An Archaeabacterial ATPase, Homologous to ATPases in the Eukaryotic 26 S Proteasome, Activates Protein Breakdown by 20 S Proteasomes*

(Received for publication, March 25, 1999, and in revised form, May 26, 1999)

Peter Zwickl‡§, David Ng‡, Kee Min Woo‡, Hans-Peter Klenk***, and Alfred L. Goldberg‡ ‡‡

From the ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 and the ‡‡Institute for Genomic Research, Rockville, Maryland 20850

In eukaryotes, the 20 S proteasome is the proteolytic core of the 26 S proteasome, which degrades ubiquitinated proteins in an ATP-dependent process. Archaea lack ubiquitin and 26 S proteasomes but do contain 20 S proteasomes. Many archaea, such as Methanococcus jannaschii, also contain a gene (S4) that is highly homologous to the six ATPases in the 19 S (PA700) component of the eukaryotic 20 S proteasome. To test if this putative ATPase may regulate proteasome function, we expressed it in Escherichia coli and purified the 50-kDa product as a 650-kDa complex with ATPase activity. When mixed with the well-characterized 20 S proteasomes from Thermoplasma acidophilum and ATP, this complex stimulated degradation of several unfolded proteins 8–25-fold. It also stimulated proteolysis by 20 S proteasomes from another archaeabacterium and mammalian. This effect required ATP hydrolysis since ADP and the nonhydrolyzable analog, 5′-adenylyl β,γ-imidophosphate, were ineffective. CTP and to a lesser extent GTP and UTP were also hydrolyzed and also stimulated proteolysis. We therefore named this complex PAN for proteasome-activating nucleotidase. However, PAN did not promote the degradation of small peptides, which, unlike proteins, should readily diffuse into the proteasome. This ATPase complex appears to have been the evolutionary precursor of the eukaryotic 19 S complex, before the coupling of proteasome function to ubiquitination.

The 26 S proteasome, which is the major site of protein breakdown in mammalian cells, is composed of the 20 S proteasome (molecular mass of 700 kDa) and two 19 S regulatory complexes (700 kDa) (1, 2). The 20 S proteasome is a cylindrical proteolytic complex composed of four stacked, seven-membered rings (3). In the presence of ATP, the 19 S complex (also called PA700) becomes associated with each end of the 20 S cylinder (4, 5). The resulting 26 S particle degrades ubiquitinated and certain non-ubiquitinated proteins in an ATP-dependent process (1). Six of the approximately 18 subunits of the 19 S complex are ATPases whose precise functions in protein degradation and in 26 S assembly remain unclear (4, 6–9).

Within the 19 S regulatory complex, these ATPases form a subcomplex (“base”), which binds directly to the 20 S core particle (10). These ATPases are all members of the large AAA family, which contains more than 100 ATPases that are involved in diverse cellular processes, including protein degradation, cell division, peroxisome biogenesis, vesicle transport, and meiosis (11, 12). Only eukaryotic cells contain ubiquitin or 26 S proteasome complexes. The initial report of the finding of ubiquitin in the archaeabacterium Thermoplasma acidophilum (13) and the cyanobacterium Anabaena variabilis (14) has not been confirmed, and the sequencing of several prokaryotic genomes has not revealed genes for ubiquitin or homologs of ubiquitin-conjugating enzymes (15). However, 20 S proteasomes are present in archaea (16–19) and in actinomycetes (20–22). The 20 S proteasome from archaea, although containing only one type of α-subunit in the outer rings and one type of β-subunit in the central two rings, is quite similar in architecture to the eukaryotic proteasome and is clearly the evolutionary ancestor of the eukaryotic particle (2), which contains seven distinct but homologous α-subunits and seven distinct but homologous β-subunits.

The present studies were undertaken to investigate whether the archaeabacterial 20 S proteasome, like the eukaryotic particle, might also function in an ATP-dependent manner in association with a regulatory ATPase complex. In bacteria such as Escherichia coli, which also lack ubiquitin, most intracellular protein degradation requires ATP and is catalyzed by large ATP-hydrolyzing proteolytic complexes (23, 24). Several of these enzymes (ClpAP, ClpXP, and HslVU) are composed of central proteolytic particles (ClpP and HslV) whose function in protein degradation requires ATP hydrolysis by an associated ring-shaped ATPase complex (ClpA, ClpX, and HslU) (24). Prior attempts to find a larger form of the archaeabacterial proteasome that functions in an ATP-dependent fashion have not been successful (25). However, the sequencing of the genome of Methanococcus jannaschii and other archaea have revealed a gene (S4) (26), whose predicted protein sequence is similar to that of the eukaryotic 26 S ATPases. Therefore, we investigated whether this protein, which we have termed “PAN” for proteasome-activating nucleotidase, can

* This work was supported by research grants from the National Institutes of Health and the Human Frontier Science Program (to A. L. G.) and a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (to P. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U67559.

‡ Present address: Dept. of Molecular Structural Biology, Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany.

¶ Present address: Dept. of Biochemistry, College of Medicine, Soonchunhyang University, Cheonan, Cheongnam 330-090, South Korea.

** Present address: Göttingen Genomics Laboratory, Institute for Microbiology and Genetics, Georg-August-University, D-37077 Göttingen, Germany.

†† To whom correspondence should be addressed: Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115-5730. Tel.: 617-432-1854; Fax: 617-232-0173; E-mail: agoldber@bcmp.med.harvard.edu.

1 The abbreviations used are: PAN, proteasome-activating nucleotidase; AAA, ATPases associated with a variety of activities; AMC, 7-ami-
stimulate protein degradation by archaeabacterial 20 S proteasomes in an ATP-dependent reaction. In these studies, we have chosen primarily to study 20 S proteasomes from *T. acidophilum* because their structure and enzymatic properties have been especially well characterized (3, 27–29).

**EXPERIMENTAL PROCEDURES**

**Materials**—All fluorogenic peptides were purchased from Bachem (Switzerland), [14C]formaldehyde from NEN Life Sciences Products, and recombinant 20 S proteasomes (*Methanosaeta thermophila*) from CalBiochem. All other reagents were purchased from Sigma. [14C]Ca-ATPase was prepared by reductive methylation and stored in 50 mM Tris/HCl, pH 7.5 (30).

**Cloning of S4 and Expression of PAN**—The *Methanococcus S4* gene was amplified from plasmid pAMJHW03 by polymerase chain reaction adding six codons for histidine residues to the 5’-end of the gene (GenBank accession number U67559) (primer 1, 5’-GGCGGGCGC- CATATGGCAGCCATATGCCACACACATCAATGGTTGAAGATTATTTTC-3’; and primer 2, 5’-GGCGGGCGATTCCTTAT-ATCTGTAGAAGACATC-CA-3’). The polymerase chain reaction product was cloned into the T7 expression vector pRSETA (Ap+) (Invitrogen) resulting in pRSETA-S4. Since 6% of the codons in the S4 gene are AGA or AGG, which are rare arginine codons in *E. coli*, we transformed *E. coli* BL21(DE3) (plasmid pitation) which carries T7 RNA polymerase (pRSETA) for the minor arginine tRNA, *M. jannaschii* S4 (31). *E. coli* BL21(DE3), pUBS520 cells were used for transformation with pRSETA-S4.

**Purification of PAN**—*E. coli* BL21(DE3), pUBS520, pRSETA-S4 cells were grown at 30 °C in 500-ml flasks. Expression of the PAN protein was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to a logarithmically (A600 = 0.7) growing culture. The cells were induced for 3 h, washed with buffer A (50 mM NaH2PO4, pH 8.0; 300 mM NaCl), and frozen at −20 °C. To purify PAN, the cells were thawed in cold water and lysed by lysozyme treatment (1 mg/ml for 30 min at 4 °C) and sonication (150 watts for 5 min at 50% duty cycle with a microtip, Branson sonifier). Membranes and cell debris were removed by centrifugation at 10,000 *g* for 10 min or at 100,000 *g* for 1 ha. Protein concentration of the clarified supernatant was determined by measurement of the absorption (λmax = 280 nm) in the 650-kDa complex, since both species were copurified together through Ni-NTA chromatography. After gel filtration, the 20-kDa PAN protein was eluted as a 650-kDa complex (Fig. 2A), as shown by SDS-polyacrylamide gel electrophoresis of the fractions (Fig. 2B). These complexes contained predominantly the 50-kDa His6-PAN protein product, whose identity was confirmed by N-terminal sequencing. Also present in lower amounts was a 40-kDa fragment of this polypeptide, whose N-terminal sequence indicated that it was formed by internal initiation at Met1 (cf. Fig. 1). The truncated form and full-length His6-PAN must be present within the same 650-kDa complex, since both species were coeluted on the Ni-NTA column. The full-length form contains the N-terminal His6 tag.

**S4 Isolated from E. coli**—The fractions containing the PAN protein had ATPase activity, as measured by the generation of inorganic phosphate from ATP (Fig. 2A). ATP hydrolysis by PAN required Mg2+ ions and was markedly inhibited by the addition of EDTA. The temperature optimum of the ATPase activity was 73 °C (data not shown), which is close to the growth optimum (85 °C) for *Methanococcus* cells (41).

**PAN Activates Breakdown of Proteins but Not Small Peptides**—To test whether PAN can stimulate the activity of archaeabacterial proteasomes, we used recombinant proteasomes from *Thermoplasma* because their biochemical properties (27) and structure (2, 3, 42) are well defined and because 20 S

---

do-4-methylcoumarin; AMP-PNP, 5’-adenyl β,γ-imidophosphate; ARC, AAA ATPase-forming Ring-shaped complexes; DTT, dithiothreitol.
proteasomes have not been isolated from \textit{Methanococcus}. However, \textit{Methanococcus} contains genes for proteasomal \(a\)- and \(b\)-subunits (26) that are very similar (50 and 39% sequence similarity, respectively) to those in \textit{T. acidophilum}. In the presence of PAN and ATP, these proteasomes degraded \([14C]\mbox{-}m\)-ethyl-\(b\)-casein 8–25 times faster (depending on the preparation of substrate) than in the absence of the nucleotide (Fig. 3). This effect requires ATP hydrolysis, since the non-hydrolyzable analog, AMP-PNP, and ADP had very little or no effect on the degradation of \(b\)-casein (Fig. 3 and Table I). In the absence of PAN, the 20 S proteasomes degraded \(b\)-casein 8–12 times more slowly, and this process was not altered by the addition of ATP.

The magnitude of this proteolytic stimulation by PAN and ATP was proportional to the amount of PAN added (Fig. 4), and maximal degradation occurred with a PAN/proteasome molar ratio of the complexes of 4:1 (subunit ratio of approximately 2:1), which was used in all of our subsequent assays. In the presence of ATP, PAN also dramatically stimulated (10–39-fold depending on the preparation) the degradation of several other \(125I\)-labeled proteins, e.g. \(a\)-lactalbumin and oxidized RNase A, which are hydrolyzed very slowly by 20 S proteasomes alone (Table II). These polypeptides had been oxidized to eliminate sulfhydryl cross-bridges and to facilitate their entry into the 20 S particle (43). Like casein, these substrates are unfolded (especially at 60 °C which was used in most assays) and have been widely used as substrates for other ATP-dependent proteases (ClpAP, HslVU, and the 26 S proteasome). Like these enzymes, PAN plus 20 S proteasomes could not degrade two native globular proteins, serum albumin or ovalbumin. Thus, PAN and ATP seem to promote the selective breakdown of unfolded polypeptides (although these substrates may have additional features allowing their recognition by PAN).

With certain ATP-dependent proteases (La or HslVU) from \textit{E. coli}, ATP binding enhances degradation of small peptide substrates as well as proteins (44), whereas with others (ClpAP or ClpXP), ATP binding and hydrolysis are necessary only for degradation of proteins (45). In contrast to the marked stimulation of \(b\)-casein degradation, PAN and ATP did not promote the cleavage of the standard fluorogenic peptide substrates of the 20 S proteasome, Suc-LLVY-AMC (where Suc is succinyl).
Archaebacterial ATPase Activates 20 S Proteasomes

**TABLE I**

| Additions               | Thermoplasma 20 S proteasomes | Methanosarcina 20 S proteasomes |
|-------------------------|-------------------------------|--------------------------------|
| None                    | 11                            | 10                             |
| PAN                     | 8                             | 8                              |
| PAN + ATP               | 99                            | 39                             |
| PAN + ADP               | 12                            | 9                             |
| PAN + AMP-PNP           | 10                            |                                |

FIG. 3. The *Methanococcus* PAN protein activates the breakdown of proteins by the *Thermoplasma* proteasome. Measurement of the proteolytic activity was performed by mixing PAN (600 ng) and *Thermoplasma* proteasomes (150 ng) at a molar ratio of the complexes of 4:1 (subunit ratio of 2:1) with 3.4 μg of β-[^14]C]casein in buffer E with 1 mM ATP (top line), with 1 mM AMP-PNP (middle line), with 1 mM ADP or control without nucleotide (lower line) in a volume of 100 μl. Data with ADP and without any nucleotide were indistinguishable. The reaction mixture was incubated at 60 °C for various periods, and the generation of radioactivity soluble in 10% trichloroacetic acid was determined by liquid scintillation counting. Proteasomes alone, incubated with the same three nucleotides or without nucleotide, had similar activity as proteasomes incubated with PAN and without any nucleotide. PAN alone had no proteolytic activity when incubated under the same conditions.

**FIG. 2.** The PAN protein forms a 650-kDa complex with ATPase activity. A, Superose 6 chromatography and ATPase activity of the recombinant PAN protein. ATPase activity was measured by incubating 50 μl of the fractions in buffer E (50 mM Tris/HCl, pH 7.5; 10 mM MgCl₂, 1 mM DTT) containing 1 mM ATP for 40 min at 60 °C in a volume of 100 μl. The production of inorganic phosphate was measured by the ascorbic acid method (34). B, SDS-polyacrylamide gel electrophoresis (12% acrylamide) of the Superose 6 fractions. The Coomassie-stained gel shows molecular weight standard proteins in lane M, and 15 μl of fractions 23–28 of the Superose 6 chromatography shown in A labeled 23–28, respectively. The N-terminal protein sequence analysis identified the 50-kDa protein as His₆-PAN (MHHHHHHVFE), whereas the 40-kDa protein is a truncated PAN protein (MKENEILRRE), which is most likely the result of an internal initiation of translation at Met⁷⁴.

(Fig. 5) or Z-GGL-AMC (where Z is carbobenzoxy), nor did PAN and ATP enhance the hydrolysis of several other peptides that are poorly cleaved by the particle (Table III). Most likely, these tetrapeptides or tripeptides are degraded in a PAN- and ATP-independent fashion because, unlike proteins, they do not require an ATP-dependent translocation step and can readily diffuse into the particle to reach the active sites located in the central chamber of the proteasome. This inability of PAN to stimulate proteasomal degradation of small fluorogenic peptides distinguishes PAN from known activators of mammalian proteasomes (PA28 and PA700), which stimulate the hydrolysis of tripeptides and, in the case of PA28, enhances degradation of oligopeptides but not proteins (1).

**PAN Stimulates 20 S Proteasomes from Other Archaebacteria and Mammals—**20 S proteasomes have also been characterized from another archaeabacterium, *M. thermophila* (16, 48). Although its α- and β-subunits are highly homologous (46–60%) to those from *Thermoplasma*, its active sites are quite different in specificity. Nevertheless, the ability of *Methanosarcina* proteasomes to degrade β-casein was stimulated several-fold by PAN and ATP but not by PAN and ADP (Table I). Surprisingly, PAN even enhanced 2–4-fold the capacity of 20 S proteasomes from rabbit muscles to degrade β-casein at 55 °C (but not at 37 °C where PAN is inactive as an ATPase). In contrast, PAN and ATP could not promote, at either temperature, β-casein degradation by the multimeric proteases from *E. coli*, ClpP, and HslV, which normally function in association with the specific ATPase complexes, ClpA or ClpX, and HslU, respectively. In addition, when these regulatory ATPases were incubated with the *Thermoplasma* proteasomes and ATP, they were unable to stimulate protein breakdown at either 37 or 55 °C. Together, these findings strongly suggest that the enhancement of protein breakdown involves a specific interaction between PAN and the 20 S particles (see below).

**Nucleotide Specificity—**In addition to ATP, PAN was found to cleave other nucleotide triphosphates at comparable rates (at 1 mM). Surprisingly, the rate of CTP hydrolysis was twice that of ATP, whereas GTP and UTP were hydrolyzed more slowly than ATP. Because of this broad nucleotide specificity, we have termed PAN a nucleotidase, rather than simply an ATPase. Moreover, these other nucleotide triphosphates were also found to support the stimulation of protein degradation by PAN (Table IV), although ATP was most effective in promoting proteolysis. CTP also had considerable activity, but since CTP is hydrolyzed faster than ATP, the coupling of nucleotide and protein hydrolysis is less efficient than with ATP (i.e., more nucleotides have to be consumed to degrade a casein molecule). Because of this ability to hydrolyze multiple nucleotide triphos-
phates, we named this complex proteasome-activating nucleotidase or PAN.

**DISCUSSION**

A fundamental feature of intracellular protein breakdown in eukaryotes and bacteria is its requirement for metabolic energy (1, 23, 24, 47). The present finding of an ATPase that markedly stimulates protein degradation by archaebacterial proteasomes reveals that a coupling of nucleotide and protein hydrolysis was established early in evolution. Accordingly, these archaebacteria also contain genes that encode homologs of the ATP-hydrolyzing protease La (Lon) (15, 18, 19, 26, 48), which is a major contributor to protein breakdown in bacteria (49) and mitochondria (50). Although the eukaryotic 26 S proteasome contains six related ATPases (2), there is only one homolog in the Methanococcus genome (26) and in the recently sequenced genomes of the archaea bacteria, *Methanobacterium thermoautotrophicum* (18), *Archaeoglobus fulgidus* (19), and *Pyrococcus horikoshii* (48). Therefore, PAN should be considered as the evolutionary precursor of the six eukaryotic 26 S ATPases, which most likely arose from one primordial gene by gene duplications during the evolution of eukaryotic cells. The eukaryotic 20 S particle also evolved through multiple gene duplications, in which the single α-subunit and single β-subunit, typical of prokaryotic proteasomes, gradually evolved into seven distinct, but homologous, α-type and seven distinct, but homologous, β-type subunits in the eukaryotic particle (51).

PAN thus appears likely to regulate 20 S function in many archaea bacteria, as shown by its ability to stimulate proteolysis by the quite different proteasomes from *Thermoplasma* and *Methanosarcina*. It is also most likely that PAN activates these particles from *Methanococcus*, which is a methanogen closely related to *Methanosarcina*. However, this protein is probably not the only mode of regulation of proteolysis in these prokaryotes. Despite several attempts, we have failed to find a PAN homolog by polymerase chain reaction in *Thermoplasma* (the source of our 20 S proteasomes) using primers synthesized according to highly conserved regions of the archaebacterial

**TABLE II**

| Proteins | Proteasomes | Proteasomes and PAN Ratio |
|----------|-------------|--------------------------|
| m mol degraded/h/mg proteasomes | | | |
| Oxidized RNase A (0.1 μM) | 4 | 145 | 39.0 |
| β-Casein (0.6 μM) | 4 | 97 | 24.0 |
| α-Lactalbumin (0.2 μM) | 17 | 197 | 12.0 |
| Bovine serum albumin | 0 | 0 |
| Ovalbumin | 0 | 0 |

**FIG. 4.** Effect of increasing amounts of PAN on casein degradation. Maximal β-[14C]casein degradation was observed with a molar ratio of four PAN complexes per proteasome (assuming PAN functions as a 650-kDa multimer). 150 ng of *Thermoplasma* 20 S proteasomes were incubated with increasing concentrations of PAN (0–1.5 μg) in the presence of buffer E, 1 mM ATP, and 2.5 μg of β-[14C]casein (100,000 cpm) in a total volume of 100 μl for 1 h at 55 °C. The reaction was stopped with 10% trichloroacetic acid, and soluble counts were measured by liquid scintillation.

**TABLE III**

| Peptides (400 μM) | Proteasomes | Proteasomes and PAN Ratio |
|-------------------|-------------|--------------------------|
| μmol hydrolyzed/h/mg proteasomes | | | |
| Suc-LLVY-AMC | 0.77 | 0.70 | 0.91 |
| Z-GGL-AMC | 3.01 | 2.93 | 0.97 |
| Suc-AAF-AMC | 1.14 | 1.13 | 0.99 |
| Z-GGR-AMC | 0.01 | 0.01 | 1.00 |
| Z-GLR-AMC | 0.01 | 0.01 | 1.00 |

**FIG. 5.** PAN does not stimulate the breakdown of peptides by *Thermoplasma* proteasomes. The hydrolysis of the fluorogenic peptide, Suc-LLVY-AMC (where Suc is succinyl) (100 μM), was measured in buffer E at 60 °C. 150 ng of proteasomes were incubated with 600 ng of PAN (−) or without PAN (−) in the presence of 1 mM ATP; proteasomes incubated without PAN or with PAN in the absence of ATP gave similar activity. The activity is expressed as arbitrary fluorescence units.

**TABLE IV**

| Nucleotide (1 mM) | Nucleotide hydrolysis | Casein hydrolysis |
|-------------------|-----------------------|-----------------|
| mmol/h | Relative rates | pmol/h | Relative rates |
| ATP | 19 | 100 | 16 | 100 |
| CTP | 39 | 205 | 13 | 81 |
| GTP | 11 | 58 | 6 | 38 |
| UTP | 11 | 58 | 2 | 13 |

**Effect of PAN on the hydrolysis of various proteins by proteasomes**

Protein degradation was assayed for 40 min at 60 °C in buffer E with 1 mM ATP or no nucleotide added. PAN (600 ng) and proteasome (150 ng) were present at a molar ratio of 4:1, which gave the maximal rate of ATP-stimulated casein hydrolysis. The results shown are the mean values of duplicate measurements in a typical experiment. Similar data were obtained in two or three separate experiments.

**Effect of increasing amounts of PAN on casein degradation**

Table II reveals that a coupling of nucleotide and protein hydrolysis was observed with a molar ratio of four PAN complexes per proteasome (assuming PAN functions as a 650-kDa multimer). 150 ng of *Thermoplasma* 20 S proteasomes were incubated with increasing concentrations of PAN (0–1.5 μg) in the presence of buffer E, 1 mM ATP, and 2.5 μg of β-[14C]casein (100,000 cpm) in a total volume of 100 μl for 1 h at 55 °C. The reaction was stopped with 10% trichloroacetic acid, and soluble counts were measured by liquid scintillation.

**TABLE II**

| Proteins | Proteasomes | Proteasomes and PAN Ratio |
|----------|-------------|--------------------------|
| m mol degraded/h/mg proteasomes | | | |
| Oxidized RNase A (0.1 μM) | 4 | 145 | 39.0 |
| β-Casein (0.6 μM) | 4 | 97 | 24.0 |
| α-Lactalbumin (0.2 μM) | 17 | 197 | 12.0 |
| Bovine serum albumin | 0 | 0 |
| Ovalbumin | 0 | 0 |

**Fig. 4.** Effect of increasing amounts of PAN on casein degradation. Maximal β-[14C]casein degradation was observed with a molar ratio of four PAN complexes per proteasome (assuming PAN functions as a 650-kDa multimer). 150 ng of *Thermoplasma* 20 S proteasomes were incubated with increasing concentrations of PAN (0–1.5 μg) in the presence of buffer E, 1 mM ATP, and 2.5 μg of β-[14C]casein (100,000 cpm) in a total volume of 100 μl for 1 h at 55 °C. The reaction was stopped with 10% trichloroacetic acid, and soluble counts were measured by liquid scintillation.

**Fig. 5.** PAN does not stimulate the breakdown of peptides by *Thermoplasma* proteasomes. The hydrolysis of the fluorogenic peptide, Suc-LLVY-AMC (where Suc is succinyl) (100 μM), was measured in buffer E at 60 °C. 150 ng of proteasomes were incubated with 600 ng of PAN (−) or without PAN (−) in the presence of 1 mM ATP; proteasomes incubated without PAN or with PAN in the absence of ATP gave similar activity. The activity is expressed as arbitrary fluorescence units.

**TABLE III**

| Peptides (400 μM) | Proteasomes | Proteasomes and PAN Ratio |
|-------------------|-------------|--------------------------|
| μmol hydrolyzed/h/mg proteasomes | | | |
| Suc-LLVY-AMC | 0.77 | 0.70 | 0.91 |
| Z-GGL-AMC | 3.01 | 2.93 | 0.97 |
| Suc-AAF-AMC | 1.14 | 1.13 | 0.99 |
| Z-GGR-AMC | 0.01 | 0.01 | 1.00 |
| Z-GLR-AMC | 0.01 | 0.01 | 1.00 |

**TABLE IV**

| Nucleotide (1 mM) | Nucleotide hydrolysis | Casein hydrolysis |
|-------------------|-----------------------|-----------------|
| mmol/h | Relative rates | pmol/h | Relative rates |
| ATP | 19 | 100 | 16 | 100 |
| CTP | 39 | 205 | 13 | 81 |
| GTP | 11 | 58 | 6 | 38 |
| UTP | 11 | 58 | 2 | 13 |
PAN and eukaryotic 26 S ATPases. Moreover the complete genome of another archaeabacterium, *Pyrococcus aerophilum*, has been sequenced; it contains proteasomal genes, but no gene similar to PAN was found nor any ClpA homolog.\(^2\) Therefore, the mode of regulation of proteasomes in these species remains uncertain but presumably involves distinct ATPase complexes.

PAN, its homologs in other archaeabacteria, and the eukaryotic 26 S ATPases share a single nucleotide binding domain, P-loop, three highly conserved domains of unknown function, and an N-terminal coiled-coil (Fig. 1). This coiled-coil region was shown to be essential for the interactions of some of the 26 S ATPases with each other (52), and they may also be important in the binding of substrates (53). Surprisingly, the truncated 40-kDa fragment of PAN lacks about half of the predicted coiled-coil, yet it was copurified in the same complex as the full-length PAN (Fig. 2). Interestingly, one of the six homologous ATPases in human and yeast 26 S proteasomes shows a low likelihood of containing a coiled-coil domain (52, 54).\(^3\) ClpA and ClpB also contain internal translation initiation sites that produce full-length and truncated forms of these proteins that differ in their catalytic properties (55, 56). However, by site-directed mutagenesis, we have recently eliminated this internal initiation site in PAN and obtained a similar sized PAN complex that contains only full-length subunits and that, surprisingly, stimulates protein breakdown almost identically to the preparations shown here.\(^3\)

It is noteworthy that ATP and PAN dramatically stimulate the degradation of several proteins (Tables I and II) but do not affect the hydrolysis of tetrapeptide and tripeptide substrates (Table III). Presumably, these small peptides can enter the 20 S particle and reach the central chamber in the absence of PAN. X-ray analysis of the *Thermoplasma* proteasome has shown that the outer \(\alpha\)-rings surround a 13-Å channel (3), which appears to be the site of entry for protein substrates (43), but this opening is closed in the crystal structure of the yeast 20 S particle (57). Perhaps the binding of PAN or the ATPases of the eukaryotic 19 S complex to the terminal \(\alpha\)-rings of the 20 S proteasome enlarges this opening in an ATP-dependent manner (8) and thus facilitates the access of proteins to the active sites localized in the central chamber (28). In addition, PAN may stimulate the uptake of a polypeptide by the proteasome by altering its conformation and orientation (see below). In fact, in more recent studies, we have demonstrated that PAN not only forms a specific complex with the 20 S proteasome (as shown by co-immunoprecipitation experiments) but PAN also interacts directly with protein substrates (as shown by their ability to enhance ATPase activity of PAN).

The stimulation of the breakdown of proteins, but not of small peptides, strongly suggests that PAN, by binding to the 20 S particle, promotes protein translocation into the 20 S proteasome. It has often been suggested that ATPases of the 26 S complex or ATP-dependent proteases function as chaperones to unfold prospective substrates. However, the substrates studied here (casein or oxidized \(\alpha\)-lactalbumin) behave as unfolded molecules at 37 °C and at 55 or 60 °C must completely lack any tertiary structure. Consequently, the primary function of PAN under these conditions must be to translocate the denatured polypeptide into the 20 S particle. Moreover, the ability of PAN to stimulate proteolysis by two different types of archaeabacterial proteasomes and even mammalian proteasomes, but not by ClpP or HslV, also supports the idea that PAN acts by forming a specific complex with the proteasome, rather than a mechanism where PAN alone binds to the substrate and releases the polypeptide in a form that then diffuses into the 20 S particle. By analogy to the other bacterial ATPase complexes that regulate proteolysis (ClpA, ClpX, and HslU) by specific proteolytic complexes (ClpP and HslV), it is attractive to assume that the 650-kDa PAN complex contains two rings, each of which is a complex of six or seven subunits that can bind to the ends of the 20 S particle. Our attempts thus far to analyze the structure of PAN by electron microscopy indicate ring-shaped structures and stacked rings, but a variety of other complexes with heterogeneous appearance were also present for reasons that are unclear.

Unlike several ATP-dependent proteases or chaperones (ClpA, ClpX, and HslU), PAN hydrolyzes all four nucleotide triphosphates at significant rates, and all four can support protein degradation. Although we assume that PAN functions primarily in an ATP-dependent fashion in *vivo*, other nucleotides (especially CTP) may also support protein breakdown. Unfortunately, information is not available on the relative concentrations of these different nucleotides in archaeabacteria under different growth conditions. This preference for ATP and CTP over GTP and UTP may even be a general feature of the AAA family members. A similar preference in nucleotide hydrolysis or in nucleotide-dependent proteolysis was found with eukaryotic 26 S proteasomes and 19 S regulatory particles (58), the *E. coli* metalloprotease FtsH (59), and the *Rhodococcus* ARC complex (60). Recently, the x-ray structure of the second ATPase domain (D2) of the human NSF protein, a member of the AAA family, was determined (61, 62). It functions with ATP, but not GTP, apparently because the active site binds ATP in a syn conformation and does not sterically admit the carbonyl and amine groups on GTP’s (purine) ring in this conformation (61, 62). Since CTP and UTP also differ in the presence of the amine and carbonyl groups on the pyrimidine ring, binding of CTP but steric hindrance of UTP binding would also appear likely.

Despite the very large evolutionary distance, the primary sequence and domain organization of PAN and those of the human and yeast 26 S ATPases are indeed very similar, and therefore, they probably function quite similarly in promoting protein breakdown by the 20 S particle. Therefore, further mechanistic and structural studies of the PAN-proteasome complex should help clarify the precise role of ATP in the formation and function of the 26 S eukaryotic complex. Certain bacteria (actinomyectes), which also lack ubiquitin and 26 S proteasomes, contain their 20 S proteasome \(\alpha\) and \(\beta\) genes in close proximity to the gene for the ATPase termed ARC (60). Presumably, this ring-shaped nucleotidease activates protein degradation by the bacterial proteasomes by a similar mechanism as PAN, although it is not a close homolog of PAN nor the 26 S ATPases. By contrast, PAN shows extensive homologies to the 26 S proteasome and is therefore the most likely evolutionary precursor to the 19 S complex and, in particular, to its base, the portion which contains its six ATPases that associates with the 20 S proteasome (10). The one notable potential difference between these ATPase complexes is that PAN does not enhance peptide hydrolysis by its 20 S particle whereas the 19 S (PA700) complex does so, perhaps because this effect may involve a non-ATPase subunit. Recent findings have suggested that the other proteins of the 19 S complex that comprise its “lid” are homologous to the COP9-signalosome-like complex, which functions in signal transduction and translation (10, 63, 64). The combination of these components with PAN must have been the critical step during the evolution of eukaryotic cells that allowed the coupling of proteasome function to ubiquitin conjugation and the establishment of the ubiquitin-proteasome pathway.

---

\(^2\) S. Fitz-Gibbon, personal communication.

\(^3\) D. Ng, P. Zwicki, and A. L. Goldberg, manuscript in preparation.
Acknowledgments—We thank R. Mattes (Universität Stuttgart) for providing us with PUBSS520; G. Pfeifer (Martinstried) for electron microscopy; and D. Finley, M. Rohrwild, and S. Lecker for critically reading the manuscript.

REFERENCES

1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847.
2. Baumeister, W., Walz, J., and Zwickl, P. (1995) Science 268, 533–539.
3. DeMartino, G. N., Moomaw, C. R., Zagnitko, O. P., Proske, R. J., Chu-Ping, M., Afendis, S. J., Swaffield, J. C., and Slaughter, C. A. (1994) J. Biol. Chem. 269, 20878–20884.
4. Peters, J. M., Cejka, Z., Harris, J. R., Potrykus, I., and Baumeister, W. (1998) Trends Cell Biol. 8, 65–71.
5. DeMartino, G. N., Moomaw, C. R., Zagnitko, O. P., Proske, R. J., Chu-Ping, M., Afendis, S. J., Swaffield, J. C., and Slaughter, C. A. (1994) J. Biol. Chem. 269, 20878–20884.
6. Durner, J., and Boger, P. (1995) FEBS Lett. 350, 340–343.
7. Kisselev, A. F., Akopian, T. N., and Goldberg, A. L. (1998) J. Biol. Chem. 273, 25190–25193.
8. Seemüller, E., Lupsa, A., Anderegg, E., Bussé, E., Hengge, H., and Huber, R. (1995) Science 268, 579–582.
9. Zwickl, P., Lottspeich, F., and Baumeister, W. (1999) FEBS Lett. 448, 245–254.
10. Brinkmann, U., Mattes, R. E., and Buckel, P. (1989) Gene (Amst.) 85, 109–114.
11. Bult, C. J., White, O., Wilson, S. J., Nierhaus, H. K., and Englemann, S. (1996) J. Mol. Biol. 258, 378–390.
12. Katz, S. B., and Baumeister, W. (1998) Trends Cell Biol. 8, 94–99.
13. Wolf, D., Grabe, J. M., and Baumeister, W. (1998) Trends Biochem. Sci. 23, 375–383.
14. Lengeler, W., and Baumeister, W. (1995) EMBO J. 14, 4593–4597.
15. Brandt, C., Sutter, C., and Baumeister, W. (1997) Trends Biochem. Sci. 22, 399–404.
16. Philips, J. M., and Baumeister, W. (1998) Trends Biochem. Sci. 23, 375–383.
17. Zwickl, P., Lottspeich, F., and Baumeister, W. (1997) Trends Biochem. Sci. 22, 399–404.
18. Smith, D. S., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Durner, J., and Boger, P. (1995) Nature Struct. Biol. 2, 199–204.
19. Patil, S., and Latterich, M. (1998) Mol. Microbiol. 29, 251–255.
20. Tamura, T., Nagy, I., Lupas, A., Murrell, D. C., and Wolf, D. (1996) Trends Biochem. Sci. 21, 96–102.
21. Richmond, C., Gorbea, C., and Rechsteiner, M. (1997) J. Biol. Chem. 272, 13403–13411.
22. Wang, W., Chen, W., and Nathans, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5808–5813.
23. Chung, C. H. (1993) Science 262, 372–374.
24. Maupin-Furlow, J. A., Aldrich, H. C., and Ferry, J. G. (1996) J. Bacteriol. 180, 1480–1487.
25. Ciechanover, A. (1994) Cell 79, 13–21.
26. Swaffield, J. C., and Purugganan, M. D. (1997) J. Mol. Evol. 45, 549–563.
27. Harrison, S. C. (1996) Cell 86, 341–343.
28. Maupin-Furlow, J. A., Aldrich, H. C., and Ferry, J. G. (1998) J. Bacteriol. 180, 1480–1487.
29. Ciechanover, A. (1994) Cell 79, 13–21.
30. Swaffield, J. C., and Purugganan, M. D. (1997) J. Mol. Evol. 45, 549–563.
31. Harrison, S. C. (1996) Cell 86, 341–343.
32. Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., and Baumeister, W. (1989) FEBS Lett. 251, 125–131.
33. Gottesman, S., Murr, D., Seeber, U., and Baumeister, W. (1997) Trends Biochem. Sci. 22, 399–404.
34. Archaebacterial ATPase Activates 20 S Proteasomes