Identification, Purification, and Characterization of a High Molecular Weight, ATP-dependent Activator (PA700) of the 20 S Proteasome*

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In order to identify protein complexes consisting of the proteasome and specific proteasome regulators, crude soluble lysates of red blood cells were fractionated by gel filtration chromatography and by velocity sedimentation centrifugation. The fractionated lysates were then tested for the relative distribution of proteasome activity, proteasome protein, and protein of a known proteasome activator, PA28. At least two proteasome complexes containing PA28 were identified. One of these complexes had an apparent molecular weight of approximately 1,750,000, and appeared to have much more proteasome activity than could be accounted for by its relative concentrations of proteasome and PA28 protein. We hypothesized that this complex contained another activator of the proteasome, and we sought to purify this activator from extracts of red blood cells. A proteasome activator with an apparent molecular weight of approximately 700,000 was identified, purified, and characterized. This activator, termed PA700, greatly stimulated the peptidase activities of the proteasome in an ATP-dependent fashion. PA700 was composed of about 16 polypeptides ranging in molecular weight from 20,000 to 100,000. The ATP-dependent activation of the proteasome by PA700 was closely linked to the formation of a high molecular weight weight complex that required no additional ATP for activated proteolysis. These results indicate that PA700 is a regulatory protein of the proteasome and is a component of at least one high molecular weight proteasome-containing complex occurring in cell extracts.

The proteasome may function in multiple cellular processes as diverse as the bulk breakdown of short-lived cytoplasmic proteins, the selective, regulated degradation of specific proteins such as cyclins, p53, Myc, Fos, and MAPK2 repressor, and the processing of antigens for presentation by class I major histocompatibility complex molecules (5-10). An enzyme involved in such diverse processes is almost certainly subject to tight regulation, and possible bases for regulation of the proteasome have been identified. For example, the proteasome probably exists in an enzymatically latent form in intact cells (11). The proteasome can be purified as a latent enzyme and activated by various biochemical treatments that may mimic the action of physiological regulators (1). In fact, recent work has identified specific proteins, including activators, that modulate proteasome function (12, 13). Several lines of evidence further indicate that the function of the proteasome is mediated by regulatory proteins. For example, immunoprecipitation of the proteasome from cell-free extracts that catalyze ATP/ubiquitin-dependent proteolysis greatly inhibit this proteolytic pathway (14). More importantly, reconstitution of purified latent proteasome, which by itself is unable to degrade any protein substrate, completely restores the ATP-dependent degradation of ubiquitinated proteins (14). The reconstitution of proteolysis is saturable with respect to the amount of added proteasome and addition of proteasome to untreated extracts has no effect on proteolysis. These results indicated that the extracts contain a factor(s) whose activity or concentration was rate-limiting with respect to proteolysis and which may have acted by mediating proteasome function. Other studies have identified an enzyme much larger than the proteasome ($M_r = 1,500,000$) that can degrade ubiquitinated proteins in an ATP-dependent fashion (5, 15, 16). This enzyme, the "26 S protease," contains proteasome subunits (15), suggesting that it is a complex of the proteasome and other proteins. Subsequent work demonstrated that an enzyme with the same catalytic and regulatory features as those of the 26 S protease could be reconstituted from the purified proteasome and two partially purified protein fractions (17). The identity and function of these proteins remain unclear, although one acts independently as a proteasome inhibitor (18). The purification of the 26 S protease and rigorous biochemical characterization its components have not been accomplished.

We have taken two approaches to understand the control of proteasome function by specific regulatory proteins. First, we have identified individual regulatory proteins by fractionating cellular extracts and testing their ability to alter activity of the purified proteasome. This approach has identified a number of proteasome activators and inhibitors (12, 13, 18-21). Second, as reported in the current work, we have identified protein complexes consisting of the proteasome and proteasome regulatory proteins. We hypothesized that such cellular complexes might dissociate into component proteins under conditions.

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used for protein purification. In order to minimize the dissociation of hypothetical complexes into component proteins, we have searched for proteasome-containing complexes in cell-free extracts that had undergone a minimal degree of biochemical manipulation. This report identifies several such complexes in extracts of red blood cells. At least two complexes contain the previously identified proteasome activator, PA28, and at least one contains a newly identified protein that activates the proteasome in an ATP-dependent fashion. This novel activator, termed PA700, has been purified and characterized.

**Materials and Methods**

**Cells and Preparation of Soluble Extracts**—Bovine blood was collected in the presence of heparin from a local meat processing plant. Blood cells were collected by centrifugation at 2000 g for 1 h. The supernatant and the buffy coat were removed by aspiration. The remaining cell pellet was resuspended in four volumes of phosphate-buffered saline and recentrifuged. The washing procedure was repeated four times. All of the following steps were carried out at 4 °C unless otherwise indicated. Cells were lysed by adding three volumes of a lysis buffer (Buffer H: 20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol) to one volume of the packed cells and stirring for 10 min. The lysate was centrifuged at 13,000 × g for 60 min. The supernatant was removed and saved. The pellet was resuspended in three volumes of Buffer H and recentrifuged. The supernatant from the second centrifugation was added to the first. Portions of this crude lysate were dialyzed against a buffer of 20% (v/v) glycerol in the same buffer. The bound proteins were eluted from the column with a linear gradient of phosphate buffer (20–200 mM, 500 ml volume). Samples of the 8-ml fractions were assayed for PA700 activity. The fractions containing peak activities (for the figures presented in the text, 11–14 for Peak I and 20–23 for Peak II) were pooled, dialyzed against Buffer H, and concentrated to approximately 1 mg/ml by ultrafiltration using a Amicon PM10 membrane. The samples could be stored at −70 °C with no detectable loss of activity. Further analysis of the PA700 activity was conducted as described in the text.

**Purification of the Proteasome and PA28**—The proteasome and PA28 were purified from bovine red blood cells or bovine heart as described previously (11, 12).

**Assay for Proteasome**—The proteasome was assayed by measuring the hydrolysis of synthetic peptide substrates by a fluorometric assay as described previously, except that assays were conducted at 37 °C (12). The production of fluorescent products was measured directly during the incubation, and hydrolytic rates were determined for the initial steady state rate. One unit of enzyme activity is defined as the change in product concentration of 1.0 nm/min at 37 °C.

**Assay for PA700 Activity**—PA700 activity was assayed by measuring the increase in proteasome activity after preincubation of PA700 and purified proteasome. The standard preincubation solution consisted of 45 mM Tris-HCl, pH 8.0, 5 mM dithiorthreitol, 50 mM ATP, 10 mM MgCl₂, 0.3 μg of purified proteasome, and PA700, as indicated in specific experiments, in a final volume of 50 μl. Unless otherwise indicated, this solution was preincubated at 37 °C for 45 min and then transferred directly to 1.0 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.5 mM dithiothreitol, and 50 μM substrate, usually Suc-Leu-Leu-Val-Tyr-AMC, for measurement of proteasome activity as described above. Routine control assays were conducted in parallel and consisted of the preincubation and subsequent assay of proteasome and PA700 individually, in the presence and absence of ATP, and proteasome and PA700 in the absence of ATP. Additional control assays were performed as described in the text. One unit of PA700 activity is defined as the increase of 1 unit of proteasome activity under the standard conditions described above.

**Velocity Sedimentation Centrifugation**—Velocity sedimentation centrifugation was carried out in glycerol gradients (10–40% glycerol) as described previously (12). Glycerol and included thyroglobulin (M, 660,000), catalase (M, 240,000), and aldolase (M, 158,000).

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis was carried out under both non-denaturing and denaturing conditions. Non-denaturing gels consisted of 4% acrylamide. The gel buffer and the electrode buffers consisted of 80 mM Tris base and 90 mM boric acid, pH 8.3. The slab gels were run at 4 °C in 5 ml at 50 mV. SDS-PAGE was conducted with 12.5% acrylamide gels as described previously (12).

**Immunoblotting**—Immunoblotting was conducted with polyclonal antibodies prepared in rabbits against human proteasome and bovine heart catalase. The antibody was detected using a 1/5000 dilution of anti-rabbit IgG linked to alkaline phosphatase, purchased from Bio-Rad and used according to the manufacturer's specifications. The antibody against human proteasome recognized the same subunits of the bovine proteasome, although not all subunits of either proteasome were recognized. Reaction was determined by the method of Bradford (22) using bovine serum albumin as the standard.

**Results**

**Identification of Proteasome-Containing Protein Complexes in Cell Extracts**—In order to identify multiprotein complexes comprised of the 20 S proteasome and specific regulatory proteins, red blood cell lysates that had not been treated with ammonium sulfate or exposed to buffers containing high concentrations of salts were fractionated by gel filtration chromatography or by velocity sedimentation centrifugation. Proteasome content of the fractionated lysates was assessed by a functional assay with a peptide substrate specific for the proteasome Suc-Leu-Leu-Val-Tyr-AMC and by an immunoassay with an antibody that recognizes multiple proteasome subunits. Each fractionation method identified two unequal peaks of proteasome activity (Figs. 1 and 2). The peak with the most of the proteasome activity had an apparent M, of approximately 1,750,000, a value much greater than that of the purified proteasome. This

1 The abbreviations used are: Suc, succinyi; AMC, 7-amino-4-methylcoumarin; PAGE, polyacrylamide gel electrophoresis.
The column was calibrated with compounds of gel filtration chromatography on Sephacryl S-400. Soluble lysates of bovine red blood cells were prepared as described under "Materials and Methods." Extract activity in the absence (M, 50 mM, 50%), using Suc-Leu-Leu-Val-Tyr-AMC substrate as described under "Materials and Methods." PA28-stimulated proteasome activity represents the total activity under this latter condition. Total protein assay, using Suc-Leu-Leu-Val-Tyr-AMC substrate as described under "Materials and Methods." Samples subjected to velocity sedimentation centrifugation as described under "Materials and Methods." Samples of the 700,000-dalton proteasome activity probably represents the 26 S protease previously identified in many laboratories (2, 5). The second peak contained much less activity and had a M, similar to that of the 20 S proteasome, i.e. approximately 700,000. Immunoblotting demonstrated that proteasome-like, the proteasome activity, also had a bimodal distribution (Fig. 3, upper panel). The first peak of proteasome activity was coincident with the 1,750,000-dalton proteasome activity. The second peak of proteasome protein had a similar, but not identical distribution to that of the 700,000-dalton proteasome activity. In repeated experiments with numerous independent preparations, the proteasome activity of the second peak always had a slightly greater M, than that of the corresponding proteasome protein. The basis for this observation is discussed below. These results also demonstrated a striking disparity between the relative levels of proteasome protein in the two peaks and the corresponding proteasome activities. Thus, even though the 1,750,000-dalton proteasome had 3-4 times more activity than the 700,000-dalton proteasome, it contained only about 20% as much proteasome protein. One explanation for these observations is that the 1,750,000-dalton proteasome activity is due to a multiprotein complex consisting of the proteasome and one or more proteins that stimulate its activity. To test this possibility, the distribution of a previously identified proteasome activator, PA28, was assessed by immunoblotting. Purified PA28 has a native M, of about 200,000, and a peak of PA28 protein was identified in the fractions corresponding to this value (Fig. 3, lower panel). However, two additional peaks of PA28 protein were also detected. One was coincident with the 1,750,000-dalton proteasome activity and the corresponding proteasome protein; this result is consistent with the hypothesis that the 1,750,000-dalton proteasome complex contains PA28. However, most of the PA28 protein had a distribution that was coincident with that of the 700,000-dalton proteasome activity. Thus, like the latter activity, the distribution of PA28 protein corresponded to a greater apparent M, than that of the proteasome protein. These results suggest that this region of column fractions contained two forms of proteasome, one associated with PA28 and one not. To examine this issue further, column fractions were reasayed for proteasome activity after supplementation with purified PA28. Exogenous PA28 had little effect on the activity of the 1,750,000-dalton proteasome but greatly stimulated the activity of the 700,000-dalton proteasome (Fig. 1). Furthermore, the distribution of proteasome activity that was stimulated by exogenous PA28 had a M, that was slightly, but reproducibly, lower than that of the endogenous activity and was coincident with that of the proteasome protein in this region of the column. Although these various results suggest that the 1,750,000-dalton proteasome complex contains an activator, PA28, they indicate that PA28 is also associated with the 700,000-dalton proteasome. Therefore, the differential association of PA28 does not, by itself, appear to account for the
ATP-dependent Proteasome Activator

Table I

ATP-dependent activation of the proteasome by a protein fraction of red blood cells

| Sample         | Preincubation | Proteasome activity |       |
|----------------|---------------|---------------------|-------|
|                |               | ~ATP                | +ATP  |
| Proteasome     | -             | 0.50                | 0.42  |
| 0–38%          | -             | 1.25                | 1.67  |
| Proteasome + 0–38% | -         | 0.04                | 0.03  |
| Proteasome     | +             | 0.48                | 0.48  |
| 0–38%          | +             | 1.35                | 1.80  |
| Proteasome + 0–38% | +         | 0.05                | 16.1  |

relative proteasome activities identified in proteasome-containing complexes. We therefore hypothesized that the 1,750,000-dalton proteasome complex contained a proteasome activator in addition to PA28.

Identification of an ATP-dependent Activator of the Proteasome—To identify a proteasome activator(s) distinct from PA28, lysates of bovine red blood cells were fractionated and assayed for their ability to activate purified 20 S proteasome. To simplify this analysis, we prepared extracts that were relatively free of both the proteasome and PA28. Previous work demonstrated that treatment of Fraction II with ammonium sulfate to 38% saturation precipitated proteins that did not include significant amounts of proteasome or PA28. This fraction, however, does contain an inhibitor of the 20 S proteasome (21), which could be responsible for the inhibition of proteasome activity (Table I). We hypothesized that variations in assay conditions might affect relative interactions among the proteasome, the inhibitor, and a hypothetical activator(s) present in the extract. In fact, one assay condition consisting of preincubation of the extract with purified proteasome, ATP, and Mg2+, resulted in a large increase in proteasome activity (Table I).

Characterization of the activation showed that it was dependent upon the time of preincubation and required both the proteasome and the extract (Table I, and see complete characterization below). These results suggested that the extract contained a protein(s) that activated the proteasome by an ATP-dependent mechanism, and that this activation was dominant with respect to the function of the proteasome inhibitor.

Purification of a High Molecular Weight, ATP-dependent Proteasome Activator (PA700)—In order to purify the hypothetical proteasome activator, Fraction II proteins that precipitated in a solution of 38% saturated ammonium sulfate were subjected to a series of chromatographic procedures, and the fractionated proteins were assayed for ATP-dependent activation of the 20 S proteasome. Gel filtration chromatography of the extract on Sephacryl S-300 identified a single peak of activity with a Mr = 700,000 (Fig. 4). Because this Mr was similar to that of the 20 S proteasome, a number of control experiments were required to establish that the activity resulted from the activation of the exogenous proteasome by proteins in the column fractions. Controls included assays of column fractions without exogenous proteasome before and after preincubation, in the presence and absence of ATP. The endogenous proteasome activity of the column fractions was low (Fig. 4) and was not significantly affected by preincubation with ATP (data not shown). Nearly all of the endogenous proteasome activity was accounted for by the 1,750,000-dalton protease, and the level of this activity was low compared to that of unfractionated extracts (see “Discussion”). To demonstrate further that the column fractions lacked significant levels of 20 S proteasome, they were assayed with exogenous PA28 and subjected to immunoblotting with anti-proteasome antibodies. These results indicated that the column fractions did not contain detectable levels of the 20 S proteasome (data not shown). The data, therefore, indicate that the 700,000-dalton protease activity in Fig. 4 resulted from the ATP-dependent activation of exogenous proteasome by a protein(s) present in the column fractions.

To further purify the proteasome activator, the column fractions containing the proteasome-activating activity were subjected to ion-exchange chromatography on DEAE-Fractogel. The activity bound to the resin and was eluted with a linear gradient of NaCl at a position corresponding to approximately 250 mM NaCl (data not shown). The eluted activity displayed the same functional characteristics as those described for the activity from the gel filtration column, i.e. there was no detectable endogenous proteasome activity in the column fractions, and all detected activity resulted from the ATP-dependent activation of exogenous proteasome during preincubation with the column fractions. The fractions containing the proteasome activating activity from the ion-exchange column were subjected to chromatography on hydroxyapatite (Fig. 5). This procedure resolved two peaks of proteasome activating activity, designated Peak I and Peak II. Peak I contained most of the activity and eluted at a position corresponding to about 75 mM phosphate. Peak II accounted for 10–25% of the total activity in various independent preparations and eluted at a position corresponding to about 115 mM phosphate.

Peak I and Peak II from the hydroxyapatite column were subjected to polyacrylamide gel electrophoresis (Fig. 6). Each activity contained a single major protein band under nondenaturing conditions. These protein bands had electrophoretic mobilities indistinguishable from one another (Fig. 6A). SDS-PAGE showed that Peak I and Peak II each contained a complex set of approximately 16 proteins that were essentially the same for the two activities (Fig. 6B). These included proteins with Mr values of 100,000, 90,000, 70,000, and 60,000, seven closely migrating proteins with Mr values between 50,000 and 40,000, and five proteins with Mr values between 39,000 and 25,000. The native sizes of Peak I and Peak II were analyzed by gel filtration chromatography on Sephacryl S-400
were pooled, dialyzed against a buffer consisting of 20 mM potassium phosphate, pH 7.6, 1 mM β-mercaptoethanol, and 20% glycerol, applied to a column of hydroxyapatite equilibrated in the same buffer, and chromatographed as described under “Materials and Methods.” Five-μl samples of the 9.0-ml fractions were assayed for PA700 activity using Suc-Leu-Leu-Val-Tyr-AMC as a substrate. The activity of the purified exogenous proteasome was 0.53 units/assay.

![Hydroxylapatite chromatography of the proteasome activator](image)

Fig. 5. Hydroxylapatite chromatography of the proteasome activator. Fraction numbers 31–37 from the DEAE-Fractogel column were pooled, dialyzed against a buffer consisting of 20 mM potassium phosphate, pH 7.6, 1 mM P-mercaptoethanol, and 20% glycerol, applied to a column of hydroxyapatite equilibrated in the same buffer, and chromatographed as described under “Materials and Methods.” Five-μl samples of the 9.0-ml fractions were assayed for PA700 activity using Suc-Leu-Leu-Val-Tyr-AMC as a substrate. The activity of the purified exogenous proteasome was 0.53 units/assay.

![Polyacrylamide gel electrophoresis of PA700](image)

Fig. 6. Polyacrylamide gel electrophoresis of PA700. PA700 Peak I and PA700 Peak II, purified through the hydroxyapatite stage, as described under “Materials and Methods” were subjected to polyacrylamide gel electrophoresis. Panel A, non-denaturing PAGE (4% polyacrylamide gels). Lane 1, 6 μg of bovine red blood cell proteasome; lane 2, 5 μg of PA700 Peak I; lane 3, 5 μg of PA700 Peak II. The proteins were electrophoresed on the same gel. PA700-II was not electrophoresed in a lane adjacent to the proteasome and PA700 Peak I and the photograph was cut and appropriately aligned. Panel B, SDS-PAGE (12.5% polyacrylamide gels). Lane 1, 4 μg of PA700 Peak I; lane 2, 3.8 μg of PA700 Peak II.

![Proteasome Activator](image)

by velocity sedimentation centrifugation through glycerol density gradients. Peak I and Peak II each eluted from the gel filtration column at a position corresponding to a M_r = 700,000 (data not shown). This value was the same as that estimated for the proteasome activating activity by the gel filtration chromatography at the initial stage of the purification (Fig. 4). Peak I and Peak II also sedimented through the glycerol density gradients at rates indistinguishable from one another at a position corresponding to a M_r of approximately 700,000 (Fig. 7, upper panel). SDS-PAGE of the fractions revealed that the characteristic set of 16 proteins described above cosegregated with each other and with the proteasome activating activity (Fig. 7, lower panel). Retrospective analysis by SDS-PAGE of column fractions from the chromatography steps employed for the purification showed that this same set of peptides comigrated with the proteasome-activating activity. Different independent preparations of the activators often contained low levels of other proteins that we judged to be contaminants because they did not comigrate with the activity and/or were not consistently observed. The most prominent and frequently observed of these was a 180,000-dalton protein (Fig. 6). We concluded that the Peak I and Peak II proteasome-activating proteins were essentially pure proteins consisting of hetero-multimers of the 16 proteins identified by SDS-PAGE. We have termed these proteins, PA700 (for Proteasome Activator of M_r 700,000) Peak I and PA700 Peak II. Each PA700 protein was abundant in blood cells and the purification produced from 2–7 mg of each in various independent preparations. A summary of a typical purification is provided in Table II.

**Characterization of PA700**—Most of the characterization described below has been conducted for both PA700 Peak I and PA700 Peak II. Other than the observation that Peak II has only about 30% of the specific activity of Peak I, no significant differences between the two activators have been detected. Therefore, for clarity of presentation, only the data for PA700 Peak I are reported here.

Proteasome activation by PA700 was dependent on preincubation of both proteins with ATP and Mg^2+^4. Preincubation of either protein alone (with or without ATP and Mg^2+^) did not result in an increase in protease activity when the protein was then assayed individually (Fig. 8) or when either individually preincubated protein was added to the other immediately prior to the hydrolytic assay (data not shown). Maximal proteasome activation by PA700 was achieved after 45-60 min of preincubation and ranged from 15- to 100-fold; variation in the calculated stimulation usually resulted from variations in the low proteasome activities measured in the unactivated samples. Proteasome activation depended on PA700 concentration, and half-maximal activation was achieved at molar ratios of about 3:1 (PA700:proteasome, Fig. 9). Proteasome activation by PA700 was completely dependent upon ATP. Significant activation was observed at 5 μM ATP, and maximal activation was achieved at 60–100 μM ATP (Fig. 10). Of the other nucleotide triphosphates tested, only CTP could also activate the proteasome. However, a given level of activation required 50–100-fold higher concentrations of CTP than ATP. Proteasome activation by PA700 was not achieved by non-hydrolyzable ATP analogs, ADP, AMP, adenosine, or PP_i (Table III). No effect of ATP was obtained in the absence of Mg^2+^4. These various results indicate that ATP hydrolysis was required for proteasome activation.

The proteasome is a multicatalytic protease whose various activities are assessed with different peptide substrates (1). Table IV shows that PA700 stimulated the hydrolysis of each of these substrates, although the degree of stimulation varied depending on the substrate. As described previously, unactivated proteasomes displays sigmoidal kinetics with respect to substrate concentration (12, 23, 24). The PA700-activated proteasome, on the other hand, displayed normal Michaelis-Menten-type kinetics. Thus, PA700 both increased the maximal reaction velocity and decreased the substrate concentration required for half-maximal reaction velocity, suggesting that it acted as a positive allosteric effector of the proteasome (Fig. 11). Despite its large effects on peptidase activities, PA700 did not affect the rate of hydrolysis of large protein substrates such as casein and lysozyme by the proteasome. A similar differential regulation of the proteasomes’s catalytic activities was observed for PA28, and the possible implications of such results have been discussed previously (12).

**The Proteasome and PA700 Form an Activated Proteasome Complex**—To investigate the biochemical mechanism by which PA700 activated the proteasome, the proteasome was isolated by velocity sedimentation centrifugation and by gel filtration chromatography after activation by PA700. In each case, the isolated proteasome was characterized by a greatly stimulated
ATP-dependent Proteasome Activator

**Fig. 7.** Velocity sedimentation centrifugation of purified PA700. PA700 Peak I and PA700 Peak II, purified through the hydroxylapatite stage, were subjected to velocity sedimentation centrifugation as described under "Materials and Methods." Fraction I represents the top of the gradient (10% glycerol), and Fraction 24 represents the bottom of the gradient (40% glycerol). Upper panel, PA700 Peak I (160 µg, ○) and PA700 Peak II (210 µg, □) were centrifuged, and 25 µl of the gradient fractions were assayed for PA700 activity. The activity of the purified exogenous proteasome was 0.96 units/assay using Suc-Leu-Leu-Val-Tyr-AMC as a substrate. Assays were also conducted for PA700 Peak I under the same conditions, but without the exogenous proteasome (○). Lower panel, 30-µl samples of the gradient fractions of PA700 Peak I (upper panel) were subjected to SDS-PAGE.

**Table II**

| Stage                   | Volume (ml) | Protein (mg) | PA700 activity (Units x 10^-3) | Units/µg |
|-------------------------|-------------|--------------|---------------------------------|---------|
| **Fraction II**         |             |              |                                  |         |
| (NH₄)₂SO₄ (0–38%)       | 30          | 590          | 2.4                             | 0.4     |
| Sephacryl S-300         | 210         | 125          | 14                              | 11.2    |
| DEAE-Fractogel          | 77          | 13           | 17                              | 134     |
| Hydroxylapatite Peak I  | 45          | 2.5          | 5.1                             | 204     |
| Peak II                 | 36          | 1.5          | 1.0                             | 67      |

Summary of the purification of PA700 from bovine red blood cells is shown. Similar results were obtained in five independent preparations.

Activity and by a size that was much greater than that of the unactivated 20 S proteasome. By gel filtration chromatography the size was about 1,750,000 daltons (data not shown), and by each method the size of the activated proteasome was similar to that of the proteasome activity isolated from the crude cellular extract (Fig. 12; compare to Fig. 2). Immunoblot analysis of the gradient fractions directly demonstrated that the activated activity contained proteasome (data not shown). No activated proteasome was identified when the proteasome and PA700 were preincubated in the absence of ATP and then centrifuged (Fig. 12). Activated rates of peptide bond hydrolysis by the isolated activity did not require, nor were they influenced by, addition of ATP to the assays (ATP concentrations in the fractionated samples should have been negligible). These results indicate that ATP promotes the formation of a complex between the proteasome and PA700, and that complex formation results in activation of the proteasome.

The size of the proteasome-PA700 complex suggests that PA700 is a component of the 1,750,000-dalton proteasome complex identified in the initial part of this work. Because PA28 also appeared to be a component of that complex and might be responsible for some of that complex's activity, the effect of PA28 on the activity of the proteasome-PA700 complex isolated by velocity sedimentation centrifugation was tested. PA28 had very little effect on this activity (Fig. 12). These results show that unlike the 20 S proteasome, the proteasome-PA700 complex could not be directly activated by PA28.

PA700 contains protein components with molecular weights similar to those of proteasome subunits and to the proteasome activator, PA28. In order to determine the possible relationship...
ATP. Proteasome activity was measured under standard conditions at blood cell lysates. In fact, PA700 was identified as a result of an "ods" with purified proteasome (0.8 pg) at 37 °C in the presence of 60 µM various proteasome-containing complexes present in crude red blood cell lysates. Identical preincubations and assays were also conducted with the proteasome alone (△) and with PA700 alone (○). The activity of the purified proteasome without preincubation was 0.65 units/assay using Suc-Leu-Leu-Val-Tyr-AMC as a substrate.

The substrate was Suc-Leu-Leu-Val-Tyr-AMC. All assays contained 0.5 µM proteasome and were preincubated for 45 min with 60 µM ATP in the presence or absence of 1.0 µg of PA700. Z, benzyloxycarbonyl; pNA, p-naphthylamide; MNA, 4-methoxy-p-naphthylamide; ADPβS, adenosine 5'-O-(2-thiodiphosphate); AMP-PNP, ββ-imidoadenosine 5'-triphosphate; AMP-PCP, ββ-methylene adenosine 5'-triphosphate.

The effect of nucleotides on PA700 activation of the proteasome was assessed as described under "Materials and Methods" with the indicated nucleotides. ATP (0.30 µg); PA700 Peak I (0.8 µg). ADPβS, adenosine 5'-O-(2-thiodiphosphate); AMP-PNP, ββ-imidoadenosine 5'-triphosphate; AMP-PCP, ββ-methylene adenosine 5'-triphosphate.

Effect of nucleotides on PA700 activation of the proteasome

| Compound     | 5 mM | 50 µM |
|--------------|------|-------|
| None         | 0.70 |       |
| ATP          | 25.0 | 36.8  |
| CTP          | 17.2 | 4.20  |
| UTP          | 1.48 | 0.61  |
| GTP          | 1.35 | 0.53  |
| ADP          | 0.73 |       |
| AMP          | 0.90 |       |
| Adenosine    | 0.67 |       |
| PP,          | 0.83 |       |
| ADPβS        | 0.89 |       |
| AMP-PNP      | 0.85 | 0.72  |
| AMP-PCP      | 1.51 |       |

Effect of ATP concentration on proteasome activation by PA700. Proteasome activation by purified PA700 Peak I was determined as described under "Materials and Methods" with the indicated concentrations of ATP (○). Similar assays were conducted with proteasome alone (△) and with PA700 alone (○). The substrate was Suc-Leu-Leu-Val-Tyr-AMC. Proteasome, 0.35 µg/assay; PA700, 0.60 µg/assay.

Effect of PA700 concentration on proteasome activation. Purified PA700 Peak I, at the indicated concentrations, was assayed for its ability to activate purified proteasome (0.3 µg/assay) as described under "Materials and Methods." The activity of the purified proteasome without preincubation was 0.9 units/assay using Suc-Leu-Leu-Val-Tyr-AMC as a substrate.

between components of PA700 and these proteins, PA700 was probed with antibodies against the proteasome and PA28. No cross-reactivity of any protein in the PA700 complex was detected with either antibody. These results indicate that neither PA28 nor subunits of the proteasome are components of the PA700 complex, although we cannot completely exclude the possibility that one or more proteasome subunit that is not recognized by our polyclonal antibody is a component of PA700.

**DISCUSSION**

This work describes the identification, large scale purification, and characterization of a new protein activator of the 20 S proteasome. This activator, termed PA700, activates peptidase activities of the proteasome, including one that is diagnostic of various proteasome-containing complexes present in crude red blood cell lysates. In fact, PA700 was identified as a result of an attempt to resolve a discrepancy between the high proteolytic activity and the low protein content of another proteasome activator, PA28, in one such complex.

PA700 activation of the proteasome required ATP. The rationale for testing ATP as a mediator of proteasome regulation by other proteins related to previous work by us (10, 14, 25) and others (5, 6, 17, 26), and the known physiological requirement of intracellular protein degradation for ATP (6, 7). The exact biochemical role of ATP in proteasome activation by PA700 is not known but seems to be closely linked to the formation of a complex between the two proteins. Thus, the hydrolysis of ATP seems to be directly involved in a mechanism that promotes the binding of PA700 to the proteasome, which in turn results in
proteasome activation. Because the isolated proteasome-PA700 complex displayed and maintained enhanced proteasome activity without an additional ATP requirement, ATP hydrolysis does not seem to be involved in, or tightly linked to, the catalytic mechanism of peptide bond hydrolysis. The estimated molar ratio of PA700:proteasome required for maximal activation may prove to be too high. From the apparent size of the PA700-proteasome complex, it seems unlikely that more than two molecules of PA700 bind to the proteasome. Furthermore, the most plausible structural model for the interaction between the two proteins is the binding of PA700 to one or both of the proteasome's terminal rings (resulting in molar ratios of 1:1 or 2:1), a structure shown for the proteasome and PA28 (27).

Recent work from a number of laboratories has identified various proteins that modulate proteasome function. These proteins include activators (12, 13) and inhibitors (18–21), as well as proteins that alter the substrate specificity and regulatory properties of the proteasome. Some of these proteins are clearly distinguishable from one another and from PA700. However, we believe that PA700 and proteins described in three other reports have sufficiently similar properties to suggest possible relationships with one another. These other proteins, termed CF-1 (17), "ball" (28), and µ particle (29), also display a number of significant differences, the basis and significance of which are unclear. Although the incomplete and/or different methods of characterization of the various proteins make a definitive comparison of their relationships impossible at the present time, a brief comparison of the most pertinent properties. Each of the proteins forms a high molecular weight protein complex with the proteasome. The estimated molar ratio of PA700:proteasome required for maximal activation by the effect of ATP on catalytic properties of the complex; proteasome-ball complexes required additional ATP for activated rates of proteolysis, while proteasome-PA700 complexes demonstrated activated rates of proteolysis without additional ATP. Obviously, much additional work will be required to understand the structure and function of each of these proteins and their relationships with one another.

Crude extracts of bovine red blood cells contained multiple protein complexes with the proteasome as a component. The fractionation of such extracts by gel filtration chromatography produced a complicated distribution of proteasome protein (detected by immunoblotting) and proteasome activity (detected by hydrolysis of a peptide substrate specific for the proteasome) (Figs. 1 and 3). By also examining the distribution of a previously identified proteasome activator, PA28, at least three forms of the proteasome were identified. The first corresponded...
to the 20 S proteasome unassociated with other proteins. This enzyme had negligible activity but was greatly stimulated by exogenous PA28. The second was associated with endogenous PA28. This complex had a higher $M_r$ than that of the "free" 20 S proteasome and probably accounted for the endogenous proteasome activity in the column fractions corresponding to an approximate $M_r$ of 700,000–800,000. Because this proteasome was already associated with PA28, its activity was not further stimulated by exogenous PA28. Thus, the difference in apparent $M_r$ between the endogenous proteasome activity and the activity stimulated by exogenous PA28 (shown in Fig. 1) represents the difference between the proteasome with and without PA28. The third form of proteasome was represented by the 1,750,000-dalton activity and probably represents a complex containing the proteasome, PA28, and other regulatory proteins. Because of its size, PA700 is an excellent candidate for a component of the 1,750,000-dalton proteasome complex, although additional reagents such as antibodies against PA700 peptides will be required to definitively establish this possibility. Interestingly, the apparent size of the proteasome-PA700 complex and the putative proteasome-PA700-PA28 complex were not detectably different, probably because of the poor resolution of very large proteins from one another by gel filtration chromatography and velocity sedimentation centrifugation. Therefore, it is also possible that the 1,750,000-dalton activity in Fig. 1 represents a heterogeneous population of proteasome-containing complexes. Additional work will be required to establish the identity and composition of the proteins responsible for this activity. Some aspects of our analysis are consistent with that provided by Rechsteiner and colleagues (28) for proteasome-containing complexes identified by electrophoretic techniques. For example, the difference between the free proteasome and the PA28-proteasome complex shown here probably corresponds to the "slow" and "fast" migrating forms of 20 S proteasome reported by them (28). Furthermore, their identification of multiple forms of the 26 S proteasome may represent proteasome-containing complexes with either PA700 or PA700 and PA28, as suggested here. In any case, these various results clearly show the existence of multiple high molecular weight proteasome-containing complexes and indicate that the view of the existence of a single 26 S proteasome may be an oversimplification. As the analysis of the type conducted here is extended to other proteasome regulatory proteins, additional multiprotein complexes of the proteasome may be identified. The exact composition of such complexes could confer upon them specific functional and regulatory properties consistent with the proteasome's multiple potential roles in different proteolytic pathways.

The number of different proteasome complexes in cell extracts raises questions as to why such complexes have not been identified more frequently in the past and what happens to them during purification of their component proteins. Our experiments have shown that certain buffer conditions employed for the purification of these component proteins promote the dissociation of the complexes. For example, chromatography of a crude cellular extract, such as is shown in Fig. 1, in the presence of 0.5 M NaCl dissociates most of the 1,750,000-dalton proteasome complex, as well as the proteasome-PA28 complex, into individual component proteins. In fact, the level of the 1,750,000-dalton proteasome activity identified in Fig. 4 represents less than 5% of the level expected from an equivalent amount of unfractonated extract (Fig. 1). Our work indicates that most of the 1,750,000-dalton complex dissociates during the preparation of Fraction II and the subsequent ammonium sulfate precipitation.

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