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Measurement of the Multiple Activities of 26S Proteasomes

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Abstract

Because proteasomes catalyze most of the protein degradation in mammalian cells, and their functioning is essential for cell homeostasis, proteasome structure, biochemical mechanisms, and regulation in normal and disease states of are now widely studied and are of major importance. In addition, inhibitors of the proteasome's peptidase activity have proven to be very valuable as research tools and in the treatment of hematologic malignancies, and a number of newer pharmacological agents that alter proteasome function are being developed. The rapid degradation of ubiquitinated proteins by the 26S proteasome involves multiple enzymatic and non-enzymatic steps, including the binding of ubiquitinated substrates to the 19S particle (Section 1), opening the gated substrate entry channel into the 20S particle (Section 2), disassembly of the Ub chain (Section 3), ATP hydrolysis, substrate unfolding and translocation, and proteolysis within the 20S particle (Section 2 and 5). Assaying each of these processes is important if we are to fully understand the physiological regulation of proteasome function and the effects of disease or drugs. Here, we describe several methods that we have found useful to measure many of these individual activities using purified proteasomes. Studies using these approaches have already provided valuable new insights into the effects of postsynthetic modifications to 26S subunits, the physiological regulation of the Ubiquitin Proteasome System (UPS), and the impairment of proteasome activity in neurodegenerative disease. These advances would not have been possible with the widely used assays of peptidase activity.

Keywords

26S proteasomes; Ubiquitin conjugate degradation; Peptide hydrolysis; ATP hydrolysis; Deubiquitination; Ubiquitin-proteasome pathway; Gate opening; Ubiquitin binding

1. Introduction

The 26S proteasome consists of the 20S core proteolytic particle and one or two 19S regulatory particles (1,2). The 20S proteasome is a hollow four-ring cylinder containing in each of its central β -rings three types of peptidase sites, which have chymotrypsin-like, trypsin-like, and caspase-like specificities (3). The outer α -rings contain a gated central channel for substrate entry (4,5). The core particle may be capped on either one or both ends with a 19S regulatory particle, which bind ubiquitin (Ub) conjugates, disassemble the Ub

chain, hydrolyze ATP, and unfold and translocate the substrate protein into the 20S particle (1,2). Most steps in the degradation process are linked to ordered cycles of ATP binding and hydrolysis (6), catalyzed by the six 19S ATPase subunits which unfold and translocate the polypeptide through their central channel and through the gated entry pore into the 20S particle, within which proteolysis occurs (1,7).

Assaying these multiple enzymatic steps is therefore essential for a full understanding of the degradative process, the actions of drugs, and the alterations in rates of protein breakdown occurring in different physiological and pathological states. Recent studies have revealed that proteasome function is regulated in multiple ways including its activation by ubiquitinated substrates, by covalent modifications of subunits (e.g. by phosphorylation) (8) and by various proteasome-associated regulatory proteins (9). Most commonly, proteasome activity in cell or tissue extracts has been determined by measurements of the hydrolysis of a fluorogenic peptide, most often suc-LLVY-amc, a substrate of the 20S's chymotrypsin-like site. Although this approach is simple and convenient, it does not actually measure the proteasomes' ability to degrade its physiological substrates, Ub conjugates. Also these peptidase activities in cell lysates can be altered by various activity factors, especially PA28 $\alpha\beta$ /11S, PA 28 γ , PA200/BLM10, the inhibitory factor PI31/PSMF1, or even by exposure to salts or detergents (10–12). The capacity of 26S particles to degrade ubiquitinated proteins depends on multiple actions of the 19S regulatory particle, including 1) the extent of binding of a Ub conjugate; 2) the rate of substrate deubiquitination; and 3) the rate of ATP hydrolysis (9). Moreover, these steps can be activated by physiological stimuli (e.g. hormones or nutrient deprivation) or pharmacological agents. For example, phosphorylation of the 19S subunit, Rpn6, is stimulated by cAMP via Protein Kinase A (PKA) and during the S-M phases of the cell cycle the Rpt3 subunit is phosphorylated by Dyrk2. These modifications activate not only 20S gate opening and peptide hydrolysis, but also the 19S ATPases (13,14) and the breakdown of Ub conjugates. Also, the ubiquitination of the 19S subunit Rpn13 blocks the binding of Ub conjugates to the particle and prevents Ub conjugate degradation without affecting the proteasome's peptidase activities (15). The recent elucidation of these regulatory mechanisms has been made possible by the development of methods to assay these specific steps in conjugate degradation, which can only be analyzed once the 26S particles are purified free of exogenous regulatory factors and soluble enzymes, especially ATPases and DUBs, present in cell lysates. We describe below the protocols that we have found useful to measure several key steps in the degradation of ubiquitinated proteins.

1. Binding of Ub proteins to the proteasome

In selectively degrading ubiquitinated proteins, the proteasome must recognize both the Ub chain on the substrate and the presence of a loosely folded region within the protein (16). This recognition of Ub conjugates proceeds by a two-step mechanism. Initially, the proteasome binds reversibly the Ub chain and then the protein's loosely folded region becomes tightly bound to the ATPases, committing the substrate to degradation. Our lab has developed an assay that distinguishes these two binding steps, and that has proven useful to clarify the roles in binding of ATP and the receptor subunits Rpn10 and Rpn13 (17), the

effects of the shuttling factors, Rad23a and Rad23b, (18), and the impairment of binding upon Rpn13 ubiquitination (15).

The initial step in proteasome degradation of ubiquitinated substrates is the reversible association of the Ub chain with the Pru domain of Rpn13/ADRM1 and the Ub-interacting motifs (UIM) of Rpn10/S5a (19,20) (although other sites on the proteasome can also contribute to Ub binding, notably the T1 region of Rpn1 (21)). This initial step does not require ATP hydrolysis (17) and therefore can occur at 4°C. This temperature also presents destructive cytosolic enzyme (e.g. DUBs or ATPases). This binding step is easily reversed by competition with an excess free UIM or by a salt wash. The second, tighter binding step can not be reversed by a UIM or a salt wash and depends on the presence of a loosely folded region in the substrate (17). This transition to tight binding requires ATP hydrolysis and thus can only occur at higher temperatures (e.g. 37°C). This step, most likely, involves the capture of the protein by the six ATPase ring, whose pore loops appear to grab the substrate and drive its translocation (22).

Our method to measure the binding of Ub conjugates assays separately the initial and tighter binding steps. In these assays, we have used several different immobilized Ub conjugates, DHFR, Nedd4, and E6AP/Ube3a (15,17,18), but because of its ease of preparation, ubiquitinated E6AP is preferred and will be discussed here. After they bind to ubiquitinated E6AP immobilized on resins, the proteasomes can be detected either by immunoblot or more rapidly, simply and quantitatively with a peptidase assay using a small fluorogenic substrate (see section 2 below). The values obtained by the two methods agree closely (17). The initial reversible and tighter binding steps can be distinguished by incubation at different temperatures. At 4°C only the initial binding step can occur, while incubation at 37°C allows the tighter binding. Also, these two steps can be distinguished by the sensitivity of the reversible proteasome binding to washing with high salt (300 mM) or UIM solutions.

2. Peptide hydrolysis and opening of the substrate entry channel into the 20S particle

The eukaryotic 20S proteasome contains three proteolytic sites on each of its central β -rings. These active sites differ in their substrate specificities. Two sites (located on $\beta 5$ /PSMB5) are 'chymotrypsin-like' and cleave after hydrophobic residues; and two sites ($\beta 2$ /PSMB7) are 'trypsin-like' and cleave after basic residues; and two (located on $\beta 1$ /PSMB6), are 'caspase-like' and cleave after acidic residues (23). In the immunoproteasomes, these constitutive β -subunits are replaced by homologous β -subunits with different specificities that favor antigen presentation (1). The activities of these individual sites can be monitored by measuring the hydrolysis of fluorogenic peptides substrates specific for each active site (24). We typically use Suc-LLVY-amc to monitor the chymotrypsin-like activity, Boc-LRR-amc for the trypsin-like activity, and Ac-nLPnLD-amc for the caspase-like activity of the proteasome.

In order to reach the proteolytic sites within the 20S particle, protein substrates are actively translocated through the ATPases' central channel (7,25) and the gated central pore in the 20S α -ring (5). Upon nucleotide binding, the ATPases' C-terminal HbYX residues bind to intersubunit pockets in the 20S's α -ring (26). Under many experimental conditions (e.g. with ATP or especially the non-hydrolyzable nucleotide, ATP γ S, present), the hydrolysis of

substrates by all three active sites is stimulated simultaneously, due to the opening of the gated entry channel. Therefore, it is important in studies of proteasome function to measure the rates of substrate cleavage by all three active sites. Often in the literature the rate of hydrolysis of substrates of the chymotrypsin-like site is taken to indicate proteasome activity. However, this assumption is not valid. In fact, the proteasome inhibitors used most widely as research tools, MG132, or in the clinic for treatment of multiple myeloma, bortezomib, preferentially block the chymotrypsin like activity and only at high doses inhibit the caspase-like site, and do not block the trypsin-like activity (27). Therefore, Bortezomib at nanomolar concentrations completely inactivates the chymotrypsin-like site, but slows protein and Ub conjugate degradation by only about 50% due to the continued functioning of the trypsin-like and caspase sites. Consequently, to effectively block proteolysis, it is essential to use much higher concentrations of these agents to inhibit multiple sites and to evaluate proteasome function it is important to assay Ub conjugate degradation rather than just peptidase activities.

3. Disassembly of Ub chains by proteasomal DUBs

During the degradation of a Ub conjugate, the Ub chain is disassembled in order that the Ub molecules can be reutilized, and that substrates can be readily translocated into the 20S particle. Three different deubiquitinating enzymes (DUBs) are associated with the 19S regulatory particle in higher eukaryotes. Two are cysteine proteases, Usp14/Ubp6 (28) and Uch37/UchL5 (29,30), and the third, Rpn11/PSMD14, is a metalloprotease (31,32). Rpn11 is an integral 19S subunit, but Usp14 and Uch37 associate reversibly with the proteasome and usually are present on the 26S in substoichiometric amounts. Deubiquitination of substrates by Rpn11 is an ATP-stimulated process (31,32) that removes the Ub chain by cleaving the isopeptide bond near to the substrate and thus facilitates the translocation of the polypeptide through the ATPase ring into the 20S particle (31).

Several approaches have been utilized to evaluate the activities of these three DUBs, but they are at best semi-quantitative. Selective assays of the contributions of these enzymes is desirable because the proteasomal DUBs have emerged as attractive drug targets for multiple diseases. However, their actual importance in conjugate degradation is quite difficult to measure. For example, Usp14 levels on the proteasome depend on the supply of Ub conjugates (33), its DUBs activity increases dramatically upon proteasome association (34). Usp14 prefers as substrates proteins bearing multiple Ub chains (35), and in addition to their DUB activities, Usp14 and Uch37 have major roles in regulating the other proteasomal activities (36–38). The most widely used substrate for monitoring DUB activities is Ub-amc, and both Usp14 and Uch37 rapidly cleave it. Alternatively, the formation of a covalent linkage between the suicide substrate HA-Ub-vinyl sulfone (VS), and the DUBs active site cysteines can be used to evaluate their activities (39). However, these Ub-based substrates are not physiological, are not Ub chains, and do not accurately reflect the deubiquitination capacity of the 26S particle because the affinities of these enzymes for free Ub and Ub chains differ widely. Also Rpn11 hydrolyzes Ub-amc poorly and does not react with HA-Ub-VS. We have found that measuring the disassembly of short poly-Ub chains, that are homogeneous in length and contain only K48 or K63 Ub chains, is a quite useful method to

monitor Rpn11's activity (38). This activity requires an active site Zn^{++} ion and thus can be selectively inhibited by o-phenanthroline, 8-thioquinoline, or its derivative capzimin (40,41).

4. ATP hydrolysis

The 26S proteasome is an ATP-dependent proteolytic machine, whose function depends on the ring of six ATPase subunits (Rpt1–6) at the base of the 19S complex. These ATPases are homologous to the bacterial ATP-dependent proteases and archaeal PAN ATPase, which are also hexameric complexes. These AAA ATPases all bind and translocate polypeptide substrates into an associated proteolytic compartment and their ATPase activities are stimulated upon binding a loosely folded polypeptide substrate (42–44). Unlike these prokaryotic enzymes, the 26S ATPases are stimulated by ubiquitinated proteins or by the simultaneous binding of a Ub chain to Usp14 or Uch37, in addition to the unstructured region of the polypeptide (which need not be covalently linked) (37). The binding of a substrate or ATP γ S to the proteasome induces a massive change in the ATPases' conformations. In the resulting degradation-competent state (7,25) the ATPases' substrate translocation channel is wider and aligned with the gated 20S pore. Also Rpn11 becomes positioned directly above the central pore so as to facilitate the degradation of the ubiquitinated substrate. ATP binding and hydrolysis thus drive almost all proteasomal steps, including substrate binding, gate-opening, deubiquitination, unfolding and translocation. Consequently, ATP consumption is directly proportional to the rate of conjugate degradation and depends on both the folding and size of the substrate (45). Here we will describe a protocol to assay the activity of the 26S ATPases using a modification of the Malachite-green assay (46) which measures the release of inorganic phosphate from ATP.

5. Degradation of ubiquitinated proteins

In studying the effects of inhibitors, post-synthetic modifications, physiological stimuli, or disease on proteasome function, the capacity of the 26S particles to degrade ubiquitinated proteins should be the major concern. The main challenge in such studies has been to generate defined ubiquitinated substrates that are easy to prepare, have defined ubiquitin chains, are easily quantitated and are degraded rapidly by isolated proteasomes. Unfortunately, most labs have followed the breakdown of only one or two ubiquitinated proteins (DHFR, cyclins, troponin I, Sic1) and non-ubiquitinated ones (e.g. casein, Sic1) as substrates.

Here, we describe a protocol for only one model substrate, PY-Sic1, based on a method originally described by Tohe and colleagues (47) that we have modified by labeling Sic1 with ^{32}P through phosphorylation with Protein Kinase A and ^{32}P -ATP. By following the hydrolysis of this labelled substrate to radioactive peptides, it is possible to precisely quantitate the degradative process. Multiple other methods are available for radiolabeling or adding fluorescent tags to the substrate that presumably could also be used in similar assays. However, this approach allows precise quantitation, is very sensitive and often advantages for kinetic studies. Most often, the degradation of ubiquitinated proteins has been assayed by monitoring the loss of the ubiquitinated substrate by immunoblot. Although simple, this approach lacks sensitivity, and substrate loss can occur through both proteasome degradation and deubiquitination without degradation. Although it can be valuable in some studies to

monitor simultaneously these two processes, quantitating each and identifying which process is changing can be problematic. In addition, the heterogeneity of Ub conjugates makes quantitative immunoblots especially challenging and measuring the loss of substrate often requires the use of low concentrations of substrate resulting in reactions that are well below the V_{max} of the enzyme. Finally, having to run samples on SDS-PAGE limits the number of experimental variables that can be studied in an experiment.

The development of fluorescently tagged substrates (48) is a useful alternative to relying on antibodies against the substrate. Even though one can still run the reactions on gels, the great advantage of fluorescence is the ability to assay degradation in real-time on a multiwell plate as described by Matouschek and colleagues (see chapter) (48). That method has the great potential for larger scale screening of proteasome activity. In using anisotropy to assay proteolysis, the substrate ideally should be labeled at a single residue, and one must ensure that the change in rotational size is the consequence of degradation rather than deubiquitination of the substrate.

The “classical” method for following degradation of ubiquitinated protein, which we describe here is to measure the conversion of the TCA-precipitable radioactive substrate to TCA-soluble (peptide) products. Similar approaches were used originally in the studies of ATP-dependent proteases (49) in bacteria and of the Ub proteasome pathway (15). Although working with radioactive tracers requires certain special precautions, it is a very sensitive and precise assay. Because this method directly measures the peptide products, radioactively labeled substrates can be used at high concentrations that are suitable for kinetic analysis and make possible the establishment of linear assay conditions. Because of these prior difficulties in preparing ubiquitinated substrates and quantitating their degradation, there have been surprisingly few kinetic studies of Ub conjugate degradation despite their potential in clarifying proteasome mechanisms and regulation.

2. Materials

2.1. Purified 26S proteasomes (100 nM) (see Note 1)

2.2. Ub(n) E6AP

1. Transform pGEX4T-E6AP into BL21(DE3). Select one Amp^R colony. Grow at 37°C in 2 L LB + Ampicillin to OD₆₀₀ 0.8. Induce with 0.5 mM IPTG at 16°C overnight. Collect the bacterial pellet by centrifugation (3,000 × g for 15 minutes), wash the pellet with 1x PBS, centrifuge again (3,000 × g for 20 minutes), and store the pellet at –80°C until ready to use.
2. Perform lysis and purification steps (2 – 5) at 4°C. Lyse pellet in 20 mL 1x PBS. Clear lysate by centrifugation at 10,000 × g for 20 minutes.
3. Bind lysate to 1.25 mL (bed volume) equilibrated glutathione-Sepharose beads by rotation for 90 minutes.
4. Wash twice with 12.5 mL 1x PBS (10 column volumes).

¹. To maintain the stability of the purified proteasome during the storage at –80°C, add 10% (v/v) glycerol to the proteasome solution.

5. Wash twice with ubiquitination buffer (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM MgCl₂)
6. Set up the ubiquitination reaction in 15 ml. (see Note 2)

Ubiquitination buffer	(20 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM MgCl ₂)
ATP	2 mM
DTT	1 mM
GST-E6AP on glutathione Sepharose	300 nM
Ube1	5 nM
UbcH5b	140 nM
Ub	30 μ M

7. Incubate overnight at 37°C with rotation. Collect the resin by centrifugation at 100 $\times$ g for 3 minutes.
8. Wash five times with 20 bed volumes of TNG buffer (50 mM Tris HCl (pH 7.5), 50 mM NaCl, 10% (v/v) glycerol). Collect the resin by centrifugation at 100 $\times$ g for 3 minutes.

2.3. Ub conjugate binding

1. Binding buffer: 25 mM HEPES-KOH (pH 8), 125 mM KAc, 1 mM DTT, 2.5 mM MgCl₂, 0.1 g/L BSA, 0.05% Triton X-100, 1 mM ATP
2. Wash Buffer I: 25 mM HEPES-KOH (pH 8), 125 mM KAc, 1 mM DTT, 2.5 mM MgCl₂, 0.05% Triton X-100, 1 mM ATP
3. Wash Buffer IIa: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 1 mM ATP
4. Wash Buffer IIb: 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP
5. Wash Buffer IIc: 50 mM Tris-HCl (pH 7.5), 5 μ M UIM, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP

2.4. Peptide hydrolysis

1. Fluorogenic substrate stock solutions: dissolve Suc-Leu-Leu-Val-Tyr-amc (Suc-LLVY-amc for the chymotrypsin-like activity), Boc-Leu-Arg-Arg-amc (Boc-LRR-amc for the trypsin-like activity) or Ac-Nle-Pro-Nle-Asp-amc (Ac-nLPnLD-amc for the caspase-like activity) in DMSO (final 20 mM).

²-It is important to also prepare a non-ubiquitinated substrate as a control. This sample does not require as many reactions, and therefore a reaction volume that is half the size of the ubiquitination reaction can be prepared.

2. Peptidase assay buffer: 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.1 mM ATP (or ATP γ S), 0.5 mM MgCl₂, 25 ng/ μ l bovine serum albumin (Sigma) with 10 μ M selected fluorogenic peptide substrate.
3. Flat bottomed black 96 well plate (Greiner Bio-one)
4. Fluorometer

2.5. ATP hydrolysis (see Note 3)

1. Solution A (5x ATP hydrolysis reaction buffer): 125 mM HEPES-KOH (pH 7.6), 625 KAc, 0.13% Triton X-100, 12.5 mM MgCl₂, 5 mM ATP, 0.5 mg/ml BSA, 2.5 mM DTT
2. Solution B (Malachite green solution): Dissolve 45 mg of malachite green hydrochloride in 100 ml of H₂O with vigorous stirring for 30 minutes at room temperature. Pass the solution through 0.45 μ m filter. This solution is stable at 4°C for at least a few months (see Note 4).
3. Solution C (4.2% ammonium molybdate in 4N HCl): 4.3 g of ammonium molybdate tetrahydrate is dissolved in 50 ml of H₂O. Slowly add 34.5 ml of concentrated HCl. Add H₂O to 100 ml. This solution is stable at 4°C for a few months.
4. 34% Citric acid in H₂O.
5. Flat bottomed clear 96 well plate (Greiner Bio-one).
6. Spectrophotometer
7. Standard inorganic phosphate solution.

nmole Phosphate	2 mM KH ₂ PO ₄ (μ l)	H ₂ O (μ l)
0	0	1000
1	50	950
2	100	900
4	200	800
6	300	700
8	400	600
10	500	500
12	600	400
14	700	300
16	800	200
18	900	100
20	1000	0

³.Do not use glassware during the preparation of reagents or the measurements in for ATPase assay.

⁴.This solution should be protected from light.

2.6. Deubiquitination

1. Deubiquitination assay buffer (5x): 125 mM HPES, pH 7.6, 500 mM KCl, 25 mM MgCl₂, 5 mM DTT and 5 mM ATP.
2. 0.25 µg/µL Tetramer Ub chains (Boston Biochem) in 50 mM Tris-HCl (pH 7.6).
3. Inhibitor of Usp14 and Uch37: 6 µM Ub-vinylsulfone (Boston Biochem)
4. Rpn11 inhibitor: 10 mM o-phenanthroline in methanol.
5. SDS-PAGE: Bolt Bis-Tris Plus Mini Gel (4–12% gradient), Bolt LDS sample buffer (4x), NuPAGE Reducing Agent (10x), and Bolt MES buffer are used with XCell SureLock Mini-Cell electrophoresis apparatus (Thermo Fisher Scientific). 1 M DTT (see Note 5).
6. Western blotting: XCell II Blot module with XCell SureLock Mini-Cell electrophoresis apparatus (Thermo Fisher Scientific). PVDF membrane (GE Healthcare) (see Note 5).
7. PBST: dilute 100 ml of 10x PBS with 890 ml of H₂O and add 10 ml of 20% Triton X-100.
8. PBSTB: dissolve 3 g of BSA in 100 ml of PBST.
9. Anti-ubiquitin antibody (Santa Cruz Biotechnology P4D1 or Life Sensors VU) (see Note 6)
10. Anti-ubiquitin antibody (Santa Cruz Biotechnology P4D1 or Life Sensors VU1) (see Note 6)

2.7. Expression, Purification, and Labeling of Sic1 (see Note 7)

1. Transform pET21-Sic1 into BL21(DE3). Select one AmpR colony. Grow at 37°C in 2 L LB + Ampicillin to OD_{600nm} 0.7. Induce with 0.5 mM IPTG at 30°C 3 h. Collect the bacterial pellet by centrifugation (3,000 × g for 15 minutes), wash the pellet with 1x PBS, centrifuge again (3,000 × g 20 minutes), and store the pellet at –80°C until ready to use.
2. Perform lysis and purification steps at 4°C. Lyse the bacteria pellet in 30 mL Lysis buffer (50 mM Na₃PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.5 mM DTT, 1 mM PMSF) by passing through the emulsiflex at 10,000 psi. Add Triton X-100 to 0.2%, RNase A to 10 µg/mL, and DNase I to 5 µg/mL. Rotate for 10 minutes.
3. Clarify the lysate by centrifugation at 10,000 × g for 20 minutes.
4. Add clarified lysate to 1 mL bed volume equilibrated Ni-NTA resin. Rotate for 1 hour.

⁵ Various kits for SDS-PAGE and membrane transferring can be used, according to the instructions of the manufacturer.

⁶ This protocol was originally established using Boston Biochem's polyclonal anti-ubiquitin antibody (A100), which is now discontinued. Anti-ubiquitin antibodies which detect mono-ubiquitin efficiently, such as P4D1 (from our experience) and VU101 (51), can be used in this assay.

⁷ This protocol is adapted with minor modifications from Saeki et al (47)

5. Pour the slurry into an empty 20 mL column body. Wash the resin twice with 10 mL wash buffer (50 mM Na₃PO₄ (pH 8.0), 300 mM NaCl, 20 mM imidazole). Wash the resin once with 10 mL wash-78 buffer (7.5 mL wash buffer and 2.5 mL elution buffer).
6. Elute four times with 1 mL elution buffer (50 mM Na₃PO₄ (pH 8.0), 300 mM NaCl, 250 mM imidazole) for 20 minutes at 4°C.
7. Determine Sic1 fractions by SDS-PAGE/Coomassie blue staining. Pool Sic1 fractions and dilute against 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM DTT)
8. Set up phosphorylation reaction (see Note 8)

10x Protein Kinase Buffer (NEB)	9 µL
500,000 U/mL CK2 (NEB)	7.5 µL
Sic1	58.5 µL
ATP, [γ -32P]- 6000Ci/mmol 10mCi/ml, 250 µCi (Perkin Elmer)	15 µL

9. Incubate at 30°C for 1 hour
10. Remove unincorporated nucleotides by adding the phosphorylation reaction to a Bio-Spin 30 (Bio-Rad) column. Spin at 1,000 × g, 4 °C for 3 minutes. Repeat the clean up by applying the flow through of the spin column to a new Bio-Spin 30 column. Collect the flow through from the second spin.
11. Set up ubiquitination reaction mixture 1. Ubiquitination buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% glycerol). 5x ATP solution (10 mM ATP, pH 7.5, 50 mM MgCl₂, 5 mM DTT in ubiquitination buffer).

Ubiquitination Buffer	1x
ATP solution	1x
Ube1	200 nM
Ubc4	6 µM
Ub	120 µM

12. Set up ubiquitination reaction mixture 2

Ubiquitination buffer	1x
ATP solution	1x

⁸.It is recommended that a mock phosphorylation reaction be set up to (1) estimate the concentration of Sic1 eluted through the Bio-Spin columns and (2) to set up a “cold” ubiquitination reaction to check the effectiveness of the ubiquitination reaction.

Rsp5	1 μ M
PY-Sic1	1 μ M

13. Incubate ubiquitination mixture 1 at 25°C for 5 minutes. Add ubiquitination mixture 2 to Ub mixture 1. Incubate at 25°C for 3 hours.

2.8. Ub (n) Sic1 Degradation

1. 2x Degradation buffer: 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 4 mM ATP, 2 mM DTT, 0.02 g/L BSA.
2. 10 g/L BSA
3. 40% TCA solution chilled on ice

3. Methods

3.1. Ub conjugate binding

1. Set up a 60 μ L reaction with 30 nm Ub(n)E6AP (see Note 9) and 10 nM 26S in 1x Binding buffer.
2. Rotate at 4°C for 30 minutes (see Note 10). Spin at 500 $\times$ g at 4°C for 3 minutes. Set aside supernatant. (see Note 11)
3. Wash the resin once with 400 μ L Wash Buffer I, rotating at 4°C for 5 minutes. Spin at 500 $\times$ g at 4°C for 3 minutes. Remove the supernatant by pipette.
4. Wash the resin twice with 400 μ L Wash Buffer II (see Note 12), rotating at 4°C for 5 minutes. Spin at 500 $\times$ g at 4°C for 3 minutes. Remove the supernatant by pipette..
5. Resuspend the resin in 310 μ L Peptidase assay buffer, aliquot 3 $\times$ 100 μ L into a 96 well plate and assay as described below.
6. Analysis involves determining the slopes for each of the binding conditions. It often takes 10 – 20 minutes for the slopes to stabilize to a linear rate. Once the slopes are determined, the background binding to non-ubiquitinated E6AP is subtracted from the initial binding, salt/UIM disrupted initial binding, and the tighter binding.

⁹.To analyze both the initial and tighter binding of the proteasome to a Ub conjugate five reactions are measured. (1) The background initial binding to a non-ubiquitinated version of the substrate (e.g. E6AP). (2) The initial binding to the ubiquitinated substrate (e.g. Ub(n)E6AP). (3) The degree of salt-sensitive binding (or UIM-disrupted binding) determined by binding at 4°C and washing with high salt. (4) The tighter binding level to the ubiquitinated substrate. (5) Any non-specific tighter binding to the non-ubiquitinated substrate.

¹⁰.A 4°C incubation will allow only initial conjugate binding, nearly all of which is sensitive to a high salt wash or competition with UIM. To achieve tighter binding, incubation at 37°C instead of 4°C is necessary.

¹¹.It is best to gently remove the supernatant by pipette, often leaving a few microliters of supernatant to avoid losing any of the resin.

¹².The initial binding assay uses a wash buffer without salt (Wash buffer IIa). To disrupt initial binding or confirm the transition to tighter binding one can use either salt disruption (Wash buffer IIb) or UIM competition (Wash buffer IIc).

3.2. Peptide hydrolysis

1. In the black 96 well, mix 1 μ l of the purified 26S proteasome (1 nM) with 99 μ l of the peptidase assay buffer.
2. Measure generation of cleaved amc at 37°C for up to 1 hour using a fluorometer ($\lambda_{\text{ex}} = 380$ nM; $\lambda_{\text{em}} = 460$ nm).
3. Calculate the peptidase activity of the proteasome by measuring slopes as arbitrary fluorescent units per minute.

3.3. Deubiquitination

1. Make deubiquitination reactions on ice

26S proteasomes	1 μ l (final 5 nM)
Deubiquitination reaction buffer	4 μ l
H ₂ O	14.5 μ l

If desired, add o-phenanthroline or Ub-vinylsulfone to inhibit different proteasomal deubiquitinases, subtract the H₂O volume by the volume of added factors and preincubate for 15 minutes at room temperature before the reaction.

2. Start the reactions by adding 0.5 μ L of tetra-Ub solution and incubate the at 37°C for 20 min.
3. Stop reactions by adding 1 μ L of DTT, 7.5 μ L of SDS-PAGE sample buffer and 1.5 μ L of H₂O. Incubate the samples at 70°C for 10 minutes.
4. Run SDS-PAGE (4–12% gradient) to resolve the proteins.
5. Transfer proteins onto PVDF membrane. (see Note 13)
6. Block membrane with 10 ml of PBSTB at room temperature for 1 hour.
7. Remove the PBSTB and incubate the membrane with 10 ml of PBST with 10 μ l of anti-ubiquitin (1/1,000 dilution) for 2 hour at room temperature or overnight at 4°C. (see Note 14)
8. Wash the membrane with 20 ml of PBST for 10 min three times.
9. Incubate the membrane with 10 ml of PBST with 1 μ l of anti-mouse IgG HRP conjugated antibody at room temperature for 1 hour. (see Note 15)
10. Wash the membrane as in step 8.
11. Incubate the membrane with 4 ml of SuperSignal West Pico Plus chemiluminescent substrate for 5 min.

¹³.PVDF binds to free and di-Ub chains much better than nitrocellulose.

¹⁴.A proper dilution of an anti-ubiquitin antibody should be determined before the assay.

¹⁵.Selection of secondary antibody and detection method can be modified.

12. Expose the membrane to X-ray film until bands of deubiquitinated products (tri-, di- and mono-ubiquitin) become evident.

3.4. ATP hydrolysis (see Note 16 and 17)

1. Prepare Malachite green assay reagent by adding 25 mL of solution C into 75 mL of solution B with vigorous stirring for 20 minutes or until precipitate disappears. Pass the solution through 0.45 μ m filter. Store well-sealed solution at room temperature
2. On ice, prepare 1.5 ml microcentrifuge tubes containing master reaction mixture (for 3 replicates) containing 8 μ L of solution A, X μ L of required drug or additional factors and H₂O to (28-X) μ L for each reaction time (e.g. 0, 15, 30, 45 and 60 minutes). (see Note 18)
3. Start the reaction by adding 4 μ L of proteasomes (10 nM final), mix briefly and place the reaction at 37°C. Reactions run for different times are started at different times; thus the 60 minute reaction starts at 0 minute, the 45 minute reaction at 15 minutes and so on) (see Note 19). Repeat this step until the last reaction.
4. At the end of the reaction (60 minute), transfer all the tubes from the water bath to ice. Let them cool down and centrifuge them briefly.
5. Put three 10 μ L aliquots of each reaction and the phosphate standard into wells of the 96 well plate.
6. Add 170 μ L of Malachite green solution to each well and wait until the color of the sample becomes dark green.
7. Add 20 μ L of citric acid to each well to stop the reactions.
8. Incubate the samples and the standard for 15 – 30 minutes at room temperature.
9. Measure the absorbance at 650 nm.
10. Calibrate readings to phosphate amounts, using the standard curve. Plot the increase in free phosphate over time and calculate the slopes, which represent ATPase activity.

3.5. Degradation of Ub(n) Sic1

1. Prepare the reaction stop tubes (one for each time point) ahead of time by aliquoting 13.3 μ L 10 g/L BSA per tube. Keep on ice.
2. Set up the degradation reaction (see Note 20)

¹⁶For proteins purified from *E. coli*, Hsp70 contamination is the major source of background activity. Incubating the affinity resin with the washing buffer containing 5 mM MgCl₂ and 1 mM ATP and then washing again with the normal washing buffer usually helps to reduce contaminating Hsp70 from the elution.

¹⁷High concentrations of glycerol can interfere the reaction. Use less than 10% glycerol in the final reactions.

¹⁸To test for ATPase contamination, prepare the sample containing 8 μ L of solution A, X μ L of drug or additional factors and (32-X) μ L of H₂O without addition of proteasomes.

¹⁹Start the reaction with the longest reaction time first. The last reaction (0 min reaction) should be mixed and used at the next step without incubation.

Master mix	1x
26S	0.5 nM
Ubiquitinated Sic1	100 nM

3. Incubate at 37°C for 0, 10, 20, 30, 40, 50, and 60 minutes. At each time, collect 6.7 μ L of the sample to 13.3 μ L 10g/L BSA on ice. Add 6.7 μ L 40% TCA. Mix and incubate on ice for at least 30 minutes.
4. Separate TCA soluble and pellet fractions by centrifugation at $10,000 \times g$ at 4°C for 10 minutes. Transfer the supernatant to 4 mL liquid scintillation fluid.
5. Resolubilize the TCA precipitate for the 0-time samples in 50 μ L 0.2 M NaOH. Add this to 4 mL liquid scintillation fluid.
6. Measure counts per minute on a liquid scintillation counter.
7. Analysis and quantification of degradation rates. Subtract from all samples the TCA soluble 0-time counts as background signal. If measuring percent substrate degraded, calculate the sample degraded per TCA-precipitated counts at time 0. Otherwise relative rates within an experiment can be compared by just determining the slope of counts per minute in the TCA-soluble fractions.

4. Conclusions

Assaying the multiple enzymatic and non-enzymatic steps of 19S and 20S components of the proteasome is essential for a full understanding of key steps in the degradation of ubiquitinated proteins. The protocols described here have proven to be useful in researches of proteasome regulation by physiological stimuli (13,39) (see chapter by VerPlank). However, assays for some proteasome functions, such as unfolding and translocation of polypeptides, have yet to be developed. For instance, the optical-trap experiment (50), which successfully assayed unfolding and translocation by ClpXP, might be suitable for analysis of the same proteasome functions.

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References

1. Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 78, 477–513 [PubMed: 19489727]
2. Smith DM, Benaroudj N, and Goldberg A (2006) Proteasomes and their associated ATPases: a destructive combination. *J Struct Biol* 156, 72–83 [PubMed: 16919475]
3. Kisselev AF, Garcia-Calvo M, Overkleeft HS, Peterson E, Pennington MW, Ploegh HL, Thornberry NA, and Goldberg AL (2003) The caspase-like sites of proteasomes, their substrate specificity, new

²⁰Typically, the degradation of Ub(n) Sic1 is measured every 10 minutes over the course of an hour. It is very important to collect the initial (time 0) point as quickly as possible, and therefore it is often advisable to stagger the start of different reactions by about 30–45 sec to ensure an accurate time course.

- inhibitors and substrates, and allosteric interactions with the trypsin-like sites. *J Biol Chem* 278, 35869–35877 [PubMed: 12815064]
4. Groll M, and Huber R (2003) Substrate access and processing by the 20S proteasome core particle. *Int J Biochem Cell Biol* 35, 606–616 [PubMed: 12672453]
  5. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, and Finley D (2000) A gated channel into the proteasome core particle. *Nat Struct Biol* 7, 1062–1067 [PubMed: 11062564]
  6. Smith DM, Fraga H, Reis C, Kafri G, and Goldberg AL (2011) ATP binds to proteasomal ATPases in pairs with distinct functional effects, implying an ordered reaction cycle. *Cell* 144, 526–538 [PubMed: 21335235]
  7. Matyskiela ME, Lander GC, and Martin A (2013) Conformational switching of the 26S proteasome enables substrate degradation. *Nat Struct Mol Biol* 20, 781–788 [PubMed: 23770819]
  8. Cui Z, Scruggs SB, Gilda JE, Ping P, and Gomes AV (2014) Regulation of cardiac proteasomes by ubiquitination, SUMOylation, and beyond. *J Mol Cell Cardiol* 71, 32–42 [PubMed: 24140722]
  9. Collins GA, and Goldberg AL (2017) The Logic of the 26S Proteasome. *Cell* 169, 792–806 [PubMed: 28525752]
  10. Li X, Thompson D, Kumar B, and DeMartino GN (2014) Molecular and cellular roles of PI31 (PSMF1) protein in regulation of proteasome function. *J Biol Chem* 289, 17392–17405 [PubMed: 24770418]
  11. Ortega J, Heymann JB, Kajava AV, Ustrell V, Rechsteiner M, and Steven AC (2005) The axial channel of the 20S proteasome opens upon binding of the PA200 activator. *J Mol Biol* 346, 1221–1227 [PubMed: 15713476]
  12. Ma CP, Slaughter CA, and DeMartino GN (1992) Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain). *J Biol Chem* 267, 10515–10523 [PubMed: 1587832]
  13. Lokireddy S, Kukushkin NV, and Goldberg AL (2015) cAMP-induced phosphorylation of 26S proteasomes on Rpn6/PSMD11 enhances their activity and the degradation of misfolded proteins. *Proc Natl Acad Sci U S A* 112, E7176–7185 [PubMed: 26669444]
  14. Guo X, Wang X, Wang Z, Banerjee S, Yang J, Huang L, and Dixon JE (2016) Site-specific proteasome phosphorylation controls cell proliferation and tumorigenesis. *Nat Cell Biol* 18, 202–212 [PubMed: 26655835]
  15. Besche HC, Sha Z, Kukushkin NV, Peth A, Hock EM, Kim W, Gygi S, Gutierrez JA, Liao H, Dick L, and Goldberg AL (2014) Autoubiquitination of the 26S proteasome on Rpn13 regulates breakdown of ubiquitin conjugates. *The EMBO journal* 33, 1159–1176 [PubMed: 24811749]
  16. Lee C, Schwartz MP, Prakash S, Iwakura M, and Matouschek A (2001) ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol Cell* 7, 627–637 [PubMed: 11463387]
  17. Peth A, Uchiki T, and Goldberg AL (2010) ATP-dependent steps in the binding of ubiquitin conjugates to the 26S proteasome that commit to degradation. *Mol Cell* 40, 671–681 [PubMed: 21095592]
  18. Nathan JA, Kim HT, Ting L, Gygi SP, and Goldberg AL (2013) Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *EMBO J* 32, 552–565 [PubMed: 23314748]
  19. Deveraux Q, Ustrell V, Pickart C, and Rechsteiner M (1994) A 26 S protease subunit that binds ubiquitin conjugates. *J Biol Chem* 269, 7059–7061 [PubMed: 8125911]
  20. Qiu XB, Ouyang SY, Li CJ, Miao S, Wang L, and Goldberg AL (2006) hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37. *The EMBO journal* 25, 5742–5753 [PubMed: 17139257]
  21. Shi Y, Chen X, Elsasser S, Stocks BB, Tian G, Lee BH, Shi Y, Zhang N, de Poot SA, Tuebing F, Sun S, Vannoy J, Tarasov SG, Engen JR, Finley D, and Walters KJ (2016) Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. *Science* 351
  22. Eralles J, Hoyt MA, Troll F, and Coffino P (2012) Functional asymmetries of proteasome translocase pore. *J Biol Chem* 287, 18535–18543 [PubMed: 22493437]

23. Kisselev AF, and Goldberg AL (2001) Proteasome inhibitors: from research tools to drug candidates. *Chem Biol* 8, 739–758 [PubMed: 11514224]
24. Kisselev AF, and Goldberg AL (2005) Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. *Methods Enzymol* 398, 364–378 [PubMed: 16275343]
25. Sledz P, Unverdorben P, Beck F, Pfeifer G, Schweitzer A, Forster F, and Baumeister W (2013) Structure of the 26S proteasome with ATP-gammaS bound provides insights into the mechanism of nucleotide-dependent substrate translocation. *Proc Natl Acad Sci U S A* 110, 7264–7269 [PubMed: 23589842]
26. Smith DM, Chang SC, Park S, Finley D, Cheng Y, and Goldberg AL (2007) Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. *Mol Cell* 27, 731–744 [PubMed: 17803938]
27. Kisselev AF, Callard A, and Goldberg AL (2006) Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem* 281, 8582–8590 [PubMed: 16455650]
28. Borodovsky A, Kessler BM, Casagrande R, Overkleeft HS, Wilkinson KD, and Ploegh HL (2001) A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *The EMBO journal* 20, 5187–5196 [PubMed: 11566882]
29. Li T, Naqvi NI, Yang H, and Teo TS (2000) Identification of a 26S proteasome-associated UCH in fission yeast. *Biochem Biophys Res Commun* 272, 270–275 [PubMed: 10872838]
30. Stone M, Hartmann-Petersen R, Seeger M, Bech-Otschir D, Wallace M, and Gordon C (2004) Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. *J Mol Biol* 344, 697–706 [PubMed: 15533439]
31. Yao T, and Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* 419, 403–407 [PubMed: 12353037]
32. Verma R, Aravind L, Oania R, McDonald WH, Yates JR, 3rd, Koonin EV, and Deshaies RJ (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298, 611–615 [PubMed: 12183636]
33. Kuo CL, and Goldberg AL (2017) Ubiquitinated proteins promote the association of proteasomes with the deubiquitinating enzyme Usp14 and the ubiquitin ligase Ube3c. *Proc Natl Acad Sci U S A* 114, E3404–E3413 [PubMed: 28396413]
34. Lee BH, Lee MJ, Park S, Oh DC, Elsasser S, Chen PC, Gartner C, Dimova N, Hanna J, Gygi SP, Wilson SM, King RW, and Finley D (2010) Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature* 467, 179–184 [PubMed: 20829789]
35. Lee BH, Lu Y, Prado MA, Shi Y, Tian G, Sun S, Elsasser S, Gygi SP, King RW, and Finley D (2016) USP14 deubiquitinates proteasome-bound substrates that are ubiquitinated at multiple sites. *Nature* 532, 398–401 [PubMed: 27074503]
36. Peth A, Besche HC, and Goldberg AL (2009) Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. *Mol Cell* 36, 794–804 [PubMed: 20005843]
37. Peth A, Kukushkin N, Bosse M, and Goldberg AL (2013) Ubiquitinated proteins activate the proteasomal ATPases by binding to Usp14 or Uch37 homologs. *J Biol Chem* 288, 7781–7790 [PubMed: 23341450]
38. Kim HT, and Goldberg AL (2017) The deubiquitinating enzyme Usp14 allosterically inhibits multiple proteasomal activities and ubiquitin-independent proteolysis. *J Biol Chem* 292, 9830–9839 [PubMed: 28416611]
39. VerPlank JJS, Lokireddy S, Feltri ML, Goldberg AL, and Wrabetz L (2018) Impairment of protein degradation and proteasome function in hereditary neuropathies. *Glia* 66, 379–395 [PubMed: 29076578]
40. Li J, Yakushi T, Parlati F, Mackinnon AL, Perez C, Ma Y, Carter KP, Colayco S, Magnuson G, Brown B, Nguyen K, Vasile S, Suyama E, Smith LH, Sergienko E, Pinkerton AB, Chung TDY, Palmer AE, Pass I, Hess S, Cohen SM, and Deshaies RJ (2017) Capzimin is a potent and specific inhibitor of proteasome isopeptidase Rpn11. *Nat Chem Biol* 13, 486–493 [PubMed: 28244987]

41. Perez C, Li J, Parlati F, Rouffet M, Ma Y, Mackinnon AL, Chou TF, Deshaies RJ, and Cohen SM (2017) Discovery of an Inhibitor of the Proteasome Subunit Rpn11. *Journal of medicinal chemistry* 60, 1343–1361 [PubMed: 28191850]
42. Benaroudj N, Zwickl P, Seemuller E, Baumeister W, and Goldberg AL (2003) ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol Cell* 11, 69–78 [PubMed: 12535522]
43. Goldberg AL (1992) The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur J Biochem* 203, 9–23 [PubMed: 1730246]
44. Menon AS, and Goldberg AL (1987) Protein substrates activate the ATP-dependent protease La by promoting nucleotide binding and release of bound ADP. *J Biol Chem* 262, 14929–14934 [PubMed: 3312197]
45. Peth A, Nathan JA, and Goldberg AL (2013) The ATP costs and time required to degrade ubiquitinated proteins by the 26 S proteasome. *J Biol Chem* 288, 29215–29222 [PubMed: 23965995]
46. Lanzetta PA, Alvarez LJ, Reinach PS, and Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. *Analytical biochemistry* 100, 95–97 [PubMed: 161695]
47. Saeki Y, Isono E, and Toh EA (2005) Preparation of ubiquitinated substrates by the PY motif-insertion method for monitoring 26S proteasome activity. *Methods Enzymol* 399, 215–227 [PubMed: 16338358]
48. Bhattacharyya S, Renn JP, Yu H, Marko JF, and Matouschek A (2016) An assay for 26S proteasome activity based on fluorescence anisotropy measurements of dye-labeled protein substrates. *Anal Biochem* 509, 50–59 [PubMed: 27296635]
49. Chung CH, and Goldberg AL (1981) The product of the lon (capR) gene in *Escherichia coli* is the ATP-dependent protease, protease La. *Proc Natl Acad Sci U S A* 78, 4931–4935 [PubMed: 6458037]
50. Iosefson O, Olivares AO, Baker TA, and Sauer RT (2015) Dissection of Axial-Pore Loop Function during Unfolding and Translocation by a AAA+ Proteolytic Machine. *Cell reports* 12, 1032–1041 [PubMed: 26235618]
51. Gilda JE, Ghosh R, Cheah JX, West TM, Bodine SC, and Gomes AV (2015) Western Blotting Inaccuracies with Unverified Antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). *PLoS One* 10, e0135392 [PubMed: 26287535]

Figure 1.

Stimulation of peptidase activities of purified mouse embryonic fibroblast 26S proteasomes by ATP γ S. Activities were assayed by measuring hydrolysis of fluorogenic peptide-amc substrates (10 μ M) in the presence of 100 μ M ATP or ATP γ S. The markedly stimulated hydrolysis of all three substrate in the presence of ATP γ S over the basal activities indicates gate-opening induced by the non-hydrolysable nucleotide. The basal peptidase activity of the proteasomes was measured in the presence of ATP. Peptidase activities are presented as the Means $\pm$ SD of at least 3 independent measurements. (Modified from Kim and Goldberg, 2017(38))

Figure 2.

Disassembly of K63 tetra Ub-chain (368 nM) by WT 26S proteasomes and lacking Usp14 (Usp14KO) was assayed after incubation at 37°C for 20 min. Proteasomes were purified from mouse embryonic fibroblast. The generation of tri-, di- and mono-Ubs was analyzed by Western blotting after electrophoresis. Usp14KO 26S proteasomes disassembled tetra-Ub chains much faster than WT 26S particles. Although Ub-vinylsulfone (Ub-VS, 1.5 μ M) did not inhibit Ub chain hydrolysis, o-phenanthroline (OPT, 1 mM) inhibits markedly and reduces deubiquitination by Usp14KO 26S to the level of WT 26S. Thus, the faster disassembly of Ub chains by the Usp14KO 26S involves the Zn^{2+} -metalloprotease Rpn11 and not Uch37. (Adopted data from Kim and Goldberg, 2017(38))

Figure 3.

ATP hydrolysis by mouse embryonic fibroblast 26S proteasomes was measured using a modified Malachite-Green assay (46). Upon addition of casein (1 μ M) and a hexa-Ub chain (1 μ M), which together mimic the binding of a Ub conjugate (37), ATP hydrolysis of WT 26S increased 2–3 fold. However, hexa-Ub alone or casein alone did not increase ATP hydrolysis. 6Ub: a linear hexa-Ub chain. Data are represented as means $\pm$ SD. (Modified from Kim and Goldberg, 2017(38))