Regulated Degradation of Yeast Ornithine Decarboxylase*

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Ornithine decarboxylase (ODC) declines in cells that accumulate an excess of polyamines, the downstream products of the enzyme. Superfluous production of polyamines is thus prevented. In animal cells, polyamines reduce ODC activity by accelerating its degradation. Similar down-regulation of ODC activity has been observed in the budding yeast Saccharomyces cerevisiae, but induced degradation has not been documented. Here we show using pulse-chase analysis that the loss of enzyme activity is the result of increased degradation of ODC. Polyamines reduce the half-life of the newly synthesized protein from 3 h to approximately 10 min. Degradation of bulk ODC pools is also accelerated by polyamines, but the absolute rate of turnover is slower, with a half-life of 5 h in untreated and 1 h in treated cells. Newly synthesized ODC polypeptide thus undergoes a process of maturation that renders it relatively resistant to both basal and polyamine-induced degradation. Proteasome mutants have a blunted or absent regulatory response, implicating both the core protease and the regulatory cap of the proteasome in induced degradation of yeast ODC.

The enzymes that control polyamine metabolism share a common characteristic: the regulation of ornithine decarboxylase (ODC) (1, 2), S-adenosylmethionine decarboxylase (3), and spermidine/spermine acetyltransferase (4) depends in large part on post-translational mechanisms. This property has perhaps been documented most fully for ODC of animal cells (1, 2). ODC catalyzes the initial step in the biosynthesis of polyamines, small, ubiquitous, abundant, and essential cellular polycations (5). When these rise to excess levels, the half-life of the enzyme becomes shorter, and its steady-state level falls. This sequence of events provides a form of feedback regulation whereby the end products of the biosynthetic pathway limit the activity of the initial enzyme of the pathway. A second protein, termed antizyme, controls the process. Production of antizyme requires a translational frameshift to align a small upstream ORF with a second ORF that encodes all known functions of the protein. Polypeptides greatly enhance frameshifting, and thus control the level of antizyme (6). Antizyme associates with ODC, disrupting the homodimeric enzyme and rendering it inactive. The ODC:antizyme heterodimer thus formed is degraded by the proteasome (7). ODC represents an unusual substrate for the proteolytic action of the proteasome. Proteasomes act predominantly on proteins that have been modified by covalent association with multiple copies of the protein ubiquitin (8), but accelerated ODC degradation depends instead on 1:1 stoichiometric noncovalent association with antizyme (9, 10).

In the budding yeast Saccharomyces cerevisiae, the mechanism of regulation of ODC (yODC) is less well understood. However, many key attributes are similar to those observed in animal cells (11, 12). Augmenting polyamines reduces yODC activity and protein, without changing the amount of yODC mRNA; changes in transcription or yODC mRNA stability are therefore implausible as mechanisms of control. Polyamines do not change the distribution of yODC mRNA on the polyribosomes, implying that translation is unaltered. Expression of the yODC open reading frame without flanking regions of the mRNA confers activity that remains under the control of polyamines, suggesting that the protein itself is subject to control (11). Degradation of yODC in response to polyamines subsists as the most probable explanation both because it is not excluded by the data and because animal cells utilize this mechanism. Direct evidence for this conclusion has, however, proven elusive. In yeast, genetic evidence has demonstrated that the proteasome digests yODC (13, 14), but polyamine excess has not been shown to influence this process. Here we provide evidence that polyamines accelerate the degradation of yODC by the proteasome in yeast.

EXPERIMENTAL PROCEDURES

Culture of Yeast—Cells were grown in liquid culture with aeration by shaking at 30 or at 25 °C for proteasome mutant strains. Synthetic minimal medium (SM) consisted of 0.67% yeast nitrogen base (Difco), 2% glucose, and amino acid omission mixtures (BIO 101, Inc.), deficient in the appropriate amino acids required for selection. Transformation of yeast was done by the lithium acetate method (15). Polyamine treatment was with 1 mM spermidine, 1 mM spermine. Yeast manipulations were carried out as described (16). General methods for DNA manipulation made use of standard procedures (17).

Strains and Plasmids—The wild type background strain used in these studies was PSY93 (matα,leu2,ura3,try1,his3) from A. Johnson, University of California, San Francisco. In the PSY93-derived strains described here, unless otherwise stated, the SPE2 gene encoding S-adenosylmethionine decarboxylase was replaced by a spe2::LEU2 disruption using a plasmid obtained from C. Tabor and H. Tabor, National Institutes of Health (18). The effect of this mutation was to prevent conversion of putrescine to the polyamines spermidine and spermine. Proteasome mutant strains were pre1-1, pre2-2 in a WCG4 strain background (19), or cim3-1 (20) backcrossed 4x to the W303 strain. Activity of yODC in each of these proteasome mutant strains was compared with that in their respective isogenic or congenic wild type control strain. The yODC (SPE1) genomic clone (21) was obtained from W. Fonzi, University of California, Irvine. A strain with three yODC gene copies (3x strain) was constructed by cloning SPE1 expressed from its native promoter in the CEN-ARS plasmids (22) pRS313 (HIS3) and pRS314 (TRP1), transforming SPE1 cells with both constructs and subsequently maintaining continuous selection for both markers. Cells with a single chromosomal copy of SPE1 are termed 1x and isogenic cells with three gene copies 3x.

yODC Enzymatic Activity—Cells were washed in H2O and resus-
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pended in 450 μl of lysis buffer (0.02% Brij, 25 mM Tris pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1% Triton X-100, 1 mM MgCl₂), disrupted by agitation with glass beads (BioSpec Bead-Beater), and the 14,000 × g supernatant recovered for determination of protein concentration (Bradford, Bio-Rad, bovine serum albumin (BSA) standard) and yODC enzymatic activity using an assay that measures release of 14CO₂ from 14C-carboxylabeled ornithine (12). Enzymatic activity units are expressed as pmol of CO₂ released/min/mg protein. A protease inhibitor mix containing phenylmethylsulfonyl fluoride, leupeptin, aprotenin, and pepstatin A (Roche Molecular Biochemicals) was included in the lysis buffer.

Metabolic Labeling of Cells and yODC Immunoprecipitation— Cultures in mid-logarithmic phase (OD₆₀₀ 0.2 to 0.5) in SM medium at 30°C were washed once in labeling medium (identical to SM, but without methionine and equimolar NH₄Cl replacing (NH₄)₂SO₄), resuspended in the same medium to a final cell density of 2 OD₆₀₀ₗ₁₆₃₅₃₃, cultured for 30–40 min, and then labeled for 3 min by addition of 5S Express label (ICN) containing [35S]methionine and [33S]-sulfate. When cells were cultured for longer periods, cold methionine and cysteine were added with 3S Express label to prolong incorporation; acid-pelletable incorporation of 35S was found to be linear for at least 3 h under these conditions. The labeling time was 3 min (pulse), followed by 80°C for subsequent analysis by SDS-PAGE and phosphoimager processing. The specificity of precipitation was verified using yODC gene disruption and spēl strain. After cell lysis, the amount of immunoreactive yODC present on the filters was assessed. We have previously reported for cells with a single gene copy, yODC activity fell with a half-life of about 1 h, declining more than 50-fold within 8 h (Fig. 1A). To confirm this inference and to determine whether regulation of protein level takes place more promptly, we used Western immunoblot analysis of extracts of cells exposed to polyamines in suspension culture for only 4 h. Immunoreactive yODC declined markedly as a result of this treatment (Fig. 1C). No immunoreactive yODC was detected in the spēl disruption strain. Because polyamines induced a comparable reduction of yODC activity and yODC protein within hours, we conclude that the loss of activity results from a diminution of protein level.

Degradation of yODC—To test directly whether polyamine treatment of cells accelerates yODC degradation, we metabolically labeled cells and measured how fast labeled yODC diminished in cells that were pretreated with polyamines or were untreated. The labeling time was 3 min (pulse), followed by subsequent incubation under conditions that prevented further labeling of newly synthesized proteins (chase). Extracts were immunoprecipitated with antibody specific for yODC, and labeled immunoprecipitated protein was visualized by SDS-PAGE and phosphoimager processing. The specificity of precipitation was verified using yODC gene disruption and overexpressor strains (Fig. 2A). yODC in 3x strain cells not pretreated with polyamines had a half-life of about 3 h (Fig. 2B). Polyamine pretreatment reduced the half-life to about 10 min. Although signal intensity declined much faster in the treated cells, the intensity of yODC labeling at the earliest time
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point observed was not strongly or consistently influenced by polyamine treatment. These results imply that polyamine treatment accelerates degradation more than ten-fold, but has little or no effect on synthesis. Pulse-chase experiments with 1x cells produced results that were qualitatively similar to those seen with the 3x strain (results not shown), but signal strength was diminished, making data analysis problematic. Consistent with the observation that activity is not regulated in a strain expressing yODC from a high copy plasmid (11), pulse-chase experiments showed that yODC has about the same 3.3-h half-life as in the 3x strain and that polyamines do not stimulate turnover (Fig. 2C).

Degradation of Steady-state yODC Pools—The pulse-chase experiments described above demonstrate that polyamine treatment labilizes newly synthesized yODC protein, those molecules metabolically labeled during the brief 3-min pulse employed. Pools of yODC that exist prior to treatment may be made similarly labile. However, it is possible instead that yODC undergoes a process of folding or other form of maturation that renders it more stable. In that case, polyamines might induce cells to accelerate the degradation only of newly synthesized yODC, whereas pre-existing pools of the molecule decay with the 3 h half-life characteristic of newly synthesized yODC in untreated cells or even remain entirely stable, decaying only through a dilution process dependent on cell growth. To test this, cells were labeled for 2.5 h before imposing polyamine treatment. Chase was initiated simultaneously with polyamine treatment, and the amount of labeled yODC was assessed during a subsequent 4-h chase period (Fig. 3). As was true for yODC populations labeled for 3 min, polyamine treatment also accelerated the degradation of long labeled yODC. However, the absolute rate of degradation after polyamine treatment is much slower in the second case. In contrast to short-labeled yODC, for which polyamines changed the half-life from 3 h to 10 min, polyamines altered the half-life of long-labeled yODC from 5 h to 60 min. In summary, in cells with either a basal or augmented level of polyamines, yODC undergoes a process of maturation that reduces its susceptibility to degradation.

Effect of Proteasome Mutations—Both mammalian ODC and yeast ODC are degraded in vitro by the yeast proteasome. In vivo, mutations in the yeast proteasome impede degradation of yODC, but these experiments have been carried out in cells not subject to treatment with polyamines. To determine whether the accelerated form of degradation induced by polyamines also depends on the proteasome, we utilized two temperature-sensitive mutants: the first, a double mutant (pre1–1,pre2–2) which impairs four of the six proteolytic sites of the 20 S proteasome core (19); the second (cim3–1), a temperature-sensitive mutant of the Rpt6 ATPase subunit of the regulatory cap of the 26 S proteasome (20). In pre1–1, pre2–2 cells, polyamines produced a 2-fold reduction, compared with a 28-fold change in the corresponding wild type cells (Table I). In cim3–1 cells, the polyamine-induced change was 0.5-fold (a small increase rather than reduction), compared with 5-fold for the wild type. Mutations of either the core proteolytic chamber or regulatory complex interfere with polyamine-induced reduction of yODC activity, presumably by interfering with polyamine-induced accelerated degradation.

FIG. 1. Loss of yeast ODC upon polyamine treatment in cells with one or three gene copies. A, time-dependent change in yODC activity. At the initial time point, spermidine and spermine at concentrations of 1 mM each were added to the medium of 1x (■) and 3x (○) strains. Portions were removed periodically for measurement of yODC activity. The data are plotted as a percent of initial activity, which was 27.8 and 60.8 pmol/min/mg of protein in the 1x and in the 3x strains, respectively. B, immunoreactive yeast ODC in cell colonies after polyamine treatment. Cells with three copies of the yODC gene (SPE1 3x) were patched to filters, treated with polyamines or left untreated, and processed to display immunoreactive yODC. Cells with the yODC gene disrupted (spe1::His3) were processed in parallel and serve as a control for nonspecific immunoreactivity. C, Western immunoblot analysis of yODC in cell extracts after polyamine treatment. Cells with the yODC gene disrupted (spe1::His3) or with three copies of the yODC gene (SPE1 3x) were untreated or treated with polyamines, as indicated, and yODC in extracts were visualized by Western blotting. Immunoreactive bands were visualized with antibody to yODC or to yeast tubulin, which serves as a loading control, as indicated.
mented yODC expression and produced highly specific antisemum (“Experimental Procedures”). These technical tools made it possible to measure changes in the level of immunoreactive yODC and to perform pulse-chase analysis to measure its rate of turnover. We found that polyamine treatment greatly reduces the amount of yODC and diminishes the half-life of newly synthesized yODC, reducing it from about 3 h to 10 min. These changes in degradation were not associated with altered synthesis; using a short pulse labeling time of 3 min, polyamines were found to cause no consistent alteration in metabolic labeling of yODC. This result is consistent with that previously found in animal cells (26). Although it is difficult to exclude small changes in translation rate, it is clear that the bulk and perhaps all of the polyamine-induced change in yODC activity results from a post-translational process. This is in contrast to the fungus *Neurospora crassa*, in which polyamines exert a repressive influence on ODC by reducing the abundance of ODC mRNA (27).

Previous investigations of yODC stability employed inhibitors of synthesis, predominantly cycloheximide, to assess the...
beled yODC in cells subjected to a prolonged label period. A 3x
concentrations of 1 mM each and a second portion in medium without
strain culture was metabolically labeled for 2.5 h. One portion of the
culture was chased in medium containing spermidine and spermine at
of the label period, most of the labeled yODC cohort present
Cells were labeled for 2.5 h before initiating a chase. At the end
These labeled yODC pools were then chased; at the time of
Experiments using inhibitors of synthesis would reveal little or
model 1 is untenable.
Model 2 offers a different resolution of the apparent
This is the case in animal cells. There the more marked effect
of ODC activity of treatment with polyamines alone, compared
is because of the polyamine-induced synthesis of the protein
polyamines. Chase samples were removed after 0, 2, and 4 h and
processed and analyzed as in Fig. 2B: □, untreated cells; ○, treated
text
TABLE I
Comparison of yODC activities
Cultures were treated with polyamines or were untreated, incubated
for 16 h at 25 °C, incubated at 34 °C for a further 4 h, and harvested for
determination of yODC activity. The yODC activities of strains with
mutations in proteasome genes were compared with those in isogenic or
congenic strains wild type for the corresponding genes.

| Proteasome allele | Polyamine treat | yODC activity | Polyamine-induced reduction |
|-------------------|----------------|---------------|-----------------------------|
| PRE1/PRE2         | −              | 3.9           | 2x                          |
|                  | +              | 0.14          | 28x                         |
| pre1–1, pre2–2   | −              | 6.9           | 2x                          |
|                  | +              | 3.5           | 2x                          |
| CIM3              | −              | 2.8           | 2x                          |
|                  | +              | 0.35          | 8x                          |
| cim3–1           | −              | 9.5           | 2x                          |
|                  | +              | 20            | 0.5x                        |

Fig. 3. Chase experiment to determine turnover of radiola-
beled yODC in cells subjected to a prolonged label period. A 3x
strain culture was metabolically labeled for 2.5 h. One portion of the
culture was chased in medium containing spermidine and spermine at
concentrations of 1 mM each and a second portion in medium without
polyamines. Chase samples were removed after 0, 2, and 4 h and
processed and analyzed as in Fig. 2B: □, untreated cells; ○, treated
cells.

The observed difference in polyamine-induced lability of
“new” versus “aged” yODC is not because of the use of a 4-h
polyamine pre-treatment before pulse-chase for the short label
experiment, versus addition of polyamines at initiation of the
chase in the long label experiment. A treatment period of 1 h is
in fact sufficient to fully establish yODC lability. That 1 h
suffices to establish lability is consistent with our data and that of
others (Ref. 12, and Fig. 1A) and directly supported by our
rate at which activity falls when synthesis of proteins is halted
(11, 12). These studies showed that by this measure of stability,
yODC has a half-life of more than an hour, regardless of
whether or not polyamines are augmented. These results are
only apparently discrepant with those reported here. Two mod-
els could reconcile these results with ours. Model 1 asserts that
yODC is subject to accelerated degradation only or predomi-
nantly during a brief period after translation, perhaps before
the newly synthesized polypeptide folds into an enzymatically
active conformation. In that case, steady-state pools of enzy-
matically active yODC, those that are determined in cyclohex-
imide chase experiments, would be predominantly in a ma-
tured form no longer susceptible to rapid degradation.
Experiments using inhibitors of synthesis would reveal little or
no effect of polyamines on degradation. To directly test this, we
carried out prolonged labeling to uniformly label yODC pools.
Cells were labeled for 2.5 h before initiating a chase. At the end
of the label period, most of the labeled yODC cohort present
will have been synthesized more than an hour ago, a calcula-
tion based on an 3-h yODC half-life and a 2-h cell division time.
These labeled yODC pools were then chased; at the time of
initiation of the chase, cells were either treated with poly-
amines or left untreated. In long labeled cells not treated with
polyamines, labeled yODC pools declined with a 5-h half-life. In
polyamine-treated cells, the half-life was reduced to 1 h. Poly-
amines therefore accelerate degradation of long-labeled yODC
5-fold. These results show that model 1 is untenable.

Proteasome mutations in either the catalytic 20 S core (29) or
19 S regulatory cap (13) interfere with yODC degradation in
cells with basal polyamine levels. The same mutations are here
shown to strongly impede polyamine-induced reduction in
yODC. Induced degradation as well as basal degradation there-
fore depends on the integrity of both these functional elements
of the proteasome, the core, and regulatory cap. As both ele-
ments of the yeast proteasome are required in vitro for prote-
olysis of mouse and yeast ODC (14), it is very likely that the
proteasome itself is the agent of induced yODC degradation.

The observed difference in polyamine-induced lability of
"new" versus “aged” yODC is not because of the use of a 4-h
polyamine pre-treatment before pulse-chase for the short label
experiment, versus addition of polyamines at initiation of the
chase in the long label experiment. A treatment period of 1 h is
in fact sufficient to fully establish yODC lability. That 1 h
suffices to establish lability is consistent with our data and that of
others (Ref. 12, and Fig. 1A) and directly supported by our

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surprisingly labile. After a 5-min pulse with \( ^3 \text{H} \)-leucine, about cultured mammalian cells that newly synthesized proteins are fall with a 1-h half-life (Ref. 12, and Fig. 1 polyamine concentrations? This treatment causes activity to induction in yODC activity observed in cells exposed to high stabilization. It is possible that yODC represents but a special high risk for degradation and then undergo time-dependent indication that a large class of nascent proteins are initially at stabilized yODC, implying that these are measuring different properties of a common molecular population. Degradation therefore accounts for loss of activity.

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