Identification, Purification, and Characterization of a PA700-dependent Activator of the Proteasome*

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The activity of the intracellular protease, the proteasome, is modulated by a number of specific regulatory proteins. One such regulator, PA700, is a 700,000-Da multisubunit protein that activates hydrolytic activities of the proteasome via a mechanism that involves the ATP-dependent formation of a proteasome-PA700 complex. Four subunits of PA700 have been shown previously to be members of a protein family that contains a consensus sequence for ATP binding, and purified PA700 expresses ATPase activity. We report here the identification, purification, and initial characterization of a new modulator of the proteasome. The modulator has no direct effect on the activity of the proteasome, but enhances PA700 activation of the proteasome by up to 8-fold. This activation is associated with the formation of a proteasome/PA700-containing complex that is significantly larger than that formed in its absence. The modulator has a native Mr of ~300,000, as determined by gel filtration chromatography, and is composed of three electrophoretically distinct subunits with Mr values of 50,000, 42,000, and 27,000 (p50, p42, and p27, respectively). Amino acid sequence analysis of the subunits shows that p50 and p42 are members of the same ATP-binding protein family found in PA700. The p50 subunit is identical to TBP1, a protein previously reported to interact with human immunodeficiency virus Tat protein (Nelbock, P., Dillion, P. J., Perkins, A., and Rosen, C. A. (1990) Science 248, 1650–1653), while the p42 subunit seems to be a new member of the family. The p27 subunit has no significant sequence similarity to any previously described protein. Both p50 and p42, but not p27, were also identified as components of PA700, increasing the number of ATP-binding protein family members in this complex to six. Thus, p50 and p42 are subunits common to two protein complexes that regulate the proteasome. The PA700-dependent proteasome activator represents a new member of a growing list of proteins that regulate proteasome activity.

The proteasome is a 700,000-Da multicatalytic protease that participates in a number of proteolytically mediated intracellular processes, including the constitutive turnover of many intracellular proteins (1), the rapid elimination of proteins with abnormal structures (2, 3), the temporal reduction in levels of critical regulatory proteins for control of the cell cycle and transcription (4–7), the proteolytic activation of the transcription factor NF-κB (8), and the processing of antigens for presentation by class I major histocompatibility complex proteins (9, 10). Despite the important role of the proteasome in these various processes, the mechanisms by which its action is controlled remain unclear. Several lines of evidence indicate that proteasome function is controlled by specific regulatory proteins. First, the proteasome can be isolated as part of a larger protein complex (Mr > 1,500,000) referred to as the “26 S protease” (11). This complex displays catalytic and regulatory properties that differ considerably from those of the purified 20 S proteasome, most likely because of regulatory influences exerted by the non-proteasome components of the complex. Second, individual proteasome regulatory proteins have been identified and purified. One of these proteins, which we call PA700 and which has been independently described in several laboratories, appears to represent the major non-proteasome component of the 26 S protease (10, 12–14). PA700 is a 700,000-Da multisubunit ATP-dependent proteasome activator. It forms a complex with the proteasome by a mechanism that requires ATP hydrolysis. The proteasome-PA700 complex has physical properties, such as molecular weight, and catalytic properties, such ATP-dependent degradation of ubiquitinated proteins, that are characteristic of the purified 26 S protease. At least four of the ~20 electrophoretically distinct subunits of PA700 are homologous to one another and are members of a large protein family that contains a consensus sequence for ATP binding (15–17). Some of these same proteins have been identified as components of the purified 26 S protease, providing additional strong evidence that the proteasome-PA700 complex is similar, if not identical, to the 26 S protease (13, 15, 16). One or more of these ATP-binding proteins may be responsible for the function of ATP in proteasome activation. In fact, purified PA700 expresses ATPase activity. Surprisingly, many of these “ATPase” subunits of PA700 have been identified independently as proteins involved in processes with no obvious relationships to proteasome function (17). These findings may be explained by new and unexpected roles for the proteasome or may indicate that a given ATPase protein has multiple cellular functions.

During the course of our continuing characterization of the function of PA700, we have identified a new protein complex that functions as a PA700-dependent activator of the proteasome. This report describes the identification, purification, and initial structural and functional characterization of this protein, which contains two members of the ATPase protein family. Furthermore, the same two proteins are identified here as

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new subunits of PA700, raising the number of family members in this complex to six.

**MATERIALS AND METHODS**

Purification and Assay of the Proteasome and PA700—The 20 S proteasome and PA700 were purified from bovine red blood cells as described previously (12, 18). Protease activity was measured by the hydrolysis of the synthetic peptide succinyl-Leu-Leu-Val-Tyr 7-amino-4-methylcoumarin, also as described previously (12). The production of hydrolysis of the synthetic peptide succinyl-Leu-Leu-Val-Tyr 7-amino-4-methylcoumarin was monitored continuously at 380 nm (excitation) and 450 nm (emission), and initial steady-state rates were assessed. One unit of proteasome activity is defined as a change in 7-amino-4-methylcoumarin concentration of 1.0 mM/min under standard assay conditions. PA700 activity was assessed by measuring the proteasome activity after preincubation with pure PA700. The preincubation contained 45 mM Tris-HCl, pH 8.0, 5.6 mM dithiothreitol, 200 mM ATP, and 10 mM MgCl₂ in a final volume of 50 μl and was carried out for 45 min at 37 °C. This solution was then added to 1.0 ml of substrate solution for the measurement of proteasome activity as described above (12).

PA700-dependent Activator (Modulator) Assay—The identification, purification, and characterization of the PA700-dependent activator (modulator) was carried out with a variation of the PA700 assay. The modulator (PA700) was heated with the purified proteasome and PA700 and then preincubated under the same conditions as the normal PA700 assay. Modulator activity is expressed as the increase in the PA700-dependent proteasome activity caused by the modulator. One unit of modulator activity is defined as an increase of 1 unit of PA700 activity.

Purification of a PA700-dependent Proteasome Activator (Modulator)—Bovine red blood cells were collected, washed, and lysed as described previously (12). Fraction II from the soluble lysate was prepared using DEAE-cellulose (DE52, Whatman). Fraction II was dialyzed against Buffer X containing 100 mM NaCl, and 10 mM MgCl₂ in a final volume of 1000 μl and was carried out for 45 min at 37 °C. This solution was then added to 1.0 ml of substrate solution for the measurement of proteasome activity as described above (12).

Identification and Purification of a PA700-dependent Modulator of the Proteasome—As part of a search for proteins that regulate proteasome function, we have been screening cell extracts for proteins that can influence one or more activities of the purified 20 S proteasome. A number of such proteins have now been identified, and they include both activators and inhibitors. Because it seemed reasonable to assume that some proteasome regulatory proteins might act in concert or might themselves be regulated by other proteins, we also have designed assays to test the effects of cell extracts on the proteasome in the presence of previously identified regulators. In the experiments described here, we tested the ability of a red blood cell extract, fractionated by ammonium sulfate precipitation and gel filtration chromatography on Sephacryl S-300, to affect the proteolytic activity of the exogenous 20 S proteasome in the presence of purified PA700, an ATP-dependent proteasome activator (12). When column fractions were added to assays containing the purified proteasome and PA700, two peaks were identified that contained more activity (3–8-fold in various preparations) than that accounted for by the purified proteasome and PA700 alone (Fig. 1). One of these peaks was coincident with the elution position of PA700 endogenous to those fractions. Therefore, this peak probably resulted from the concentration-dependent increase in proteasome activity from increased PA700 in the assay. The second peak of enhanced proteasome activity was identified in fractions with an apparent M₀ of ~300,000. Control assays indicated that this peak did not result from endogenous proteasome activity, which was very low in these column fractions (<1.5 units/assay) and was

1 The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
were coincident with PA700-dependent proteasome activation. 3) had elution profiles indistinguishable from one another and absence of PA700, the modulator had no effect on proteasome ized with respect to its effect on proteasome activity. In the tor(forsimplicity,hereaftertermed Activator—

FIG. 1. Identification of a PA700-dependent activator (modulator) of the proteasome by gel filtration chromatography. Proteins from Fraction II that precipitated between 0 and 38% saturated ammonium sulfate (see "Materials and Methods") were solubilized and chromatographed on Sephacryl S-300. Column fractions were assayed for PA700 activity (●) and PA700-dependent activator (modulator) activity (○) as described under "Materials and Methods." PA700 activity was assessed using 5 μl of column fractions and the purified exogenous 20 S proteasome (0.25 μg/assay, 0.4 units). Modulator activity was assessed using 5 μl of column fractions and the purified exogenous proteasome (0.25 μg/glass) and purified exogenous PA700 (0.64 μg/glass, 7.6 units). Control assays for the endogenous proteasome in column fractions had <1.5 units/assay (not shown). The column fractions were also subjected to Western blotting with anti-TBP1 (top panel). To show all fractions, results from two different blots are shown. Standards of TBP1 on each blot produced bands of equal intensity.

not observed unless exogenous proteasome, PA700, and MgATP were present in the assays (see further characteriza-

The fractions containing the PA700-dependent proteasome-activating activity were pooled and subjected to further purifi-
cation by ion-exchange chromatography on DEAE-Fractogel as described under "Materials and Methods." The PA700-dependent proteasome-activating activity bound to this resin and was eluted as a single peak at a position corresponding to ~100 M NaCl (Fig. 2). The fractions containing the peak activities were pooled and subjected to hydroxylapatite column chromatogra-
phy. The PA700-dependent proteasome-activating activity eluted from this resin as a single peak (Fig. 3). SDS-PAGE analysis of the fractions from the hydroxylapatite column showed that three major proteins (denoted with arrows in Fig. 3) had elution profiles indistinguishable from one another and were coincident with PA700-dependent proteasome activation. These proteins had apparent Mₐ values of 50,000, 42,000, and 27,000. Retrospective analysis of the column fractions from the Fractogel ion-exchange chromatography by SDS-PAGE also showed that these three proteins coeluted with one another and with modulator activity (data not shown). The activity from the hydroxylapatite chromatography was subjected to a second Sephacryl S-300 chromatography step. The activity eluted at a position corresponding to its originally estimated Mₐ of 300,000 and was coincident with the three proteins described above (data not shown). Therefore, we conclude that red blood cell extracts contain a PA700-dependent proteasome activator composed of protein subunits with Mₐ values of 50,000, 42,000, and 27,000.

The Modulator Functions as a PA700-dependent Proteasome Activator—The purified PA700-dependent proteasome activa-
tor (for simplicity, hereafter termed modulator) was character-
ized with respect to its effect on proteasome activity. In the absence of PA700, the modulator had no effect on proteasome

activity (Fig. 4). However, the modulator stimulated PA700-de-
pendent proteasome activity up to 8-fold. The magnitude of this effect was decreased at very high concentrations of PA700 (Fig. 4; see "Discussion"). We previously showed that PA700 activa-
tion of the proteasome required preincubation of both proteins in the presence of ATP (12). The modulator’s effect on protea-
some activity required the simultaneous preincubation of all three proteins with ATP. Preincubation of any individual protein or various combinations of any two proteins, followed by the addition of the other(s) immediately prior to the addition of the substrate for assay of proteasome activity, did not result in activated rates of PA700-dependent proteasome activity (data not shown). The modulator did not affect the rate of proteasome activation compared with that promoted by PA700 alone (data
The modulator was tested for its ability to activate the purified proteasome. The indicated amounts of modulator were preincubated for 45 min with 0.25 μg of proteasome in the presence (●) or absence (▲) of PA700 (0.64, 2.5, or 5.0 μg assay) and in the presence of 100 μM ATP prior to the assay for proteasome activity using succinyl-Leu-Leu-Val-Tyr 7-amino-4-methylcoumarin as a substrate.

The Modulator Promotes Formation of an Activated Proteasome-Containing Complex—To determine possible mechanisms of action of the modulator, proteasome/PA700-containing complexes formed in the presence and absence of the modulator were isolated by glycerol density gradient centrifugation. As reported previously, the proteasome and PA700 form a complex that is activated with respect to proteasome and that is much larger than either individual protein. Preincubation of all three proteins resulted in a complex that was 2–4-fold more active and significantly larger than the complex formed after preincubation of the proteasome and PA700 alone (Fig. 5).

The Modulator Contains Homologous Members of an ATP-Binding Protein Family—To learn about the structural basis of modulator function, we subjected each of its subunit proteins to amino acid sequence analysis. The p50, p42, and p27 proteins were isolated by HPLC and digested with trypsin or Lys-C protease. The resulting peptides were isolated by HPLC and sequenced by automated Edman degradation as described under "Materials and Methods." Sequences were obtained for 12 peptides of the p50 subunit; these sequences contained 211 amino acids. Comparison of these sequences with those in current data bases showed that they exactly matched the sequence of a previously described human protein, TBP1 (human immunodeficiency virus Tat-binding protein) (21) (Fig. 6). TBP1 is a member of a large protein family that contains a consensus sequence for nucleotide binding (see below). Sequences were obtained for five peptides of the p42 subunit, containing 88 amino acids. Comparison of these sequences with proteins in current data bases indicated that p42 had significant sequence similarity to TBP1 (p50) as well as to other members of this protein family (Fig. 7 and data not shown). It is not possible from these partial data to determine whether p42 is the clear homolog of any one member of the family. Eight tryptic peptides of the p27 subunit of the modulator were isolated and sequenced. 90 amino acids were identified, and these sequences had no significant similarity to those of any protein listed in current data bases (data not shown).

p50 (TBP1) and p42 Are Common Subunits of Two Different Proteasome Regulatory Complexes: PA700 and the Modulator—Four members of a protein family containing a consensus sequence for ATP binding have been identified as components of PA700 or of the 26 S protease, a large proteasome-containing complex that contains PA700 as its major, if not its only, non-proteasome component. These proteins include S4 (22), MSS1 (23, 24), TBP7 (15, 25), and p45 (15, 26). Although TBP1, a member of this family originally identified as a human immunodeficiency virus Tat-binding protein (21), has not been identified as a component of PA700 by direct sequencing, an antibody prepared against human TBP1 was shown to cross-react with a 50,000-Da subunit of PA700 from rat liver (6). We used this antibody to examine the bovine modulator and PA700 proteins. Fractions from the Sephacryl S-300 column on which PA700 and the modulator were first isolated contained a single immunoreactive band of 50,000 Da. This band was present in two peaks that were coincident with PA700 and the modulator, respectively (Fig. 1). Interestingly, more immunoreactive protein was present in the modulator peak than in the PA700 peak. The same immunoreactive band was observed in the purified PA700 and modulator proteins (Fig. 8). These results indicate that TBP1 (p50) is a subunit of each protein. To provide direct evidence for this conclusion, the PA700 subunit that reacted with the anti-TBP1 antibody was isolated by a two-dimensional procedure involving HPLC and SDS-PAGE (15). Its retention time on HPLC was identical to that of the p50 (TBP1) subunit of the modulator (peak 4 of Fig. 9 and data not shown). The isolated PA700 subunit was subjected to digestion by Lys-C protease. The resulting peptides were isolated by HPLC and sequenced by automated Edman degradation as described under "Materials and Methods." Sequences were obtained for six peptides containing a total of 80 amino acids. Each of these sequences was identical to sequences of TBP1,
thereby establishing the identity of the p50 subunit of PA700 as TBP1 and demonstrating that this protein is a subunit of both PA700 and the modulator (Fig. 6). In light of this surprising result, we next determined whether the other two modulator subunits, p42 and p27, might also be components of PA700.

Fig. 6. The p50 subunits of the modulator and PA700 are identical to one another and to TBP1. The p50 subunit of the modulator was isolated by HPLC and SDS-PAGE and subjected to amino acid sequencing as described under "Materials and Methods." The sequences of 12 peptides produced by Lys-C digestion were determined and are shown aligned with the complete sequence of human TBP1 (21). The p50 subunit of PA700, identified by its reactivity to an anti-TBP1 antibody, was isolated and subjected to sequencing in the same manner as the modulator subunit. The sequences of five peptides were determined and are shown aligned with TBP1.

DISCUSSION
We have identified a new multisubunit protein complex that regulates proteasome function. Unlike previously identified proteasome regulators, this new protein, referred to here as the modulator, does not directly influence proteasome activities, but enhances, by up to 8-fold, the effect of PA700, an ATP-de-
The possible origin of the modulator as a dissociated subcomplex of PA700 subunits, and we never observed PA700 always migrated coincidently with PA700 activity and for the ability to be stimulated by the purified exogenous modulator. In all cases, both of these activities were exactly coincident with one another and with all PA700 subunits (including p50 and p42) detected by SDS-PAGE. Such results would not be expected if the modulator had a selective effect on a subpopulation of PA700 that lacked p50 and p42 because such a population should have a lower molecular weight than native PA700. Thus, we have been unable to provide any evidence that the modulator is derived from dissociation of PA700, although our current analysis cannot rigorously exclude this possibility.

Could the identification of p50 and p42 in PA700 preparations represent contamination by the modulator, either nonspecifically or as the result of a specific interaction between PA700 and the modulator? Nonspecific contamination seems unlikely because PA700 and the modulator were well separated from each other early in the purification (Fig. 1) and differed significantly in their chromatographic behavior on several additional columns. Furthermore, both p50 and p42 represented major PA700 components (Fig. 9). Although we cannot completely exclude the possibility that our PA700 preparations contained complexes formed by the specific interaction of the modulator and PA700, the failure to dissociate the modulator from PA700 preparations (as described above) seems to argue against this possibility. Therefore, these various results support the conclusion that the modulator represents a distinct protein complex that shares two subunits, p50 (TBPI) and p42, with PA700.

The sharing of p50 and p42 between two different proteins suggests that these subunits could have functions common to each of the complexes. Because p50 and p42 are members of the ATP-binding protein family, such a function might involve the role of ATP in proteasome activation, particularly in the assembly of the proteasome-containing complex. We are currently examining this possibility. In any case, another member of the ATP-binding protein family, p45, has been identified in at least two different multiprotein complexes: PA700 (15) and the transcriptional mediator complex that contains Suglp1, the homolog of p45 in yeast (26–28). It seems possible that other members of this ATP-binding protein family will be shared among different protein complexes.

The regulation of proteasome function by the combined action of two proteins, as shown here, is reminiscent of early reports by Hershko and co-workers (29) showing the requirement for two factors, termed CF1 and CF2, for proteasome activation. Therefore, it is reasonable to question the possible relationship between PA700 and the modulator, on the one hand, and CF1 and CF2, on the other. Direct comparison between these protein pairs is difficult because CF1 and CF2 are not identical, to PA700 (12). In contrast, there are significant differences between the modulator and CF2. Goldberg and co-workers (30) purified a protein with functional properties indistinguishable from CF2 (i.e., the ability to activate the proteasome in a CF1-dependent manner in a reconstitution system where proteasome activation required both proteins). In the absence of CF1, this CF2 protein functioned as a proteasome activator. Because the modulator's stimulatory effect magnifies a 50–200-fold stimulatory effect by PA700, its influence on total proteasome activity is very large. The mechanism by which the modulator exerts its effect is presently unclear. PA700 activation of the proteasome involves the formation of a proteasome-PA700 complex in which PA700 binds to one or both of the proteasome's terminal rings. The finding that the modulator promotes the formation of a larger complex than that which is formed in its absence (Fig. 5) suggests two possible mechanisms for its action, which are not mutually exclusive and do not represent all possible mechanisms. First, the modulator might form a ternary complex with PA700 and the proteasome. Alternatively, the modulator could promote the formation of more complexes in which the proteasome is bound to two, rather than just one, PA700 molecule. Each of these models would account for the larger proteasome-containing complex caused by the modulator, and structural changes associated with each presumably result in increased proteasome activation.

Two modulator subunits, p50 and p42, are homologous to one another and are members of a large protein family that contains a consensus sequence for ATP binding (17). The p50 subunit is identical to TBP1, previously identified as a human immunodeficiency virus Tat-binding protein (21), while p42 seems to be a new family member. We are currently determining the complete primary structure of p42 to confirm this latter conclusion. In any case, this work demonstrates that p50 and p42 modulator subunits are also subunits of PA700. Four other members of this protein family, S4, MSS1, p45, and TBP7, previously were shown to be subunits of PA700. Thus, the results presented here demonstrate that PA700 contains at least six members of this ATPase family.

The surprising finding that p50 and p42 are common subunits of two distinct proteasome regulators raises obvious questions regarding the origin of the modulator protein, on the one hand, and the basis for the identification of p50 and p42 as PA700 subunits, on the other. For example, could the modulator represent a subcomplex of PA700, derived from the disassociation of PA700? To address this question, we attempted to generate the modulator from purified PA700 by treating purified PA700 (which contains p50 and p42 as major components) as judged by staining intensity; Fig. 9) with a variety of chaotropic and other agents (including 38% ammonium sulfate) and then subjected the protein to gel filtration chromatography or to density gradient centrifugation. The p50 and p42 subunits of PA700 always migrated coincidently with PA700 activity and with the rest of the PA700 subunits, and we never observed formation of the modulator from PA700 in these experiments.2

some inhibitor and was judged identical to a previously identified proteasome inhibitor with a subunit size of 40,000 Da (31). We have failed to detect inhibition of the proteasome by the modulator under a variety of assay conditions.2 Furthermore, the 40,000-Da inhibitor was subsequently shown to have the same structural, functional, and immunological properties as δ-aminolevulinic-acid dehydratase (32). These various findings clearly distinguish the modulator described here from previously described CF2 proteins and suggest that the modulator may be one of several proteins that regulate proteasome function indirectly through PA700.

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