

Title

Structural elements of the ubiquitin-independent proteasome degron of ornithine decarboxylase

Junko Takeuchi, Hui Chen*, Martin A. Hoyt and Philip Coffino

Department of Microbiology and Immunology, University of California, San Francisco, California 94143, USA

*Present address: Department of Biopharmaceutical Sciences, University of California, San Francisco, CA 94143, USA

Corresponding author: P. Coffino

Tel: +1 415 516 6515

Fax: +1 415 476 8201

E-mail: Philip.Coffino@ucsf.edu

Short title: Structural elements of a ubiquitin-independent proteasome degron

Keywords: proteasome, ornithine decarboxylase, degradation, ubiquitin

Abbreviations: AZ1: antizyme 1, DHFR: dihydrofolate reductase, DTT: dithiothreitol, ME: mercaptoethanol, ODC: ornithine decarboxylase, TCEP: Tris (2-carboxyethyl) phosphine

Abstract

Mouse ornithine decarboxylase (ODC) is quickly degraded by the 26S proteasome in mammalian and fungal cells. Its degradation is independent of ubiquitin but requires a degradation signal composed of residues 425-461 at the ODC C terminus, cODC. Mutational analysis of cODC revealed the presence of two essential elements in the degradation signal. The first consists of cysteine and alanine at residues 441 and 442. The second element is the C terminus distal to residue 442; it has little or no sequence specificity, but is intolerant of insertions or deletions that alter its span. Reducing conditions, which preclude all well-characterized chemical reactions of the Cys⁴⁴¹ thiol, are essential for in vitro degradation. These experiments imply that the degradative function of Cys⁴⁴¹ does not involve its participation in chemical reaction; it instead functions within a structural element for recognition by the 26S proteasome.

INTRODUCTION

The proteasome plays a central role in intracellular protein degradation. Most of its substrates are recognized via polyubiquitin chains [1]. In contrast, the enzyme ornithine decarboxylase (ODC) is recognized by the proteasome through a mechanism independent of ubiquitination [2]. Mouse ODC contains a degradation signal comprised of its carboxy terminal 37 amino acids (cODC) [3]. This domain, which is conserved in vertebrates (Figure 1), acts as a portable degradation tag when added to the C terminus of various proteins, such as GFP. The cODC degron also functions when expressed exogenously in the cells of non-vertebrate species, including fungi [4] and plants [5]. Mutation of ODC cysteine 441 (Cys⁴⁴¹), within cODC, was found decades ago to create stabilizing alleles [6]. The mutation of Cys⁴⁴¹ also stabilizes ODC protein expressed in yeast [4]. Thus, it is apparent that there is a conserved mechanism by which the 26S proteasome recognizes ODC through Cys⁴⁴¹. However the biochemical function of Cys⁴⁴¹ in degradation and the specific sequence context and conditions required for this function remain unclear. In our previous study, we showed that removing the Cys⁴⁴¹ thiol by mutation (C441A) or replacing it with a hydroxyl (C441S) impairs an early step of degradation, recognition by the 26S proteasome [7, 8]. One possible role of Cys⁴⁴¹ in facilitating interaction with the 26S proteasome may be structural. It may simply be used as a recognition element, in a manner that does not depend on its chemical modification. Alternatively, proper cODC function may require chemical alteration of the thiol group. We show here that reducing conditions are necessary for degradation of substrates dependent on cODC, but not for substrates that utilized polyubiquitin as a degradation signal. Mutation of the adjacent alanine 442 also impaired function, implying that residue Cys⁴⁴¹ and Ala⁴⁴² each contributes to recognition. Truncation and deletions within the C-terminal end of cODC distal to Cys⁴⁴¹ Ala⁴⁴² showed that moving the C terminus closer or further from this pair also impaired degradative function. Although the length of the free C terminus is constrained, its sequence is not.

EXPERIMENTAL

Strain and plasmids The yeast strain MHY501; *his3-D200*, *leu2-3,112*, *lys2-801*, *trp1-1*, *ura3-52*, *MAT α* was used as the wild type for this study. Point mutations in ODC were introduced by overlap PCR. The coding region of ODC was amplified and cloned onto p416ADH1 yeast expression vector [9]. Amino acid sequence of cODC^{C441A} random is -EFHGPNLVPAAMVTIADEEAMDRSPAGDQASCPERESQ-. The last ten amino acids of above sequence were inserted between 450 and 451 to make ODC^{451::10}, and SCPER were inserted to make ODC^{451::5}.

Recombinant protein expression

The expression vector for ODC (*Mus musculus*) and AZ1 (*Rattus norvegicus*) was pQE30 (Qiagen, GmbH, Germany) with a His₆ tag at the N-terminus as described previously [10]. DHFR-cODC (the human DHFR fused by the last 37 amino acids of ODC C-terminus) and DHFR were cloned into the pQE30 vector and expressed in *E. coli* [7]. Site-directed mutagenesis was carried out using the mega-primer method [11]. All PCR products used for *in vitro* translation were verified by sequencing.

ODC degradation assay in reticulocyte lysate

The site-directed mutated ODC was *in vitro* transcribed and translated in reticulocyte lysate (TNT, Promega, Madison WI). All the PCR products used for *in vitro* translation contained a T7 promoter and Met-Gly-His₆ positioned upstream of the ODC second amino acid. AZ1-stimulated ODC degradation was conducted in reticulocyte lysate [12]. Briefly, ³⁵S-labeled ODC was prepared by *in vitro* translation and incubated with recombinant AZ1 or in the absence of AZ1 (in a buffer containing 50 mM Tris-Cl pH7.5, 150 mM NaCl, 0.05% Tween20 and 1 mM DTT) and an ATP-regenerating system at 37 °C for 1 hour. The reaction was stopped by addition of SDS-PAGE loading buffer, followed by SDS-PAGE analysis. The autoradiogram was scanned and quantified with the *TotalLab* software from Nonlinear Dynamics (UK). ATP regenerating buffer contained 30 mM Tris-Cl pH7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM ATP, 10 mM phosphocreatine and 1.6 mg/ml creatine phosphokinase final concentration. Where indicated, DTT was eliminated from the ATP regenerating buffer. The percent degradation of mutant forms of ODC (ODCmut) compared to that of wild type ODC

(ODCwt) (figure 2) is calculated as $100 \times (1 - \text{ODCmut} + \text{AZ} / \text{ODCmut} - \text{AZ}) / (1 - \text{ODCwt} + \text{AZ} / \text{ODCwt} - \text{AZ})$, where, for example, ODCmut+AZ is the intensity of the mutant ODC autoradiographic band after incubation with AZ1.

Protein degradation using purified components

Purified ^{35}S methionine-labeled recombinant His₆-TEV-FLAG-ODC was made and degraded *in vitro* using purified components as described previously [10]. The percent of ODC degradation was determined by dividing the trichloroacetic acid soluble counts by the total input ODC counts. His₆ tagged Ub₅DHFR degradation was carried out in the presence of 1.2 μM Ubiquitin-aldehyde (Boston Biochem) and detected by 1:3000 dilution of anti-His₆ antibody (Amersham/Pharmacia). Mammalian 26S proteasomes were purchased from Biomol (Plymouth Meeting, PA).

Pulse chase experiments

Yeast manipulations followed standard protocols [13]. Other procedures were as described previously [4]. For pulse chase experiments, cells in exponential growth phase were harvested, washed with SD medium, resuspended in SD-Met containing 0.2 mCi of ^{35}S methionine/cysteine (NEN) and labeled for 5 min at 30° C. Cells were harvested, resuspended in 400 μl of SD-Ura containing 10 mM methionine/cysteine, 0.5 mg/ml cycloheximide and continuously incubated at 30° C. Samples of 100 μl were taken periodically and suspended in lysis buffer (50 mM HEPES pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). Cells were immediately disrupted by shaking with glass beads in a Bead Beater (Biospec, Bartlesville, OK) followed by centrifugation at 18,000xg 10 min. Anti-Flag M2 agarose beads (Sigma, St Louis MO) were added to the supernatant followed by 40 min of incubation at 4 °C. The volume of sample for immunoprecipitation was adjusted to contain equal acid insoluble counts. The resin was then washed four times with lysis buffer and boiled with SDS-PAGE sample buffer.

Non-reducing PAGE and blotting of biotinylated proteins

To examine the effect of reducing conditions on disulfide bonds, a small molecule, Profound Mts-Atf-Biotin label transfer reagent (Pierce, Rockford IL), was conjugated to recombinant Sem1 protein, as described in the manufacturer's protocol. This reagent contains a disulfide, which is required to maintain association of a biotin group with the target protein. After incubation with various reducing reagents, protein samples were mixed with SDS-PAGE sample buffer free of reducing reagent. Protein sample was then boiled for 10 minutes and subjected to SDS-PAGE, followed by transfer to nitrocellulose membrane. The membrane was blocked by 7% non-fat dry milk in TBST, incubated with streptavidin-peroxidase conjugate (Roch, Penzberg Germany). Signal was detected by ECL detection kit (GE Healthcare, Uppsala Sweden).

RESULTS

Mutation of individual residues surrounding Cys⁴⁴¹ does not impair cODC deprotonation function

As C441A and C441S mutations, the most conservative available, prevented degradation of ODC, we investigated the possibility that Cys⁴⁴¹ forms a recognition element in conjunction with other residues that lie nearby in the primary sequence. To do so we individually mutated to alanine each non-alanine amino acid within the region encompassing residues Thr⁴³⁶ to Met⁴⁴⁷. We tested the proteasome-mediated turnover of each mutant. Although cODC functions as an autonomous degradation tag, in the context of native ODC, its degradative activity is enhanced by the protein antizyme 1 (AZ1), which forms an AZ1:ODC heterodimer in which cODC is more accessible than in the native ODC:ODC homodimer. The AZ1-stimulated turnover of each of the above mutants was examined in a reconstituted degradation system using rabbit reticulocyte lysate as a source of proteasomes. If Cys⁴⁴¹ takes part in functionally significant sidechain interactions with an amino acid in this local neighborhood, one or more of these alanine substitutions should be stabilizing. However, none of the alanine substitution mutants tested stabilized ODC, except for C441A, which profoundly reduced activity (Figure 2). We conclude that no other residue in the region examined makes sidechain interactions with Cys⁴⁴¹ that are critical for ODC degradation, or that are independently required for ODC degradation. Additionally, we tested the effect of an H450A mutation, a possible proton donor; this mutation also did not impair turnover. These investigations exclude the most plausible interactions of Cys⁴⁴¹ with other residues within cODC.

Cysteine 441 is intolerant of positional change

To assess whether Cys⁴⁴¹ can function only in its precise molecular context, we moved Cys⁴⁴¹ to each adjacent position in cODC. Since the sequence of the local region is Ser⁴⁴⁰/Cys⁴⁴¹/Ala⁴⁴² in wild type cODC, we substituted a cysteine at position 440 of a C441S mutant, or at position 442 of a C441A mutant, thus forming ODC with the mutations S440C/C441S and C441A/A442C. As shown in Figure 3A, swapping cysteine with either of the two adjacent residues stabilizes ODC, reducing degradation in the

experiment shown from 25% to less than 5% in each case. The effect of these sequence changes was also examined by pulse-chase analysis of ODC turnover in yeast cells. Introduction of a cysteine at either position 440 or 442 failed to restore degradative function of Cys⁴⁴¹ mutants (Figure 3B). Two possible mechanisms could account for the stabilization of ODC in these mutants: stabilization depends on either the loss of Cys⁴⁴¹ or a dominant negative effect of Cys at position 440 or 442. To distinguish these possibilities, we re-introduced Cys at position 441 in the above mutants to create Ser⁴⁴⁰/Cys⁴⁴¹/Cys⁴⁴² and Cys⁴⁴⁰/Cys⁴⁴¹/Ala⁴⁴². Using pulse chase analysis in yeast cells, we found that a cysteine substitution at 440 does not impair degradation, but cysteine at 442 stabilizes ODC. This result suggested a potential role for Ala⁴⁴², the single local residue not previously susceptible to analysis by alanine scanning mutagenesis. We therefore examined the A442P mutant and found it to be stable. The role of Ala⁴⁴² was further studied by creating the more conservative mutations A442G and A442L. As shown in Figure 3C, A442G was slowly degraded; in contrast, A442L was extremely stable. Thus we conclude that Ala⁴⁴² cannot be changed to residues with a more bulky sidechain. Taken together, the results above imply that Cys⁴⁴¹ must be precisely positioned in the native context to act as a degradation signal and may be recognized together with the adjacent Ala⁴⁴² residue. Considering the requirement of an adjacent Ala⁴⁴² after Cys⁴⁴¹, we made the Cys⁴⁴⁰/Ala⁴⁴¹/Ala⁴⁴² mutant to clarify whether Cys⁴⁴¹ is movable when it accompanies Ala. Surprisingly, this mutant was completely stable, suggested fixed positioning of the Cys⁴⁴¹/Ala⁴⁴² pair is necessary for degradation (Figure 3D)

Cys⁴⁴¹ does not form an intra-molecular disulfide bond.

The thiol group of cysteine acts as the catalytic center of some enzymes, by undergoing an oxidative reaction, by acting as a proton donor or as a thiolate anion [14]. Cys⁴⁴¹ may act catalytically or, alternatively, Cys⁴⁴¹ may simply be a part of a cODC structural element important for proteasome recognition. We had previously made a series of mutations intended to distinguish these molecular mechanisms. The previously described C441A mutation effectively removes the thiol of Cys⁴⁴¹, and the C441S mutation substitutes a hydroxyl group for the thiol. Among the natural amino acids, the C441S

alteration best preserves the size, hydrophobicity and polarity of the cysteine residue. The stabilization of ODC by the C441S mutation [7] implies that the hydroxyl group cannot replicate the functional properties of the thiol of Cys⁴⁴¹. Under permissive reduction-oxidation conditions, a pair of cysteines can form a disulfide bond. Perhaps a disulfide bond, or a cycle of bond formation and reduction involving Cys⁴⁴¹, is essential for cODC-mediated degradation. Mouse ODC contains 12 cysteine residues but most can be excluded as possible disulfide partners of Cys⁴⁴¹, because the cODC portable degradation tag contains only two cysteines, Cys⁴⁴¹ and Cys⁴⁵⁴. Thus within cODC the possible partner for bond formation with Cys⁴⁴¹ is restricted to Cys⁴⁵⁴. To test whether disulfide bonding between Cys⁴⁴¹ and Cys⁴⁵⁴ is needed for degradation, we changed Cys⁴⁵⁴ to alanine and subjected the mutant to an *in vitro* degradation assay using reticulocyte lysate as a proteasome source. C454A did not impair degradation, eliminating the potential for disulfide bond formation within cODC as critical for its function (Figure 4A). A pulse chase experiment also revealed a similar result (data not shown). Another possible bonding partner of Cys⁴⁴¹ is a cysteine thiol within a proteasome protein. We examined the AZ1-stimulated degradation of ODC with and without dithiothreitol (DTT), a reagent that can prevent or reverse disulfide bond formation. Using purified rat 26S proteasomes we measured the production of acid insoluble proteolysis products from ³⁵S-labeled ODC. To our surprise, degradation was observed only under reducing conditions (Figure 4B). Next, in order to assess whether reducing conditions are generally required for proteasome function, we tested another substrate of the 26S proteasome, oligo-ubiquitylated dihydrofolate reductase (Ub₅-DHFR), a previously described substrate whose degradation occurs through a K48-linked N-terminal penta-ubiquitin chain [15]. As shown in Figure 4C, this ubiquitin-dependent substrate was degraded equally effectively in the presence of 0.1 mM or 2 mM of DTT; the lower DTT concentration is insufficient to sustain degradation of ODC. *In vitro* degradation of ubiquitylated substrates in the absence of reducing agents has been reported previously (e.g. [16]) consistent with the present result. In addition, we tested the degradation of a substrate that is tethered to the proteasome and requires neither polyubiquitin nor cODC for association [8]. Rpn10-GFPcODC^{C441A} degradation by the rpn10Δ 26S proteasome did not require reducing conditions (data not shown). Strong reducing conditions therefore favor the

turnover of ODC, but this is not a general requirement for the proteolytic function of proteasomes. Taken as a whole, these data support the conclusion that ODC degradation does not require that Cys⁴⁴¹ participate in disulfide bond formation or other chemical processes involving the oxidation of its thiol group, and indeed suggest that chemical modification of the thiol precludes its degradative function.

cODC mediated degradation requires reducing conditions

The observation that ODC turnover requires reducing conditions led us to examine whether this property also applies to proteins that are made labile by appending the cODC degradation tag. We tested this using a DHFR-cODC construct in which the cODC tag was appended to the C terminus of the human DHFR open reading frame [7]. As described above, the degradation of Ub₅-DHFR, which is recognized through a ubiquitin chain, is DTT-independent *in vitro*. However, as shown for ODC, the degradation of DHFR-cODC is also strongly promoted by reducing conditions (Figure 5A). Thus, the requirement of proteasome-mediated degradation of ODC for reducing conditions is a property inherent in its C-terminal 37 amino acids. One possible role of Cys⁴⁴¹ in cODC function is that it forms a thio-ester bond, which may be tolerant to 2 mM DTT. Such a putative bond is not likely to be a cyclic association with the carboxyl of an acidic residue within cODC: the alanine scanning result excluded interaction of Cys⁴⁴¹ with the local structure of cODC; the known protein intramolecular thioesters, e.g., in complement protein C3, involve cyclization with a nearby acidic residue [17]. However, degradation may require that Cys⁴⁴¹ form a thioester with a protein of the 26S proteasome. We increased the concentration of DTT to 10 mM, aiming to cleave any thio-ester bond (or poorly accessible disulfide bond) and examined whether this condition impaired degradation of DHFR-cODC. Degradation persisted in the presence of 10 mM DTT, suggesting thioester bond formation between cODC and the proteasome was not required. In addition to disulfide or thio-ester bond cleavage, 10 mM DTT is also sufficient for reduction of cysteines oxidized to the sulfonic or sulfinic state. These results taken together effectively eliminated all but the simplest hypothesis: The sulfhydryl group of Cys⁴⁴¹ must be maintained in a reduced state to act as a recognition signal for the 26S proteasome, and does not act as a bonding partner with other residues.

Additionally, the structurally unrelated reducing agents 2-mercaptoethanol and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) also supported degradation (Figure 5B). To more readily evaluate the redox status of disulfide bonds under various experimental conditions, we utilized a protein attached to biotin through a disulfide bond. Strongly reducing conditions supported degradation and fully cleaved biotin via disulfide reduction of the model protein. In contrast, 0.1 mM DTT and 0.2 mM 2-mercaptoethanol, did not support degradation of ODC, and reduced the disulfide bond of the protein-linked biotin only partially (Figure 5C). Thus we conclude that the degradation demands disulfide reduction.

Spacing between Cys⁴⁴¹ and other elements of cODC

Our previous studies revealed that cODC-mediated proteasome association and peptide entry are two separable events. Within cODC, Cys⁴⁴¹ functions as a proteasome association element [8], while the C-terminal end of cODC initiates entry into the proteasome [18]. If each of these must interact with a distinct specific site within the proteasome, changing the native spacing between these elements- 20 residues intervenes between Cys⁴⁴¹ and the cODC terminus in the native primary sequence- could have functional consequences. We first asked whether increasing the spacing interfered with function. To move the end further from a functional copy of Cys⁴⁴¹, we duplicated cODC, forming ODC with a tandem cODC-cODC^{C441A} at its C-terminus. In this construct, the first ODC copy is wild type and includes a wild type Cys⁴⁴¹, and the distal C-terminal copy of cODC carries a mutant, C441A. As controls, we also constructed and compared forms of ODC terminated by cODC-cODC, cODC^{C441A}-cODC and cODC^{C441A}-cODC^{C441A}. As expected, cODC^{C441A}-cODC was normally degraded, but cODC-cODC^{C441A} was stable (Figure 6A). Also as expected, the duplicate wild type copies were functional, and the duplicated mutant copies, cODC^{C441A}-cODC^{C441A}, were not. This result suggests that Cys⁴⁴¹ requires a nearby free end, and that it cannot collaborate with a too-distant cODC copy that is made defective in its Cys⁴⁴¹ association element, one that is displaced from its native position by an extra 37 residues. Therefore cODC cannot function in the interior of the molecule. To rule out the possibility that cODC^{C441A} retains a structure that confers a dominant stabilizing effect in cODC-cODC^{C441A}, we

randomized the sequence of cODC^{C441A}, retaining its amino acid composition. The cODC-cODC^{C441A}_{random}, like cODC-cODC^{C441A}, was stable, implying that it is excess distance rather than an inhibitory function of the distal mutant copy that precludes collaboration. To test whether a lesser displacement also precludes functional interaction, we inserted a random 10 amino acid sequence between residues 451-452 of ODC. This insertion also stabilized in contrast to rapid degradation of wild type Flag-ODC, but a smaller, insertion of five amino acids, did not stabilize (Figure 6B). We next decreased the distance between the end of cODC and Cys⁴⁴¹. Truncation of the last four residues (458-461) stabilized, as did deletion of the last five (data not shown). Both an internal deletion of the next five, residues in 452-457, and the next five, residues 447-451, were also stabilizing (Figure 7). Because deleting each tract of five residues had similar effects, as did a ten residue insertion, we concluded that polypeptide chain length between Cys⁴⁴¹ and the C-terminus is strongly constrained. To ask whether sequence as well as size matters, we randomized the last five amino acids from ARINV to VINAR. We found that this mutant, designated (457-461)random in Figure 7, is normally degraded. We also tested an inversion of the last five and the second last five amino acid sequences, shown as (457-461)-(452-456). This mutant was again rapidly degraded. These results imply that the sequence is not the determinant of stability, but the length of the C-terminus is important. We tested the additional point mutations A457W and S456A and found neither stabilized ODC (data not shown).

DISCUSSION

Substrates of the proteasome must be attracted to the proteasome and once there present an unstructured region that serves as an entry point and site of initial degradation [19]. For most substrates a polyubiquitin chain serves the first function, but there is a subset that uses an alternate degron [20, 21]. The ODC degron cODC has been previously shown to subsume both required functions within a 37 amino acid tract [8]. Cys⁴⁴¹ within cODC is critical for proteasome association. Here we show that the thiol group of Cys⁴⁴¹ does not require oxidative chemical change, but instead functions within a structural motif that includes the adjacent Ala⁴⁴². Mutations at the Ala⁴⁴² residue adjacent to Cys⁴⁴¹

that increase the size of the alanine sidechain were found to impair function. Taken in conjunction with our previous work characterizing the function of Cys⁴⁴¹, our present mutagenesis data and chemical reduction data imply that Cys⁴⁴¹ and Ala⁴⁴² function together as a recognition element in which the cysteine thiol must be reduced and the sidechain of the next residue must be no larger than a methyl group. We further show that spacing between the Cys⁴⁴¹ Ala⁴⁴² pair and the C-terminal end of cODC, about 20 residues in the native sequence, must be maintained. Their functional interaction was disturbed by moving them ten amino acids further apart or five residues closer together. However, milder changes, deleting one amino acid or inserting five amino acids did not perturb degradation. Additionally, deletions within cODC on the N-terminal side of Cys⁴⁴¹, performed as block deletions of 5 amino acids, failed to abolish its degradative effect in the context of GFP-cODC (data not shown).

Previously it has been reported that deletion of ODC residues 447-451 [22] in mammalian cells was not stabilizing. However, we here found that the same deletion of ODC to be stabilizing in yeast cells. The tandem cODC degron experiment, as well as our earlier studies [23] show cODC does not work as a degradation signal when in a non-terminal position. These data imply that a proximal free ODC C-terminus is required for Cys⁴⁴¹ dependent degradation.

The C-terminus of ODC has not been detected by X-ray structural analysis [24, 25]. This suggests that cODC is unstructured, or assumes a structure induced by interaction with its unknown binding partner, one important for recognition by the 26S proteasome. Alternately, the lack of electron density attributable to cODC may indicate that it has a defined structure, but is highly mobile with respect to the rest of the ODC polypeptide. The portable nature of cODC, which seemingly confers lability when attached to the C terminus of any protein, implies that its structure, if any, can act autonomously.

Ubiquitin chains and cODC compete for recognition by proteasomes [7]. Such studies, based on functional competition of cODC with ubiquitin chains, imply identity or overlap of their binding sites, and suggest that cODC can act as a molecular mimic of ubiquitin

chains. Ubiquitin binding motifs require a hydrophobic patch on the ubiquitin surface; alanine substitutions therein abolish binding ability [26]. However, our mutational analysis showed that individual hydrophobic residues around Cys⁴⁴¹ can be either deleted or mutated without changing protein degradation.

Cysteine is widely involved in the ubiquitin-proteasome system. The catalytic center of deubiquitinating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases all contain a cysteine residue [1]. Some E2 enzymes are known to build ubiquitin chains on its catalytic center through a thio-ester bond [27, 28]. Also ubiquitin chain formation on a cysteine residue has been reported [29]. These chemical modifications, which depend on thioester formation, are destroyed by 10 mM DTT, thus clearly excluding a role for this structure in the function of Cys⁴⁴¹ in ODC. A co-factor AZ1 binds to ODC and enhances degradation, but this is not an essential event for ODC degradation in yeast cells or animal cells. Also, ODC with a C441A mutation, which is extremely stable, functionally binds to AZ1 in the yeast two hybrid assay (our unpublished data). The Cys⁴⁴¹ and adjacent Ala⁴⁴² are invariant among vertebrates, however other conserved aminoacids of cODC were revealed not to be important for degradation.

ACKNOWLEDGEMENTS

Ub₅-DHFR was a gift from Cecile Pickart. JT was supported by a fellowship from the Uehara Memorial Foundation. This work was supported by NIH grants GM45335 and GM074760 to PC.

Stage 2(a) POST-PRINT

REFERENCES

- 1 Hershko, A., Ciechanover, A. and Varshavsky, A. (2000) The ubiquitin system. *Nat. Med.* **6**, 1073-1081
- 2 Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360**, 597-599
- 3 Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D. and Coffino, P. (1989) Prevention of Rapid Intracellular Degradation of ODC by a Carboxyl-Terminal Truncation. *Science* **243**, 1493-1495
- 4 Hoyt, M. A., Zhang, M. and Coffino, P. (2003) Ubiquitin-independent mechanisms of mouse ornithine decarboxylase degradation are conserved between mammalian and fungal cells. *J. Biol. Chem.* **278**, 12135-12143
- 5 DeScenzo, R. A. and Minocha, S. C. (1993) Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. *Plant Mol. Biol.* **22**, 113-127
- 6 Miyazaki, Y., Matsufuji, S., Murakami, Y. and Hayashi, S. (1993) Single amino-acid replacement is responsible for the stabilization of ornithine decarboxylase in HMOA cells. *Eur. J. Biochem.* **214**, 837-844
- 7 Zhang, M., Pickart, C. M. and Coffino, P. (2003) Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate. *EMBO J.* **22**, 1488-1496
- 8 Takeuchi, J., Chen, H. and Coffino, P. (2007) Proteasome substrate degradation requires association plus extended peptide. *EMBO J.* **26**, 123-131
- 9 Mumberg, D., Muller, R. and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**, 119-122.
- 10 Chen, H., MacDonald, A. and Coffino, P. (2002) Structural elements of antizymes 1 and 2 required for proteasomal degradation of ornithine decarboxylase. *J. Biol. Chem.* **277**, 45957-45961
- 11 Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

- 12 Zhu, C., Lang, D. W. and Coffino, P. (1999) Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport. *J. Biol. Chem.* **274**, 26425-26430
- 13 Guthrie, C. and Fink, G. R., eds. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego
- 14 Leung-Toung, R., Li, W., Tam, T. F. and Karimian, K. (2002) Thiol-dependent enzymes and their inhibitors: a review. *Curr. Med. Chem.* **9**, 979-1002
- 15 Thrower, J. S., Hoffman, L., Rechsteiner, M. and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**, 94-102
- 16 Verma, R. and Deshaies, R. J. (2005) Assaying degradation and deubiquitination of a ubiquitinated substrate by purified 26S proteasomes. *Methods in Enzymology* **398**, 391-399
- 17 Law, S. K. and Dodds, A. W. (1997) The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Sci.* **6**, 263-274.
- 18 Zhang, M., MacDonald, A. I., Hoyt, M. A. and Coffino, P. (2004) Proteasomes begin ornithine decarboxylase digestion at the C terminus. *J. Biol. Chem.* **279**, 20959-20965
- 19 Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E. and Matouschek, A. (2004) An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat. Struct. Mol. Biol.* **11**, 830-837
- 20 Hoyt, M. A. and Coffino, P. (2004) Ubiquitin-free routes into the proteasome. *Cell. Mol. Life Sci.* **61**, 1596-1600
- 21 Verma, R. and Deshaies, R. J. (2000) A proteasome howdunit: the case of the missing signal. *Cell* **101**, 341-344
- 22 Ghoda, L., Sidney, D., Macrae, M. and Coffino, P. (1992) Structural elements of ornithine decarboxylase required for intracellular degradation and polyamine-dependent regulation. *Mol. Cell. Biol.* **12**, 2178-2185
- 23 Li, X. and Coffino, P. (1993) Degradation of Ornithine Decarboxylase: Exposure of the C-Terminal Target by a Polyamine-Inducible Inhibitory Protein. *Mol. Cell. Biol.* **13**, 2377-2383

- 24 Kern, A. D., Oliveira, M. A., Coffino, P. and Hackert, M. L. (1999) Structure of mammalian ornithine decarboxylase at 1.6 Å resolution: stereochemical implications of PLP-dependent amino acid decarboxylases. *Structure* **7**, 567-581
- 25 Almrud, J. J., Oliveira, M. A., Kern, A. D., Grishin, N. V., Phillips, M. A. and Hackert, M. L. (2000) Crystal structure of human ornithine decarboxylase at 2.1 Å resolution: structural insights to antizyme binding. *J. Mol. Biol.* **295**, 7-16.
- 26 Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M. and Pickart, C. (1996) Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 861-866.
- 27 Ravid, T. and Hochstrasser, M. (2007) Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nat. Cell Biol.* **9**, 422-427
- 28 Li, W., Tu, D., Brunger, A. T. and Ye, Y. (2007) A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. *Nature* **446**, 333-337
- 29 Cadwell, K. and Coscoy, L. (2005) Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science* **309**, 127-130

FIGURE LEGENDS

Figure 1. Alignment of vertebrate cODC sequences. Cys⁴⁴¹ is marked by an asterisk.

Figure 2. Alanine scanning mutagenesis within the sequence surrounding Cys⁴⁴¹. The amino acids from Thr⁴³⁶ to Met⁴⁴⁷ were individually mutated to Ala. The effect of each mutation was analyzed by comparing the amount of ³⁵S-radiolabeled ODC present after incubation with AZ1 plus proteasomes with that present in an identical control reaction without AZ1. Incubation was for 1 hr. and proteasomes were provided by a reticulocyte lysate. The percent degradation of mutant forms of ODC compared to that of wild type ODC is calculated as described in **EXPERIMENTAL**. As the native residue at position 442 is alanine, the bar corresponding to that position (marked by asterisk) represents the AZ1-induced degradation of wild type ODC.

Figure 3. Mutation of Cys⁴⁴¹ and adjacent amino acids.

A. In vitro degradation of ³⁵S -labeled ODC and indicated mutants. Degradation was analyzed as in Figure 2. **B-D.** Pulse chase analysis of ODC and indicated mutants. Each ODC allele was expressed in yeast cells, pulse labeled with ³⁵S and chased for the indicated time period.

Figure 4. Effect of disrupting potential disulfides.

A. Effect of mutation of Cys⁴⁴¹ and its potential disulfide partner Cys⁴⁵⁴. In vitro degradation was examined as in Figure 2. Arrow indicates full-length ODC. The lower band is a C-terminal truncated form of ODC that is generated to a variable extent in the in vitro translation reaction. **B.** Requirement for reducing conditions for in vitro degradation of ODC. Filled and open bars represent degradation in the absence and presence of AZ1. The production of acid-soluble radiolabeled peptides from ³⁵S -labeled ODC was monitored. **C.** Requirement for reducing conditions for in vitro degradation of Ub₅-DHFR. After incubation with purified rat 26S proteasomes with or without DTT, remaining substrate protein was detected by Western blotting.

Figure 5. Comparison of biochemical conditions required for disulfide bond reduction and cODC-dependent degradation.

A. Dependence of degradation on concentration of the reducing agent DTT. ³⁵S-labeled DHFR or DHFR-cODC were incubated with AZ1 and rat 26S proteasomes and acid-soluble counts then measured. MG132: proteasome inhibitor was used at the concentration of 0.2 mM. **B.** Comparison of diverse reducing agents, analyzed as in 5A. 2-ME: 2-mercaptoethanol. **C.** Efficacy of reduction of disulfide bonds. A protein linked to biotin through a disulfide bond was subjected to reduction as indicated and the residual protein-associated biotin assessed by SDS-PAGE, blotting and development with enzyme-linked streptavidin

Figure 6, cODC functions only at the C-terminus.

A, ODC with a cODC duplication and its mutational variants were expressed in yeast and stability examined by pulse chase analysis. Cells were labeled with ³⁵S and chased for the indicated time. **B,** ODC and mutants with 5 or 10 amino acid inserted were analyzed by pulse chase.

Figure 7. Deletion analysis of the C-terminal region of cODC.

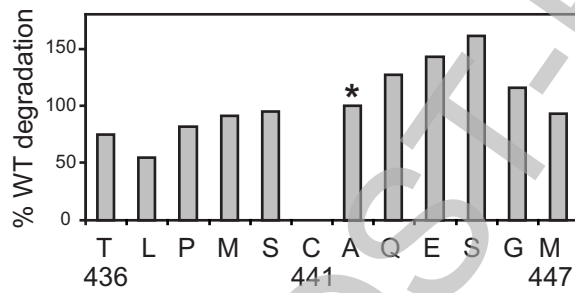
The indicated C-terminal deletions of Flag-ODC were expressed in yeast and their stability examined by pulse chase analysis. The C-terminal sequence of each construct is shown.

*

mouse	425	FPPEVVEEQDDGTLPMSCAQESGMDRHPAACASARINV	461
human	425	FPPEVVEEQDASTLPVSCAWESGMKRHRAACASASINV	461
chicken	415	FLAEVVEEQDVASLPLSCACESGIE-YPATCASASINV	450
Xenopus	426	ILPEVP--DLSALHVSQAQESGMELAPAVCTAASINV	460

I	identical
C	conservative
S	block of similar
W	weakly similar

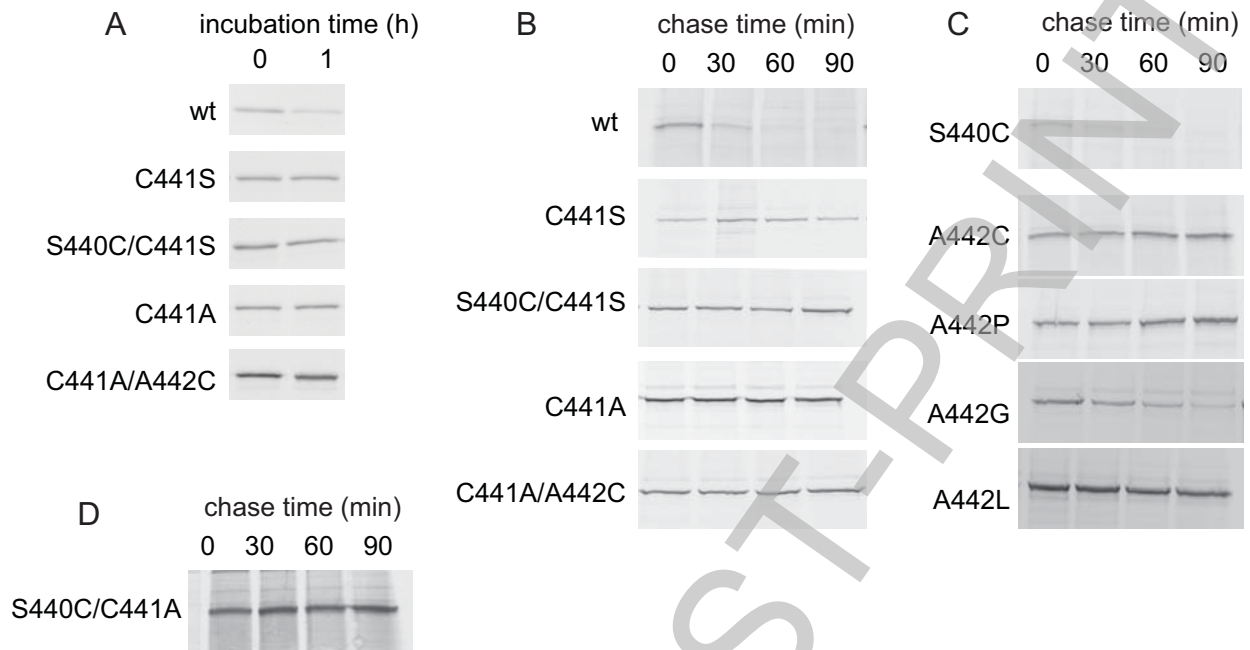
Figure 1, Takeuchi et al.



Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071239

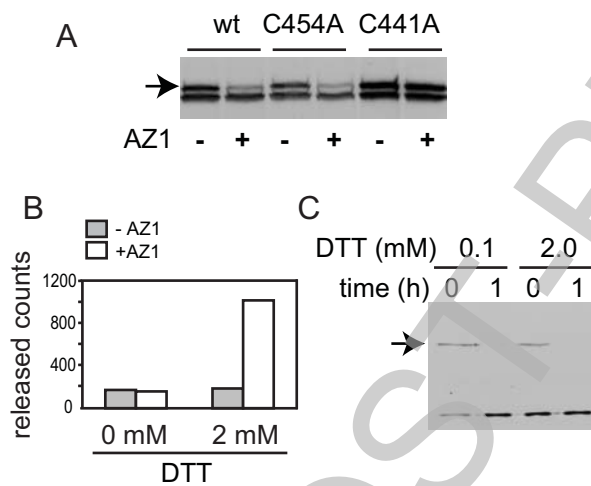
Takeuchi et al., Figure 2



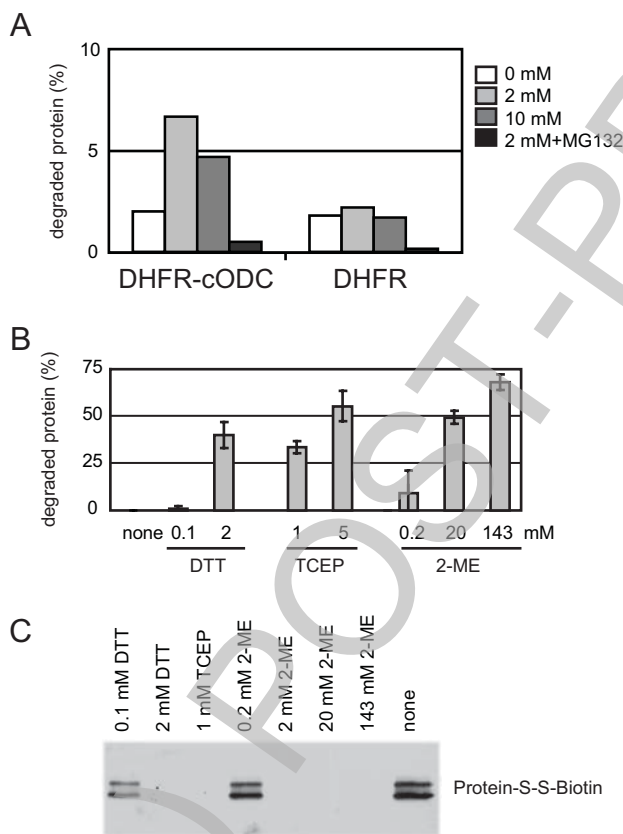
Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071239

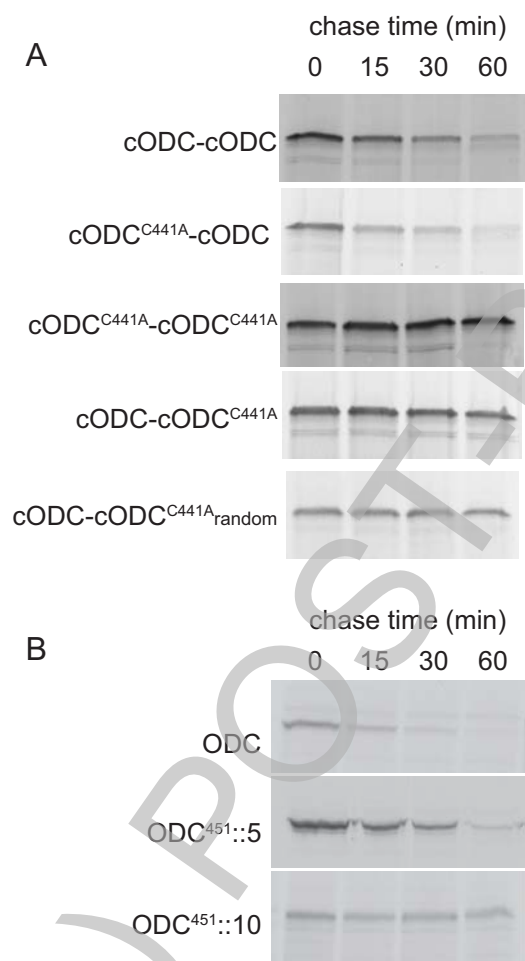
Takeuchi et al., Figure 3



Takeuchi et al., Figure 4



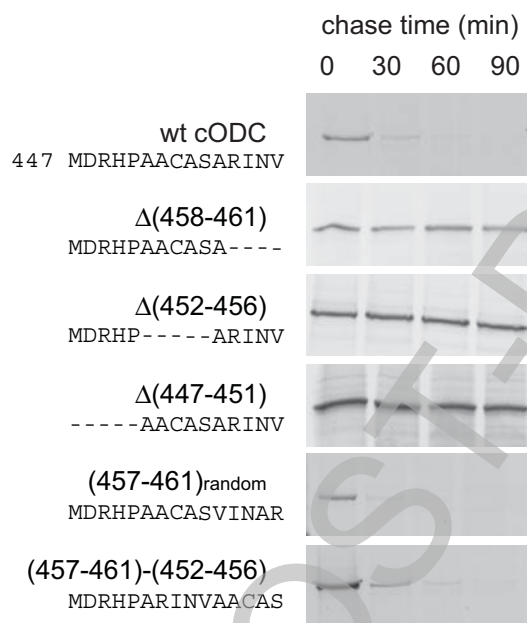
Takeuchi et al., Figure 5



Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071239

Takeuchi et al., Figure 6



Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071239

Takeuchi et al., Figure 7