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Research paper

Immunological methods to quantify and characterize proteasome complexes: Development and application ☆

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Abstract

The ubiquitin-proteasome pathway plays major roles in all aspects of biology and contributes to various disease processes. Due to the lack of assays that permit proteasome quantification in crude cell extracts, its concentrations in health and disease states as well as the relationship between free 20S core particles (20S) and 26S proteasomes (26S) that consist of 20S singly or doubly capped with 19S regulator complexes (19S) are still largely unknown. Thus, we established a 20S ELISA for the detection of total 20S, and developed a specific 26S ELISA. The latter utilizes the ATP/Mg^{2+} requirement for 26S stability and shows no cross-reactivity with 20S. Both ELISAs demonstrate intra- and inter-assay variations between 4.9% and 9.4% and recoveries of 105%–109%. Initial application showed that maintenance of the physiological ATP concentration is essential for accurate 26S assessment. Measurements in erythrocyte and peripheral blood mononuclear cell (PBMNC) extracts revealed that the concentrations of 20S were 15-fold and of 26S 130-fold higher in PBMNCs, and suggested that the 26S is the physiological relevant form in PBMNCs (molar ratio 20S/26S 1.1 ± 0.4), whereas free 20S is predominant in erythrocytes (molar ratio 20S/26S: 11.5 ± 4.0). During storage of packed red blood cell units spontaneous 26S assembly was detectable while specific 26S enzyme activities decreased, indicating that these assays are useful to assess the dynamic interplay between the 20S and 19S. During 26S assay development we further observed that solid phase affinity immobilization (SPAI) of 26S enables quantification of its dissociation into 20S and 19S. Utilizing the SPAI-26S method in combination with the non-hydrolyzable analogue ATP[β,γ-NH] and Mg^{2+} depletion, we provided evidence that ATP binding without hydrolysis via a high affinity binding site (Kd 4–6 μM) as well as ATP binding with hydrolysis via a low affinity binding site that is virtually not saturable under physiological conditions is required to fully stabilize the 26S. Application of these immunological techniques is expected to facilitate proteasome analyses, and may help to better understand its roles in health and disease processes.

Keywords: 20S core particle; 19S regulator complex; 26S proteasome; ATP binding sites; ATP hydrolysis; Solid phase affinity immobilization

1. Introduction

The ubiquitin-proteasome pathway (UPP) is the principal non-lysosomal proteolytic system (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002; Ciechanover, 2003). In this pathway, ubiquitin-protein ligase systems, which traditionally consist of a ubiquitin-

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activating enzyme, a ubiquitin carrier or conjugating enzyme and a ubiquitin-protein ligase, catalyze the covalent ligation of ubiquitin to intracellular proteins (=ubiquitination or ubiquitylation) (Hershko and Ciechanover, 1998). The ubiquitylated protein is then destined for degradation by the multicatalytic 26S proteasome. The 26S proteasome is formed from a ~700 kDa cylinder-shaped multimeric protein complex referred to as the 20S proteasome core particle and either singly or doubly capped at its ends by a regulatory component termed the 19S regulator complex, which confers Mg^{2+}/ATP dependency and ubiquitylated substrate specificity (Orlowski, 1990; Rivett, 1993; Baumeister et al., 1998; Hershko and Ciechanover, 1998; Groll and Huber, 2004). The 20S core particle is composed of four stacked rings. Each ring consists of seven α- and β-type subunits ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$). In eukaryotic 20S core particles, the proteolytic active sites are located in the subunits β1, β2 and β5 of the inner rings (Groll and Huber, 2004). While Mg^{2+}/ATP is required for assembly and function of the 26S complex, the 20S core particle alone is involved in the degradation of misfolded and damaged proteins, independent of Mg^{2+}/ATP or ubiquitylation (Eytan et al., 1993; Orlowski and Wilk, 2003). The 700 kDa 19S regulator complex consists of at least 17 subunits. It is arranged as a base consisting of six AAA (*A*TPases *a*ssociated with different cellular *a*ctivities family) ATPase subunits (Rpt1–6) and three non-ATPase subunits (Rpn1, 2 and 10) that interacts with the 20S core particle, and a lid consisting of eight non-ATPase subunits (Glickman et al., 1998; Neuwald et al., 1999).

The major biological relevance of the UPP is well accepted and studies on its role in disease processes accumulated exponentially during the past decade. While enzyme linked immunosorbent assays (ELISA) for the quantification of the 20S proteasome core particle in human plasma and serum have been described previously (Dutaud et al., 2002; Egerer et al., 2002; Roth et al., 2005), applicability of theses methods for cell extracts or crude enzyme preparations has not been shown and methods to quantify 26S proteasome complexes are not available. To fill this gap, we established a 20S proteasome sandwich ELISA for the use in cell extracts, and developed a highly specific sandwich ELISA for 26S complexes. The 26S ELISA utilizes the ATP/Mg^{2+} requirement for 26S complex stability. During assay development for 26S complexes we further observed that solid phase affinity immobilization (SPAI) of the 26S proteasome complex can also be used to characterize its dissociation into the 19S regulator complex and the 20S proteasome core particle, thus providing a new tool to analyze proteasome characteristics.

2. Materials and methods

2.1. Antibodies

The following commercially available mono- and polyclonal antibodies (mAb/pAb) were used: mAb 20S subunit α6 (PW8100), pAb 20S "core subunits (α5, α7, β1, β5, β5i, β7)" (PW8155), mAb 19S subunit Rpt5 (PW8770) (all from Biomol, Plymouth Meeting, PA), pAb 19S subunit Rpn2 (AP-104, Boston Biochem, Boston, MA), horseradish peroxidase (HRP) linked donkey anti-rabbit and sheep anti-mouse IgG (NA934V and NA931V, GE Healthcare, Piscataway, NJ).

2.2. General ELISA protocol

A single protocol was used for the 20S and 26S proteasome ELISA. The individual antibody combinations and dilutions are shown in Table 1. Microtiter plates (Nunc Maxisorb, Nalge Nunc International, Rochester, NY) were coated with the capture antibodies diluted in phosphate buffer saline (PBS), pH 7.4 overnight at 4 °C. After coating plates were blocked with PBS, 1% bovine serum albumin (Sigma, PBS–BSA) for 1 h at room temperature. Standard curves were prepared employing highly purified 20S (PW8270) and 26S proteasomes (PW 9310, both from Biomol) in a concentration of 5000 ng/mL to 7.5 ng/mL diluted in PBS–BSA. The 26S proteasome preparations contain singly and doubly capped 20S core particles in a ratio of 1/1.5 (Biomol). Cell extracts were diluted 1/5–1/500 in PBS–BSA. 100 μL of standards and samples were placed in the wells and incubated for 2 h at room temperature. After washing with PBS, 0.05% tween 20, the secondary antibody diluted in PBS–BSA was added to the wells and incubated for 1 h at room temperature. The plates were washed again and HRP-labeled anti-rabbit or anti-mouse diluted in PBS–BSA was added and incubated for 1 h at room temperature. Plates were washed again and the bound antibodies were detected using tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO). The reaction was stopped by addition of 50 μL 2 N HCl and the optical

Table 1
Antibody combinations and dilutions

ELISA	Capture Ab (dilution factor)	Secondary Ab (dilution factor)	Conjugate (dilution factor)
20S	mAb 20S subunit α6 (1/15,000)	pAb 20S "core" (1/2000)	Goat anti-rabbit (1/2000)
26S	pAb 19S subunit Rpn2 (1/500)	mAb 20S subunit α6 (1/1000)	Sheep anti-mouse (1/1000)