

Proteasomes Begin Ornithine Decarboxylase Digestion at the C Terminus*

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Proteasomes denature folded protein substrates and thread them through a narrow pore that leads to the sequestered sites of proteolysis. Whether a protein substrate initiates insertion from its N or C terminus or in a random orientation has not been determined for any natural substrate. We used the labile enzyme ornithine decarboxylase (ODC), which is recognized by the proteasome via a 37-residue C-terminal tag, to answer this question. Three independent approaches were used to assess orientation as follows. 1) The 461-residue ODC protein chain was interrupted at position 305. The C-terminal fragment was degraded by purified proteasomes, but because processivity requires continuity of the polypeptide chain, the N-terminal fragment was spared. 2) A proteasome-inhibitory viral sequence prevented degradation when introduced near the C terminus but not when inserted elsewhere in ODC. 3) A bulky tightly folded protein obstructed *in vivo* degradation most effectively when positioned near the C terminus. These data demonstrate that the proteasome initiates degradation of this native substrate at the C terminus. The co-localization of entry site and degradation tag to the ODC C terminus suggests that recognition tags determine the site for initiating entry. Flexibility of a polypeptide terminus may promote the initiation of degradation.

Selective proteasome-mediated protein degradation plays a pivotal role in cellular life and function. The eukaryotic 26 S proteasome is an ATP-dependant, multisubunit, self-compartmentalizing protease (1). It performs regulated turnover of short-lived proteins involved in cell cycle control, cellular differentiation, signal transduction, and antigen presentation. It also ensures the efficient destruction of misfolded or aggregated proteins whose presence might damage the cell. The majority of proteins are targeted to the proteasome by the conjugation of multiple copies of ubiquitin, a 76-amino acid protein (2). These ubiquitin chains attach to one or more lysine residues of the proteasome substrate. A few proteins are degraded through *cis*-acting recognition signals that function independently of ubiquitin (3).

The 26 S proteasome consists of a 20 S cylinder capped at one

or both ends by a 19 S regulatory particle (1). The proteolytic sites of the 20 S proteasome are sequestered in the interior of a nano-chamber formed by a stack of four seven-member rings. Entry into the chamber takes place through sites positioned at the central axis of the distal rings. A 19 S regulatory particle, containing six distinct but homologous ATPases and about a dozen other proteins, is juxtaposed to one or both ends of the 20 S proteasome. The regulatory particle is involved in substrate recognition, unfolding, and translocation into the proteolytic chamber. Because the entry portals of the 20 S cylinder are narrow, proteins must be in an unfolded state if they are to be threaded into the proteolytic chamber (4). In the present paper, we consider whether a substrate protein assumes a preferred orientation when entering the proteasome. In the simplest case, one end of the substrate polypeptide chain, either the N or C terminus, is recognized as the exclusive or strongly preferred site of entry. This end engages the portal, entry begins, and the rest of the protein then follows continuously, until ingestion and degradation are complete. In this model, merely specifying a privileged end of the polypeptide chain suffices as a complete description of orientation and processivity. Is this a generally adequate description of how proteasomes interact with genuine native substrates? Or do these behave instead according to less constrained models? Is directionality indeed restricted and processivity sustained?

Although a variety of experimental approaches have previously been used to investigate these questions, they have employed artificially constructed model proteins (5–7) or disordered proteins (8, 9) rather than those that are folded native substrates of the proteasome. In the present studies we have investigated the processing of ornithine decarboxylase (ODC),¹ a rapidly degraded native substrate of the eukaryotic proteasome (10). Its degradation does not utilize ubiquitin conjugation. The terminal 37 amino acids of ODC constitute a proteasome recognition signal that mimics polyubiquitin and that can function when artificially appended to other proteins (11). We constructed variants of ODC and used these to determine whether the C terminus, the structural element for proteasome recognition of ODC, is also the site of entry. Three independent experimental approaches showed that degradation of ODC begins at the C terminus.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All plasmid constructions utilized standard molecular biology techniques (12). Constructions that utilized PCR steps were verified by sequencing; constructions that relied on restriction-ligation utilized fully sequenced constituents. Primer sequences are available on request. Vectors for expression of mouse ODC in *Escherichia coli* were generated from vector pQE30 (Qiagen) as described (11). In yeast (*Saccharomyces cerevisiae*) the *CEN/ARS* plasmid

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¹ The abbreviations used are: ODC, ornithine decarboxylase; AZ1, antizyme 1; CHX, cycloheximide; DHFR, dihydrofolate reductase; GAR, glycine-alanine repeat sequence; HA, hemagglutinin; MTX, methotrexate.

p414ADH, containing a *TRP1* selectable marker, was used for expression of ODC proteins under control of an *ADH* promoter (13).

Recombinant ODC with the polypeptide interrupted at residue 305 was produced in *E. coli* using the pQE30 (Qiagen) plasmid encoding a bi-cistronic message (14). The vector sequence provides an in-frame His₆ tag. ODC codons 306–461 followed the His₆ tag and were placed upstream of ODC codons 1–305. The ODC-coding regions were connected (by splice overlap extension PCR) through an 18-nucleotide untranslated linker region (TAAGAGGAGAAAATTAAC) containing a TAA stop codon immediately after ODC codon 461 and a ribosome binding site. A construct with a C-terminal five-residue truncation and one with His₆ on the N-terminal fragment (instead of the C-terminal fragment) were similarly made. The above linker region was omitted to make topologically inverted ODC, in which the chain is interrupted at 305 and residue 461 is connected to residue 1. ODC activity and its inhibition by AZ1 was measured as described (15).

Mouse ODC containing internal insertions of a Gly-Ala repeat or DHFR (ODC_N::GAR and ODC_N::DHFR fusion proteins, where N = the position of insertion) were each made by a two-step process. In the first step, common to both classes of fusion proteins, splice overlap PCR extension was used to create ODC with additional unique MluI and NdeI restriction endonuclease sites corresponding to regions near the N terminus (between codons 32 and 33), near the C terminus (between codons 424 and 425), or near the middle (between codons 297 and 298) of the protein. The MluI and NdeI restriction sites were used for in-frame insertion of modules encoding the required glycine-alanine repeat (GAR) or DHFR proteins.

ODC_N::GAR constructs were made as described (16). The synthetic protein module inserted into ODC encodes the following (single-letter amino acid code): GSTR (GAR linker 1)-DYKDDDDK (FLAG epitope)-AGAGGGAGAGGAGGAGGAGGAGGAGGAGGAGGAG (Gly-Ala repeat)-YPYDVPDYA (HA tag)-HMID (GAR linker 2). The 30-residue GA repeat is identical to the amino acid sequence of human EBNA-1 protein, strain B95–8, GI:59074, residues 196–225 (17, 18).

ODC_N::DHFR constructs were made similarly. Inserts encoding human DHFR were PCR-generated using the plasmid pKT7HDR (19) as a template. The sense primer contained a flanking MluI restriction site followed by a short peptide linker arm and a FLAG epitope tag. The antisense primer contained a flanking NdeI site followed by a short peptide linker arm and a hemagglutinin (HA) epitope tag. The PCR fragment was cut with MluI and NdeI restriction endonucleases and ligated into the equivalent positions in the ODC constructs containing unique MluI and NdeI sites. This resulted in the insertion of a module consisting of DHFR flanked by linkers and epitope tags as follows: GGGGSGGGGSGGGG (DHFR linker 1)-DYKDDDDK (FLAG epitope)-DHFR protein, 187 residues)-YPYDVPDYA (HA tag)-GGGGSGGGGSGGGG (DHFR linker 2). ODC_N::DHFR fusion proteins with a truncation at the C terminus were made similarly using a C-terminal PCR antisense primer that had the 5 terminal codons of ODC deleted.

Recombinant Substrate Preparation, *In Vitro* Degradation by Proteasomes—Expression, purification, and ³⁵S-radiolabeling of proteins in *E. coli* for preparation of proteasome substrates and purification of proteasomes from rat liver were done exactly as described (11). Assays of the degradation of ³⁵S-labeled ODC and variants were performed in a volume of 30 μ l at 37 °C and contained 50 mM Tris-HCl pH 7.5, 1 mM ATP, 10 mM KCl, 10% glycerol, an ATP regenerating system (2 mM dithiothreitol, 10 mM creatine phosphate, 1.6 mg/ml creatine phosphate), 2 mg/ml bovine serum albumin, and 50 nM rat 26 S proteasomes. At time point 0, 1, and 2 h, 10- μ l aliquots were removed, and the reaction was stopped by the addition of SDS-PAGE loading buffer. The samples were fractionated by 4–20% gradient SDS-PAGE gels (for chain-interrupted ODC) or 10% gels (for all other ODC variants). Radiolabeled proteins were visualized by autoradiography.

Yeast Growth and Harvesting—Yeast expression vectors carrying ODC::DHFR fusion proteins were transformed into strain Y13 (*Mata his3 leu2 trp1 ura3 spe1 Δ ::hisG spe2 Δ ::LEU2*), a polyamine auxotroph with a mutation in the endogenous *SPE1* ODC gene. 1 μ M spermidine was added to cultures to support growth. Transformation, cell growth at 30 °C, and preparation of extracts were as previously described (20).

Cycloheximide Chase Experiments—Yeast cultures expressing each ODC::DHFR fusion protein or FLAG-ODC were grown to mid-logarithmic phase (A_{600} 0.5–0.8) at 30 °C. A cycloheximide chase was initiated by adding cycloheximide (CHX) to a final concentration of 200 μ g/ml. Cells additionally treated with 20 μ M methotrexate (MTX, Sigma) were preincubated with that reagent for 30 min before the addition of CHX. After preparation of cell extracts, protein concentration was determined (protein assay reagent, Bio-Rad), and 15 μ g of protein extract per lane was fractionated by 12% SDS-PAGE. ODC::DHFR fusion proteins were

TABLE I

Activity and AZ1 inhibition of ODC and interrupted chain variants

Recombinant ODC proteins A–D correspond to those used in Fig. 1, A–D. Purity after one-step affinity purification was estimated by SDS-PAGE and Coomassie staining. A, wild type ODC with His₆ at the N terminus. B, ODC chain interrupted after residue 305, His₆ preceding residue 306. C, as in B, but the terminal 37 amino acids truncated. D, as in B, but with chain continuity restored by joining amino acid 461 to amino acid 1. AZ1 is an inhibitor of ODC enzymatic activity; inhibition of activity, therefore, provides a measure of ODC/AZ1 association. Determinations of enzymatic activity were performed in 200- μ l reaction volumes containing ~20 ng of ODC. Inhibition of activity was carried out using a stoichiometric excess of recombinant AZ1.

Protein	Approximate purity	ODC activity		% Inhibition by AZ1
		%	μ mol/h/mg protein	
A	>90	120	80	
B	>90	95	75	
C	>90	108	79	
D	~70	55	72	

detected by Western blot analysis with mouse anti-FLAG M2 primary antibody (Sigma, 1:2,000 dilution), sheep anti-mouse Ig-horseradish peroxidase secondary antibody (Amersham Biosciences, 1:15,000 dilution), and the Amersham ECL detection kit. After stripping, immunoblots were reprobed with rabbit anti-HA primary antibody (Santa Cruz Biotechnology, 1:1,000) and goat anti-rabbit Ig-horseradish peroxidase secondary antibody (Sigma, 1:15,000).

Metabolic Labeling of Yeast and Immunoprecipitation—Pulse-chase experiments were carried out as previously described (20). 20 μ M MTX was added to stabilize the DHFR portion of each ODC::DHFR fusion protein as required. SDS-PAGE autoradiogram band intensity was quantitated by scanning and analyzing with TotalLab Version 2.00 software (Phoretix) in fully automatic mode using rolling circle background subtraction.

Thermolysin Protection Experiments—Yeast cells were pulse-labeled as described above and then immediately harvested and lysed. ODC::DHFR fusion proteins were affinity-purified with anti-FLAG M2 affinity gel by incubation for 2 h at 4 °C on a rocking platform. The buffer consisted of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Fusion proteins were eluted in 100 mM HEPES, pH 7.5, and 2 mM CaCl₂ containing 150 ng/ μ l FLAG peptide (Sigma) for 1 h at 4 °C. 0, 5, 100, or 1000 ng thermolysin (Sigma) was added to 16 μ l of immunopurified sample. MTX was added to a concentration of 20 μ M as required. After digestion at 25 °C for 30 min, SDS loading buffer was added, and samples were heated to 100 °C for 5 min and analyzed by 12% SDS-PAGE followed by autoradiography.

RESULTS

Chain Interruption—We interrupted the 461-residue ODC polypeptide at residue 305 by co-expressing two proteins in *E. coli*, one containing amino acids 1–305 and the second containing amino acids 306–461. Previously published observations (14) led us to expect that the two protein fragments would self-assemble into active ODC enzyme. This proved to be true; affinity purification (using a His₆ tag positioned before either residue 1 or residue 306) yielded an enzymatically active protein containing both component polypeptides (Table I) that was reactive with antizyme 1 (AZ1), an endogenous regulator of ODC turnover (21). Upon incubation with purified rat 26 S proteasomes under conditions that promote ATP-dependent degradation, proteolysis of intact ODC depended on AZ1 and was inhibited by the proteasome inhibitor MG132 (Fig. 1A), as previously described (11). When ODC(1–305 + 306–461) with an interruption of the polypeptide chain at position 305 (and His₆ positioned before residue 306) was incubated under identical conditions, the C-terminal 306–461 fragment was degraded by rat 26 S proteasomes, but the N-terminal 1–305 fragment was not (Fig. 1B). Altering the position of the His₆ tag from residue 306 to residue 1 did not change this outcome (results not shown). A 37-residue C-terminal truncation, which stabilizes native ODC, also stabilized the C-terminal fragment

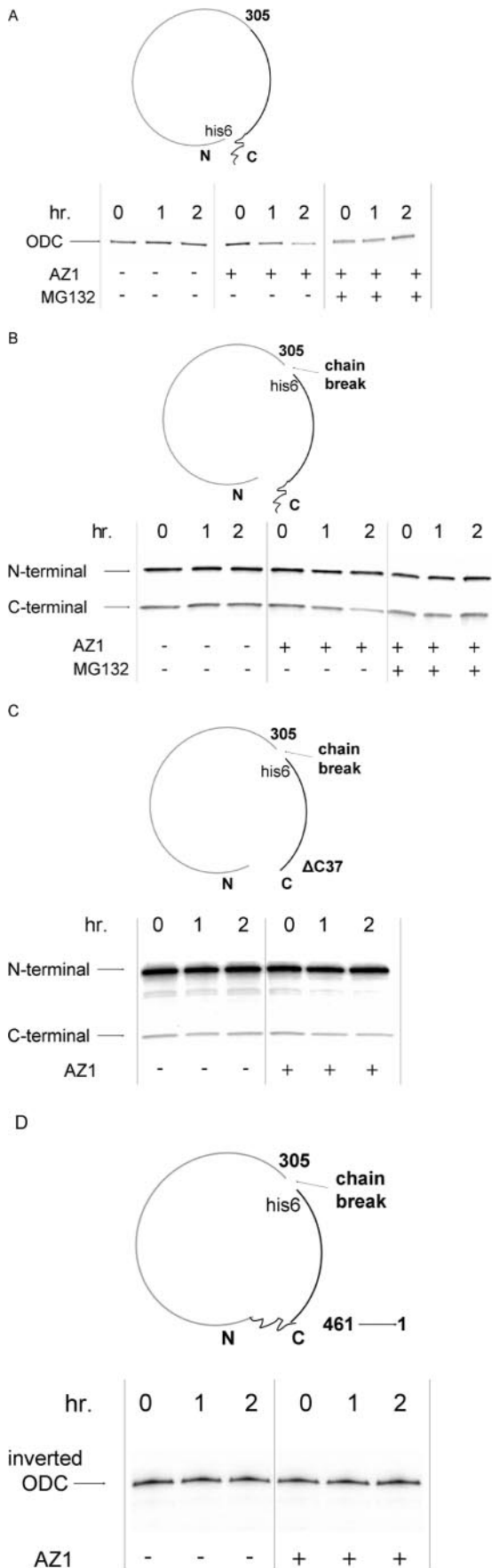


FIG. 1. Proteasome degradation of C-terminal fragment of interrupted ODC chain. Structurally related ODC recombinant proteins with diverse elements or topology, as indicated by the associated cartoon representation, were incubated with purified 26 S proteasomes

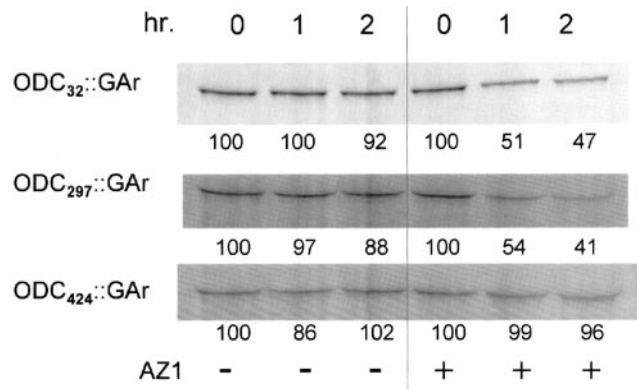


FIG. 2. Position-dependent inhibition of ODC degradation by viral glycine-alanine repeat insertion. *In vitro* degradation was performed as in Fig. 1. A modular insert consisting of a viral 30-residue glycine-alanine repeat sequence flanked by FLAG and HA epitopes was inserted after ODC residue 32, 297, or 424. The number below each lane shows the percent residual substrate remaining.

of the interrupted ODC (Fig. 1C), demonstrating that the presence of an artificial end (at residue 306, whether or not preceded by His₆) is not sufficient to initiate processing of fragment 306–461. When continuity of the interrupted ODC-(1–305 + 306–461) protein was restored by joining residue 461 (the terminal amino acid of native ODC) to residue 1, the resulting topologically inverted single-chain protein was also enzymatically active and reactive with AZ1 but was not degraded (Fig. 1D). This confirms that the free N terminus at residue 306 cannot be used to initiate the processing of interrupted ODC and demonstrates that a free C terminus at residue 461 is required for initiating degradation. This series of experiments demonstrates in a reaction using purified components that chain continuity is needed to sustain processive degradation from beginning to end of ODC and that the process begins in the C-terminal portion of the protein.

Virus-derived Inhibitory Sequence Inserts—We recently reported (16) that a viral sequence (22) composed solely of 30 glycine and alanine residues (GAR) inhibits proteasome degradation when inserted after ODC residue 424 (ODC₄₂₄::GAR). In yeast cells the proteasome removes ~20, 30, or 45 amino acids from the C terminus of ODC₄₂₄::GAR, and degradation halts just short of the viral sequence. Using purified rat proteasomes and the reaction conditions described in Fig. 1, we also reported that insertion of the GAR at ODC position 424 inhibits *in vitro* degradation. Because the insertion of GAR does not perturb the initiation of degradation (16), this result is consistent with a process of ODC degradation that begins at the C-terminal end and can be impaired by the presence of the repeat sequence near that end. To further test this, we compared the effect of inserting GAR after ODC residue 32, 297, or 424. We carried out these experiments both *in vitro* and in yeast cells. As previously reported, GAR insertion at position 424 prevented *in vitro* proteolysis. In marked contrast, insertion at positions 32 or 297 had no apparent effect; degradation of ODC₃₂::GAR and ODC₂₉₇::GAR was unimpaired (Fig. 2). The observed position-dependent inhibition of degradation by GAR is consistent with a degradation process that begins at the C-terminal end. A

for the times indicated, and the extent of degradation in the presence or absence of AZ1 was determined by SDS-PAGE. Where indicated, the effect of the proteasome inhibitor MG132 was examined. *A*, wild type ODC with His₆ at the N terminus. *B*, ODC chain interrupted after residue 305, His₆ preceding residue 306. *C*, as in *B*, but with the terminal 37 amino acids truncated. *D*, as in *B*, but with chain continuity restored by joining amino acid 461 to amino acid 1.

similar position-dependent inhibitory effect was seen when these constructs were expressed in yeast. In yeast, ODC₄₂₄::GAR proteolysis initiates but then halts before reaching the repeat sequence (16). In contrast, ODC₃₂::GAR and ODC₂₉₇::GAR were fully degraded in yeast (results not shown). Thus, both *in vitro* and *in vivo* results using GAR inserts are consistent with C-terminal initiation of degradation.

Position-dependent Obstructive Inserts—Bulky adducts that interfere with proteasome processing or insertion can be used to obtain information about the direction and processivity of degradation by the proteasome (structural prediction algorithms suggest and NMR studies demonstrate (22) that a viral GAR assumes an unfolded conformation and, therefore, cannot inhibit by creating a bulky obstruction to insertion). A suitable candidate for providing an obstruction should fold into a globular form, should not impair folding of the “host” protein when embedded in it, and should be stabilized (hence forming a more effective obstruction) when bound by a small ligand. DHFR is a well characterized molecule that fulfills these requirements (11, 23, 24). Its folding properties are well characterized, and it can be stabilized by the ligand MTX (25). A DHFR cassette was made that consisted of DHFR flanked on its N terminus by a FLAG epitope and on its C terminus by a HA tag. In addition, a linker was added at each end to form an additional separation between ODC and DHFR. We inserted this module after the same three positions described above, 32, 297, or 424, to form ODC₃₂::DHFR, ODC₂₉₇::DHFR, and ODC₄₂₄::DHFR; these contain inserts positioned, respectively, near the N terminus, the middle, or the C terminus of the protein. All three regions chosen for insertion are disordered in the crystal structure (26), are poorly conserved, and should tolerate bulky inserts that do not obstruct formation of the ODC homodimer, the active form of the enzyme.

Each of the three proteins containing the DHFR insert was expressed in yeast, as was a control ODC without insert but carrying a FLAG epitope tag at the N terminus. We needed first to determine whether DHFR within the fusion proteins could fold properly and be stabilized by MTX. We used resistance to thermolysin proteolysis to test the integrity of folding. ODC::DHFR fusion proteins were purified from yeast and treated with various amounts of thermolysin, and the digestion products were examined by SDS-PAGE (Fig. 3). The MTX-treated samples containing each of the three ODC::DHFR fusion proteins yielded a band with slightly less mobility than that of DHFR. Samples not treated with MTX or ODC without DHFR (results not shown) did not produce this band. All fusions showed similar protection (the thermolysin digest product was less highly labeled than the full-length parent protein because there are 22 methionines in the fusion protein *versus* only six in DHFR). We conclude that each of the three engineered ODC proteins contains a DHFR moiety in a native configuration competent to bind MTX.

To assess protein stability *in vivo*, we used Western blot analysis with antisera to FLAG or HA epitope tags (which flank DHFR) and measured the extent of change in protein levels after treatment for 1 h with CHX, an inhibitor of protein synthesis. FLAG-ODC without DHFR served as a control. Cells were additionally treated with MTX or were left untreated to determine whether ligand-mediated stabilization of DHFR conformation could impair degradation. All three fusion proteins were efficiently degraded in the absence of MTX, as was the FLAG-ODC control (Fig. 4A). However, when cells were preincubated with MTX for 30 min before the addition of CHX and maintained in MTX during the CHX chase period, a difference in degradation patterns among the fusion protein was seen. ODC₄₂₄::DHFR was made fully stable by MTX. In contrast,

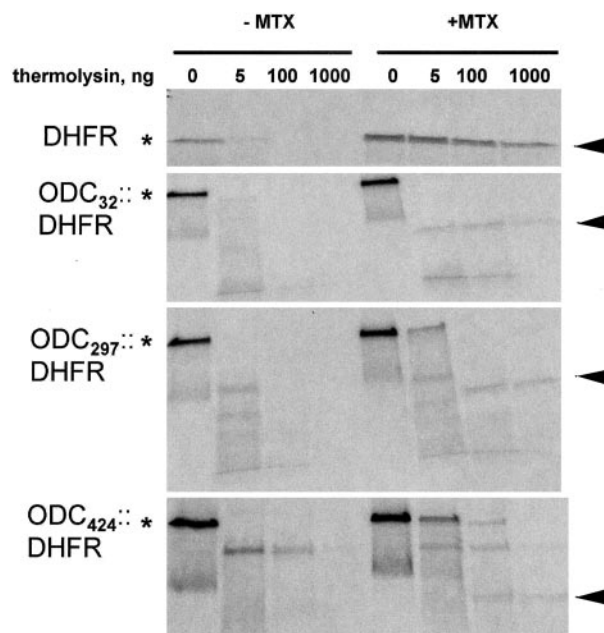


FIG. 3. Methotrexate stabilization of DHFR insert. DHFR or each of three ODC::DHFR fusion proteins was metabolically labeled in yeast and purified, and the DHFR moiety was tested for resistance to proteolysis using various concentrations of thermolysin. Asterisks indicate input proteins, and arrowheads mark the position of the methotrexate-stabilized protease-resistant DHFR fragment.

degradation of ODC₃₂::DHFR was only slightly impaired by MTX, and ODC₂₉₇::DHFR not at all impaired. The changes in protein level are quantified in Fig. 4B. Similar results were observed using antiserum to the HA epitope tag (results not shown). The position-dependent effect of insertion of stabilized DHFR provides further evidence for a C- to N-terminal directionality of degradation.

Metabolic pulse-chase analysis was used to further test the effect of DHFR position on turnover (Fig. 5, A and B). The experiment was performed with MTX present in the medium for 30 min before metabolic labeling. Over a chase period of 1 h the degradation of ODC₄₂₄::DHFR was slower and less extensive than that of ODC₃₂::DHFR or ODC₂₉₇::DHFR. The pulse-chase data are, thus, consistent with the findings using CHX chase and Western blot analysis. Inserting DHFR has some inhibitory effect regardless of its position; turnover of ODC₃₂::DHFR and ODC₂₉₇::DHFR is somewhat prolonged, with a half-life of about 30 min, compared with that of ODC with no DHFR, for which the half-life is about 8–15 min in yeast cells (20).

It is important to show that the degradation of composite ODC::DHFR proteins follows the proteasome-mediated ubiquitin-independent pathway normal for ODC and is not due to unrelated intracellular protease activity in yeast or to ubiquitin-mediated proteasomal proteolysis associated with protein misfolding. The C-terminal 37-amino acid tail of ODC is a critical signal for ubiquitin-independent degradation by the proteasome. Moreover, deletion of just the terminal 5 amino acids abrogates degradation (20, 27). Therefore, a five-amino acid deletion at the C terminus was introduced into each ODC::DHFR fusion protein, and CHX chase was used to examine their degradation (Fig. 6). Unlike the corresponding full-length fusion proteins, the truncated forms were stable regardless of whether MTX was added or not. Full-length FLAG-ODC was efficiently degraded, whereas FLAG-ODC was also stabilized by the C-terminal deletion. Therefore, because the intact ODC C-terminal domain provides a signal specific for the nor-

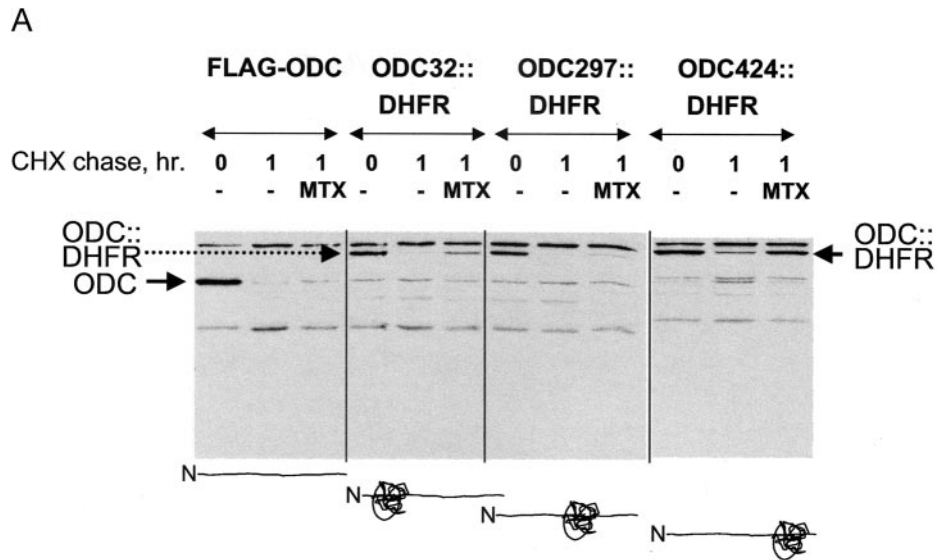


FIG. 4. Western blot CHX chase analysis of position- and ligand-dependent inhibition of ODC degradation by DHFR insertion. *A*, yeast expressing each of the indicated proteins (represented in schematic form below the gel with DHFR as a folded curlicue) were treated with CHX or with CHX plus methotrexate, and the effect of these treatments on ODC and ODC::DHFR protein levels was determined by Western blotting. *Arrows* indicate the positions of migration of ODC and ODC::DHFR proteins. *B*, densitometric analysis of band intensities, normalized to 100% density for each individual protein at the initial time point.

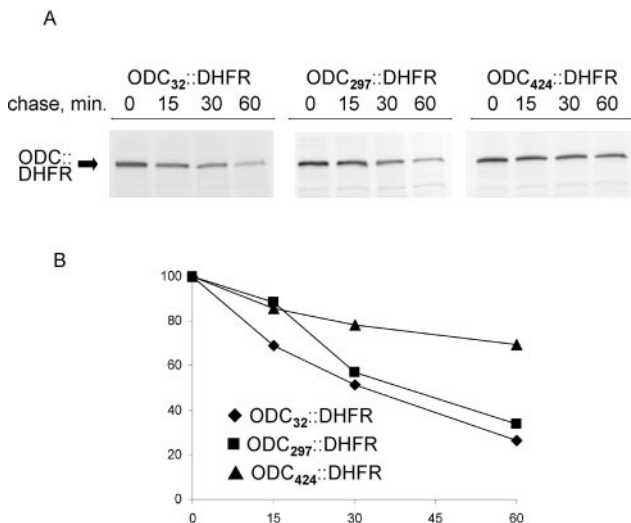
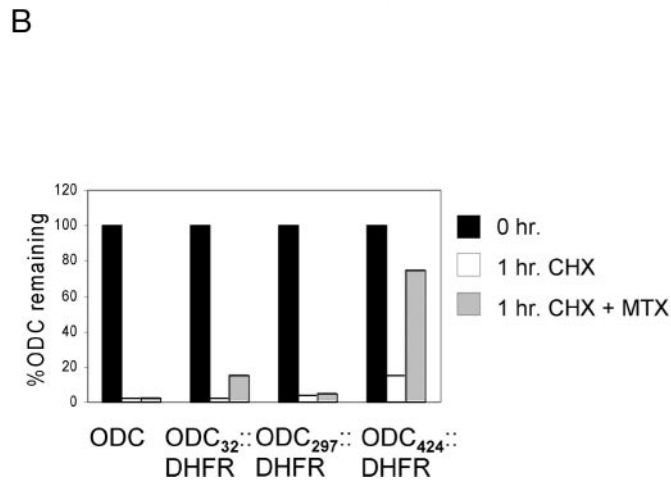


FIG. 5. Pulse-chase analysis of position- and ligand-dependent inhibition of ODC degradation by DHFR insertion. *A*, ODC::DHFR proteins were expressed in yeast as in Fig. 4, and protein turnover kinetics were determined by pulse-chase analysis. *B*, densitometric analysis of band intensities, normalized to 100% density for each individual protein at the initial time point.

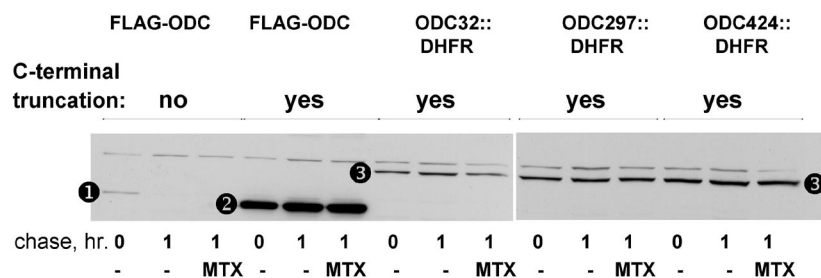
mal mode of ODC turnover (20), we conclude that the degradation of full-length ODC::DHFR fusion proteins utilizes that pathway.

DISCUSSION

The proteasome must coordinate at least three tasks to degrade a native protein substrate: recognition, unfolding, and insertion. Recognition usually takes place through a polyubiquitin chain. The degradation signal of ODC cross-competes with polyubiquitin for proteasome recognition (11); it thus presents the proteasome with a molecular mimic of the ubiquitin chain. The 37-amino acid C-terminal tail of ODC constitutes a physiologically functional signal for proteasome degradation (10, 11, 28). Mammalian proteasomes recognize this as an association signal; interaction of ODC with the protein AZ1, a negative feedback regulator of the polyamine biosynthetic pathway initiated by ODC, improves proteasome-substrate binding about 8-fold.

Because the structural features of ODC required for its proteasome interactions are well characterized, it provides a favorable model substrate for investigating general attributes of processing. The work described here uses ODC to address the following question. Are recognition, unfolding, and insertion highly coupled such that the proteasome 19 S regulatory complex grasps the degradation tag and pulls on it, thus coordinately unfolding the substrate and inserting it into the entry site of the 20 S catalytic core? According to such a view, a degradation tag should lead the way into the catalytic chamber. Although this model parsimoniously links critical events in degradation, supporting data are presently fragmentary. Artificially constructed N-end rule substrates carrying polyubiquitin near the N terminus are degraded *in vitro* starting at that

FIG. 6. Stabilization of ODC::DHFR fusion proteins by C-terminal truncation. ODC and ODC::DHFR proteins were expressed and analyzed as in Fig. 5, but where indicated the five terminal residues were truncated. Symbols mark the mobility of FLAG-ODC (1), truncated FLAG-ODC, and (2) truncated ODC::DHFR proteins (3).



end (5). Efficient *in vivo* co-translational degradation of an N-end rule substrate (6) is also consistent with that interpretation because C termini are unavailable during translation. Using hybrid archaeal proteasomes formed from the *Thermoplasma acidophilum* 20 S complex and the *Methanococcus jannaschii* proteasome-activating nucleotidase (PAN) ATPase complex, engineered substrates with the bacterial ssrA degradation tag at their C terminus were found to start proteolysis at that end of the molecule (7). Other evidence suggests, however, that under suitable conditions recognition, unfolding and insertion are dissociable (7, 29). The co-localization of a degradation tag and proteasome entry site is consistent with a model (4) whereby the degradation tag is used to initiate protein unraveling by an ATP-powered machine. Data supporting this attractive model have heretofore been restricted to substrates utilizing artificially appended tags and, in some cases, non-eukaryotic hybrid proteasomes assembled from components derived from unlike species. The model therefore needs further experimental tests that employ more physiologically relevant constituents. Using ODC, a protein with an intrinsic C-terminal degradation tag, we now provide both *in vitro* and *in vivo* evidence that initiation of proteolysis also localizes to the C terminus.

ODC has the remarkable capacity to self-assemble into a functional enzyme if produced in bacteria as two co-expressed unconnected fragments consisting of residues 1–305 + 306–461 (14). This makes it possible to produce ODC with a native structure but a sundered polypeptide backbone. Utilizing the well substantiated finding that proteasomes act *in cis* on individual proteins within a multi-protein complex but fail to degrade those proteins within a complex that are not themselves covalently associated with a degradation signal (30–32), we subjected bi-partite ODC-(1–305 + 306–461) to AZ1-stimulated degradation by purified 26 S rat proteasomes. Only the polypeptide fragment with residues 306–461 was degraded. Control conditions that prevented degradation included omitting AZ1 or adding the proteasome inhibitor MG132 or truncating the terminal 37 amino acids of the 306–461 polypeptide. The possibility that degradation of the 306–461 polypeptide occurs because of the presence of an unnatural N terminus in 306–461 was ruled out by the observation that the structurally similar single-chain circularly permuted ODC was not degraded. We infer, therefore, that native ODC degradation initiates within residues 306–461. We postulate that as the bi-partite ODC protein enters the proteasome and progressively unfolds, the tight association between the two fragments, which depends on their native conformation (14), is interrupted, and the N-terminal fragment dissociates and so escapes proteolysis. These data imply that ODC degradation is oriented and progressive, that it initiates within the last third of the molecule, likely at the C-terminal end, and that processivity demands polypeptide chain continuity.

Our second approach used a different strategy to interrupt proteolysis. We have recently shown (16) that a 30-residue Epstein-Barr virus glycine-alanine repeat sequence (GAR) stops degradation when placed after amino acid 424 of ODC. In

yeast cells, ODC₄₂₄::GAR undergoes partial proteolysis that removes a portion of the C terminus but stops short of the GAR. This result showed that in yeast cells ODC₄₂₄::GAR processing initiates at the C terminus. *In vitro*, purified proteasomes failed to degrade the composite ODC₄₂₄::GAR protein, even though the initial interaction step was not impaired by the GAR insert. We have here compared the effect of embedding the GAR sequence after ODC residue 32, 297, or 424. Using purified proteasomes, we found that insertions at 32 or 297 were not inhibitory, in contrast to the complete inhibition observed by insertion of GAR at position 424. Comparison of the three composite proteins in yeast yielded similar results; that is, inhibition only when the GAR was placed at position 424, not 32 or 297. This position-dependent inhibitory effect is consistent with C terminus-first proteolysis of native ODC.

The third experimental approach to establishing the direction of ODC degradation was to place bulky obstructions within ODC that would allow degradation to initiate but then stop or pause. DHFR is well suited to this purpose. It folds into a globular form, folding is usually not impaired by embedding the DHFR sequences within another protein (33), and methotrexate, a cell-permeable reagent, binds DHFR tightly and further stabilizes folding. The rate of degradation of proteasome substrates that incorporate DHFR can be sharply reduced by DHFR ligands; this is true regardless of whether polyubiquitin (23, 24) or the ODC C terminus (11, 34) is the recognition signal. Importantly, an embedded DHFR moiety has been shown to stop substrate transfer into the proteasome (5), as does avidin when placed near a degradation signal (7). The utility of DHFR for the intended purpose is, therefore, well established.

DHFR was inserted into ODC after positions 32, 297, or 424. Expression was initially evaluated in three systems. 1) In *E. coli* the proteins were expressed but were largely insoluble (in contrast to similar constructs without DHFR inserts), strongly suggesting misfolding. Denaturation/renaturation did not solve this problem. 2) Translation *in vitro* using a rabbit reticulocyte lysate resulted in expression of soluble proteins with DHFR domains that could be protected from thermolysin proteolysis by methotrexate. However, AZ1 did not bind to these proteins, making them unsuited for *in vitro* degradation studies. 3) Expression in *S. cerevisiae* (where mouse ODC degradation is only slightly stimulated by AZ1 (20)) resulted in production of soluble proteins with DHFR inserts at each of the three selected positions. Turnover of all three fusion proteins was rapid (as assessed by *in vivo* cycloheximide chase or [³⁵S]methionine pulse/chase experiments) and required that the C-terminal five amino acids of ODC be present, a characteristic of proteasome dependence. Degradation of the protein with DHFR at residue 424 was strongly impaired by *in vivo* treatment with methotrexate, but methotrexate had little or no effect on the turnover of ODC with inserts at 32 or 297 or of control ODC with no insert. Protease protection experiments with thermolysin and methotrexate confirmed the presence of a native DHFR fold in all three composite proteins expressed in yeast. These findings imply that DHFR is properly folded

within the composite proteins, that a cell-permeable ligand can be used to make that folding tighter, and that stabilizing DHFR folding prevents degradation by the proteasome if DHFR is near the C terminus, but not otherwise. As a whole these observations with ODC::DHFR proteins are consistent with a C terminus-first mode of inserting ODC. The products anticipated to result from paused processing of the constructs with inserts at positions 32 and 297 were not found in cell extracts. If partial degradation generates these products, they almost surely become misfolded once formed, then are likely subjected to rapid ubiquitin conjugation and consequent degradation, thus eluding detection.

Proteins that are intrinsically unstructured or contain domains with that property have been recognized as a functionally important subset of protein "structure" (35). Natively unfolded or partially folded structures often have functional importance, but the significance of this property in proteasome degradation is uncertain. Substrate denaturation promotes processing by the proteasome (36). Disorder of the ODC C terminus may promote an orientational freedom needed to sustain simultaneous interactions of the two critical elements of the ODC degradation tag, cysteine 441 and the terminal five amino acids (11). A deletion of residues 447–451 has no apparent effect on ODC degradation (27), indicating that maintaining a fixed spacing between these elements can play no part in processing. Crystallographic studies suggest that the last 40 amino acids of ODC lack defined structure (26), a result consistent with this expectation. Solution NMR spectroscopy was used to compare uniformly ^{15}N -enriched samples of full-length ODC and ODC with a 37-amino acid C-terminal truncation.² ^1H - ^{15}N -correlated NMR spectra provided evidence that at least some of the C-terminal 37 amino acids of ODC are located in a flexible part of the protein structure.

If proteolysis begins at or near an end-positioned tag, the location of one should predict that of the other. The lysine residues that support ubiquitin conjugation of Sic1, a proteasome substrate, are distributed throughout the molecule, but mutation of the six N-terminal lysines suffices to prevent turnover (38). One or more of these six must, therefore, play a vital role. Each of the six deleted lysines was individually restored to determine the effect on turnover of a single ubiquitin chain in the critical region. Individual ubiquitin chains destabilized the protein, but those located closer to the N terminus did so more effectively. We are aware of no data on the direction of Sic1 degradation, but the model under discussion predicts that Sic1 proteolysis starts at the N terminus and is initiated more efficiently by a ubiquitin chain positioned closer to that end. Some ATP-dependent bacterial proteases, e.g. ClpXP and ClpAP, have poor homology to proteasomes but share a similar architecture (39) consisting of a regulatory complex containing an ATPase hexamer that recognizes, unfolds, and transfers substrate proteins into a structurally distinct co-axially aligned catalytic chamber where proteolysis takes place (40). These

bacterial proteases also initiate polypeptide proteolysis at the end bearing a recognition tag (5, 37). We have shown that degradation tag and entry site co-localize in ODC, the first native labile protein characterized in this way. Testing the generality of this conclusion for ATP-dependent proteases will require experimental manipulations directed toward dissociating signals for recognition from those specifying unfolding and insertion.

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² D. Hoffman (University of Texas, Austin, TX) and P. Coffino, unpublished observations.