

[32] Assaying Degradation and Deubiquitination of a Ubiquitinated Substrate by Purified 26S Proteasomes

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Abstract

The 26S proteasome is a multisubunit complex that catalyzes ATP-dependent proteolysis of cellular proteins. It eliminates misfolded proteins, as well as labile regulatory proteins, thereby serving a central role in maintaining cellular homeostasis. The bulk of the known substrates of the 26S proteasome are earmarked for proteolysis by covalent modification with a multiubiquitin chain, which is recognized by specific receptors. Once targeted, the substrate is deubiquitinated and degraded by the 26S proteasome. This chapter describes assays that monitor ATP- and ubiquitin-dependent proteolysis of the S-Cdk inhibitor Sic1.

Introduction

The 26S proteasome is a 2-MDa complex that comprises a 20S proteolytic core that is sealed off from the cellular milieu at both ends by the 19S cap (Pickart and Cohen, 2004). The 19S cap contains (minimally) 20 proteins present in stoichiometric amounts and numerous other proteasome-interacting proteins (PIPs) present in substoichiometric amounts (Verma *et al.*, 2000). Known activities resident in the 19S cap are receptors such as Rpn10 that bind the multiubiquitin chain as a prelude to substrate degradation (Verma *et al.*, 2004), an isopeptidase activity (Rpn11) that removes the polyubiquitin chain (Verma *et al.*, 2002; Yao and Cohen, 2002), and six ATPases (Rpts 1–6) that contribute to the unfolding and translocation of the substrate into the 20S proteolytic core (Rubin *et al.*, 1998). The 20S protease itself is a cylinder composed of two seven-membered outer (α) rings that are catalytically inactive and two seven-membered inner (β) rings. In each of the β rings, three of the subunits possess a peptidase active site (Chen and Hochstrasser, 1996).

Purification of 26S Proteasomes

The following method is a more detailed description of the protocol described previously (Verma *et al.*, 2000).

Reagents

1. 5× lysis buffer (buffer A): 250 mM Tris, pH 7.5, 750 mM NaCl, 50% glycerol, 25 mM MgCl₂, 5 mM ATP
2. 10× ATP regenerating system: 10 mg/ml creatine phosphokinase, 100 mM ATP, 200 mM HEPES, pH 7.2, 200 mM magnesium acetate, 1.5 M creatine phosphate

Individual Component Preparation

Creatine phosphokinase (Sigma) stock is made up as 10 mg/ml in 50 mM NaCl, 20 mM HEPES, pH 7.2, 2 mM MgCl₂, 1 mM EDTA, 50% glycerol and stored at -20°

Creatine phosphate, Sigma, is made up in water and stored at -80°
ATP is prepared from ATP sodium salt (Calbiochem). Dissolve 5 g ATP in 90.7 ml water and pH to 7.5 with NaOH

3. 2× no-salt buffer (buffer B): 50 mM Tris, pH 7.5, 20 mM MgCl₂
4. Washed anti-FLAG M2 affinity gel: Anti-FLAG affinity resin (Product No. A2220, Sigma) is washed with 0.1 M glycine, pH 3.5, and Tris-buffered saline as detailed in the manufacturer's protocol and resuspended in 1× buffer A as a 50% slurry
5. 20% Triton X-100
6. 26S elution buffer: 25 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 15% glycerol
7. 100× Flag peptide: 10 mg/ml of NH₂-ASP-TYR-LYS-ASP-ASP-ASP-ASP-LYS-COOH in water. Store in small aliquots at -20°

Growth Medium Preparation

10× synthetic medium (SD) containing 6.7% yeast nitrogen base minus amino acids, 5% casamino acids, 20% dextrose, 0.02% adenine sulfate, and 0.02% tryptophan

Strain. Purification of 26S proteasome complexes from yeast cells whose endogenous PRE1 locus (PRE1 encodes a 20S β subunit) is modified to encode a FLAG-tagged polypeptide (RJD1144).

Protocol

1. Inoculate 25 ml of SD medium with a single colony of RJD1144 growing on a SD-Ura plate. Grow culture to saturation (~28 h) at 30°.
2. Dilute 1.5 ml of saturated culture into each of six 2.8-liter Fernbach flasks containing 1.5 liter of 1× SD and grow to an OD₆₀₀ of 3.0.
3. Harvest cells in six 1-liter Nalgene centrifuge bottles in a Sorvall RC 3B Plus centrifuge using the HBB6 rotor (6K rpm for 20 min). Collect

pellet into four 50-ml centrifuge tubes by washing cells in a total volume of 200 ml ice-cold sterile water.

4. Flash freeze in liquid nitrogen the cell pellet obtained by centrifuging the 50-ml tubes in the Sorvall H1000B tabletop rotor at 5000 rpm for 5 min and store at -80° for a minimum of 2 h such that a frozen cell pellet that can be ground is obtained.

5. Grind cell pellet manually using a mortar and pestle, with the mortar nestled inside an ice bucket filled with dry ice. The procedure typically takes 10–20 min (depending on the amount being ground) so keep the pellet cold by the periodic addition of liquid nitrogen every 2 min. Transfer the ground powder to a 50-ml centrifuge tube up to the 20-ml mark and flash freeze in liquid nitrogen. Store tubes at -80° until ready to use.

6. Add 10 ml of $1\times$ lysis buffer per tube and supplement with ATP to 5 mM and ARS to $1\times$. Thaw cell powder on ice, while gently shaking occasionally to help bring material into suspension.

7. Transfer thawed lysate into a 30-ml screw-cap (i.e., Oak Ridge) centrifuge tube and centrifuge in a Sorvall SS34 rotor at 17,000 rpm for 20 min.

8. Collect pooled supernatants into a 50-ml tube and supplement again with ATP and $MgCl_2$ to 5 mM final concentrations. Add 800 μ l of washed FLAG beads (50% slurry) to a clean 15-ml centrifuge tube.

9. Add 13 ml of supernatant (typically 130 mg protein) from step 8 and incubate at 4° on a rotating wheel for 1.5 h.

10. Centrifuge tubes at 3000 rpm in a Sorvall H1000B rotor for 5 min and aspirate all but 1 ml of supernatant.

11. Transfer bead suspension to a 2-ml microcentrifuge tube.

12. Centrifuge beads at 7000 rpm in a microfuge for 30 s and wash beads three times with buffer A containing 0.2% Triton and an additional 2 mM ATP.

13. Wash twice with buffer B containing 2 mM ATP. Collect beads by centrifugation and use a 25-gauge needle to remove all traces of wash buffer.

14. Add a volume of 26S elution buffer that is three times the volume of the bead pellet. Add ATP to 2 mM (taking bead volume into account) and FLAG peptide to $1\times$. Elute at 4° for 3 h on a rotator.

15. Pellet beads in a microcentrifuge and collect 12 μ l supernatant for SDS-PAGE analysis (12% gel). Bands should be visible by Coomassie blue staining if a typical yield is obtained (300–400 μ g 26S/130 mg lysate). Flash freeze remaining supernatant and store at -80° . In addition to the aforementioned protocol, there are several other published procedures for purifying 26S proteasomes by one-step affinity chromatography. They are listed in [Table I](#).

TABLE I
OTHER PROTOCOLS FOR ONE-STEP AFFINITY PURIFICATION OF 26S PROTEASOMES

Nature of epitope tag	Chromatographic step	Assay	Comments	Ref.
1. <i>PRE1-TEV-MYC9</i>	Anti-myc 9E10 antibody coupled to protein A-Sepharose	Degradation of UbSic1	Elution using TEV protease, thus avoiding Flag peptide, which may interfere with peptidase assays	Petroski and Deshaies (2003)
2. <i>RPN11-TEV-PROTEIN A</i>	IgG	—	Elution using TEV protease	Leggett <i>et al.</i> (2002)
3. <i>RPN11-3XFLAG</i>	Anti-Flag M2 agarose	Degradation of T7Sic1PY	Elution with Flag peptide; good yield of doubly capped 26S proteasomes	Sone <i>et al.</i> (2004)

Monitoring Activity of Purified 26S Proteasome

Degradation

Substrate. Mbp-Sic1-MycHis6 is purified from *Escherichia coli* (Verma *et al.*, 1997b) or the trimeric Sic1 complex comprising Gst-Cdc28, Clb5, and Sic1 is purified from Hi5 insect cells. Purified Sic1 is phosphorylated by the G1 Cdk complex and ubiquitinated by the SCF ubiquitin ligase complex in the presence of yeast E1, Cdc34 (E2), ubiquitin, 1× ARS, and magnesium acetate as described previously (Seol *et al.*, 1999; Verma *et al.*, 2001) [see Petroski and Deshaies (2005) for a detailed protocol for the preparation of ubiquitinated Sic1].

Assay. A typical degradation reaction contains 50 μl purified 26S proteasome (75–100 nM final concentration), 6 μl 10× ARS, and 1 μl 200 mM magnesium acetate and is assembled on ice. The reaction is initiated by the addition of 2–3 μl of ubiquitinated Sic1 (UbSic1; 250–300 nM final concentration) and is incubated at 30° for 3 min (trimeric UbSic1) or 5 min (UbMbpSic1). The reaction is stopped by the addition of 5× Laemmli SDS–PAGE buffer. A 15- μl aliquot is resolved on an 8% (or 10%) polyacrylamide gel, and proteins are transferred to nitrocellulose, taking care to retain the stacker gel. The reaction is monitored by developing the nitrocellulose blot with a polyclonal antibody to Sic1. The ATP dependence of the degradation reaction can be assessed by pretreating 26S preparations and UbSic1 with apyrase (15 U/ml at 30° for 5 min) or glucose/hexokinase (5 U/ml hexokinase plus 30 mM glucose) to deplete ATP. Ubiquitin dependence can be monitored by mixing in an equimolar amount of unmodified Sic1 (generated by a reaction equivalent to a ubiquitination reaction but lacking ubiquitin and E1) (Verma *et al.*, 2001).

Deubiquitination

The deubiquitinating activity of the 26S proteasome can be unmasked when degradation is inhibited. Degradation can be blocked by using inhibitors of the 20S core peptidase activity (Meng *et al.*, 1999). Lactacystin and MG132 were ineffective at 200 μM (not shown), whereas both epoxomicin and YU101 were effective in blocking degradation of UbSic1 at 100 μM (Fig. 1). A time course experiment demonstrated that purified 26S proteasomes had to be preincubated with the inhibitor at 30° for a minimum of 20 min for maximal inhibition (Fig. 2). An epoxomicin dosage experiment demonstrated that the IC₅₀ was approximately 50 μM (Fig. 3). This value is much higher than the reported IC₅₀ of epoxomicin (40–80 nM) for inhibiting chymotryptic activity of the proteasome (Meng *et al.*, 1999). However, it has been argued that inhibition of the chymotryptic site by

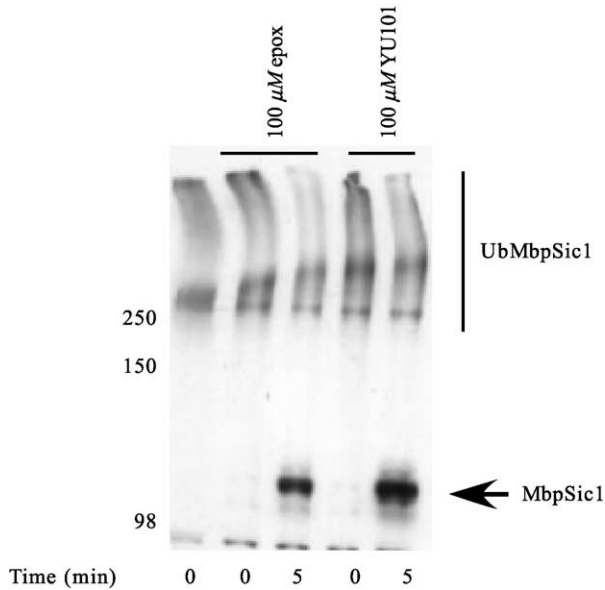


FIG. 1. UbSic1 is deubiquitinated but not degraded in the presence of the 20S peptidase inhibitors epoxomicin and YU101. 26S proteasomes were preincubated with either epoxomicin or YU101. The assay was initiated by the addition of UbSic1 and was terminated either immediately (lanes 2 and 4) or after a 5-min incubation at 30° (lanes 3 and 5). Lane 1 is time 0 with no added inhibitor.

itself causes little inhibition of protein breakdown and that either the tryptic or the caspase-like active site has to be inhibited in addition to the chymotryptic active site to achieve significant inhibition of protein breakdown (see [Kisselev and Goldberg, 2005](#)). At 100 μM epoxomicin, all three active sites of the proteasome should be inhibited.

In our hands, the deubiquitination assay serves as a useful surrogate for measuring protein breakdown by the proteasome. Like proteolysis of UbSic1, its deubiquitination requires (i) intact 26S proteasome, (ii) ATP, (iii) multiubiquitin chain receptor function (provided by either Rpn10 or Rad23), and (iv) an intact Rpn11 active site. An advantage of assaying deubiquitination as opposed to degradation is that instead of monitoring the disappearance of high molecular weight UbSic1, one can instead monitor the appearance of a discrete, lower molecular weight species of a deubiquitinated substrate. This renders the assay much more sensitive, which makes it easier to see subtle defects in substrate processing (e.g.,

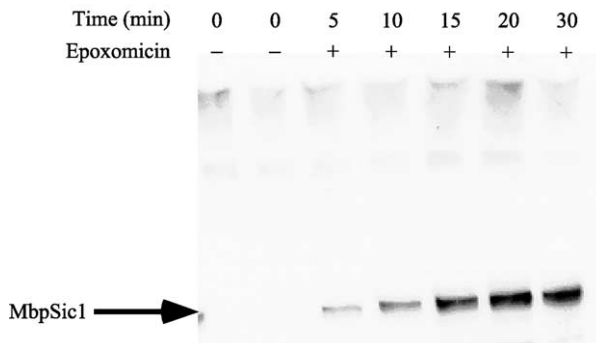


FIG. 2. Time course of deubiquitination. 26S proteasomes were pretreated with $100 \mu\text{M}$ epoxomicin at 30° for different lengths of time as indicated. The reaction was then initiated by the addition of UbSic1 and was terminated after 5 min.

Epoxomicin (μM):	0	0	100	1	10	25	50	100
Time (min):	0	5	5	5	5	5	5	5

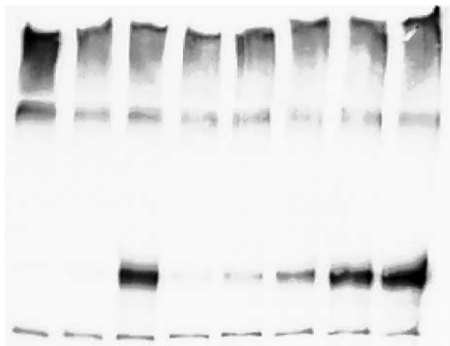


FIG. 3. Determination of IC_{50} for epoxomicin. 26S proteasomes were pretreated with varying concentrations of epoxomicin for 45 min at 30° before addition of substrate.

such as that shown by 26S proteasomes isolated from *rad23* Δ cells; see Verma *et al.*, 2004).

Assay. A standard deubiquitination reaction is set up by incubating $50 \mu\text{l}$ of purified 26S proteasome with $100 \mu\text{M}$ epoxomicin ($0.5 \mu\text{l}$ of a 10mM stock in dimethyl sulfoxide; store in aliquots at -20°) at 30° for 45 min. Ubiquitin aldehyde (UbA1) can also be added at a final concentration of $2.5 \mu\text{M}$ (Chung and Baek, 1999) if contaminating cysteine isopeptidases are a problem. The preincubated 26S proteasomes are put on ice, and the

reaction is assembled, initiated, and monitored as described earlier. The deubiquitination activity is dependent on the metalloisopeptidase Rpn11, a proteasomal subunit that contains the highly conserved JAMM motif (EX_nHXHX₁₀D) (Verma *et al.*, 2002). The specificity of the deubiquitination reaction can thus be determined by assaying in the presence of the metal chelator 1,10-phenanthroline (1 mM) or by assaying activity with 26S proteasomes prepared from *rpn11ts/rpn11AXA* mutant strains (Verma *et al.*, 2002).

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[33] Probing the Ubiquitin/Proteasome System with Ornithine Decarboxylase, a Ubiquitin-Independent Substrate

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Abstract

Ornithine decarboxylase (ODC) is an unusual proteasome substrate—ubiquitin conjugation plays no part in its turnover. It can therefore be used as a probe to distinguish proteasome-mediated actions that do or do not depend on the activity of the ubiquitin system. A 37 residue region of ODC suffices for proteasome interactions, and within this sequence functionally critical residues have been identified. Because no posttranslational modifications are required for substrate preparation, ODC and derived constructs can be readily generated as substrates for either *in vitro* or *in vivo* studies. This chapter describes methodologies that allow the use of ODC as a reporter to examine the ubiquitin-proteasome system, both in reconstituted *in vitro* systems and in living cells.

Introduction

Ornithine decarboxylase is degraded by the proteasome, but ubiquitin conjugation plays no role in this process. ODC is an enzyme required for polyamine biosynthesis. Proteasome degradation is an important element of its complex regulation. The protein has several characteristics that make it a favorable experimental substrate for studying proteasome action.

1. Active substrates are easily prepared without need for performing a complex series of enzymatic transfers of ubiquitin.
2. A small domain of ODC suffices as a degradation tag and functions autonomously upon transfer to other proteins.
3. The tag is conserved and recognized among diverse eukaryotes.