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[25] Purification and Analysis of Recombinant 11S Activators of the 20S Proteasome: *Trypanosoma brucei* PA26 and Human PA28α, PA28β, and PA28γ

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Abstract

Proteasomes perform the bulk of nonlysosomal degradation of aberrant, damaged, misfolded, and naturally short-lived regulatory proteins in eukaryotic cells. They are ~700-kDa assemblies whose hollow architecture sequesters the proteolytic sites inside a central chamber, thereby ensuring that the activity of isolated proteasomes is repressed. *In vivo*, proteasomes are activated by protein complexes, including the 11S activators (PA28 and PA26), which bind to one or both ends of the barrel-shaped structure. This chapter describes protocols for the purification of recombinant 11S regulators, characterization of their ability to stimulate proteasome activity, and crystallization of proteasome complexes.

Introduction

The proteasome (also called 20S proteasome or core particle) performs the bulk of nonlysosomal protein degradation in eukaryotes. This activity is of central importance for a myriad of cellular processes, including housekeeping functions and regulatory pathways. Crystal structures of archaeal (Löwe *et al*., 1995) and eukaryotic proteasome (Groll *et al*., 1997; Unno *et al*., 2002) revealed a barrel-shaped, ~700-kDa assembly that is composed of 28 subunits arranged in four heptameric rings, with the end rings composed of α subunits and the two central rings composed of β subunits. Archaeal proteasomes generally consist of only one type of α and one type of β subunit, each repeated seven times in their respective rings. In contrast, eukaryotic proteasomes contain seven different α subunits

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(α1–α7) and seven different β subunits (β1–β7), with each distinct subunit occupying a unique position in its ring.

The mechanism by which the proteasome avoids indiscriminate degradation of inappropriate substrates was revealed by the crystal structures, which showed that the proteolytic active sites are sequestered inside a central catalytic chamber that is formed by the double ring of β subunits. Substrates enter the proteasome through the α-annulus (Wenzel and Baumeister, 1995), which has a diameter of 17 Å (measured between atom centers), and products are believed to exit through this same opening also. This narrow opening prevents the entry of folded proteins. Access is further restricted by the N-terminal tails of α subunits, which in the case of eukaryotic proteasomes are ordered and form a precisely closed gate structure. For archaeal proteasomes, the α tails are generally disordered but still provide a barrier to entry of protein substrates (Benaroudj *et al*., 2003; Förster *et al*., 2003).

Proteasomes are activated *in vivo* by three different types of activator: 19S/PA700/RC (DeMartino and Slaughter, 1999; Voges *et al*., 1999), PA200 (Ustrell *et al*., 2002), and 11S (Dubiel *et al*., 1992; Hill *et al*., 2002; Ma *et al*., 1992). This chapter concerns the 11S activators, which are heptamers of ~30-kDa subunits (Johnston *et al*., 1997; Zhang *et al*., 1999) and are broadly distributed in metazoans but appear to be absent in yeasts and plants. Jawed vertebrates encode three homologs known as PA28α, PA28β, and PA28γ (also known as REGα, REGβ, and REGγ) that typically share ~45% sequence identity between PA28α and PA28β, whereas the PA28γ shares ~30% sequence identity with the α and β homologs. Simpler species encode a single PA28 that is most closely related to PA28γ. The most distantly related 11S homolog is PA26 of *Trypanosoma brucei* (Yao *et al*., 1999), which is an outlier within the family and shares 133 structurally equivalent residues, from a total of 231, with its closest relative, PA28α. The RMSD for overlap of PA26 and PA28α on Cα atoms is 1.7 Å, and only 9% of these residues are invariant between PA26 and all three human homologs (Förster *et al.*, 2005).

The biological roles of 11S activators are not entirely clear (Rechsteiner and Hill, 2005). Several observations suggest that PA28α and PA28β function in the production of peptide ligands for MHC class I molecules. These data are not entirely consistent, however, and possible roles for PA28γ and the single homolog of simpler species are not obvious. PA28 is known to form hybrid proteasomes in which 11S and 19S activators are bound to opposite ends of the same proteasome molecule, and given the relative abundance of 11S, 19S, and proteasome, it seems likely that 11S activators normally function as hybrid proteasomes (Hendil *et al*., 1998; Tanahashi *et al*., 2000). In this context, 11S activators may function to

localize the 19S–proteasome complex to a specific intracellular site or substrate or they might enhance proteasome activity, for example, by facilitating product release. Regardless of their biological roles, biochemical and structural studies of 11S activators have revealed fundamental principles of the proteasome mechanism.

Here we provide details for the purification of recombinant 11S activators, biochemical assays of their binding to proteasome and stimulation of the peptidase activity of the proteasome, generation of mutants with altered activities, and protocols for crystallization of human PA28α and PA26 complexes with yeast and archaeal proteasomes.

Activity Assays

PA28 was first identified and purified from bovine liver and heart (Ma *et al*., 1992) and from human red blood cells (Dubiel *et al*., 1992) on the basis of its ability to stimulate the hydrolysis of small fluorogenic peptide substrates by the proteasome. This assay uses model peptide substrates attached covalently by their C terminus to groups that fluoresce when the bond connecting peptide and fluorophor is cleaved. This assay is quick, convenient, and monitors the three distinct active sites of eukaryotic proteasomes. It is the method of choice for monitoring activator peaks during purification procedures. There is some concern, however, that it does not fairly report on the activity against larger, potentially more physiologically relevant substrates. Consequently, HPLC/MS assays are sometimes employed to monitor the degradation of longer peptide substrates (Dick *et al*., 1996; Li *et al*., 2001; Zhang *et al*., 1998c). Another recently applied assay uses fluorescamine to quantify the number of amino groups liberated during proteolysis (Cascio *et al*., 2002). It is important when interpreting the results of these various assays to remember that the connection between the biochemically observed stimulation of peptidase activity and the potential biological roles of 11S activators is currently unknown.

Fluorogenic Peptide Activity Assays

Fluorogenic peptide substrates are usually modified at their N terminus with *t*-butyloxcarbonyl (Boc), succinyl (Suc), or benzyloxycarbonyl (Cbz). The C-terminal leaving groups are usually 7-amido-4-methylcoumarin (MCA) or β-naphthylamide (βNA). The substrates are commonly written according to the one-letter amino acid code. The identity of the C-terminal residue reports on each of the three distinct active sites of eukaryotic proteasomes, which are located at N termini of β1 (PGPH), β2 (T-like),

and β5 (C-like), and preferentially cleave following acid, basic, and hydrophobic residues, respectively (Bochtler *et al*., 1999). Thus, commonly used substrates include Cbz-LLE-βNA for the PGPH site, Boc-FSR-MCA, PFR-MCA, and Boc-VLK-MCA for the T-like site, and Suc-LLVY-MCA, Suc-AAF-MCA, and Suc-LY-MCA for the C-like site. These reagents can be purchased from Sigma or Peptides International. Note that although preference for the P1 site of the short peptide substrates provides a useful tool, other determinants are also important for specificity in the hydrolysis of longer substrates (Bogyo *et al*., 1998; Groll *et al*., 2002; Harris *et al*., 2001; Wang *et al*., 2003).

Fluorogenic substrates, with the exception of Cbz-LLE-βNA, are prepared by dissolving the powdered solid in dimethyl sulfoxide (DMSO) to make a 10 m*M* stock, which is stored in aliquots at –80°. Immediately before use, a 10 m*M* substrate aliquot is thawed and brought to 200 μM, typically in TSD, pH 8.8 [10 m*M* Tris-HCl, 25 m*M* KCl, 10 m*M* NaCl, 1.1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT)]. Cbz-LLE-βNA is prepared by dissolving the powdered solid in DMSO to make a 10 m*M* stock and then storing at room temperature. Substrate solutions must be prepared fresh shortly before use or frozen aliquots must be used soon after thawing.

A typical assay incubates 170 ng proteasome with various amounts of activator (e.g., 100 nmol) for 10 min in 50 μl of buffer (e.g., 10 m*M* Tris, pH 7.5). The reaction is performed at a defined temperature, usually 37°. The reference reaction omits an activator. Enzymatic reactions are initiated by adding 50 μl of a 200 μM solution of fluorogenic peptide substrate with mixing by pipeting up and down gently. After incubation for various time intervals, reactions are quenched by the addition of 200 μl of ice-cold ethanol with mixing. One hundred microliters of the quenched reaction mixture is transferred to a glass culture tube (6 $\times$ 50 mm, VWR), and the fluorescence signal is measured on a LS–5 fluorescence spectrophotometer (Beckman). For MCA substrates, the instrument is set to excitation at 380 nm and emission at 440 nm, with a slit width of 3. βNA substrates require settings of excitation at 335 nm, emission at 410 nm, and a slit width of 3.

Results are expressed as fold-stimulation, i.e., ratios of the rate of fluorescence generation in the presence of 11S compared to the reference reaction. For examples, see Realini *et al*. (1997). Higher concentrations are typically used for PA28β (e.g., 440 nmol), which at lower concentrations shows significantly diminished activity, presumably because homomeric PA28β has a much weaker heptamerization affinity (Realini *et al*., 1997).

A simple version of this assay is used to identify chromatography fractions that contain an activator. Fifty-microliter reactions are made up with 10 μl of each fraction and 150 ng of purified proteasome in TSD, pH 8.8. A reference reaction with only TSD, pH 8.8, and 150 ng proteasome is also prepared. Reactions are incubated at 37° for 20 min, quenched, and fluorescence read as described earlier. Fractions corresponding to increased activity represent the activator peak.

HPLC Analysis of Longer Peptides

An HPLC/MS method has been applied to assay proteasome stimulation by PA28/REG (Li *et al*., 2001; Zhang *et al*., 1998c). For example, see Zhang *et al*. (1998c), in which two substrates were used: P21, a 21 residue peptide (SADPELALALRVSMEEQRQRQ), and BBC1, a 49 residue peptide (MKKEKARVITEEEKNFKAFASLRMARANARLFGIRAK-RAKEAAEQDGSG). Substrates are incubated with proteasome and PA28, and samples are taken at various time points (e.g., 10 min, 30 min, 135 min, 5 h, and 12 h). Reaction products are separated on a C18 HPLC column and eluted with a gradient of 0–45% acetonitrile containing 0.1% trifluoroacetic acid. Product peptide masses are analyzed by submitting fractions to mass spectrometry. This assay has revealed more complex behavior than apparent from the fluorogenic peptide release assays. For example, PA28γ decreases the rate of P21 hydrolysis but increases the rate of BBC1 hydrolysis, whereas PA28α accelerated degradation of both P21 and BBC1.

Expression and Purification of Recombinant 11S Activators

The biochemical characterization of 11S activators has been facilitated greatly by the ability to express recombinant proteins in *Escherichia coli*, including homologs from rat (Song *et al*., 1997). As described later, bacterial expression and purification procedures have been developed for recombinant human PA28α, PA28β, and PA28γ, and we have utilized procedures for *T. brucei* PA26 that were initially developed elsewhere (Yao *et al*., 1999).

Purification of PA26

All of our studies with PA26 have utilized the recombinant protein expressed in *E. coli*. This protein includes a hexahistidine tag inserted after the initiator methionine. This tag has not been removed for the subsequent structural or biochemical studies. The expression plasmid, pBtpa, was created by Yao *et al*. (1999) and includes a threonine in place of the

authentic serine residue at position 226. Our protocol is a minor modification of the published method. A 4-liter culture will normally yield ~25 mg of purified protein.

The following solutions and reagents are prepared for the purification of PA26:

1. Solutions for *E. coli* culture in low phosphate media:
   a. 10^6 micronutrient stock. A 10-ml solution of deionized water, 37 mg of ammonium molybdate, 158 mg manganese chloride tetrahydrate, 247 mg boric acid, 84 mg cobalt chloride, 25 mg copper sulfate, and 18 mg zinc sulfate.
   b. 10× MOPS salts. A 1-liter solution is prepared with 0.4 *M* MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.4, 40 m*M* tricine, 0.5 *M* sodium chloride, 95 m*M* ammonium chloride, 0.1 m*M* ferrous sulfate, 2.76 m*M* potassium sulfate, 0.005 m*M* calcium chloride. Finally, 10 μl of the 10^6 micronutrient stock is added.
   c. 5× low phosphate media. A 1-liter solution is prepared with 500 ml 10× MOPS salts, 20 g glucose, 10 g casamino acids (Difco), 0.5 ml of 1 *M* potassium phosphate, pH 7.5, 5 ml of 1.5 m*M* thiamine, and 0.1 g adenine. The solution is sterile filtered with a 0.2-μm filter (Nalgene) into an autoclaved bottle and stored at 4°.
   d. Low phosphate media. Prepare shortly before use by adding 200 ml of sterile filtered 5× low phosphate media to 800 ml of sterile water. Ampicillin is added to 75 μg/ml and media swirled to mix.
2. Solutions for protein purification (all are 0.2 μm filtered and degassed for use on a chromatography workstation).
   a. TBS: 20 m*M* Tris-HCl, pH 7.9, 150 m*M* NaCl
   b. TBSI: TBS made up with 0.5 *M* imidazole
   c. TS_{200}: 10 m*M* Tris-HCl, pH 7.1, 190 m*M* KCl, 10 m*M* NaCl, 1.1 m*M* $MgCl_2$, 0.1 m*M* EDTA
3. Columns for purification: 20 ml Ni-NTA (Qiagen), HiPrep Superdex 200 26/60 HR (Amersham Biosciences).

Escherichia coli BL21(DE3) is transformed with pBtpa and grown overnight on a LB agar plate with 50 μg/ml ampicillin at 37° (Yao *et al.*, 1999). A 10-ml starter culture is initiated by inoculating a single colony into low phosphate media. The culture is grown overnight with constant shaking at 37° and centrifuged to pellet cells. The supernatant is decanted and cells are resuspended in 10 ml of fresh media. Four 2-liter culture flasks are each prepared with 1 liter of low phosphate media and 1 ml of the starter culture. Cells are grown at 37° with constant shaking (200 rpm) until reaching a density of $OD_{600} = 1.6$ (typically after 20–24 h) before harvesting by centrifugation at 8000*g* (4°, 15 min). Cell pellets are stored at –80°.

Cells are thawed on ice and resuspended in TBS (3–5 ml TBS/g of cell paste) to which a protease inhibitor cocktail tablet (complete, EDTA-free, protease inhibitor cocktail tablets, Roche) is added. Cells are lysed by a French pressure cell (two passes, minimum 5000 psi) followed by three rounds of 30 s of sonication interspersed with 30 s on ice. The lysate is clarified by centrifugation (39,000*g*, 30 min, 4°) and the supernatant is 0.2 μm filtered. All subsequent steps are performed at 4°.

The clarified supernatant is applied to a 20-ml Ni-NTA column equilibrated in TBS. The column is washed with 5 column volumes (CV) of TBS followed by 5 CV of 95% TBS:5% TBSI. Protein is eluted with 5 CV of 100% TBSI. Fractions (5 ml) are monitored by SDS–PAGE, and fractions corresponding to the main peak of PA26 are pooled and dialyzed against 2 liters of TS_{200} + 4 m*M* EDTA for at least 2 h, followed by dialysis against 2 liters of TS_{200} + 2 m*M* DTT for at least 2 h. The protein is concentrated by ultrafiltration (Amicon Stirred Cell with YM30 filter, Millipore) to not more than 20 mg/ml (Bradford method). Up to 1 ml of the concentrated sample is loaded onto the Superdex 200 column that has been equilibrated in TS_{200} + 1 m*M* DTT, with multiple runs performed as needed for protein volumes in excess of 1 ml. Protein is eluted in TS_{200} + 1 m*M* DTT at a flow rate of 0.5 ml/min; 2-ml fractions are collected. The protein elutes ~180 ml after injection, and the purest fractions (by SDS–PAGE) are pooled and the protein stored at 4°. For example chromatograms and gels, see Yao *et al.* (1999). We have always used the protein within a few days of completing the preparation and have not attempted longer term storage.

Purification of Recombinant Human PA28α, PA28β, and PA28γ

Our procedure for the purification of recombinant human PA28α/REGα, PA28β/REGβ, and PA28γ/REGγ is a minor adaptation of the published method (Realini *et al.*, 1997). These recombinant proteins do not have extraneous sequences, such as affinity tags. A 2-liter culture will typically yield ~10 mg of ~95% pure PA28.

The following solutions and reagents are prepared for the purification of PA28.

1. Solutions for protein purification (all are 0.2 μm filtered and degassed for use on a chromatography workstation).
   a. TSD, pH 8.8: 10 m*M* Tris-HCl, 25 m*M* KCl, 10 m*M* NaCl, 1.1 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 1 m*M* DTT
   b. TSD400, pH 8.8: TSD, pH 8.8, + 365 m*M* KCl
   c. TSD pH 7.2: TSD, pH 8.8, with pH adjusted to pH 7.2
   d. TSD200, pH 7.2: TSD, pH 7.2, + 165 m*M* KCl

2. Columns for purification: DEAE (HR10/30 column packed with DEAE-Sepharose Fast Flow resin, Amersham Biosciences) and HiPrep Superdex 200 26/60 (Amersham Biosciences)

Escherichia coli BL21(DE3) is transformed with the appropriate plasmid (pAED4-REGα, pAED4-REGβ, pAED4-REGγ) (Realini *et al.*, 1997) and plated onto LB agar, ampicillin (50 μg/ml). Fifty milliliters of LB medium, supplemented with 100 μg/ml of ampicillin, is inoculated with a single colony. The culture is grown overnight (30°, 200 rpm) and is used to inoculate 2 liters of LB medium with ampicillin at 100 μg/ml. Following growth (30°, 200 rpm) to an OD_{600} of 0.3, isopropyl-β-D-thiogalactopyranoside (IPTG) is added to 0.5 m*M*. Two hours postinduction, cells are harvested by centrifugation (4°, 15 min, 4000*g*) and resuspended in cold (4°) TSD, pH 8.8. Cells are harvested by centrifugation at 3700*g* for 15 min and stored at –80°.

Cells are thawed on ice and resuspended in TSD, pH 8.8, to a final volume of ~45 ml to which a protease inhibitor cocktail tablet (complete protease inhibitor cocktail tablets, Roche) is added. Lysis and clarification are performed as described earlier for PA26. All subsequent steps are performed at 4°.

The clarified, filtered lysate is applied to the DEAE column. The loaded column is washed with 1 CV of TSD, pH 8.8. A gradient is run over 26 CV from TSD pH 8.8, to TSD400, pH 8.8. Five-milliliter fractions are collected, and the purest fractions, as estimated by SDS–PAGE, are pooled and concentrated by ultrafiltration (Amicon Stirred Cell with YM30 filter, Millipore), without exceeding a protein concentration of 20 mg/ml (determined by the Bradford assay). For example gels and chromatograms for these preparations, see Realini *et al.* (1997). Overconcentration at this stage can lead to the formation of soluble aggregates, as revealed by dynamic light scattering. Up to 2 ml of the concentrated sample is loaded onto the Superdex 200 column equilibrated in TSD200, pH 7.2, with additional runs performed as necessary for volumes larger than 2 ml. The protein is eluted with the same solution at 0.5 ml/min. After 100 ml of elution, 5-ml fractions are collected until 300 ml, with protein elution monitored by A_{280}. In the case of PA28α, PA28γ, and heteroligomeric PA28α/β, it is common to see three distinguishable peaks: a small peak corresponding to soluble aggregates eluting in the void volume, a predominant peak corresponding to the heptameric state eluting at ~160 ml, and a small peak representing monomeric protein eluting after 200 ml. In the case of PA28β, the majority of protein elutes at retention volumes expected for a dimer or monomer state. Fractions are assayed for activity and SDS–PAGE is used to identify the purest fractions. Recombinant human

PA28 proteins run as single bands at ~32 kDa on SDS–PAGE. Fractions showing the greatest purity are pooled and concentration is determined by the Bradford method. The protein is typically dialyzed against TSD, pH 7.2, and stored at 4°. The protein can also be dialyzed against TSD, pH 7.2, + 10% glycerol, flash frozen in liquid nitrogen, and stored at –80°.

Preparation of Heteroligomeric PA28 Complexes

PA28α and PA28β copurify from tissues (Mott *et al*., 1994), and the recombinant proteins preferentially form a heteroligomer (Realini *et al*., 1997). Preparation of heteroligomers by mixing the separately purified recombinant proteins has been used to investigate the roles of the separate subunits (Song *et al*., 1997; Zhang *et al*., 1998b) and the heteroligomer stoichiometry (Zhang *et al*., 1999). This approach has also been used to investigate the potential for ''dominant-negative'' effects that might be achieved by incorporating one or a few defective subunits into an assembled heptamer (Zhang *et al*., 1998a). Typically, equal amounts of purified recombinant PA28α and PA28β are mixed and incubated overnight at 4°. The complex is purified on a Superdex 200 column as described earlier for homomeric complexes.

Generation and Selection of PA28 Activity Mutants

An efficient error-prone polymerase chain reaction (PCR) screen has been developed to generate PA28 single residue mutants that show diminished activity or altered specificity. The major advantage of this approach is that it rapidly surveys the importance of essentially all 11S residues without reliance upon prior mechanistic assumptions. For example, the first use of this method (Zhang *et al*., 1998a) identified an internal nine-residue loop (the ''activation loop'') as being especially important for the stimulation of proteasome activity without making dramatic contributions to binding affinity. This analysis was performed prior to determination of the PA28α crystal structure and contributed significantly to mechanistic interpretation of the structure (Knowlton *et al*., 1997). An adaptation of this approach was also used to find a point mutant of PA28γ that displayed altered specificity for the hydrolysis of model substrates (Li *et al*., 2001). For an illustration of the method, see Zhang *et al*. (1998a).

Identification of the activation loop (Zhang *et al*., 1998a) used pAED4-REGα as the template for PCR with the following components: 0.2 m*M* each of dGTP and dATP; 1 m*M* each of dCTP and dTTP; 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 7 m*M* $MgCl_2$, 0.01% gelatin, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer) for 100-μl reactions. After an initial melting at

94° for 4 min, 30 cycles of denaturation (1 min at 94°), annealing (1 min at 50°), and extension (3 min at 72°) are performed. Subsequent analysis demonstrated that this procedure generates mostly (60% of transformants) single site point mutants.

For the identification of active and inactive constructs, PCR products are ligated into pET11a, transformed, and colonies selected to inoculate 50-μl cultures (H medium: 10 tryptone, 8 g NaCl per liter water) in a 96-well microtiter plate and grown overnight at 30°. Aliquots from each well are transferred to the corresponding wells of another 96-well microtiter plate and induced with 0.4 m*M* IPTG at 30° for 2 h. Each aliquot of the induced cells is lysed by the addition of 30 μl of 20 m*M* Tris-HCl, pH 7.5, 1% Triton X–100, and 0.6 mg/ml polymixin B sulfate. Ten microliters of 17 ng/μl proteasome and 50 μl of 200 m*M* *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Sigma) are added to each well. The tray is incubated at 37° for 30 min and is then visualized under near-UV illumination. Colonies producing active PA28α are highly fluorescent after about 30 min of incubation at 37°; those producing inactive PA28α remain dark.

To distinguish valid nonactive mutants from clones that fail to express PA28α, reaction mixtures are transferred to a nitrocellulose membrane with a dot blot apparatus and anti-PA28α antibodies are used in a standard Western blot protocol (Harlow, 1988). To further validate the results, clones that are inactive in the peptide degradation assay yet positive for expression are rescreened using the same protocol. Clones passing the second round of screening are further characterized by sequencing their plasmids.

11S–Proteasome Binding Assays

The use of purified proteins in peptide hydrolysis assays indicates that the activator and the proteasome bind to each other. Other more direct binding assays have also proven useful. Sizing chromatography is not generally helpful because dissociation usually occurs on the column. Competition activity assays, in which nonactivating mutant PA28 proteins are tested for their ability to impair stimulation by wild-type PA28, suffer from the possibility of subunit mixing between a wild-type and a mutant activator. Our two preferred approaches are (1) an ELISA, which has the advantage that it can provide estimates of binding constants, but requires specific antibodies for proteasome and activator and (2) velocity ultracentrifugation, which can be used for any proteasome–activator pair, without need for antibodies, and is especially valuable for studies with archaeal proteasomes, which have high constitutive peptidase activity and therefore

do not display large levels of further stimulation upon addition of activator in fluorogenic peptide assays.

ELISA Binding Assay

Briefly, proteasomes are tethered to the well of an ELISA tray and incubated with a wild type or mutant activator. Unbound proteins are washed away. Bound activator is eluted with high salt buffer and quantified by immunoblot. The assay relies on a monoclonal antibody, MCP20 (a kind gift from K. Hendil, University of Copenhagen), which binds native proteasome without interfering with activator binding. This antibody is now available commercially (Affiniti Research Products).

The ELISA tray is prepared by coating the microtiter well with 200 μl of goat antimouse IgG at 20 μg/ml in 0.05 *M* carbonate, pH 9.6. Coated wells are rinsed three times in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and blocked with 200 μl of 1.5% nonfat milk in TBS-T for 2 h. Wells are filled with 200 μl of a 1:2500 dilution of ascites fluid containing MCP20 and incubated overnight at 4°. After three washes with TBS-T, each well is filled with 200 μl of human red cell proteasomes at 30 μg/ml in TBS-T and incubated overnight. The wells are washed three times with TBS-T and incubated with activator in 10 m*M* Tris, pH 7.5, for 20 min at 37° followed by an additional 150 min at 4°. Each well is quickly rinsed twice with an excess of cold 10 m*M* Tris, pH 7.5, 0.1% Tween 20 and once with 10 m*M* Tris, pH 7.5. The bound activator proteins are eluted with 200 μl of 0.5 *M* NaCl in 20 m*M* Tris, pH 7.5, and the high salt eluate is dot blotted onto nitrocellulose for subsequent detection with PA28-specific antibodies. Specific monoclonal antibodies to PA28α, β, and γ are available commercially from a number of sources, including Affiniti Research Products (Exeter, United Kingdom).

This technique can be used to determine the relative binding affinities for different activators through a competitive binding assay (Realini *et al.*, 1997). For example, this technique shows that PA28γ has a greater affinity for proteasome than PA28α because PA28γ outcompetes PA28α when an equimolar mix is allowed to bind to the tethered proteasome. This approach has also been used to demonstrate that although PA28β alone has a low affinity for proteasome, presumably because of its low heptamerization affinity, the heteroligomeric PA28α/β complex has a greater affinity for proteasome than PA28γ.

Sedimentation Velocity Binding Assay

We collect sedimentation velocity data on a Beckman Optima XL-I analytical ultracentrifuge. Immediately before the centrifugation run,

samples are dialyzed extensively against 20 m*M* Tris, pH 7.5, 200 m*M* NaCl, and 2 m*M* DTT and the used dialysis buffer is retained as a blank for background correction. Samples (proteasome alone, activator alone, various proteasome:activator ratios; final protein concentration 0.9 mg/ml) are centrifuged at 20° at a rotor speed of 42,000 rpm, and 200 interference measurements are recorded at 30-s intervals. Interference data are averaged and corrected for background against the blank. The program dcdt+ (Philo, 2000) is used for g(s*) analysis to determine values for the sedimentation coefficients. Proteasomes and activators from different species display different s* values; typical values are activator alone 8.5 s*, proteasome alone 18 s*, single capped proteasome–activator complex 22 s*, and double-capped proteasome–activator complex 24 s*.

Crystallization

We have made extensive efforts at crystallizing 11S activators and their complexes with proteasomes. Efforts at cocrystallization have been assisted by the remarkable lack of species specificity for proteasome–11S interactions; proteasome from almost any species will be stimulated by almost any activator homolog. This observation is explained by the high degree of structural and sequence conservation in proteasome residues that contact 11S activators (Förster *et al*., 2005). We have therefore employed a combinatorial approach to cocrystallization that screens many of the available 11S activators with proteasomes from a variety of species. Thus far we have succeeded in crystallizing human PA28α (Knowlton *et al*., 1997) and complexes of PA26 with yeast (*S. cerevisiae*) (Whitby *et al*., 2000) and archaeal (*Thermoplasma acidophilum*) proteasomes (Förster *et al*., 2005).

Crystallization of Recombinant Human PA28α

PA28α, expressed and purified as described earlier, is dialyzed into 10 m*M* MOPS, pH 7.2, 0.2 m*M* EDTA, and 1 m*M* DTT (precrystallization buffer) at 4°. Note that it is important to maintain the protein at less than 20 mg/ml throughout the purification, as higher concentrations seem to induce the formation of soluble aggregates that poison crystallization (monitored by dynamic light scattering). The protein is concentrated to 10 mg/ml using YM30 Centripep concentrators (Millipore), with concentration determined by Bradford assay. Crystals grow at 4° in sitting drops against a reservoir of 500 μl of 12% polyethylene glycol 6000 (PEG 6000), 0.1 *M* MOPS, pH 7.2, 2 *M* NaCl, and 0.9 m*M* $ZnCl_2$. The drops are 2 μl of protein solution mixed with 2 μl of reservoir solution. Crystals normally grow in 5–7 days as thin plates. The highest quality crystals are grown from protein that has been freshly purified. Crystals are transferred to a

cryoprotectant solution of 20% glycerol, 13.5% PEG 6000, 2 *M* NaCl, and 100 m*M* MOPS, pH 7.1, suspended in a rayon loop, and cooled for data collection by plunging into liquid nitrogen.

Crystallization of PA26–Yeast Proteasome Complex

Recombinant *T. brucei* PA26 is purified as described earlier, and the histidine-tagged yeast proteasome is prepared as described previously (Whitby *et al*., 2000). PA26 and proteasome are combined in a molar ratio of 2.5:1, dialyzed against 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 1 m*M* DTT (precrystal buffer), and concentrated to 10 mg/ml (determined by Bradford) with YM30 Centripep concentrators (Millipore) that have been washed in fresh precrystal buffer. It is important that PA26 is concentrated in the presence of proteasome, as it is prone to precipitate out of solution in isolation. Crystals are grown in sitting drops at 4° using a 500-μl reservoir of 0.1 *M* sodium HEPES, pH 7.5, 40% 2,4-methylpentanediol, and 0.2 *M* NaCl. The drop is composed of 4 μl of protein and 4 μl of reservoir solution. Crystals grow as rectangular blocks of 200 μm in the longest dimension and 50 μm in the smaller dimension after ~5–6 weeks of undisturbed growth. It is important not to move the trays during this time. Crystal growth is infrequent. Many preparations never yield crystals and even for those that do, X-ray grade crystals are generally only found in ~1 out of 24 drops. Crystals are prepared for data collection by suspending in a nylon loop directly from the drop and plunging into liquid nitrogen.

Crystallization of PA26–Archaeal Proteasome Complexes

PA26 and *T. acidophilum* proteasome are mixed at a molar ratio of 2.5:1 and concentrated to 10 mg/ml. Crystals grow at 21° in hanging drops. Optimization of conditions utilized the additive approach of Majeed *et al.* (2003). The reservoir solution is 90% (0.1 *M* Na citrate/phosphate, pH 4.2, 0.2 *M* Li_2SO_4, 15% PEG 1000) + 10% (1 *M* imidazole, pH 7.0). Sometimes, the initial crystals can be further improved by ''feeding'' (Bergfors, 2003), in which 2 μl of fresh protein solution is added to drops that contain a spray of tiny crystals. For data collection, crystals are transferred to 0.1 *M* Na citrate/phosphate, pH 5.7, 0.2 *M* Li_2SO_4, 20% PEG 1000, and 30% glycerol, suspended in a nylon loop, and plunged into liquid nitrogen.

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