Difference between PA700-like proteasome activator complex and the regulatory complex dissociated from the 26S proteasome implies the involvement of modulating factors in the 26S proteasome assembly

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Abstract The PA700-like proteasome activator complex was highly purified from porcine erythrocytes, and its properties were compared with those of the regulatory complex disassembled from the purified 26S proteasome. The molecular mass of the PA700-like complex, which comprises 25–110-kDa subunits, was estimated to be 800 kDa by Superose 6 gel filtration. This complex showed neither ATPase activity nor peptidase activity toward Suc–Leu–Leu–Val–Tyr–MCA. Nevertheless, it was possible to make a high molecular mass complex from the purified PA700-like complex by incubating with the 20S proteasome in the presence of ATP. In contrast, the regulatory complex dissociated from the 26S proteasome did not reconstitute a larger complex under the same conditions. The subunit composition of the PA700-like complex was similar but not identical to that of the regulator complex dissociated from the 26S proteasome: the former complex had a 25-kDa subunit which is absent in the latter, whereas the latter had two or three 43-kDa subunits lacking in the former. These results indicate that the purified PA700-like proteasome activator complex is structurally and functionally distinct from the regulatory complex dissociated from the 26S proteasome, implying the involvement of modulating factors in the 26S proteasome assembly.

Key words: ATPase; Ubiquitin; Protease; Proteasome; Multicatalytic; Activator; Erythrocyte

1. Introduction

Intracellular short-lived proteins or stress-induced abnormal proteins are degraded by a ubiquitin (Ub[1])-proteasome pathway [see reviews: [1–7]. In this pathway, substrate proteins are specifically recognized and tagged with multi-Ub chains by E1, E2 and E3, and they are subsequently degraded by the 26S proteasome [1–7]. Both ubiquitination and proteolysis require ATP hydrolysis [1–7].

The 26S proteasome comprises two subcomplexes: the 20S proteasome and the 19–22S regulatory complex [1,5–7]. The former is a cylindrical 700-kDa proteolytic core complex stacked by four 7-subunit rings composed of 20–33-kDa subunits, while the latter is a 700–1000-kDa regulatory complex containing ATPase subunits (S4 [8], S6 (TBP7) [9], S7 (MSS1) [10,11], TBP1 [12], p45 [13], and p42 [12,14]), an RNA-processing enzyme subunit (S1) [15], and a multi-Ub chain recognizing subunit (S5a) [[16], see reviews: [7,17]]. The regulatory complex is currently known by different names such as an ATPase complex [18], μ particle [19], 19S cap complex [20], or PA700 [21], but it is not clear whether these subcomplexes are mutually identical at the molecular level.

Hershko and coworkers [22] first reported that the 26S proteasome can be disassembled into three factors, CF-1 (600 kDa), CF-2 (250 kDa) and CF-3 (650 kDa), in ATP-depleted reticulocyte lysates, and that these partially purified preparations are able to reconstitute the 26S proteasome in an ATP-dependent manner. In addition, they reported that the assembled 26S complex possesses ATPase activity but the disassembled subcomplexes do not [23]. Later, CF-3 and CF-2 were identified as the 20S proteasome [24] and an ATP-stabilized inhibitor against the 20S proteasome [25], respectively; the latter is proposed to be a hexameric 40-kDa δ-aminolevulinic acid dehydratase [25]. In contrast to CF-2 and CF-3, CF-1 has not yet been completely purified, though it is thought to be an activator for the 20S proteasome [1].

Taken together, it seems likely that the 26S proteasome may dissociate into different subcomplexes under different experimental conditions. In addition to these issues, it is also unclear whether the regulatory complex and the 20S proteasome, both of which are once disassembled from the purified 26S proteasome, are capable of reconstituting the 26S proteasome without the addition of any factor(s).

In order to clarify these issues, we first attempted to reconstitute the 26S proteasome from the regulatory complex and the 20S proteasome, both of which had been isolated from the purified 26S proteasome by non-denaturing PAGE [26]. However, we failed to reconstitute the 26S proteasome from these two subcomplexes. Since the purified PA700 is reported to be capable of binding to the 20S proteasome, we assumed that a proteasome activator (PA700) may be a complex functionally distinct from the regulatory complex dissociated from the 26S proteasome (dRC), although these two complexes are similar in terms of subunit composition.

In the present study, therefore, we attempted to purify the PA700-like proteasome activator complex from porcine erythrocytes according to the procedure described by Chu-Ping et al. [21] with slight modification, and we compared its properties with those of dRC. The results clearly showed that our purified PA700-like complex has a proteasome activator activity and an ability to make up a higher molecular mass complex with the 20S proteasome in an ATP-dependent manner. In addition, it was found that this activator complex

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Abbreviations: AMC, 7-amino-4-methylcoumarin; dRC, regulatory complex dissociated from the 26S proteasome; DTT, dithiothreitol; MCA, 4-methylcoumaryl-7-amide; NEPHGE, non-equilibrium pH gradient gel electrophoresis; PA700, 700-kDa proteasome activator; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Suc, succinyl; 2D, 2-dimensional; Ub, ubiquitin

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possesses a unique 25-kDa subunit, which is absent in dRC, and that it lacks 43-kDa subunits, which are present in dRC. Taken together, we propose that putative modulating factors, which are not incorporated into the assembled 26S proteasome, may function in the 26S proteasome assembly.

2. Materials and methods

2.1. Materials

Suc–Leu–Leu–Val–Tyr–MCA and AMC were purchased from the Peptide Institute Inc. (Osaka, Japan). DEAE–cellulose (DE-32) and phosphocellulose (P-11) are products of Whatman BioSystems. The FPLC system equipped with Mono Q (HR 5/5) and Superose 6 (HR 10/30) is a product of Pharmacia AB Biotechnology. Hydroxylapatite column (Pentax column, 0.75×10 cm) was obtained from TOSO. Prep Cell apparatus model 491 is a product of Bio-Rad Laboratories.

2.2. Buffers

Buffer A, 20 mM Tris-HCl (pH 7.6) containing 0.5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol; Buffer B, 20 mM sodium phosphate (pH 7.6) containing 1 mM 2-mercaptoethanol, and 10% glycerol; Buffer C, 200 mM sodium phosphate (pH 7.6) containing 1 mM 2-mercaptoethanol, and 10% glycerol.

2.3. Assay for enzymatic activities

Hydrolysis of Suc–Leu–Leu–Val–Tyr–MCA was determined as previously described [26]. ATPase activity was assayed by quantifying the inorganic phosphate, which is released by ATP hydrolysis, essentially according to the method of Fiske and Sabbarow with some modifications [26].

2.4. Assay for proteasome activator activity

Proteasome activator activity, which was MgATP-dependent, was assayed essentially according to [21]. Briefly, the activator fraction was previously incubated with the 20S proteasome at 37°C for 60 min, and then proteasome activity was measured as described above.

2.5. Protein determination and electrophoresis

Protein concentration was determined by the method of Bradford [27] with bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli [28]. In order to compare the subunit compositions among the PA700-like activator complex, dRC, and the 26S proteasome, 2D-PAGE (first dimension, NEPHGE; second dimension, SDS-PAGE) was carried out as described by O'Farrell et al. [29] with slight modifications [26].

2.6. Preparation of porcine erythrocyte lysates

Porcine fresh blood (3 l) was collected in a plastic vessel, in which had been placed one-fiftieth volume (60 ml) of 100 U/ml heparin dissolved in PBS, and the solution was gently mixed on ice. The blood was centrifuged at 2000×*g* for 10 min, and the resulting precipitate was washed 4 times with 3 volumes of PBS by centrifugation at 2000×*g* for 10 min. At this time, the buffy coat (mainly platelets and leukocytes) was removed by a pipette. The resulting precipitates, containing packed erythrocytes, were mixed with 2 volumes of 1 mM DTT and the solution was stirred for 30 min at 4°C. The erythrocyte lysates thus obtained were centrifuged at 10000×*g* for 1 h, and the supernatant was dialyzed against Buffer A containing 20 mM NaCl and used as a crude extract.

2.7. Purification of 20S and 26S proteasomes from porcine erythrocytes

The 20S and 26S proteasomes were purified from the crude extract of porcine erythrocyte lysates essentially according to the method described previously from Fraction II [30].

2.8. Isolation of the regulatory complex from the 26S proteasome purified from porcine erythrocytes

The regulatory complex (dRC) was isolated from the purified 26S proteasome by preparative non-denaturing PAGE according to the method of Laemmli [28], but in the absence of SDS, using a Bio-Rad model 491 Prep Cell apparatus as previously described [26].

2.9. Purification of the PA700-like proteasome activator complex from porcine erythrocytes

To the crude extract obtained from 3 l of blood, 1 l of DEAE-cellulose wet gel equilibrated with Buffer A containing 20 mM NaCl was added. After stirring for 30 min, the gel was washed with the equilibration buffer using a glass filter, and the gel was then packed into a column (10×13 cm) followed by washing with 2.5 l of the equilibration buffer. The adsorbed materials were eluted with 2 l of Buffer A containing 500 mM NaCl. To the eluate (2.3 l, so-called Fraction II), ammonium sulfate was added at 38% saturation and stirred for 30 min. The insoluble materials were collected by centrifugation (10000×*g*, 30 min) and dissolved in 30 ml of Buffer A containing 100 mM NaCl. This solution was divided into two parts, and each portion was applied to a Sephacryl S-300 column (3.6×55 cm) that had been previously equilibrated with Buffer A containing 100 mM NaCl. Chromatography was carried out at a flow rate of 60 ml/h, and 10-ml fractions were collected. Fractions showing proteasome activator activity were collected and adsorbed to a Mono Q column (0.5×5 cm) that had been equilibrated with Buffer A containing 100 mM NaCl. After the column had been washed with the equilibration buffer, the adsorbed materials were eluted with 250 mM NaCl and then with a linear gradient of NaCl (250–400 mM; total volume, 8 ml) dissolved in Buffer A. The flow rate was 0.25 ml/min, and 1-ml fractions were collected. Activator activity was detected at about 330–350 mM NaCl. Active fractions were collected, dialyzed against Buffer B, and applied to a hydroxylapatite column (0.75×10 cm) equilibrated with Buffer B. After washing with the equilibration buffer, the adsorbed materials were eluted with a linear gradient generated by mixing Buffer C into Buffer B (total volume, 25 ml). The flow rate was 0.25 ml/min, and 1-ml fractions were collected. Activator activity was detected at a phosphate concentration of 90 mM, and active fractions were concentrated with Centricon-10 up to 0.5 ml and placed on a Superose 6 column (1×30 cm) that had been equilibrated with Buffer A containing 100 mM NaCl. The column was developed at a flow rate of 0.25 ml/min, collecting 0.5 ml in each tube. Activator activity was detected as a symmetrical peak, and active fractions were used as a purified preparation of the PA700-like activator complex.

2.10. Molecular mass estimation

The molecular mass of the PA700-like activator complex was estimated by Superose 6 HR10/30 FPLC using thyroglobulin (667 kDa), apoferritin (443 kDa) and alcohol dehydrogenase (150 kDa) as molecular mass standards. The column was equilibrated and developed with buffer C at a flow rate of 0.2 ml/min, and 0.5-ml fractions were collected.

Table 1
Properties of PA700-like activator complex, dRC, 26S proteasome and 20S proteasome purified from porcine erythrocytes

Properties	PA700-like complex	dRC[a]	26S proteasome	20S proteasome
Molecular size (kDa)	800	1100	~2000	700
Subunit (kDa)	25–110	28–110	23–110	23–32
Hydrolytic activity				
Suc–LLVY–MCA	−	−	++	+
ATP	±	−	++	−
Activator activity toward 20S proteasome	Yes	No		
Binding activity to 20S proteasome	Yes	No		
25-kDa subunit	Present	Absent	Absent?	Absent
43-kDa subunits	Absent	Present	Present	Absent

[a]Ref. [26].

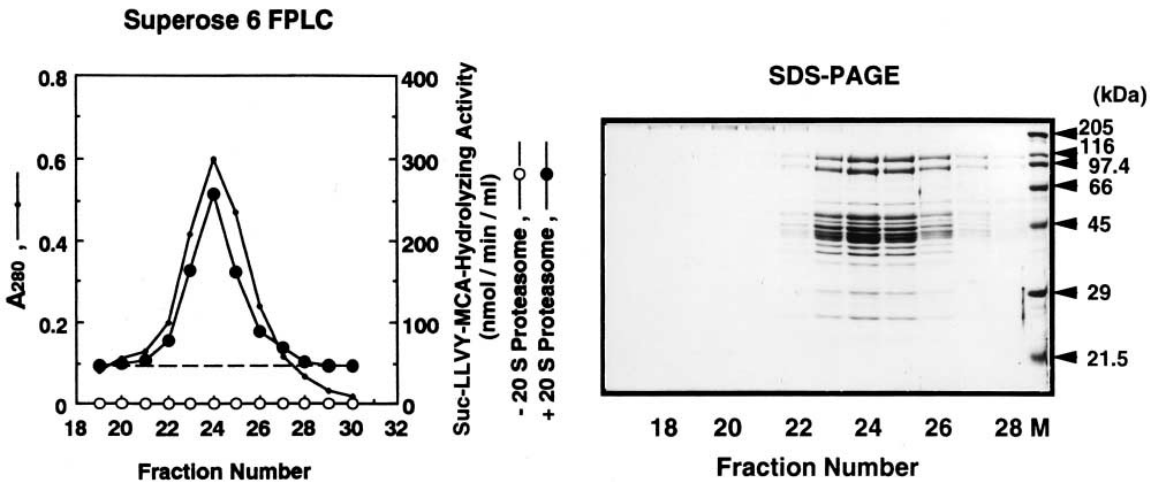

Fig. 1. Superose 6 FPLC of the PA700-like activator complex obtained from the hydroxylapatite column. Left panel: The activator fractions eluted at about 90 mM phosphate on hydroxylapatite chromatography were collected and applied to a Superose 6 column (1×30 cm) that had been equilibrated with Buffer A containing 100 mM NaCl. Fractions (500 μl) were collected at a constant flow rate of 0.25 ml/min. Each fraction was incubated at 37°C for 60 min with 20S proteasome, and proteasome activity toward Suc–Leu–Leu–Val–Tyr–MCA was measured as described in Section 2. Absorbance at 280 nm (– · –); Suc–Leu–Leu–Val–Tyr–MCA-hydrolyzing activity in the presence (– ● –) or absence (– ○ –) of the 20S proteasome. The dashed line indicates the activity level of the 20S proteasome exogenously added. Right panel: SDS-PAGE of the purified PA700-like activator complex. Fifteen microliters of each Superose 6 fractions was subjected to SDS-PAGE (12.5% gel) according to the method of Laemmli [28].

3. Results

3.1. Purification and purity of the PA700-like proteasome activator complex from porcine erythrocytes

We first attempted to purify the PA700- or PA700-like proteasome activator complex according to the method of Chu-Ping et al. [21] with some modifications.

The PA700-like proteasome activator complex was highly purified from porcine red blood cells by DEAE-cellulose chromatography, 0–38% ammonium sulfate fractionation, Sephacryl S-300 gel filtration, hydroxylapatite chromatography, Mono Q FPLC and Superose 6 FPLC. From 3 l of porcine fresh blood, approximately 750 μg of the purified proteasome activator complex was obtained. In the last purification step on Superose 6 FPLC, proteasome activator activity was coincidently eluted with the protein peak, showing a constant specific activity (Fig. 1, left panel).

Purity of the activator was examined by SDS-PAGE. As shown in Fig. 1 (right panel), the purified preparation gave several bands, all of which were eluted together at fractions 23–25 (the highest amounts at fraction 24). This suggests that these subunit components are mutually associated and that the purity of our activator preparation is high.

3.2. Characterization of the PA700-like activator complex

The molecular mass of the activator complex was estimated to be 800 kDa by Superose 6 FPLC (see Fig. 1, left panel). SDS-PAGE gave at least 13 bands consisting of 4 bands with molecular masses of 110, 100, 29, and 25 kDa, and at least 9 bands between 32 and 60 kDa (see Fig. 2, right panel). This SDS-PAGE pattern is very similar to that of PA700 reported previously [12,21] but not to that of dRC [26], since dRC does not have the 25-kDa subunit. It is reported that PA700 preparation purified from bovine erythrocytes also contains the 25-kDa subunit [21].

The purified activator preparation showed no activity toward Suc–Leu–Leu–Val–Tyr–MCA, a preferred substrate for the 26S and 20S proteasomes, indicating that there is no contamination of the 26S or the 20S proteasome in this preparation. In addition, little or no ATPase activity (< 1% of the activity of the 26S proteasome) was detected in the PA700-like activator complex.

In contrast to the report that ATPase activity is copurified with PA700 [21], Ganoth et al. [22] failed to detect significant ATPase activity in the disassembled CF-1, a similar complex to PA700. In connection to this, it should be noted that dRC also has no detectable ATPase activity, i.e. less than 0.1% of the ATPase activity of the 26S proteasome [26].

3.3. Comparison of subunit compositions among PA700-like activator complex, dRC, and 26S proteasome

The subunit composition of the PA700-like activator complex of porcine erythrocytes was analyzed by 2D-PAGE (first dimension, NEPHGE; second dimension, SDS-PAGE) and was compared with those of porcine erythrocyte dRC and 26S proteasome. As shown in Fig. 2, the subunit composition of the PA700-like activator complex was distinct from that of dRC. The main differences are as follows: first, the 25-kDa subunit (p*I*, ≈5.7) detected in the PA700-like complex is absent in dRC, and second, two or three 43-kDa subunits with slightly different isoelectric points (p*I*, ≈6.5–7.0) detected in dRC and the 26S proteasome are absent in the PA700-like complex.

3.4. The PA700-like activator complex but not the regulatory complex dissociated from 26S proteasome is capable of reconstituting a higher molecular mass complex with 20S proteasome

It has been reported that the PA700 is capable of making up a higher molecular mass complex with the 20S proteasome in the presence of ATP [12]. Thus, we examined whether our PA700-like activator complex and the 20S proteasome can reconstitute the 26S proteasome (or a 26S proteasome-like high molecular mass complex) in the presence of ATP. As

depicted in Fig. 3, proteasome activity was detected at a higher molecular size position (fraction 22), which corresponds to that of the 26S proteasome, than that of the 20S proteasome (elution position; fraction 25–26). In contrast to this, dRC was unable to form a higher molecular mass complex with the 20S proteasome (data not shown). These results indicate that the PA700-like activator complex, unlike dRC, can make a higher molecular mass complex with the 20S proteasome.

Taken together, we conclude that the PA700-like activator complex is functionally and structurally different from the regulatory complex once dissociated from the 26S proteasome, although these two complexes are similar in terms of their subunit compositions.

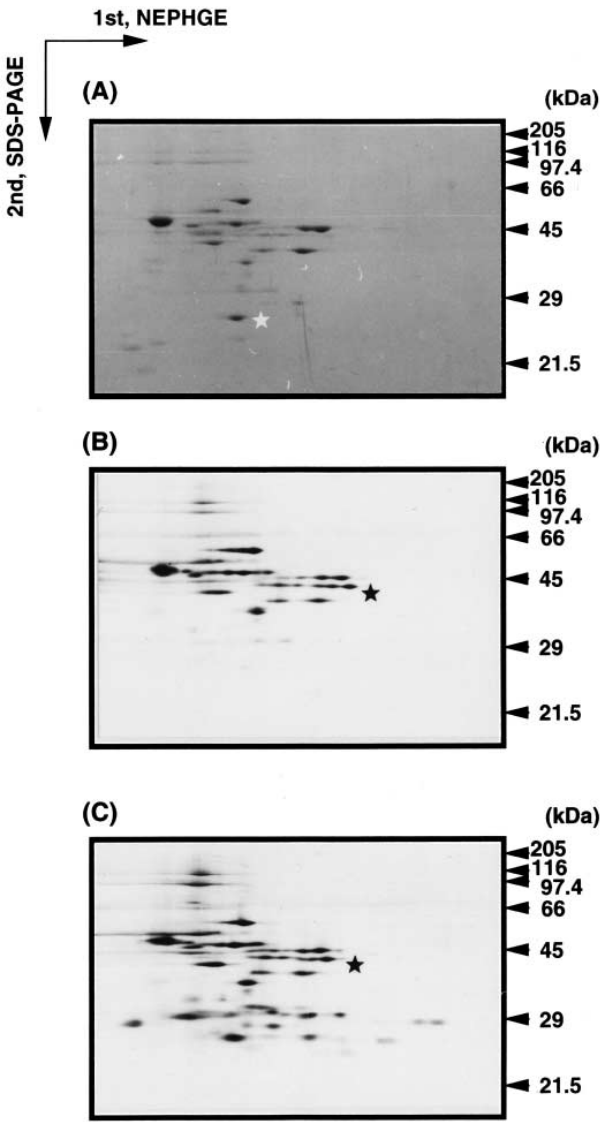

Fig. 2. 2D-PAGE (first dimension, NEPHGE; second dimension, SDS-PAGE) patterns of the PA700-like activator complex (A), dRC (B), and the 26S proteasome (C), all of which are purified preparations from porcine erythrocytes. On NEPHGE, pH gradient was made between pH 4 (left) and pH 9 (right). Note that the 25-kDa subunit (☆; p*I*, ≈5.7) observed in the PA700-like complex is absent in dRC or the 26S proteasome, while two or three 43-kDa subunits (★; p*I*, ≈6.5–7.0) observed in the dRC and the 26S proteasome are not detected in the PA700-like complex.

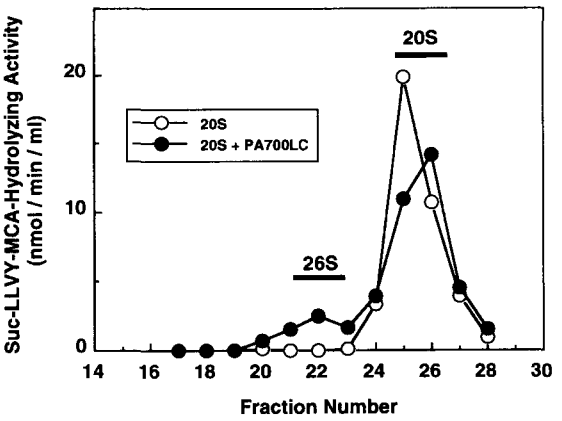

Fig. 3. Formation of a higher molecular mass complex by incubating the 20S proteasome with (– ● –) or without (– ○ –) the PA700-like activator complex. The purified 20S proteasome (120 units) was mixed with or without 150 μg of the PA700-like complex followed by incubation at 37°C for 60 min. These samples were subjected to Superose 6 FPLC in Buffer A containing 100 mM NaCl. Note that the higher molecular mass complex showing Suc–Leu–Leu–Val–Tyr–MCA-hydrolyzing activity, which corresponds to the elution position of the 26S proteasome, was newly formed by incubating the 20S proteasome with the PA700-like complex, suggesting that the purified 20S proteasome and PA700-like complex preparations are capable of being associated with each other in the presence of ATP.

4. Discussion

From porcine erythrocytes, we succeeded in purifying a PA700-like proteasome activator complex with a molecular mass of 800 kDa, which is capable of forming a higher molecular mass complex by incubation with the 20S proteasome in the presence of ATP. We also showed that the purified PA700-like activator complex (800 kDa) is similar but not identical to the regulatory complex (1100 kDa) dissociated from the 26S proteasome reported previously [26], on the bases of molecular size and subunit composition: the dRC lacks a 25-kDa subunit detected in the PA700-like complex, while the former contained 43-kDa subunits lacking in the latter (Table 1).

In our experimental conditions, little or no ATPase was detected in the completely purified PA700-like complex preparation. In the purified dRC preparation also, we could not detect any ATPase activity in spite of the existence of ATPase subunits [26]. In contrast, a high ATPase activity was observed in the purified porcine erythrocyte 26S proteasome (this study) and in the bovine erythrocyte PA700 [31]. The following three reasons are offered as possible explanations for this discrepancy in ATPase activity. Firstly, the ATPase activity in our PA700-like complex may have been diminished by inactivation or removal of some of the ATPase subunits during purification. Secondly, the ATPase activity contained in the PA700 preparation [31] may have been contaminated by non-related ATPases. Thirdly, ATPase activities may have been masked in our PA700-like complex and dRC, and these masked ATPases may have been activated after association with the 20S proteasome. Further study is necessary to clarify these points.

The 20S proteasome was only partially shifted to the elution position of 26S proteasome on Superose 6 gel filtration after its incubation with PA700-like complex (Fig. 3). This

appears to be due to the partial dissociation of the high molecular mass proteasome complex, which was formed from the 20S proteasome and PA700-like complex, since our preliminary data showed that the purified preparation of the 26S proteasome is partially dissociated into the constituent subcomplexes on Superose 6 gel filtration even in the presence of 0.1 mM ATP and 10% glycerol. Treatment of the assembled complex with cross-linking reagents or addition of an excess amount of PA700-like complex into the 20S proteasome would increase a yield of the higher molecular mass complex. Alternatively, the 43-kDa component of dRC- or ATP-dependent modulator [12] may be necessary for the formation of stable complex.

CF-1 (600 kDa) has not yet been purified, but CF-2 (250 kDa) has been purified and identified as a homo-hexamer of 40 kDa. The purified PA700-like activator complex does not contain the two or three 43-kDa subunits that are present in dRC and the 26S proteasome. These results led us to assume that the PA700-like complex may correspond to a complex of CF-1 plus the 25-kDa subunit, and that the 43-kDa subunits that are absent in the PA700-like complex may correspond to CF-2, i.e. δ-aminolevulinic acid dehydratase. The 25-kDa subunit appears to be distinct from p27, a subunit of ATP-dependent modulator of PA700 [12], albeit they are similar to each other in molecular mass, since it is reported that the purified PA700 does not contain p27 subunit [12]. In connection to these issues, one intriguing hypothesis is that the 25-kDa subunit may be a kind of molecular chaperon that stimulates the assembly of the 26S proteasome complex. Further investigations on the identification of the above 25- and 43-kDa subunits, as well as molecular elucidation of reconstitution of the 26S proteasome from the PA700-like complex and 20S proteasome, are now in progress in our laboratory.

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