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Assembly of proteasome subunits into non-canonical complexes *in vivo*

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ABSTRACT

Proteasomes exist in all domains of life. In general, they are comprised of a compartmentalized protease whose activity is modulated by one or more regulatory complexes with which it interacts. The quaternary structure of this compartmentalized protease, called the 20S proteasome, is absolutely conserved and consists of four heptameric rings stacked coaxially. The rings are made of structurally related α and β subunits. In eukaryotes, assembly factors chaperone the α and β subunits during 20S biogenesis. Here we demonstrate that proteasome subunits can assemble into structures other than the canonical 20S proteasome *in vivo*. Specifically, the yeast α4 subunit forms high molecular weight complexes whose abundance increases when proteasome function is compromised. Results from a disulfide crosslinking approach are consistent with these complexes being ring-shaped. Though several eukaryotic α subunits can form rings when expressed recombinantly in bacteria, this is the first evidence that such non-canonical complexes exist *in vivo*.

1. Introduction

The 26S proteasome comprises two major sub assemblies: the 20S proteasome, also known as the core particle (CP), and a 19S regulatory particle (RP) [1,2]. The CP consists of four heptameric rings built up from two structurally related subunits, α and β, in an $\alpha_7\beta_7\beta_7\alpha_7$ arrangement [3–5]. In eukaryotes, the α and β rings contain seven different subunits, and peptide hydrolysis activity resides within the β1, β2, and β5 subunits. Assembly of the CP is guided by structural features intrinsic to the CP subunits and by extrinsic factors in the form of dedicated assembly factors [6]. There are five such factors in eukaryotes, Ump1 and Pba1–4 (called PAC1–4 in mammals).

Assembly of the CP begins with the formation of α-rings which serve as a platform for the subsequent entry of β subunits until a half-proteasome (i.e. $\alpha_7\beta_7$) intermediate is formed [7]. Two half-proteasomes dimerize to give rise to the CP, a process that coincides with the autocatalytic removal of propeptides on the enzymatically active β subunits [8]. The assembly factors carry out several functions, including: ensuring the correct placement of subunits within the rings [9], performing key checkpoint functions [10], preventing non-desirable interactions between CP subunits themselves [11,12] and preventing binding of RP to immature CP species [13]. The end result is a fully functional CP with all subunits occupying a defined position. However, evidence has been mounting that the structure of the CP need not be considered fixed. In the yeast *Saccharomyces cerevisiae*, the α3 subunit is not essential for viability; in α3Δ yeast cells, an alternative CP is formed in which a second copy of α4 occupies the position normally held by α3 [14]. These "α4–α4 proteasomes", so-called because their α-rings now contain two neighboring α4 subunits, also arise in yeast when the Pba3-Pba4 assembly factor is absent [9] even though α3 is still present. Recently, α4–α4 proteasomes were shown to exist in mammalian cells, arguing that formation of this alternative CP is evolutionarily conserved [15]. Here, we present additional evidence that the CP structure need not be considered immutable. In addition to enabling the formation of α4–α4 proteasomes, we find that α4 assembles into high molecular weight complexes (HMWCs) that are most likely α4 rings. Some eukaryotic α subunits can form non-canonical rings when expressed recombinantly in bacteria but the relevance of these structures was not clear [16–19]. This is the first report of such structures existing in wild-type cells *in vivo*, raising the possibility of functional significance.

Abbreviations: CP, core particle; HMWC, high molecular weight complex; RP, regulatory particle; ICAR, immobilized-cobalt affinity resin.

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2. Materials and methods

2.1. Strains and yeast culture

Yeast strains are listed in Supplementary Table 1. The α2HF strain (*MAT*a) was generated by backcrossing the α2HF *MAT*α strain kindly provided by Mark Hochstrasser. The crosslinkable α4 (α4CC) was engineered as described [14], except we employed a C-terminal Flag tag. One liter yeast cultures were grown in YPD at 30 °C to mid-log phase. Yeast cells were harvested by centrifugation at 4000 × *g* and the pellets washed with 40 ml of H_2O prior to storage at −80 °C.

2.2. Yeast lysis and Flag purification

Yeast cell pellets were thawed in cold water and gently and resuspended in 48 ml of Buffer A (40 mM Hepes-NaOH pH 7.5, 10% (v/v) glycerol, 350 mM NaCl, 0.1% (v/v) Tween-20 supplemented with yeast protease inhibitors (Sigma) according to manufacturer's instructions). The suspension was transferred to a 50 ml bead beater lysis chamber along with 0.5 mm glass beads (BioSpec). The yeast cells were lysed via bead beating for 15 cycles (1 min beating followed by 1 min rest/cooling). Total lysates were centrifuged at 11,000 rpm for 1 h at 4 °C in a Beckman J2-21M centrifuge using a JA-20 rotor. The supernatant (soluble lysate) was transferred to a fresh 50 ml conical tube and the protein concentration measured via Bradford Assay or BCA Protein Assay Kit (ThermoScientific). When processing parallel samples, equal amounts of protein from the soluble lysate were incubated with 200 μl of anti-Flag agarose resin (Sigma) overnight at 4 °C. The resin was collected in a 30 ml gravity column (Bio-Rad), washed with 60 ml of Buffer A, transferred to a fresh microcentrifuge tube, and centrifuged for 30 s at 4000 × *g*. Excess Buffer A was aspirated, and the Flag-tagged proteins were eluted with 300 μl of Flag peptide (Sigma), at a concentration of 5 μg/μl in Tris-buffered saline (TBS), for 30 min at 4 °C. The eluted proteins were collected by transferring the resin mixture to a Pierce Micro-spin column (ThermoScientific) and centrifuging at 10,000 × *g* for 3 min.

2.3. Electrophoresis

Samples were subjected to SDS-PAGE and native PAGE as described [20,21] except 4–15% non-denaturing polyacrylamide gradient gels, as well as 10%, 12% and 11–15% step gradient SDS-PAGE gels were used as indicated. For all gels, the migration of molecular size standards is indicated to the left of each gel image in the figures. The 4–15% gradient gels were precast Mini-PROTEAN TGX (Bio-Rad) while all the others were poured in lab. For native PAGE, purified protein (20 μg) was mixed with 5 × non-denaturing sample buffer (0.5 M Tris-HCl, pH 8.8, 50% (v/v) glycerol, traces of bromophenol blue). Non-denaturing gels were run at 60 V for 10 h at 4 °C. Substrate overlay assay was carried out as described [20]. Native gels were stained with Imperial Protein Stain (ThermoScientific). Loading control samples were run on reducing 12% SDS-PAGE. All SDS-PAGE gels were stained with GelCode blue (ThermoScientific).

For Western blotting, samples were run on 10% SDS-PAGE gels and transferred to PVDF membrane. The membrane was blocked overnight with 5% (w/v) non-fat milk in TBS then incubated with anti-Flag antibody (Sigma), diluted 1:2000 in 5% (w/v) non-fat milk in TBS, for 1 h at room temperature with gentle rocking. The membrane was washed 3 times with TT Buffer (0.1% (v/v) Tween-20 in TBS) for 5 min and then incubated for 1 h at room temperature with gentle rocking in TT buffer containing secondary goat anti-mouse IgG_1 antibody (SouthernBiotech) diluted 1:5000. The membrane was again washed 3 times as above before adding ECL substrate (ThermoFisher) and exposing to film.

2.4. Disulfide crosslinking

Disulfide crosslinking was performed as described [20]. Crosslinked and non-crosslinked samples were mixed with 2 × SDS sample buffer without DTT and loaded on to 11–15% SDS-PAGE step gradient gels. Where indicated, a 25 μl aliquot of each sample was reduced with 2 μl of 1M DTT at room temperature for 15 min.

2.5. Depletion assay

Yeast cell pellets were lysed and CP purified with anti-Flag agarose resin as above. The Flag eluates were subjected to depletion via immobilized-cobalt affinity resin (ICAR) as described [21], with the following modifications. The samples were applied to 150 μl of resin (Talon resin; Clontech) for 1 h at 4 °C with gentle rocking. The flow through from the first ICAR depletion was subjected to a second round of ICAR using a fresh 150 μl of resin.

2.6. Proteomic analysis

Slices cut from indicated gels were submitted to the Indiana University School of Medicine Proteomics Core Facility (IUSM-PCF) to identify proteins by LC-MS/MS. Summarized and annotated data are presented in table format throughout the manuscript and supplementary information. The data analysis files provided by IUSM-PCF in Microsoft Excel format can be found in the accompanying Data In Brief article [22].

3. Results

Initially, we set out to study the formation and function of α4–α4 proteasomes in yeast. We employed a crosslinking strategy [14] using an engineered α4 subunit (α4CC) with a C-terminal Flag epitope (Fig. 1A). When α4–α4 proteasomes are present, the α4CC subunit gives rise to a diagnostic α4 dimer on non-reducing SDS-PAGE under mildly oxidizing conditions. This dimer was readily observed when CP was purified from α3Δ yeast (Fig. 1B, lane 4). However, the α4 dimer was not observed in the wild-type sample (lane 2) consistent with previous observations that α4–α4 proteasomes are not detectable (at least using this approach) in wild-type yeast cells [9,14].

Interestingly, additional bands appeared near the top of the SDS-PAGE gel under non-reducing conditions. Like the α4 dimer, these species required the presence of α4CC and disappeared under reducing conditions (Fig. 1B); they also failed to form in the absence of added oxidant (Fig. 1C). This argues that these slowly migrating species were the result of the engineered disulfide crosslink, just like the α4 dimer. However, unlike the α4 dimer, these species were also present in wild-type cells, albeit at lower levels compared to α3Δ cells (Fig. 1B, lanes 2 versus 4). LC-MS/MS analysis confirmed the presence of α4 in these bands (not shown) consistent with the slowly migrating species being a multimer (or multimers) of α4 crosslinked to itself.

To determine if these slowly migrating species represented actual α4-containing high molecular weight complexes (HMWCs), we analyzed Flag-purified CP samples by native PAGE. The major complex in each sample, migrating near the 670 kDa size standard, was the CP (Supplementary Fig. 1). Species migrating slower than the CP were likely complexes of CP and Blm10; these were more abundant in the α3Δ samples because CP lacking α3 are constitutively open and Blm10 preferentially binds to CP with an open (or disordered) gate [23]. Species migrating faster than the CP were also observed, some of which were likely CP assembly

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