Recombinant ATPases of the yeast 26S proteasome activate protein degradation by the 20S proteasome

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Abstract The 26S proteasome contains a proteolytic core, 20S proteasome, and its regulatory particle, 19S complex. That regulatory particle contains six ATPases that are involved in unfolding and translocation of substrates to the 20S proteasome's catalytic chamber. We expressed ATPase-encoding genes of the regulatory particle of *Saccharomyces cerevisiae* and found that some recombinant ATPases can self-assemble into a high-molecular-weight protein complex in *Escherichia coli*. Purification of the Rpt1Rpt2 hetero-complex and the Rpt4 homo-complex for functional characterization demonstrated their contribution to energy-dependent protein degradation. Our finding, production of a functional subunit of the 19S regulatory particle in bacteria, is a simpler and technically advanced system to functionally characterize individual subunits.

Keywords: 26S proteasome; AAA-ATPase

1. Introduction

The 26S proteasome, which plays diverse roles in intra-cellular proteolysis, is a highly conserved protease complex among eukaryotes [1]. It comprises two major complexes: catalytic 20S proteasome and 19S regulatory particles. The 20S proteasome is a multicatalytic protease complex that sequesters catalytic centers in the inner space of the complex [1,2]. The 19S regulatory particle binds one or both ends of the 20S proteasome and confers substrate selectivity; thereby, the 26S proteasome degrades ubiquitinated proteins. The 19S regulatory particle is responsible for binding to the ubiquitin-chain-tagged substrates, unfolding, translocation into the 20S proteasome and refolding of the substrates [1,3]. The primary components comprising the regulatory particle are the base and the lid [4]. The base, which contains six ATPases and two non-ATPases, is connected to the 20S proteasome. Six ATPases belong to the AAA-ATPase family and they are homologous to each other [5]. Most AAA-ATPases generally form an oligomer and hydrolyze ATP at the interfaces of neighboring molecules [6]. The lid comprises a number of non-ATPases and is bound to the base [7].

Archaebacteria and eubacteria also have proteasomes that are well conserved with the 20S proteasome of eukaryotes [8]. The proteasome acts with an ATPase complex and degrades protein substrates in an ATP-dependent fashion. One example is the S4 homolog from *Methanococcus jannaschii*, named PAN, which forms a homo-oligomeric structure and acts as an activator of the proteasome [9]. Bacterial energy-dependent protein degradation systems are often associated with AAA-ATPase complexes as well as proteasomes; ClpAP is composed of a ClpP protease complex and an ATPase complex, ClpA. ClpXP is composed of ClpP and another ATPase complex, ClpX [10,11]. The HslUV system comprises a protease complex, HslV, and an ATPase complex, HslU [12]. In eukaryotes, the activator of the proteasome is more differentiated, but the ancestor of yeast 26S proteasome could be simply the 20S proteasome and an ATPase complex, like a bacterial and archaeal system. It seems possible to re-construct a simplified yeast proteolytic system in vitro using the 20S proteasome and an ATPase homo-oligomeric complex.

2. Materials and methods

2.1. Strains, plasmids, media and antibodies

We PCR-amplified *RPT1*, *RPT2*, *RPT3*, *RPT4*, *RPT5*, and *RPT6* genes of *Saccharomyces cerevisiae* (W303 strain). Subsequently, each DNA fragment was inserted into the *Nde*I/*Xho*I gap of pET28b (Novagen Corp., Madison, WI) to yield N-terminally 6His-tagged protein. A gene cassette containing T7 promoter, *RPT1*, and T7 terminator was inserted into the *Bgl*II site of *6His-RPT2*-expression vector to express *RPT1* and *6His-RPT2* simultaneously. Dr. Ishii (RIKEN) kindly provided a GroEL/S expression vector, pT-groE. We purchased Anti-Rpt polyclonal antibodies (Affiniti Research Products, Ltd., UK), anti-6His monoclonal antibody (Covance Inc., Richmond, CA), and anti-GroEL polyclonal antibody (Sigma–Aldrich Corp., St. Louis, MO). Alkaline phosphatase-conjugated secondary antibodies were purchased from Promega Corp. (Madison, WI).

2.2. Expression and purification of recombinant Rpt proteins

Recombinant proteins were expressed in *E. coli* BL21(DE3) carrying pT-groE [13] and experimental plasmid(s). Genes were induced at 16 °C for 20 h; then 6His-tagged proteins were isolated using Ni-NTA superflow (Qiagen, GmbH, Germany) according to the manufacturer's instructions. To isolate Rpt4, the fractions containing Rpt4 proteins were pooled and adjusted to the final buffer concentration of 0.1 M phosphate, 10% glycerol and 0.5 mM DTT and subjected to a hydroxyapatite (Bio-gel HTP; Bio-Rad Laboratories, Inc., Hercules, CA)

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column pre-equilibrated with buffer A (0.1 M potassium phosphate, pH 7.0, 10% glycerol, and 0.5 mM DTT). After washing the column with buffer A and buffer B (0.2 M potassium phosphate, pH 7.0, 10% glycerol, and 0.5 mM DTT), Rpt4 was eluted with buffer C (0.5 M potassium phosphate, pH 7.0, 10% glycerol, and 0.5 mM DTT). The eluate was diluted immediately with buffer D (20 mM HEPES–NaOH, pH 8.0, 10% glycerol, and 0.5 mM DTT) to 1/2, followed by addition of Tween 20 to a final concentration of 0.02%. After dialysis against buffer D, the sample was concentrated and subjected to 10–40% glycerol density gradient centrifugation ($85,000 \times g$ 22 h) prepared with buffer E (20 mM HEPES–NaOH, pH 8.0, 0.5 mM DTT, 0.1 mM ATP, 0.02% Tween 20 and glycerol). From a 10-l culture of *E. coli* cells, 0.3 mg of Rpt4 was isolated. To isolate the Rpt1Rpt2 complexes, glycerol density gradient centrifugation was performed before hydroxyapatite chromatography. The Rpt1Rpt2 complex (133 μg) was isolated from a 10-l culture of *E. coli*.

3. Results and discussion

3.1. Expression of recombinant ATPases

RPT1, *RPT2*, *RPT3*, *RPT4*, *RPT5*, and *RPT6* were expressed in the same *E. coli* cell to make an artificial complex of Rpt ATPases. Recombinant proteins were purified using Ni-NTA resin followed by fractionation using glycerol density gradient centrifugation. Western blotting analyses of fractions revealed that all ATPases sedimented only in low-density fractions, although they were independently expressed (data not shown). We accordingly expressed an individual ATPase. Western blot analysis of the fractions from each glycerol density gradient centrifugation revealed that sedimentation profiles of respective ATPases are different (Fig. 1). Rpt1, Rpt4 and Rpt5 were distributed in two peaks, in fractions 5–7 and 13–15. Rpt3 peaked in fractions 13–15. Rpt2 and Rpt6 were detected only in low-density fractions. Rpt1, Rpt3, Rpt4 and Rpt5 seemed to form oligomeric complexes independently, compared to GroEL/S, which forms an approximately 900 kDa complex, peaked between fractions 13 and 17. Rpt4 was the most suitable material for the substantial preparation. It was used as a representative ATPase. On native-PAGE, the migration profile of Rpt4 was nearly identical to that of the 20S proteasome (Fig. 2A). These results indicate that Rpt4 assembles into a high molecular weight complex; Rpt4 sedimented in glycerol density gradient at the similar value of

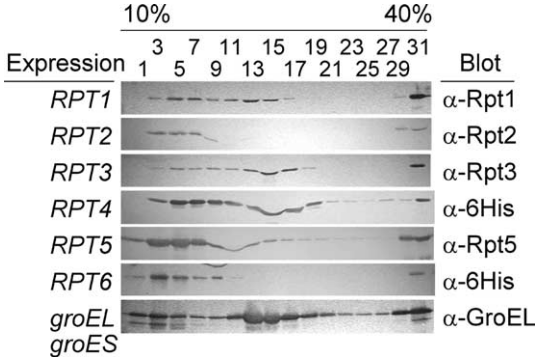

Fig. 1. Recombinant Rpt proteins form high-molecular-weight complexes. Recombinant 6His-Rpt proteins were batch-purified individually and separated by glycerol density gradient centrifugation. The fractions were subjected to Western blot analysis using anti-Rpt (to detect Rpt1, Rpt2, Rpt3, and Rpt5) or anti-6His (to detect Rpt4 and Rpt6) antibodies. Expressed genes in each experiment (left) and antibodies used for Western blot analyses (right) are shown.

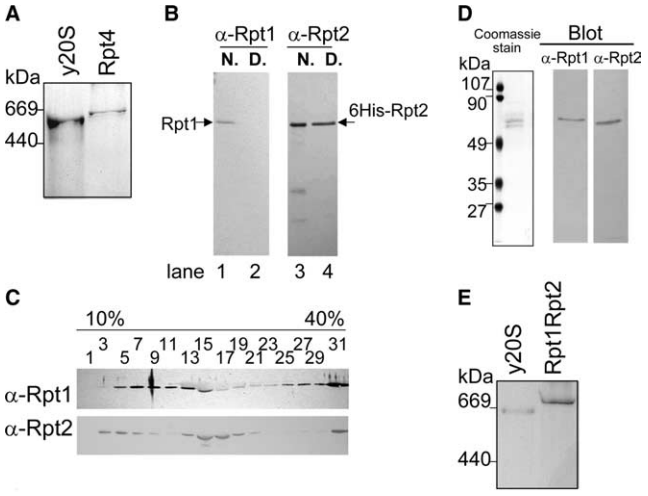

Fig. 2. Characterization of Rpt4 and Rpt1Rpt2. (A) Native-PAGE analysis of the Rpt4 complex. Purified Rpt4 protein (3.2 μg) and yeast 20S proteasome (1.2 μg) were subjected to a native-PAGE (4–20% gradient of acrylamide) analysis. (B) Co-purification of Rpt1 and Rpt2. Extract from cells expressing *6His-RPT2* and untagged *RPT1* was prepared and proteins from soluble fraction were batch-purified using Ni-NTA (N: lanes 1 and 3). Insoluble materials were dissolved in 8 M urea, followed by purification (D: lanes 2 and 4). Eluates were analyzed by Western blotting (left, anti-Rpt1; right, anti-Rpt2). (C) Co-sedimentation of Rpt1 and Rpt2. *RPT1* and *6His-RPT2* were co-expressed in *E. coli* cells and purified by Ni-NTA chromatography followed by glycerol density gradient centrifugation. Fractions of the gradient were subjected to Western blot analysis using anti-Rpt1 and anti-Rpt2 antibodies. (D) Purification of the Rpt1Rpt2 complex. Fractions 14–19 of figure (C) (over 669 kDa) were pooled and subjected to hydroxyapatite chromatography. Purified proteins were subjected to SDS–PAGE and Western blot analyses using anti-Rpt1 and anti-Rpt2 antibodies. (E) Native-PAGE analysis of the Rpt1Rpt2 complex. The yeast 20S proteasome (2.0 μg) and the Rpt1Rpt2 complex (2.9 μg) were analyzed by native-PAGE.

GroEL (Fig. 1). Another representative material was Rpt2 because Rpt2 was well characterized by genetic studies [14,15]. Attempts to express *RPT2* with a gene encoding other ATPase components, Rpt3, Rpt4, Rpt5 and Rpt1, were performed because Rpt2 could not self-assemble into a complex as stated above. We found that Rpt2 was solubilized remarkably and formed a complex only when it was co-expressed with *RPT1*. As shown in Fig. 2C, both Rpt1 and Rpt2 were detected in fractions 13 and 15 of the glycerol density gradient centrifugation. Co-expression of *6His-RPT2* and untagged *RPT1* showed that Rpt1 was detected in the eluate only when proteins were isolated under a native condition, whereas 6His-Rpt2 was eluted substantially from the Ni-NTA column under both native and denaturing conditions (Fig. 2B). Co-purification of Rpt1 and Rpt2 was also confirmed by a complementary experiment, using cells co-expressing *6His-RPT1* and untagged *RPT2* (data not shown). Reportedly, S4, a mammalian homolog of Rpt2, interacted with S7, a mammalian homolog of Rpt1, in both biochemical experiment and a yeast two-hybrid system [16,17], suggesting that Rpt2 is positioned next to Rpt1 in the 19S regulatory particle. The purified complex from high-density fractions of glycerol density gradient centrifugation (Fig. 2C) contains two proteins that were confirmed to be identical to Rpt1 and 6His-Rpt2 by Western blot analysis (Fig. 2D). The protein-staining profile implies 1:1 stoichiometry of Rpt1 and Rpt2 in the complex, suggesting

that Rpt1 and Rpt2 are incorporated alternately into a high-molecular weight complex. On native-PAGE, the Rpt1Rpt2 complex migrated a little slower than the 20S proteasome did (Fig. 2E). The Rpt1Rpt2 complex sedimented in glycerol density gradient at the same fraction of Rpt4 complex. Both Rpt1Rpt2 and Rpt4 complexes are of similar size as PAN, which forms a dimerized ring structure of 650 kDa in size [9,18]. Further purification of the Rpt4 and Rpt1Rpt2 complex was not successful because Rpt proteins are easily unstabilized and aggregated. Precise determination of molecular size and stoichiometry of the components in the complexes was not accomplished. However, because AAA-ATPase is thought to hydrolyze ATP at the interface between neighboring subunits and the structure between adjacent subunits is critical [6], it can be inferred that Rpt1Rpt2 and Rpt4 complexes have ring-shaped structures as do other AAA-ATPases complexes, e.g., HslU and PAN [5,18].

3.2. Rpt oligomer exhibits ATP-hydrolyzing activity

The ATPase activities of the purified Rpt1Rpt2 complex and Rpt4 complex were examined. Results indicated that the specific activities of Rpt1Rpt2 and Rpt4 were 0.88 and 0.13 μmol Pi/h/mg, respectively. The exhibited ATPase activity of the Rpt1Rpt2 complex was nearly the same level as that of the 19S regulatory particle from mammalian cells [19]. When the reaction mixture contained α-casein, which is known to be degraded by the proteasome, the ATPase activity of both Rpt1Rpt2 and Rpt4 was not activated. However, when the reaction mixture contained both of α-casein and the proteasome from *Thermoplasma*, the ATPase activity of Rpt1Rpt2 complex was increased markedly (2.14 μmol Pi/h/mg) and that of Rpt4 was increased slightly (Fig. 3). In contrast to α-casein, the experiment used proteasome-resistant polypeptide ECFP showed that the ATPase activity was not stimulated (data not shown). These results suggested that the ATPase activity is coupled with casein degradation. In contrast to the archaeal proteasome, we found that Tween 20 in the buffer inhibited the yeast proteasomal peptidase activity in a dose-dependent manner (data not shown). The mode of inhibitory action by Tween 20 is unknown, but we hypothesized that Tween 20 may interfere with the entrance of the 20S proteasome because 0.05% of SDS restored the activity under Tween 20. Therefore, we used proteasome from *Thermoplasma* for characterization of ATPase complex.

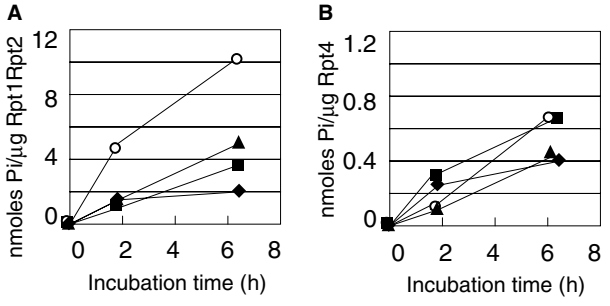

Fig. 3. ATP-hydrolyzing activity of Rpt proteins. ATPase activities of the Rpt1Rpt2 complex (A) and Rpt4 complex (B) were measured. Rpt1Rpt2 (0.05 μg) or Rpt4 (0.6 μg) was incubated with 2 mM ATP and 10 mM $MgCl_2$ in 16 mM HEPES–NaOH, pH 8.0, at 37 °C (diamonds) in the presence of 6 μg of α-casein (triangles) or 2.9 μg of *Thermoplasma* proteasomes (boxes), or both of them (open circles). The amount of released inorganic phosphate was determined by measurement of absorption ($\chi_{640\,nm}$) [20]. Experiments were repeated three times and averages of those values were plotted.

3.3. Rpt complexes activate protein degradation by the archaeal proteasome

Although α-casein was slowly hydrolyzed by proteasomes alone, the degradation-rate of α-casein increased when the Rpt1Rpt2 complex was present in the reaction mixture (Fig. 4A); in this experiment, the rate of degradation of α-casein reflected the ATP-hydrolyzing activity (Figs. 3A and 4A). When non-hydrolyzable ATP analog, ATP-γS, was used instead of ATP, the Rpt1Rpt2 complex promoted the degradation of α-casein less effectively than under the condition with ATP. This result suggests that ATP binding to Rpt1Rpt2 stimulates proteolytic activity, although ATP hydrolysis facilitates the protein degradation process.

When an Rpt4 complex was incubated with *Thermoplasma* proteasome, the rate of α-casein degradation by the proteasome also increased. Nevertheless, it was completely abolished by addition of ATP-γS (Fig. 4B). These results indicate that the activation of proteolysis by Rpt4 is entirely hydrolysis-dependent. Results presented in Figs. 4A and B show that both Rpt1Rpt2 and Rpt4 accelerated the degradation of α-casein at

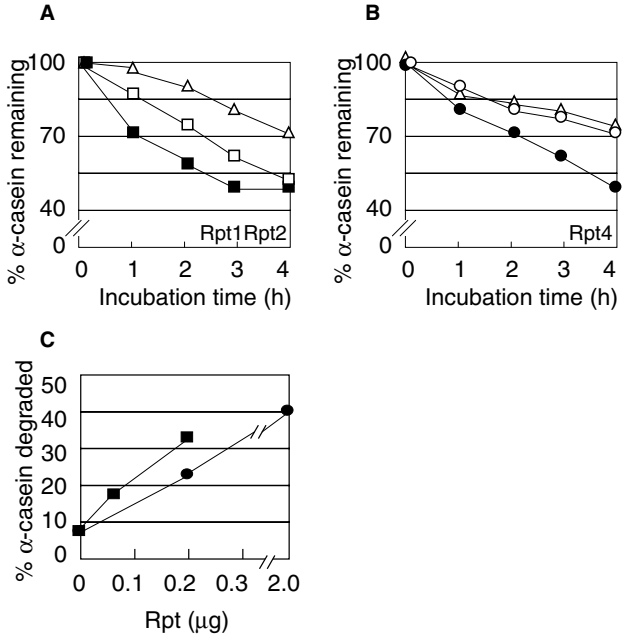

Fig. 4. Breakdown of α-casein by the proteasome is accelerated by the Rpt complex. (A) Rpt1Rpt2. α-Casein (10 μg) was incubated with 3.5 μg of *Thermoplasma* proteasome and 0.2 μg of Rpt1Rpt2 complex at 37 °C in 80 μl of 15 mM HEPES–NaOH, pH 8.0, in the presence of 2 mM ATP and 5 mM $MgCl_2$ (closed boxes). A 15-μl sample of reaction mixture was collected every hour; the remaining α-casein was quantified using a molecular imager (FX; Bio-Rad Laboratories, Inc.) after SDS–PAGE analysis. Open boxes: ATP-γS was used instead of ATP. Open triangles: the reaction mix was lacking Rpt1Rpt2. (B) Rpt4. Experiments were performed as described in (A) except for use of Rpt4 instead of Rpt1Rpt2. Every circle corresponds to every box of experiment A. (C) Comparison of initial rates of α-casein degradation by different amounts of Rpt1Rpt2 and Rpt4. α-Casein (10 μg) was incubated with 3.5 μg of proteasome, 2 mM ATP, 5 mM $MgCl_2$ and various amounts of Rpt1Rpt2 complex (0–0.2 μg; closed boxes) or Rpt4 (0–0.2 μg; closed circles). Total volume was adjusted to 80 μl with buffer E of 20% glycerol and the mixture was incubated at 37 °C. The degraded casein was quantified as described in the legend for (A).

a two-times faster rate than that by the proteasome itself and that α-casein degradation is the dose-dependent effect of Rpt (Fig. 4C). It is noteworthy that co-existence of Rpt1Rpt2 and Rpt4 did not increase the rate of degradation of α-casein co-operatively.

Overall, the profile of proteasomal ATPase is similar to that of PAN [21]. It has been reported that PAN from *Methanococcus* promotes the breakdown of casein by 20S proteasomes from other sources, *Thermoplasma acidophilum*, *Methanosarcina thermophila* and rabbit muscle [9]. These observations strongly suggest that primary sequences and domain organizations are conserved between PAN and those of human and yeast Rpt proteins, despite their very large evolutional distance. The isolation of the complex of Rpts and proteasome was not successful due to their weak interaction. Further characterization of the mode of interaction of Rpts and proteasome is required.

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