase inhibitor p21 (54) and ODC (14). We have here tested the capacity of yeast proteasomes to recognize phylogenetically distant ubiquitin-independent degradation signals, those associated with mODC.

Mamroud-Kidron and Kahana (21) showed that mODC was rapidly degraded in yeast and provided genetic evidence that the 26 S proteasome was the protease responsible. We show now that mODC degradation has similar structural requirements in yeast and animal cells, namely its COOH-terminal five amino acids and Cys<sup>441</sup>, that these elements can be exported to an otherwise stable protein, that degradation occurs without prior ubiquitinylation in yeast as well as animal cells, and that purified yeast proteasomes have similar capabilities. The present studies therefore demonstrate both *in vivo* and *in vitro* that *S. cerevisiae* proteasomes conserve salient characteristics of mODC recognition.

Our present studies contribute to answering a further question. Can degradation of mODC take place without AZ1? *In vivo* evidence has been difficult to gather because it is hard to fully exclude the action of residual AZ1 in animal cells. Using yeast as a foreign host resolves this issue. There is no obvious AZ1 homolog in *S. cerevisiae*, and the unidentified yeast ODC regulatory protein does not act on mODC when induced by polyamine addition, yet mODC is rapidly degraded in these cells. This degradation shows the same structural requirements as in animal cells. Thus, in a cell type lacking any obvious AZ1 or other polyamine-responsive regulatory activity that can act on mODC, this protein is subject to rapid degradation by the proteasome.

*In vitro* evidence for AZ-independent degradation of mODC has also been difficult to obtain, likely because of the use of assay methods insufficiently sensitive to accurately measure limited substrate degradation. We have now demonstrated in a purified system that the degradation of mODC by yeast or rat proteasomes occurs *in vitro* in the absence of AZ1. Given the relatively low levels of AZ1-independent mODC degradation using our *in vitro* assays (<10%), only the combined use of a released radiolabel assay, a substrate of high specific radioactivity, and concentrated proteasomes allow its detection. Because the occurrence of AZ-independent degradation of ODC has previously been hard to establish, the question of whether it requires ubiquitin has also been difficult to answer. This question is now clearly resolved: regardless of whether AZ1 is or is not present, degradation of ODC takes place without the participation of ubiquitin.

Although AZ1 is dispensable for mODC degradation in yeast cells, AZ1 expression does produce an increase in the rate of mODC degradation. This 2–3-fold stimulation of mODC degradation is more modest than the ~10-fold effect seen in animal cells (55). However, the 10-min half-life of mODC in yeast is also more rapid than in the ~60-min half-life of this protein in animal cells in the absence of AZ1 induction. Perhaps the more rapid basal degradation of mODC in yeast cells mutes its response to AZ1. Strikingly, the AZ1 stimulation of mODC turnover is lost in our purified *in vitro* system, but retained in purified systems utilizing proteasomes from mammalian sources (14).

The structural features of mammalian ODC that are required for its degradation are not present in the otherwise highly conserved yeast homolog. When the two proteins are aligned, mODC is seen to contain a COOH-terminal extension, not present in yODC. Conversely, yODC contains an NH<sub>2</sub>-terminal extension not found in the mammalian enzyme. The mODC degradation signal resides in its COOH terminus (38, 40), and the NH<sub>2</sub> terminus of yODC is required for its rapid degradation (42). Although the mODC degradation signal is recognized by both mammalian and yeast proteasomes, yODC is stable in mammalian cells (42), suggesting either that its degradation signal is specific for yeast proteasomes, or that interaction with the yeast polyamine regulator is indispensable for yODC degradation.

Even though no AZ homolog has yet been identified in *S. cerevisiae*, a polyamine-ODC-AZ regulatory circuit may yet exist in this organism. Several observations strengthen this conjecture. Polyamines increase the turnover of ODC in yeast cells (19), this regulation requires new protein synthesis (20), and, as shown here, the regulated turnover of yODC is ubiquitin-independent. It has also been recently reported that the basal turnover rate of yODC (in the absence of excess polyamines) is not impaired in mutants that alter ubiquitin metabolism (42). The only other substrate of the proteasome shown to be degraded in *S. cerevisiae* independently of ubiquitylation is

Rpn4. Rpn4 is a transcriptional activator of genes encoding subunits of the proteasome as well as other genes (56, 57). The Rpn4 protein is short-lived and interacts with the Rpn2 subunit of the base of the 19 S regulatory particle (58).

The finding that yeast provides an appropriate milieu for studies of mammalian ODC degradation by the 26 S proteasome will facilitate future studies. For example, the apparent discrepancy between the capacity of AZ1 to accelerate degradation *in vivo* but not *in vitro* suggests the participation of additional components of the degradative system that are excluded upon purification. Utilizing the genetic methods available in yeast should help to reveal the identity and roles of ancillary factors that influence degradation.

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