A Model for the Quaternary Structure of the Proteasome Activator PA28*

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PA28 is a protein activator of the 20S proteasome. It has a native molecular weight of approximately 200,000 and is composed of six 28,000-dalton subunits arranged in a ring-shaped complex. Purified preparations of PA28 contain two polypeptides, α and β, which are about 50% identical in primary structure. It has been unclear whether native PA28 consists of two distinct homohexameric proteins or of a single protein containing both α and β subunits. To distinguish between these possibilities, we prepared antibodies that reacted specifically with either the α or β subunit and used these subunit-specific antibodies in two types of experiments designed to elucidate PA28 quaternary structure. In the first experiment, the α and β subunits were completely co-immunoprecipitated by each subunit-specific antibody, indicating that both subunits were part of a single protein complex. In the second experiment, PA28 was chemically cross-linked using bis(sulfosuccinimidyl)suberate. When the cross-linked products were immunoblotted after SDS-polyacrylamide gel electrophoresis, indistinguishable patterns were obtained with each subunit-specific antibody. These results confirm that the α and β subunits were part of the same protein complex. The pattern of cross-linked products also provided insight as to the relative abundance and arrangement of the subunits within the PA28 complex and indicated that the ring-shaped PA28 hexamer may be composed of alternating α and β subunits with a stoichiometry of (αβ)₆. PA28 was inactivated by treatment with carboxypeptidase Y, which cleaved Tyr and Ile residues from the carboxyl terminus of the α subunit but had very little effect on the β subunit. This selective and limited proteolysis prevented binding of both α and β subunits to the proteasome and therefore provides additional evidence of the heterodimeric nature of PA28. These results indicate that a short carboxyl-terminal sequence of the α subunit is critical for binding of native PA28 to the proteasome. To learn about the relative functions of the α and β subunits, PA28α was expressed in Escherichia coli and purified to homogeneity. Purified PA28α stimulated proteasome activity but required 5–10-fold greater concentrations than the heterodimeric PA28 to achieve a given level of activity. These results suggest that the heterodimeric structure of PA28 is required for maximal proteasome activation.

The proteasome is a 700,000-dalton, multisubunit protease complex found in bacteria (1), archaea (2), and all examined higher eukaryotes (3, 4). The overall architecture of the proteasome is remarkably similar among all sources, consisting of four stacked rings of seven subunits each (5). This arrangement results in a cylinder-shaped particle comprising 28 subunits. In archaea the subunits represent the products of two homologous gene products, α and β (6). The α subunits form the two outer rings, whereas the β subunits form the two inner rings (7). Proteasomes in higher eukaryotes demonstrate a greater subunit complexity and in a given source are composed of 14 different gene products, 7 of which are homologous to the archaeobacterial α subunit and 7 of which are homologous to the archaeobacterial β subunit (8, 9). Nevertheless, the relative arrangement of these subunits is the same as in archaea with the α type subunits constituting the outer rings and the β type subunits constituting the inner rings (10). Recent analysis of the crystal structure of the archaeobacterial proteasome indicates that the β subunits contain the active sites of the proteasome and that these sites are located in the interior of the cylinder (11).

The proteasome associates with a number of other protein complexes, which function as regulators of its activity. For example, one large regulator, which we call PA700, activates the proteasome’s ability to degrade ubiquitinated proteins as well as small synthetic peptides (12–15). Another protein, called PA28, also activates the proteasome’s hydrolysis of small peptides but does not promote its degradation of ubiquitinated proteins (16, 17). Although these distinct regulatory proteins do not share common subunits (18, 19), they each interact with the proteasome by binding to its terminal α rings. Electron microscopic studies have shown that PA28 is itself a ring-shaped structure composed of 6 or 7 subunits (20). The binding of PA28 to the proteasome seems to depend on the carboxyl terminus of PA28, because carboxypeptidase-treated PA28 fails to bind to the proteasome (21).

Recent work in our laboratory demonstrated that purified preparations of PA28 contain two polypeptides, termed α and β (22). These proteins represent distinct gene products but are about 50% identical to one another in primary structure (23). Although α and β are present in approximately equal abundance in purified preparations of PA28, their relative organization in the PA28 quaternary structure is unclear. For example, it is possible that native PA28 consists of two different homohexamers, each composed exclusively of one type of subunit. Alternatively, PA28 might consist of both α and β subunits in either fixed or variable stoichiometry. Because of the

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structural similarities of the α and β subunits, our previous biochemical characterization of PA28 was not able to distinguish between these possibilities, and new reagents were required to address this issue. Therefore, we have generated antibodies that are specific for each PA28 polypeptide and have used these antibodies to investigate the quaternary structure of PA28. The data reported here provide strong support for a heterodimeric model of PA28 quaternary structure. This model of PA28 structure prompted us to examine the biochemical nature of the carboxyl-terminal modification of PA28 that results in loss of binding to the proteasome, a phenomenon identified before PA28 was recognized to contain the distinct α and β subunits. Interestingly, carboxypeptidase treatment selectively alters the α subunit.

MATERIALS AND METHODS

Production of Antibodies—Antibodies against purified PA28 from bovine red blood cells were produced in rabbits as described previously (21). These antibodies recognize both the α and β subunits of PA28. Subunit-specific antibodies were prepared using synthetic peptides corresponding to 0.04% of the linking β subunits of the rat PA28. Two peptides, corresponding to the amino and carboxyl termini, were synthesized for each subunit as follows: PA28α amino terminus, TLRVH-PEAQAKVDV; PA28α carboxyl terminus, EKLKPKRGETKGM; PA28β amino terminus, KPSGVRSLGAEKRQV; PA28β carboxyl terminus, EKIVNPRGKEEKP5MY. Each peptide was synthesized with an additional cysteine residue for one of the subsequent coupling procedures. The cysteine was added to the carboxyl terminus of the amino-terminal peptides and to the amino terminus of the carboxyl-terminal peptides. The peptides were coupled to keyhole limpet hemocyanin (KLH)1 by each of two procedures. In the first procedure, a solution of KLH (1 mg/mL) and a given peptide (2.5 mg/mL) in 20 mM sodium phosphate buffer, pH 7.2, was treated with glacial acetic acid at a final concentration of 0.04% by gentle shaking at room temperature for 16 h. The solution was dialyzed extensively against water. In the second procedure, peptides were coupled to KLH with the cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester as described previously (24). Rabbits were injected subcutaneously with 300 μg of peptide-coupled KLH emulsified with Freund’s complete adjuvant. Booster injections were given 21 and 41 days after the first injection and consisted of 150 μg and 75 μg, respectively, of peptide-coupled KLH emulsified with Freund’s incomplete adjuvant. Rabbits were bled 51 days after the first injection. The sera were collected and stored at −70°C. Analysis of some of these sera is described in the text.

Immunoprecipitation of PA28—Immunoprecipitation of native PA28 was conducted with the antisera described above and the co-precipitant, protein A-Sepharose 4B (Sigma). Serum (10 μl) was preincubated with 250 μl of a 20% suspension of protein A-Sepharose in TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.05% Tween 20 for 1 h at 4°C. After the preincubation, the protein A-Sepharose was collected by centrifugation was performed as described previously (16, 21). In brief, protein samples were layered on 4.5-ml gradients (10–40% glycerol) in 30-250 quadrupole mass spectrometer (Micromass Inc., Altrincham, United Kingdom) and ionized by electrospray. The mass analyzer was mass calibrated using the VG Instruments Ti50.1 rotor. Each tube was fractionated into 24 200-μl samples and assayed as described in the text.

Digestion with Carboxypeptidase Y—Carboxypeptidase-catalyzed removal of carboxyl-terminal residues from PA28 and from recombinant PA28 was monitored quantitatively by mass spectrometry. PA28 (382 ng/μl) or PA28α (150 ng/μl) was digested with carboxypeptidase Y (Boehringer Mannheim; sequencing grade) at enzyme:substrate ratios of 1:2170 (PA28) or 1:480 (PA28α) for varying periods. Samples of digested protein were tested for their capacity to bind to and activate 20S proteasomes (16, 21) and for the loss of amino acids from their carboxyl termini. For the latter purpose, digestion was terminated by adding trifluoroacetic acid to a final concentration of 0.9% for PA28 or 0.02% for PA28α and then freezing at −70°C. The protein was desalted by reverse phase HPLC (22), and subunits were collected manually, always in the same volume for a given quantity of protein injected. 10 μl (1.4–3.6 μg) of each aliquot was introduced by loop injection into a VG 30-250 quadrupole mass spectrometer (Micromass Inc., Altrincham, United Kingdom) and ionized by electrospray. The mass analyzer was scanned over the m/z range of 1,000–2,000 at 1,000, and the data were transformed automatically using the manufacturer’s standard LAB-BASE™ protocol. The relative quantities of the intact protein and its digestion products were estimated by comparison of peak areas in the transformed mass spectra.

Plasmid Construction—The PA28α plasmid was constructed using the pET16b vector (Novagen) containing the complete rat PA28α sequence (23). The plasmid-based sequence encoding the His-Tag was removed by digestion with NcoI and Ndel (all restriction and modifying enzymes were obtained from Life Technologies, Inc.). The 5′- and 3′-overhangs were removed using S1 nuclease. After religation using T4 DNA ligase, the plasmid was transformed into Escherichia coli BL21. Expression of PA28α in E. coli—50 ml of SOC medium (25) containing 100 μg/ml ampicillin was inoculated with E. coli BL21 (DE3) carrying the construct described above and was grown overnight at 30°C. This culture was used to inoculate 1000 ml of the same medium to an A500nm value of 0.2. The cells were grown to an A500nm value of 0.5, and then expression of PA28α was induced with 0.75 mM isopropyl-1-thio-β-D-galactopyranoside. After 4 h, the cells were harvested by centrifugation, washed with Buffer H, and frozen at −70°C. The pellet was thawed, resuspended in 20 ml of Buffer H containing 600 μg/ml lysozyme, and incubated at 4°C for 30 min. The cells were sonicated and centrifuged at 20,000 × g for 10 min. A small portion of the soluble supernatant containing the PA28α protein was analyzed for PA28 activity and for PA28α protein. PA28α was purified to homogeneity from the remaining supernatant using sephacryl column chromatography procedures using DEAE ion-exchange chromatography and hydroxylapatite chromatography. For each column, PA28α was monitored by activity and immunoblotting. Amino-terminal amino acid sequencing of the purified PA28α was automated Edman degradation yielded the homogenous sequence ATLRVHPEAQAKVDV. This is the expected sequence for rat PA28α with the initiating methionine removed. The purified

1 The abbreviations used are: KLH, keyhole limpet hemocyanin; TBS, Tris-buffered saline; BS3, bis(sulfosuccinimidyl)suberate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
Characterization of Antibodies Specific for PA28α and PA28β—To examine the subunit composition of the proteasome activator PA28, we raised antibodies against each of the protein’s two components, α and β. These polypeptides are both present in purified preparations of PA28 from bovine and rabbit tissues and represent the products of two distinct but homologous gene products that are about 50% identical in primary structure. As described under “Materials and Methods,” we used synthesized peptides corresponding to the amino- and carboxyl-terminal regions of each protein to serve as antigens.

Two of the resulting antisera were used for the present work: one generated against the carboxyl terminus of the α protein (cross-linked by the m-maleimidobenzoyl-N-hydroxysuccinimide ester method). Right, blotted with anti-β antisemur.

Results

Characterization of Antibodies Specific for PA28α and PA28β—Characterization of PA28 subunit-specific antisera. Antiserum against carboxyl-terminal peptides of PA28α and PA28β were prepared as described under “Materials and Methods.” Two identical sets of samples of PA28 were electrophoresed by SDS-PAGE and subjected to immunoblotting as described under “Materials and Methods.” Lanes denoted by PA28 contain 20 ng of purified PA28. Lanes denoted by PA28α contain 20 ng of PA28α isolated by HPLC. Lanes denoted by PA28β contain 20 ng of PA28β isolated by HPLC. Left, blotted with anti-α antisemur. Right, blotted with anti-β antisemur.

PA28α was concentrated using a PM10 membrane in an Amicon concentrator. The protein was dialyzed extensively against Buffer H and stored frozen at −70 °C until use.

As shown in Fig. 2, each of the subunit-specific antisera completely immunoprecipitated PA28. Thus, no PA28 recognized by any of the three antisera remained in the supernatant of the immunoprecipitation reactions. In contrast, the immunoprecipitated protein reacted with both the α- and β-specific antisera, regardless of which of these antisera had been used for the immunoprecipitation protocol but that included a nonimmune serum or no serum, respectively. The large band at the 50 K marker represents IgG from the immunoprecipitation.

Chemical Cross-linking of α and β Subunits—To confirm and extend the evidence of the physical association between α and β subunits, we subjected native PA28 to chemical cross-linking using BS3. The products of this reaction were then analyzed by Western blotting, using each of the subunit-specific antisera.

As shown in Fig. 3, treatment of PA28 with BS3 resulted in a time-dependent loss of PA28 protein migrating with its characteristic molecular weight of approximately 30,000 and a concurrent appearance of new protein bands with higher molecular weights. After 5 min of exposure to BS3, two prominent bands with apparent molecular weights of 59,000 and 67,000 and two minor bands with apparent molecular weights of 54,000 and 73,000 were observed. The sizes of the major bands are consistent with the formation of dimers from the 28,000-
The results strongly support the conclusion that native PA28 molecules are prepared against purified intact PA28 (data not shown). A pattern of cross-linked products was also detected using the anti-minor contaminating protein (data not shown). The same pattern was obtained for intact PA28 molecules in solution or between PA28 and any proteins added to the cross-linking reaction; such results excluded the possibility that cross-linking occurred between dissimilar proteins and that the molecular weight products were formed, the largest of which migrated with a molecular weight of approximately 200,000. This value is similar to that of native PA28 and, therefore, is consistent with the expected size of the largest possible cross-linked structure. Again, each subunit-specific antiserum detected the same pattern of cross-linked products. Similar results were obtained at several different concentrations of PA28 and in the presence or absence of other proteins added to the cross-linking reaction; such results excluded the possibility that cross-linking occurred between distinct PA28 molecules in solution or between PA28 and any contaminating protein (data not shown). The same pattern of cross-linked products was also detected using the antibody prepared against purified intact PA28 (data not shown). These results strongly support the conclusion that native PA28 contains both α and β subunits within the same protein complex. They also suggest that the spatial arrangement of the subunits within the PA28 complex is such that the α and β subunits are arranged alternately in the ring (Fig. 4; also see “Discussion”).

**Limited Carboxyl-terminal Proteolysis of the α Subunit Inactivates PA28**—We previously reported that native PA28 was inactivated by carboxypeptidases and that this inactivation was associated with the inability of the proteolyzed PA28 to bind to the proteasome (21). The proteolytic modification at the carboxyl terminus must involve a very short sequence, because there is no detectable difference in mobility of the native and proteolyzed PA28 on SDS-PAGE (21). Our original studies of the effects of carboxypeptidase inactivation of PA28 were conducted before it was clear that PA28 was composed of both α and β subunits. Therefore, to examine the structural basis for inactivation of PA28 by carboxypeptidases, native PA28 was treated with carboxypeptidase Y for various periods and examined by mass spectrometry. Samples from carboxypeptidase reaction mixtures were desalted for electrospray ionization by reverse phase HPLC. Protein elution was monitored by absorbance at 214 nm, and quantities measured according to peak height were found to be constant from aliquot to aliquot (± 10%). Subunits were collected manually in equal volumes for each sample and introduced into the mass spectrometer by loop injection. Because protein concentrations were constant, signals in the mass spectrometer could be compared directly for a series of time points. In preliminary experiments in which the digestion of the PA28α subunit was studied, measurements were made of ion current per scan due to each protein peak in the transformed mass spectrum. The sum of these ion currents was found to be constant (±2.3%) during the course of digestion. Because as much as 72% of intact α chain had been consumed during the digestion, this observation indicated that the various digestion products ionized with efficiencies equal to the intact PA28 subunit. This enabled the relative quantities of protein species in digestion mixtures to be measured from the transformed spectra by direct comparison of peak areas.

Mass spectra acquired at different stages of digestion are shown in Fig. 5. They revealed progressive loss of the full-length α and β subunits. However, the rate of loss was much higher for α than for β. Under the conditions used, full-length α subunits were replaced by polypeptides lacking the carboxyl-terminal tyrosine residue and by polypeptides lacking both carboxyl-terminal tyrosine and the penultimate isoleucine. Full-length β subunits were replaced to a limited extent by polypeptides lacking the carboxyl-terminal tyrosine only. A quantitative analysis relating time of digestion with both changes in proteasome-activating activity and changes in the abundance of subunits lacking different numbers of residues is shown in Fig. 6. The data show that loss of activity occurs much more rapidly than loss of the full-length β subunit. For example, after 20 min of digestion, only 37% of PA28 activity remains, yet 97% of β subunits are uncleaved. Assuming that each molecule of PA28 contains three β subunits (Fig. 4) and that their probability of being digested by carboxypeptidase Y is equal and independent, only 9% of PA28 molecules would be expected to lack their full complement of intact β subunits at this stage of digestion. This suggests that degradation of α rather than β subunits is responsible for activity loss. Although the rate of activity loss is also significantly faster than the rate of loss of intact α subunits (for example, after 20 min of digestion, 75% of intact α subunits remain), only 42% of PA28 molecules are expected to contain their full complement of intact α subunits at this stage of digestion. The data are therefore consistent with PA28 activity loss being due to the digestion of one or more α subunits in the complex. In this study, measurements of the specific activities of PA28 molecules lacking one or two residues from a single α subunit could not be measured directly or compared with the specific activities of molecules lacking various combinations of carboxyl-terminal residues from two or more of their α subunits. It is therefore presently impossible to assign functional contributions to the carboxyl-terminal tyrosine and isoleucine residues individually. Both α and β Subunits of Carboxypeptidase Y-treated PA28 Fail to Bind to the Proteasome—The effect of carboxypeptidase digestion on the binding of PA28 subunits to the proteasome was also investigated. Samples of PA28 that had lost more than 80% of their proteasome-activating activity through digestion with carboxypeptidase Y were used in these experiments, and electrospray mass spectrometry was performed to verify that only the α subunits had been degraded. Aliquots of PA28 treated in this way, as well as untreated controls, were incubated briefly with the 20S proteasome and subjected to glycerol density gradient centrifugation. Fig. 7 shows the distribution of...
PA28 subunits following centrifugation, as revealed by Western blotting of gradient fractions using subunit-specific antisera. Untreated PA28 showed both the α and β subunits in the fractions coincident with the proteasome, indicating that both subunits bind to the 20S proteasome. As expected, carboxypeptidase Y-treated PA28 showed only a minor amount of PA28 associated with the proteasome and indicated that the degree of proteasomal activation was low. The majority of both the α and β subunits of PA28 was detected in gradient fractions in which uncomplexed PA28 migrates. This indicates that the binding of both the α and β subunits to the 20S proteasome is prevented by degradation of the α subunit only and therefore provides additional support for the model of PA28 structure in which both subunits are part of the same protein complex.

The α Subunit Is Sufficient for Proteasome Activation—The results described above indicate that the α subunit plays an important role in the interaction between PA28 and the proteasome. To examine the relative roles of the α and β subunits in PA28 function, we expressed PA28α in E. coli and purified the protein to homogeneity (Fig. 8). The purified α subunit stimulated the proteasome to the same extent as did native PA28 (Fig. 9). However, the specific activity of the isolated subunit was 5–10 times lower than that of the native PA28 protein containing both subunits. These results indicate that the isolated α subunit is competent for proteasome activation but suggest that both subunits are required for maximal activation. Alternatively, some of the expressed α subunit may be incorrectly folded or otherwise functionally inactive.

PA28α Is Inactivated by Carboxypeptidase Y—To examine further the proteolytic modification of PA28α by carboxypeptidase, the purified α subunit was treated with carboxypeptidase Y. Mass spectral analysis of PA28α revealed at least two subforms (Fig. 10). The mass of the smaller one was in excellent agreement with the value of 28,503 Da expected for the recombinant rat protein lacking the amino-terminal methionine residue (see “Materials and Methods”). The other species was 75.6 Da larger. This difference is consistent with the presence in the preparation of two proteins differing in amino acid sequence by the substitution of a Phe for an Ala residue (76.1 Da) or a Tyr for a Ser residue (76.1 Da) or may be the result of a presently unidentified posttranslational modification. Mass spectra of PA28 digested with carboxypeptidase. Transformed electrospray mass spectra are shown for the α and β subunits of PA28 after digestion with carboxypeptidase Y for 0, 20, 45, and 90 min. For the α subunit, peaks within the mass interval 28,602–28,609 Da represent intact polypeptide, those of mass 28,431–28,445 Da result from loss of the carboxyl-terminal tyrosine, and those of mass 28,327–28,335 Da result from the further loss of the penultimate isoleucine. For the β subunit, the peaks of mass 27,290–27,297 Da represent the intact polypeptide, and those of mass 27,120–27,131 Da result from loss of the carboxyl-terminal tyrosine. FS, full scale.
s spectra of samples digested with carboxypeptidase Y were consistent with the progressive loss of tyrosine and isoleucine from both subforms (Fig. 11), indicating that each subform has the same carboxy-terminal sequence as native PA28. Quantitative comparison of proteasome-activating activity and molecular mass (Fig. 11) shows a close correlation between the loss of the full-length protein and loss of activity. As observed with native PA28, however, activity loss occurs at a rate faster than the consumption of the full-length polypeptide, suggesting that recombinant PA28α may also exist as a multimeric protein. To test this possibility, samples of PA28α were subjected both to gel filtration chromatography and to density gradient centrifugation. These results indicate that, in the absence of β subunits, α subunits can associate in a multimeric complex (data not shown).

[Graph showing proteasome activity as a function of fraction number]

**FIG. 7.** Effect of carboxypeptidase digestion on the binding of PA28 to the proteasome. Purified bovine PA28 (30 μg) was incubated with and without carboxypeptidase Y for 60 min. The carboxypeptidase-treated PA28 lost 87% of its proteasome-activating activity. Samples of each digestion reaction were mixed with purified bovine proteasome, activate purified proteasome using Suc-Leu-Leu-Val-Tyr-AMC as described under “Materials and Methods.” The same carboxyl-terminal sequence as native PA28 was centrifuged in separate tubes, and their respective sedimentation positions are indicated (arrows).

**FIG. 8.** Expression of PA28α in E. coli. E. coli containing the pET16b vector with PA28α was treated as described under “Materials and Methods.” Left, Coomassie Blue-stained proteins. Right, immunoblot with anti-PA28α antibody. Lane 1, 20 μg of E. coli extract from noninduced cells; lane 2, 20 μg of E. coli extract from cells induced with isopropyl-1-thio-β-D-galactopyranoside; lane 3, 1.5 μg of purified recombinant PA28α; lane 4, 1.5 μg of purified native PA28 from bovine red blood cells.

**DISCUSSION**

The current work presents three lines of evidence that demonstrate that the proteasome activator PA28 is composed of a complex containing two distinct protein subunits, α and β. Two lines of evidence were made possible by the production of antitibodies specific for one or the other of these highly homologous proteins. First, the α and β subunits were co-immunoprecipitated by each of the subunit-specific antibodies. Second, chemical cross-linking of native PA28 generated products that were recognized equally by each of the subunit-specific antibodies. Finally, selective proteolytic modification of the α subunit prevented binding of both subunits to the proteasome, indicating that these subunits are part of the same complex. These various results exclude an alternative model for the PA28 quaternary structure in which PA28 exists as two distinct protein complexes, each composed exclusively of either α or β subunits.

The current results provide the basis for a structural model of PA28. As shown in Fig. 4, PA28 may consist of a hexameric ring comprising alternating α and β subunits. The ring-shaped structure of PA28 has been observed by electron microscopy, although those images do not unambiguously distinguish between hexameric and heptomeric structures (20). Because the terminal rings of the proteasome to which PA28 binds contain seven subunits, PA28 might also be expected to contain 7-fold symmetry. Nevertheless, there are several examples of symmetry mismatch between interacting proteins, the most relevant of which is the ClpAP protease in E. coli. The ClpAP protease has a number of structural and functional similarities to the...
proteasome and its regulatory proteins (26). For example, ClpAP is assembled from two different subcomplexes, a protease called ClpP, and an ATPase regulatory protein called ClpA. ClpP is composed of two stacked ring-shaped structures, each comprising seven 21,500-dalton subunits, whereas ClpA consists of two stacked ring-shaped structures, each comprising six 84,000-dalton subunits. Each seven-membered ring of ClpP associates with a six-membered ring of ClpA, resulting in a complex with an overall structural organization remarkably similar to that of the proteasome and its ATPase regulator PA700 (26). Although the number of PA700 subunits that interact with the proteasome is unclear, the mismatch of symmetries in the ClpAP complex is similar to that proposed here for the proteasome and PA28.

The relative abundance and spatial arrangement of the α and β subunits depicted in the model shown in Fig. 4 are indicated by several experimental findings. First, in numerous independent preparations, purified PA28 contained approximately equal quantities of α and β proteins, suggesting that they occur in a fixed and equal stoichiometry (22). Similar conclusions were reached in experiments in which α and β proteins were quantitated by immunoblotting of crude extracts of several different cell types.2 Second, the pattern of cross-linked products detected by each of the subunit-specific antibodies was the same (Fig. 3). The latter results are consistent with two possible models: 1) a single form of PA28 composed of alternating α and β subunits; and 2) multiple forms of PA28 consisting of α and β subunits arranged in random order and stoichiometry within the ring. The existence of two prominent bands in the size range characteristic of dimers, which is consistent with both models, may represent the cross-linking between the two different faces of adjacent subunits or could represent different degrees of intramolecular cross-linking that affect product mobility in SDS-PAGE and is consistent with all models. The results of the cross-linking experiments by themselves cannot completely exclude a third model in which PA28 exists as two distinct homohexamers of α and β subunits, respectively, because the electrophoretic patterns of the cross-linked products of such complexes might be indistinguishable from one another (just as the α and β monomers are electrophoretically indistinguishable from one another). However, the immunoprecipitation and carboxypeptidase experiments clearly exclude such a model. Although the various data cannot formally distinguish between the models 1 and 2, we consider model 1 to be more attractive.

In addition to the structural model for PA28, the current work also provides considerable insight about the relative functions of the α and β subunits. The sequence similarity of these proteins suggests that they may have similar functions. As reported here and elsewhere, the isolated α subunit, produced by expression in E. coli, stimulates proteasome activity to the same extent as does the native heterodimeric PA28 (Fig. 9 and Ref. 27) but requires 5–10-fold greater concentrations of protein to do so. These findings may indicate that the β subunit enhances the function of the α subunit, perhaps by increasing the binding affinity of the complex to the proteasome. Unfor-

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2 X. Song and G. N. DeMartino, unpublished observations.
fortunately, we have not yet purified a recombinant β subunit to test its interaction with the proteasome either as an isolated protein or after reconstitution with the α subunit.

The current work also provides information regarding the structural basis for the interaction of PA28 with the proteasome. We have significantly extended our previous observation, which indicated that the carboxyl terminus of PA28 was involved in such an interaction (21). That observation, however, was made before the heterodimeric nature of PA28 was recognized. The current work demonstrates that the carboxyl-terminal modification is limited to the α subunit. Because this selective modification prevents binding of both subunits to the proteasome, these data provide additional evidence of the heterodimeric nature of PA28. Furthermore, the surprising finding that the loss of only two, or possibly even one, amino acids on one type of subunit prevents PA28 from binding to the proteasome indicates a critical functional role for this limited region of the PA28 molecule. Finally, the present data on the rate of loss of PA28 activity by carboxypeptidase Y are consistent with the possibility that not all of the α subunits within a PA28 molecule need to be modified for the molecule to lose significant function.

PA28α and PA28β subunits are homologous to another protein, called Ki antigen, the function of which is unknown (28). Although we have not detected Ki antigen sequences in our preparations of PA28, it is possible that this protein is a minor component of the complex. We have used an antibody specific for the Ki antigen to probe our purified PA28 preparations for Ki antigen. We have failed to identify this protein in PA28 at the limits of detection for this antibody.3 Moreover, none of the anti-PA28 antibodies reacted with the Ki antigen protein (data not shown). These results indicate that the Ki antigen protein is not a part of purified bovine PA28, but they cannot exclude the possibility that this protein may play a role in proteasome activation or be a component of another subpopulation of PA28 that is not represented in our purified preparations. Additional work will be required to learn the functional relationship of the Ki antigen to PA28.

3 X. Song, K. Tanaka, and G. N. DeMartino, unpublished observations.

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