ATP Hydrolysis by the Proteasome
Regulatory Complex PAN Serves Multiple Functions in Protein Degradation

Nadia Benaroudj,1,3 Peter Zwickl,2 Erika Seemu¨lter,2 Wolfgang Baumeister,2 and Alfred L. Goldberg 1,*

1Harvard Medical School
Department of Cell Biology
240 Longwood Avenue
Boston, Massachusetts 02115

2Max-Planck-Institute for Biochemistry
Department of Molecular Structural Biology
Am Klopferspitz 18a
D-82152 Martinsried
Germany

Summary

To clarify the role of ATP in proteolysis, we studied archaeal 20S proteasomes and the PAN (proteasome-activating nucleotidase) regulatory complex, a homolog of the eukaryotic 19S ATPases. PAN’s ATPase activity was stimulated similarly by globular (GFPssrA) and unfolded (casein) substrates, and by the ssrA recognition peptide. Denaturation of GFPssrA did not accelerate its degradation or eliminate the requirement for PAN and ATP. During degradation of one molecule of globular or unfolded substrates, 300–400 ATP molecules were hydrolyzed. An N-terminal deletion in the 20S α subunits caused opening of the substrate-entry channel and rapid degradation of unfolded proteins without PAN; however, degradation of globular GFPssrA still required PAN’s ATPase activity, even after PAN-catalyzed unfolding. Thus, substrate binding activates ATP hydrolysis, which promotes three processes: substrate unfolding, gate opening in the 20S, and protein translocation.

Introduction

In all cells, degradation of intracellular proteins requires ATP hydrolysis (Goldberg, 1992). In eukaryotes, ATP hydrolysis is necessary for ubiquitination of protein substrates, as well as for the proteolytic activity of 26S proteasomes, the principal site of proteolysis in the cytosol and nucleus (Coux et al., 1996). 26S proteasomes contain six homologous ATPase subunits in their 19S regulatory particles. The precise roles of those ATPases in proteasome function, as well as the amount of ATP consumed during proteolysis, are uncertain. It has been proposed that the ATPases catalyze unfolding of protein substrates and promote their translocation into the central chamber of 20S proteasomes where protein degradation occurs (Lupas et al., 1993). More recently, it has been shown that bacterial ATP-dependent proteases (ClpAP and ClpXP) and the PAN (proteasome-activating nucleotidase) complex (the archaeal 19S homolog) unfold globular proteins (Benaroudj and Goldberg, 2000; Hoskins et al., 2000; Kim et al., 2000; Navon and Goldberg, 2001; Singh et al., 2000; Weber-Ban et al., 1999). The amount of ATP consumed and the specific functions of ATP hydrolysis are particularly difficult to analyze with 26S proteasomes due to the requirement for ubiquitin conjugation to most substrates, the low intrinsic stability of the 19S and its complexity (it contains at least 17 distinct subunits), and the presence of six different ATPases that probably serve distinct functions (Glickman et al., 1998; Holzl et al., 2000; Rubin et al., 1998).

Although prokaryotes lack ubiquitin and 26S proteasomes, archaeabacteria and certain eubacteria contain 20S proteasomes whose structural organization and catalytic mechanism are very similar to those of the eukaryotic 20S particles (Baumeister et al., 1998; De Mot et al., 1998; Zwickl et al., 2000). Consequently, studies of archaeal proteasomes have provided important insights into the structure and enzymatic mechanisms of their eukaryotic homologs. 20S particles have a barrel-shaped structure composed of two outer α rings and two central β rings, which contain the proteolytic active sites sequestered within a central chamber (Lowe et al., 1995; Wenzel and Baumeister, 1995). Because of the tight packing of subunits, substrates enter through a narrow channel in the α ring which only permits entrance of unfolded polypeptide chains or short peptides (Lowe et al., 1995). In the native form of yeast 20S particles, this channel is blocked by the amino-terminal extremities of the α subunits (Groll et al., 1997). As a consequence, eukaryotic 20S particles exhibit low peptide activity because substrates cannot readily enter the internal chamber. Deletion of the N termini of the α3 subunits (residues 2–10) in yeast 20S prevents closing of the channel and greatly enhances peptide hydrolysis (Groll et al., 2000). Recent studies have suggested that the 19S ATPase Rpt2 is particularly important in opening the axial pore (Kohler et al., 2001). In contrast to the eukaryotic particles, the X-ray diffraction analysis of the 20S particles from Thermoplasma acidophilum did not reveal a closed channel, presumably because the amino-terminal extremities of the α subunits are disordered and therefore not visible (Lowe et al., 1995). Moreover, these particles by themselves appear fully active in degradation of fluorogenic tetrapeptides, but not of proteins. Therefore, it has been generally assumed that the channel formed by the α rings of the archaeal 20S particles is in an open state (Groll et al., 2000; Kohler et al., 2001; Lowe et al., 1995).

We have identified a 650 kDa ATPase complex from the archaeabacterium Methanococcus jannaschii named PAN, which is probably the evolutionary precursor of the eukaryotic 19S (Zwickl et al., 1999). It is composed of identical subunits closely homologous to the six ATPases of the 19S and stimulates markedly the degradation of proteins, but not of tetrapeptides, by archaeal 20S proteasomes. This process requires hydrolysis of ATP (Zwickl et al., 1999), although the mechanisms linking ATP hydrolysis and protein degradation are unknown. Because of its simple structural organization, lack of requirement for substrate ubiquitination, and ease of expression in...
E. coli, PAN offers many advantages for mechanistic studies. PAN exhibits certain activities characteristic of molecular chaperones, e.g., it prevents aggregation and promotes the refolding of denatured proteins (Benaroudj and Goldberg, 2000). In addition, in the presence of ATP, PAN catalyzes the unfolding of the globular green fluorescent protein when its C terminus is fused to the 11 residue ssrA peptide (GFPssrA) (Benaroudj and Goldberg, 2000). This ATP-dependent “unfoldase” activity is essential for the degradation of GFPssrA by archaean 20S proteasomes, which otherwise do not degrade GFPssrA. Like ATP-dependent proteases in E. coli and other molecular chaperones, PAN’s ATPase activity is stimulated by protein substrates (our unpublished data), but the mechanism of this substrate-activated ATP hydrolysis and its role in translocation and degradation of proteins remain unclear.

In the present studies, we have used the PAN complex to analyze further the coupling between ATP hydrolysis, substrate unfolding, and translocation. We have investigated how different types of protein substrates influence ATP hydrolysis by PAN and the actual amount of ATP consumed during hydrolysis of globular and denatured proteins. In order to clarify the process of protein entry into 20S particles, we have investigated whether archaean 20S proteasomes, like eukaryotic 20S, have a functional gate that precludes protein entry. We have analyzed the degradation of different proteins by a 20S proteasomes deletion mutant Δ(2-12)20S that lacks the 11 residue ssrA peptide (GFPssrA) (Benaroudj and Goldberg, 2000). When the ssrA peptide did not stimulate PAN’s ATPase activity. It is very likely that substrate-stimulated ATPase activity is stimulated by protein substrates (our unpublished data). However, upon addition of PAN to a form that has a higher maximal ability to this activation, we compared the rates of ATP hydrolysis by PAN in the presence of casein, which contains little secondary or tertiary structures (Cremer et al., 1981), and GFPssrA, a globular protein that is unfolded by PAN (Benaroudj and Goldberg, 2000). These experiments were performed with a 1000-fold molar excess of these substrates, which causes maximal stimulation of PAN’s ATPase activity (data not shown). Casein stimulated the ATPase activity of PAN 5-fold. Surprisingly, PAN’s ATPase activity was stimulated by GFPssrA to a similar extent as casein (Figure 1A). Therefore, the maximal stimulation of ATP hydrolysis is the same whether or not the substrate is globular. Prior observations suggested that only proteolytic substrates of the PAN-20S complex enhance ATP hydrolysis (our unpublished data). Accordingly, native GFP (i.e., lacking the ssrA peptide), which is not unfolded by PAN (Benaroudj and Goldberg, 2000), did not stimulate PAN’s ATPase activity. When a 5-fold molar excess of 20S proteasomes was added so that all PAN should be bound to proteasomes, the basal and the substrate-stimulated ATPase activity of PAN were not significantly affected (Figure 1B). Thus, the protein-activated PAN’s ATPase activity is not increased further when PAN binds to 20S and when the substrate is translocated into the proteolytic chamber and degraded.

Peptide Binding to PAN Stimulates Its ATPase Activity
Native GFP lacking the ssrA peptide at its C terminus is not unfolded or degraded by PAN and 20S proteasomes (Benaroudj and Goldberg, 2000), and did not stimulate ATP hydrolysis by PAN, presumably because it does not interact with PAN. Thus, the binding of PAN to GFPssrA seems to depend on the 11 residue ssrA extension. We first tested whether this peptide could interact with PAN and inhibit its ability to unfold GFPssrA. Upon transfer of GFPssrA to 45°C, there was an immediate small drop in its fluorescence signal (Figure 1C, closed circle), which by 5 min reached a new steady state corresponding to GFPssrA fluorescence at 45°C. This drop reflected the effects of high temperature on the quantum efficiency of fluorescence and does not indicate any unfolding of GFP, which is a very thermostable protein (Tm > 65°C) (Bokman and Ward, 1981). However, upon addition of PAN (Figure 1C, closed triangle), there was a large further decrease in fluorescence, indicating an ATP-dependent unfolding of GFPssrA catalyzed by PAN (Benaroudj and Goldberg, 2000). When the ssrA peptide was added to the reaction containing GFPssrA-PAN and ATP, the fall was prevented. Thus, the ssrA peptide inhibited the unfolding of GFPssrA by PAN, presumably because this peptide binds to the same site on PAN as GFPssrA. Interestingly, the ssrA peptide also inhibited the PAN-stimulated degradation of casein by 20S particles (Figure 1D), suggesting that these two very different protein substrates bind to the same site on PAN.

We then tested whether the ssrA peptide could stimulate PAN’s ATPase activity. By itself, the ssrA peptide stimulated PAN’s ATPase activity to a comparable extent as GFPssrA or casein when used at the same concentration (Figures 1E). Together, these findings indicate that the binding of a peptide to PAN is sufficient to stimulate its ATPase activity. It is very likely that substrate binding induces a change in the conformation of PAN to a form that has a higher maximal ability to hydrolyze ATP.

Substrate Denaturation Does Not Eliminate the Requirement for PAN and ATP in Degradation by 20S Proteasomes
Archaeal 20S proteasomes by themselves can degrade certain unfolded proteins, e.g., the fully reduced α-lactalbumin (Wenzel and Baumeister, 1995). However, rapid degradation of even the loosely folded casein, like the breakdown of the globular GFPssrA, required PAN’s ATPase activity (Benaroudj and Goldberg, 2000; Zwickl et al., 1999). To further investigate the influence of the substrate’s folding status, we tested whether denaturation of GFPssrA would make it competent for degradation by 20S independently of PAN and ATP. GFPssrA was denatured by treatment with acid, which causes a complete loss of fluorescence, modification of the...
absorption spectrum, and a disruption of the secondary structure of GFP, as measured by circular dichroism (Bokman and Ward, 1981; Ward and Bokman, 1982) (data not shown). Surprisingly, the acid-denatured GFPssrA was not degraded faster than globular GFPssrA by 20S proteasomes (Figure 2). The addition of PAN and ATP led to a similar stimulation of degradation of denatured GFPssrA as was found for globular GFPssrA. Therefore, unfolding of this substrate does not appear to be the rate-limiting step in its degradation by 20S. It is noteworthy that the rate of casein degradation was three to four times higher than those of globular and denatured GFPssrA, although casein and GFPssrA activated PAN’s ATPase activity to a similar extent and have comparable molecular weights (24 and 30 kDa, respectively). The reason for the faster degradation of casein is still unclear.

ATP Consumption during Degradation of Globular and Unfolded Proteins
In order to determine how many ATP molecules are consumed when a protein is degraded by the PAN-20S complex, the rates of degradation of different proteins and of ATP hydrolysis (in the presence of protein substrates) were measured simultaneously with different concentrations of the PAN-20S complex. In initial experiments, conditions were established where hydrolysis of both ATP and protein were linear with time. In order to ensure that each PAN complex interacted with a 20S particle (i.e., that PAN’s activated ATPase activity was linked to proteolysis), these experiments were performed with a 5-fold molar excess of 20S proteasomes over PAN. The rates of ATP hydrolysis were then plotted against those of protein degradation (Figure 3). During degradation of 1 molecule of casein, 312 molecules of ATP were hydrolyzed (upper panel).

In similar experiments, when a molecule of globular GFPssrA was degraded, 331 molecules of ATP were hydrolyzed (Figure 3, middle panel). Thus, surprisingly, ATP consumption during degradation by the PAN-20S complex appears to be similar for a loosely folded and a tight globular substrate. To test further if ATP consumption is in fact independent of the folding state of the substrate, GFPssrA was fully denatured by acid
Figure 2. PAN Stimulates the Degradation of Globular and Unfolded Proteins by 20S Proteasomes

The degradation of 1 μM of 14C-GFPssrA, acid-denatured 14C-GFPssrA, or 14C-casein was followed with 2.1 nM of 20S proteasomes alone (open bars), with 9.3 nM of PAN complex added (striped bars), or with 9.3 nM of PAN complex and 1 mM ATP added (filled bars) at 45°C in 50 mM Tris (pH 7.5), 1 mM DTT, and 10 mM MgCl₂. Values are means plus standard deviations of three independent experiments. Treatment just before incubation with PAN and 20S proteasomes. Three hundred and seventy three molecules of ATP were hydrolyzed during the degradation of a molecule of acid-denatured GFPssrA (Figure 3, lower panel). Together, these findings indicate that a large number of ATP molecules (at least 300) are hydrolyzed during degradation of a protein substrate, regardless of its folding status. These findings are consistent with our observation that folded and unfolded protein substrates stimulate PAN’s ATPase activity to a similar extent.

This calculation of energy cost for protein breakdown includes the basal and the protein-activated ATP hydrolysis. This analysis assumed that all the ATP consumption in the presence of substrate is linked to proteolysis. An alternative assumption would be that only the increase in ATP hydrolysis upon addition of substrate is linked to proteolysis. Therefore, the basal ATP hydrolysis (35% of the total ATP hydrolysis on the average) should be subtracted. On this basis, the actual ATP cost during degradation of a molecule of those three substrates is then about 220 molecules.

Entry of Unfolded Proteins Is Gated by the Amino Termini of the 20S α Subunits and Regulated by PAN

One apparent difference between the X-ray structure of the prokaryotic and eukaryotic 20S particles lies in the α rings. In the X-ray structure of 20S from T. acidophilum, an axial channel with a diameter of 1.3 nm appeared to connect the surrounding medium with the internal chamber of the particle (Lowe et al., 1995). Such a channel through the α rings was not observed in the X-ray structure of yeast 20S proteasomes (Groll et al., 1997). Indeed, entry of small peptides into the eukaryotic particle appeared to be blocked by the N-terminal extremities of the α subunits that act as a gate for the channel (Groll et al., 2000). However, these differences between archaeal and yeast 20S might only reflect differences between the forms of the particles crystallized in these studies.

To investigate whether protein entry in the archaeal 20S is limited by a similar gate that was missed in prior studies, we have deleted the residues 2–12 of the α subunits in archaeal 20S particles. These residues correspond to those found to block the axial pore of the yeast 20S and whose deletion led to rapid substrate entry and hydrolysis (Groll et al., 2000). The α subunits of the wild-type and Δα(2-12)20S proteasomes were expressed in E. coli, and the resulting particles were analyzed by electron microscopy. Previous studies showed that expression of the archaeal α subunits alone leads to formation of seven-membered rings which can assemble further into double rings (Zwickl et al., 1994). Accordingly, both wild-type and Δα(2-12) proteasome α subunits form...
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Figure 4. Deletion of Amino-Terminal Residues of 20S α Subunits Opens the Axial Channel of α Rings

Electron microscopy top views of the α subunits of wild-type (A) and Δα(2-12) 20S proteasomes (B) were performed as described in Experimental Procedures. (C) represents the difference mapping of the averaged pictures. The diameter of both complexes is approximately 11 nm.

particles with 7-fold symmetry (Figures 4A and 4B). Difference maps of averaged images of the wild-type and the Δα(2-12) α ring showed the presence of mass in the center of the α rings formed by the wild-type 20S α subunits, which was lacking in the center of the Δα(2-12) α ring (Figure 4C). Therefore, the wild-type archaeal proteasomes normally have their axial channel in a closed form, and deletion of residues 2–12 in the α subunits led to the opening of this channel.

The Δα(2-12) 20S variant enabled us to examine the effect of gate opening as an isolated process, and thus the ability of the Δα(2-12) 20S variant to degrade peptides, unfolded proteins, or globular proteins was tested. In contrast to eukaryotic 20S particles, which degrade small peptides at a slow rate when their axial channel is closed, the wild-type and the Δα(2-12) archaeal 20S particles did not differ in degrading tetrapeptides (Figure 5A). However, the Δα(2-12) 20S degraded casein 29 times and acid-denatured GFPssrA 46 times faster than the wild-type 20S proteasomes (Figure 5B). In fact, the Δα(2-12) 20S degraded protein substrates even more efficiently than the wild-type 20S in the presence of PAN and ATP (three and five times more for casein and denatured GFPssrA, respectively). Moreover, degradation of casein and acid-denatured GFPssrA by the Δα(2-12) 20S variant was not stimulated further by the addition of PAN and ATP. However, the Δα(2-12) 20S, like the wild-type 20S, was not able to degrade the globular GFPssrA, in the absence of PAN or ATP (Figures 5B and 5C). Presumably, the folded GFPssrA molecule, unlike the unfolded one, is incapable of entering the 20S particles even when the channel is open. Therefore, although the amino-terminal extremities of the α subunits are not visible in the X-ray structure of archaeal proteasomes, they function in gating, as they do in eukaryotic 20S. The axial channel of archaeal 20S proteasomes in its native state is still permeable to tetrapeptides, but is not large enough to accommodate poly-peptides, which require the presence of PAN and ATP for efficient degradation. Once the channel of the 20S proteasome is opened by deleting the N-terminal extremities of the α subunits, the dependence on PAN and ATP is abrogated for unfolded proteins. Therefore, we conclude that PAN, in the presence of ATP, facilitates the opening of the gate in the 20S proteasome, enabling the translocation of unfolded polypeptides.
Figure 6. After Unfolding of GFPssrA by PAN, Hydrolysis of ATP by PAN Is Still Essential for Its Degradation by the Δα([2-12]20S) Proteasome Variant

(A) The time course of fluorescence change of 250 nM of GFPssrA was followed in 50 mM Tris (pH 7.5), 1 mM DTT, 1 mM ATP, and 10 mM MgCl₂ at 45°C in the presence of 50 nM of PAN (●, ▲). After 15 min of incubation to allow unfolding of GFPssrA, 0 nM (●) or 20 nM of Δα([2-12]20S) was added to the samples together with 2 mM of ATP-γ-S (●) or the equivalent volume of buffer (▲). If Δα([2-12]20S) were present together with PAN and ATP from the onset (○), only buffer was added. (B) GFPssrA was incubated as in (A), and at the indicated times, aliquots were withdrawn, loaded on a 12% SDS-PAGE, and analyzed by Western blot using an anti-histidine antibody. The antibody also recognizes the β subunit of 20S proteasomes which carries a (His)₆ tag. (C) GFPssrA amount on (B) was quantified and relative amounts were determined using the 0 time point as 100%. Relative amounts of GFPssrA in lanes 1–3 (○), 4–6 (▲), 7–9 (●), and 10–12 (●) are plotted as indicated.

addition of an excess of ATP-γ-S that totally inhibited PAN’s ATPase activity, as well as its ability to unfold GFPssrA (data not shown). Then, the Δα([2-12]20S) was added, and degradation of GFPssrA was assayed by monitoring its fluorescence (Figure 6A) and the amount of the protein on a SDS-PAGE gel (Figures 6B and 6C).

After incubating GFPssrA with PAN and ATP, when the 20S variant was added to the preincubated mixture of GFPssrA, PAN, and ATP (Figure, 6, closed triangles, lanes 7–9) degradation of GFPssrA occurred to a similar extent as when the Δα([2-12]20S), PAN, and ATP were all present from the onset (open circles, lanes 1–3). However, when the Δα([2-12]20S) was added in the presence of ATP-γ-S to prevent further ATP hydrolysis, GFPssrA was not degraded (closed circles, lanes 10–12), even though most of it had been unfolded by PAN during the preincubation (open triangles). Similar results were obtained with wild-type 20S proteasomes (data not shown). Some disappearance of GFPssrA was observed on the electrophoretic gel at 15 min just after the addition of the Δα([2-12]20S) (lanes 7–9). This decrease corresponds probably to inevitable degradation that occurs between the withdrawing of the sample from the fluorescence cuvette just after addition of 20S and the loading on the electrophoretic gel due to the very high ability of this variant to degrade unfolded substrates as noted in Figure 5B. Such degradation was minimized in experiments using the wild-type 20S and when aliquots were withdrawn before addition of proteasomes at 15 min (data not shown). Thus, although GFPssrA is unfolded by PAN, its degradation by 20S still requires ATP hydrolysis, even when the axial channel in the proteasome α ring is in an open state.

This finding strongly suggests that ATP hydrolysis by PAN serves an additional function after substrate unfolding and gate opening. This additional function very likely is translocation of unfolded substrates. One alternative possible explanation of this lack of degradation of unfolded GFPssrA by the open channel 20S variant in the presence of ATP-γ-S would be that, in the absence of ATP hydrolysis, the unfolded substrate forms a stable association with PAN, which precludes entry of GFPssrA into the proteolytic chamber. However, prior work has established that the complex between GFPssrA and PAN is quite short lived, since PAN and GFPssrA, even when it is acid denatured, could not be
coimmunoprecipitated, even in the presence of ATPγS (Navon and Goldberg, 2001) (our unpublished data). Accordingly, PAN could not be retained by a GFPssrA-containing resin (our unpublished data). In addition, when PAN was added without ATP, it did not affect the degradation of acid-denatured GFPssrA or of casein (Figure 5B), as would be expected if these substrates remained bound to PAN in the absence of ATP hydrolysis. This observation also allows us to rule out a model whereby PAN hinders the entry of unfolded substrates into the open channel 20S particle in the presence of ATPγS.

Thus, once a globular protein has been unfolded by PAN, it is not simply released into the medium and does not reach the 20S by passive diffusion. Instead, the unfolded polypeptide has to be actively translocated by PAN into 20S proteasomes, by a process that requires ATP hydrolysis, even if 20S proteasomes have their axial channel sufficiently open to digest the denatured protein.

Discussion

ATP Consumption during Protein Degradation

Although it has long been appreciated that intracellular proteolysis requires ATP hydrolysis, the actual amount of ATP required for the breakdown of a protein by proteasomes is not known. The present studies show that when the protein-activated ATP hydrolysis and proteolysis proceed at linear rates, there is an apparent stoichiometry between the amount of ATP molecules hydrolyzed and protein molecules degraded that appears independent of the nature of the protein substrate. At least 300 ATP molecules are hydrolyzed by PAN when proteasomes degrade 1 molecule of the protein substrate. This surprisingly high number indicates that protein unfolding and translocation into the 20S particle requires many cycles of ATP hydrolysis. Even more surprising was the finding that the amount of ATP hydrolysis per protein degraded is similar with the globular and unfolded substrates. Thus, the rates of ATP hydrolysis by PAN are independent of whether or not PAN has to catalyze substrate unfolding. This conclusion is also supported by our finding that globular and unfolded substrates stimulate ATP hydrolysis to similar extents. ATP-driven changes in the conformation of PAN must underlie both the unfolding and translocation through the ATPase ring. With PAN alone, the protein is probably released into the solution. With 20S particles present, these events are linked to substrate injection into the proteolytic chamber. ATP consumption was identical whether or not proteasomes were present. In contrast, with the ATP-dependent proteases from E. coli, the ATPase activity is altered by the proteolytic component; e.g., ATP consumption by ClpA and ClpX decreases upon addition of the ClpP protease (Burton et al., 2001; Hwang et al., 1988) while ATP hydrolysis by HslU increases in the presence of HslV (Seol et al., 1997).

Because the 11 residue recognition sequence in GFPssrA (ssrA peptide) by itself stimulated ATP hydrolysis to a similar extent as GFPpsrA or casein, the activation of ATP consumption must be triggered by the interaction between the ATPase and the substrate. Although no prior studies have addressed the energy costs for proteasome function, the energy costs for protein breakdown by two E. coli ATP-dependent proteases have been investigated. It has been shown that during degradation of Arc-ssrA, 150 mol of ATP were hydrolyzed by ClpXP, and this number was independent of the substrate’s thermodynamic stability (Burton et al., 2001). Thus, in degrading this small globular protein (about 75 residues), ClpXP consumed almost as much ATP as found here for PAN-20S proteasome degrading the much larger polypeptides casein and GFPpsrA (220 and 268 residues). Menon et al. (1987) found that ATP hydrolysis by the lon protease is proportional to the number of cleavages made in the substrate, but with this type of enzyme (which contains ATPase and proteolytic active sites in the same polypeptide chain and where these processes may be more tightly coupled), significantly less ATP is consumed than by PAN-20S complex.

The Rate-Limiting Step in Protein Degradation by Proteasomes

Because acid treatment of GFPssrA destroyed its globular conformation and its ability to fluoresce, but did not accelerate its rate of degradation, gross unfolding of this protein can not be the rate-limiting step in its degradation. One alternative explanation of this result might be that once the acid-denatured GFPssrA was diluted into the reaction buffer (pH 7.5) at 45°C, it refolded rapidly and therefore showed similar rates of proteolysis and ATP dependence as globular GFPpsrA. However, several findings make this trivial explanation very unlikely. First, after removing the acid, the denatured GFPpsrA did not regain its fluorescence, and after 2 hr, its absorption spectrum was still indistinguishable from that of denatured GFPssrA maintained in acid (data not shown). Second, the yield of GFP refolding at 50°C is only 19% of that at 37°C (Makino, 1997). Perhaps the strongest evidence against refolding was that the acid-denatured GFPssrA, unlike globular GFPpsrA, was rapidly degraded by the Δ(2-12)20S proteasome. Therefore, its structure must differ from that of the globular GFPpsrA at 45°C. Although gross unfolding of globular substrates does not appear to be the rate-limiting step in degradation, translocation of the unfolded polypeptide through the ATPase ring into the 20S particle could be rate limiting. Consistent with this conclusion was the finding that deletion of the N-terminal residues of the α subunit that obstruct entry into the 20S led to much faster degradation of unfolded proteins, even faster than with PAN and ATP.

It has been generally assumed that protein unfolding is the rate-limiting step in the functioning of ATP-dependent proteases. For example, it has been shown that when isolated ClpX ATPase complex catalyzes the unfolding of GFPpsrA, fluorescence of GFPpsrA decreases at a similar rate as GFPpsrA is degraded by the holoenzyme ClpXP (Kim et al., 2000). The authors concluded that unfolding is the rate-limiting step during ClpXP-mediated proteolysis. On the other hand, Singh et al. (2000) observed a difference in the rates of GFPpsrA unfolding and degradation. Nevertheless, both studies suggested that the rate-limiting step precedes peptide bond cleavage, in agreement with our conclusion that the rate-limiting step occurs after protein unfolding, and
Figure 7. Model for the Energy-Dependent Steps of PAN and Proteasomes-Mediated Protein Degradation

Cut side views of 20S particles are schematized with the active sites represented as blue dots in the internal chamber. PAN is schematized as a hexameric ring. The circle and pentagonal shape of the subunits represent the substrate-free and substrate-bound forms of PAN, respectively. The gate which precludes entry of protein into 20S particles is represented in red.

thus probably is the translocation into the 20S particle. For the unfolded proteins, translocation into the 20S is clearly rate limiting, since their degradation was accelerated dramatically by deletion that enlarges the gate and facilitates entry into the 20S (see below).

Gating of the Archaeal 20S Proteasomes
These studies have demonstrated that archaeal 20S proteasomes contain a functional gate in their outer ring whose opening appears to be regulated by PAN. Indeed, substrate entry into the archaeal 20S is normally blocked by the N termini of the α subunits, since deletion of residues 2–12 of those subunits dramatically increased the degradation of unfolded proteins and led to an opening in the α ring visible by electron microscopy analysis. Those residues are highly conserved among prokaryotic and eukaryotic proteasomes, and the present findings are similar to those obtained with analogous deletions in yeast 20S particles (Groll et al., 2000). With eukaryotic 20S proteasomes in their native state, entry and exit of small peptides requires gate opening by the 19S regulatory particle (Kohler et al., 2001) or by PA28-related activators (Whitby et al., 2000). By contrast, entry of tetrapeptide into archaeal 20S particles occurs readily since the Δω(2-12) 20S variant and the wild-type 20S-PAN complex did not degrade these small peptides faster than the wild-type 20S proteasome (this work; Zwickl et al., 1999). Thus, although archaeal proteasomes show ATP-regulated gating that prevents entry of polypeptides, in the basal state these particles do appear open to small peptides, unlike the eukaryotic 20S.

ATP-Dependent Gate Opening and Substrate Translocation
The most fundamental function of the PAN ATPase is to facilitate entry of protein substrates into proteasomes for degradation. Since the axial channel in the α ring has a gate that normally prevents protein entry, it is not surprising that PAN and ATP trigger gate opening. However, such a function was unexpected because the X-ray structure of archaeal proteasomes showed an open axial channel (Lowe et al., 1995). By interacting with 20S particles, PAN probably induces an ATP-dependent movement of the N termini of the α subunits that displaces them from the surface of the axial pore. A similar mechanism has been suggested for the activation of 20S proteasomes by PA28 (Whitby et al., 2000). When the channel into the 20S was opened by the Δω(2-12) deletion, degradation of casein and acid-denatured GFPssrA were rapid and independent of ATP and PAN, while in the wild-type, where the channel was in its closed state, degradation of these unfolded substrates required PAN’s ATPase activity. Therefore, PAN appears to open the gate in the α ring. Interestingly, the rates of degradation of unfolded proteins by the PAN-wild-type 20S complex was lower than those by the Δω(2-12) 20S. Therefore, protein binding to PAN and ATP-dependent translocation through the ATPase ring into the 20S particle appear to be slower than diffusion directly through the open axial channel, perhaps because the PAN-induced opening in the α ring may not be as large as in the deletion mutant.

In eukaryotes, a homologous ATPase in the 19S particle activates proteolysis apparently by promoting the opening of the gate into the 20S particles. Mutation in one of the six ATPases (rpt2RF) strongly reduced the ability of 20S particles to hydrolyze peptides (Rubin et al., 1998). This defect could be suppressed by a mutation in the 20S particle that leads to the opening of the channel in the α ring (Kohler et al., 2001). Therefore, Rpt2 appears to be particularly important in regulating opening of the axial channel and peptide entry, although analogous mutations in the other five ATPase subunits were not analyzed.

Even after its unfolding by PAN, and even when the axial channel of the 20S particles was open, hydrolysis of ATP by PAN was still required for degradation of globular GFPssrA. In contrast, when the GFPssrA was denatured with acid, PAN was not required for its translocation into the Δω(2-12)20S. This difference in the requirement for PAN between GFPssrA unfolded by PAN
and GFPssrA denatured by acid is intriguing. Perhaps, when PAN unfolds this substrate, it does not adopt a stable extended conformation that can diffuse readily into the 20S particles. The gross unfolding of GFPssrA occurs on the surface of PAN’s ring, and probably precedes its PAN-mediated substrate threading through PAN’s central opening into the 20S (Navon and Goldberg, 2001). Perhaps, this ATP-dependent threading through PAN might also eliminate residual secondary structural elements of GFPssrA that prevent its diffusion into Δ匆(2-12)20S particles.

In summary, these findings allow us to dissect several distinct steps where PAN is required during protein degradation (Figure 7). The substrate binds to PAN, which triggers a change in its conformation leading to an increased capacity to hydrolyze ATP. Substrate binding to PAN can occur on the isolated PAN or on the PAN-20S complex. This substrate-activated ATPase (i) facilitates unfolding of the substrate, probably at the surface of the ATPase ring, (ii) favors an open form of the 20S axial channel, and (iii) promotes substrate translocation into the internal chamber of the 20S particles where peptide bond cleavage occurs. (Because ATP hydrolysis by PAN can also occur in the absence of a protein substrate, although at a lower rate, it seems likely that gate opening in the 20S by PAN occurs also in the absence of a substrate.) These distinct functions of PAN’s ATPase activity are very likely to apply to the closely homologous 19S ATPases, although in addition one ATPase subunit in the 19S is involved in interacting with ubiquitinated proteins (Lam et al., 2002).

PAN belongs to the large family of AAA ATPases (Ogura and Wilkinson, 2001), many of which have been shown to catalyze protein remodeling. The present findings on substrate-activated ATPase activity, the energy costs of unfolding and translocation, and the multiple roles of ATP hydrolysis are likely to apply also to these related multimeric ATPases. Analogous studies of these AAA family members may also help clarify their molecular mechanisms.

Experimental Procedures

Proteins and Peptides
PAN and archaeal 20S proteasomes were purified as previously described (Benaroudj and Goldberg, 2000; Zwickl et al., 1999). Molar concentrations of PAN were expressed on the basis of a 650 kDa complex (Zwickl et al., 1999). (His)6-GFP and (His)10-GFPssrA were purified as described earlier (Benaroudj and Goldberg, 2000) with the following modifications. After purification on an Ni-NTA agarose column, the fractions containing GFP proteins were pooled and subjected to a MonoQ HR5/5 (Amersham Pharmacia Biotech) anion exchange column equilibrated with 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. A linear gradient of NaCl from 0 to 500 mM was applied, and the GFP proteins eluted at approximately 150–200 mM. Fractions containing (His)6-GFP or (His)10-GFPssrA were pooled and dialyzed against 50 mM Tris-HCl (pH 7.5). The ssrA peptide (NH2-CAANDENYALAA-CO2H) was a generous gift from Y.I. Kim (Kim et al., 2000). Casein was purchased from Sigma.

Construction of Δ匆(2-12)20S Proteasome Variant
Deletion of residues 2–12 in the α subunits of archaeal 20S proteasomes was performed by reverse PCR mutagenesis using the pRSETα-α expression vector as described earlier (Seemuller et al., 1995; Zwickl et al., 1992). The resulting pRSETα-α was then introduced into E. coli BL21(DE3) cells, and the Δ匆(2-12)20S proteasome variant was purified as the wild-type 20S proteasomes.

Expression and Purification of 20S α Rings
The α gene was elongated at the 3′ end by an affinity tag that codes for 6 histidine residues and cloned into pRSETα expression vector. Then, the tagged α gene was deleted for 33 nucleotides at the 5′ end, which codes for residues 2–12, thus generating the Δ匆(2-12)α gene. Wild-type and mutant α proteins were expressed in BL21(DE3) and purified as 20S proteasomes.

Hydrolysis of ATP
Hydrolysis of ATP was assayed by following production of inorganic phosphate at 45°C as described elsewhere (Ames, 1966; Zwickl et al., 1999).

Degradation of [14C]-Radiolabeled Proteins
[14C]-Radiolabeled casein and GFPssrA were prepared by reducing alklylation, a gentle procedure that does not produce dramatic changes in protein structure (Rice and Means, 1971) and stored in 50 mM Tris-HCl (pH 7.5). The radiolabeling of GFPssrA did not alter its fluorescence properties. Breakdown of these [14C]-radiolabeled proteins was conducted in the presence of 20S proteasomes, PAN, and ATP as indicated in 50 mM Tris (pH 7.5), 1 mM DTT, and 10 mM MgCl2 at 45°C. After 40–60 min incubation, trichloroacetic acid at a final concentration of 12% was added together with 100 μg of BSA as a carrier. Acid-soluble radioactive peptides products were quantified by scintillation counting.

Peptides Hydrolysis
100 μM of fluorogenic Suc-LLVY-amc peptide diluted in 100% DMSO was incubated with 420 mM 20S proteasomes at 45°C in 50 mM Tris (pH 7.5), 1 mM DTT, and 10 mM MgCl2. Hydrolysis of the peptide was followed by measuring the release of amc (7-amido-4-methylcoumarin) in a spectrophotofluorimeter (λex 380 nm; λem 440 nm).

GFPssrA Fluorescence
Measurements of GFPssrA fluorescence were obtained at 45°C with excitation at 400 nm and emission at 510 nm. GFPssrA was diluted in 50 mM Tris (pH 7.5), 1 mM DTT, and 10 mM MgCl2, in the presence or the absence of PAN, 20S proteasomes, and the indicated nucleotide.

Denaturation of GFPssrA
GFPssrA or [14C]-GFPssrA were incubated with 50 mM HCl (pH 2.0) for 10 min at room temperature. Denatured GFPssrA was diluted directly into reaction mixture at 45°C and used immediately.

Electron Microscopy and Image Analysis
Purified proteins were negatively stained with 2% uranyl acetate. Measurements containing (His) 6-GFP or (His 10)-GFPssrA were pooled and subjected to a MonoQ HR5/5 anion exchange column. After performing size exclusion, the projections were interpreted according to their coordinates along the dominating first factor.

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