

ATP Binding to PAN or the 26S ATPases Causes Association with the 20S Proteasome, Gate Opening, and Translocation of Unfolded Proteins

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Summary

The archaeal ATPase complex PAN, the homolog of the eukaryotic 26S proteasome-regulatory ATPases, was shown to associate transiently with the 20S proteasome upon binding of ATP or ATP γ S, but not ADP. By electron microscopy (EM), PAN appears as a two-ring structure, capping the 20S, and resembles two densities in the 19S complex. The N termini of the archaeal 20S α subunits were found to function as a gate that prevents entry of seven-residue peptides but allows entry of tetrapeptides. Upon association with the 20S particle, PAN stimulates gate opening. Although degradation of globular proteins requires ATP hydrolysis, the PAN-20S complex with ATP γ S translocates and degrades unfolded and denatured proteins. Rabbit 26S proteasomes also degrade these unfolded proteins upon ATP binding, without hydrolysis. Thus, although unfolding requires energy from ATP hydrolysis, ATP binding alone supports ATPase-20S association, gate opening, and translocation of unfolded substrates into the proteasome, which can occur by facilitated diffusion through the ATPase.

Introduction

A fundamental feature of protein breakdown in both prokaryotes and eukaryotes is its requirement for ATP (Goldberg and St John, 1976). Much of our present knowledge about intracellular proteolysis came from studies seeking to understand the biochemical basis of this surprising requirement (Ciechanover, 2005; Goldberg, 2005). The degradation of most proteins in eukaryotic and prokaryotic cells is catalyzed by large proteolytic complexes that hydrolyze ATP and proteins in linked reactions (Baumeister et al., 1998). In eukaryotes, ATP is required both for ubiquitin conjugation to substrates and for the functioning of the 26S proteasome, which catalyzes the breakdown of the ubiquitinated and certain nonubiquitinated polypeptides (Ciechanover, 2005; Goldberg, 2005; Voges et al., 1999). Recent studies have identified multiple steps in proteasomal function where ATP is required (Benaroudj et al., 2003), but the precise roles of ATP are not clear, nor is it clear how these steps function together to catalyze efficient protein degradation (Ogura and Tanaka, 2003).

The 26S proteasome is composed of one or two 19S regulatory complexes and the central 20S proteasome (Voges et al., 1999), which is a hollow proteolytic cylinder. Its two outer α rings and two inner β rings are

each composed of seven subunits. Three of these β subunits contain the proteolytic sites, which are sequestered in the hollow interior of the particle (Groll et al., 1997). Substrates enter the 20S through a narrow channel formed by the α subunits, whose N termini can either obstruct or allow substrate entry and thus function as a gate (Groll et al., 2000; Groll and Huber, 2003). This entry channel is narrow and only permits passage of unfolded, linearized polypeptides (Groll et al., 1997). The 19S complex is composed of two subcomplexes: the lid, which seems to bind and disassemble the ubiquitin-conjugated substrate, and the base. The base contains six homologous ATPase subunits (termed Rpt1–Rpt6 in yeast) (Voges et al., 1999), all of which are members of the AAA family of ATPases (Patel and Latterich, 1998), plus two non-ATPases: Rpn1 and Rpn2. For a globular protein to be degraded, it must associate with the 19S ATPases and undergo ATP-dependent unfolding followed by translocation into the 20S particle, which requires opening of the gate in the α ring (Kohler et al., 2001).

Studying these processes and their mechanisms is difficult or impossible with the 26S complex because of its structural complexity, multiple enzymatic activities, and ubiquitin requirement (Glickman et al., 1998). The closest known homolog of the six ATPases in the 26S proteasome is PAN, the proteasome-activating nucleotidase complex from archaea (Zwickl et al., 1999). Based on its homology to other AAA complexes, PAN is presumably a hexameric ring complex that functions like the six 19S ATPases with which it shares 41%–45% similarity (Zwickl et al., 2000). However, in archaea, degradation of proteins occurs without ubiquitin conjugation (Zwickl et al., 1999). Nevertheless, this process still seems to involve substrate binding, ATP-dependant unfolding, translocation, and opening of the gated channel in the proteasome (Benaroudj et al., 2003; Ogura and Tanaka, 2003). The present studies have utilized PAN to clarify the roles of ATP binding and hydrolysis in each of these steps.

When PAN is mixed with archaeal 20S proteasomes and ATP, it stimulates the degradation of proteins that lack tight tertiary structures as well as stable globular proteins (Benaroudj and Goldberg, 2000; Navon and Goldberg, 2001). Because protein degradation by eukaryotic proteasomes occurs while the 19S and the 20S are complexed, it is likely that PAN also associates with the 20S. However, several groups (including our lab) have failed to demonstrate an association between PAN and the 20S (Forster et al., 2003) even when PAN and the 20S were from the same species. Consequently, some have suggested a mechanism that differs sharply from that postulated for the 26S ATPases in which PAN makes substrates competent for proteasome entry through its unfolding activity without having to contact the proteasome directly (Forster et al., 2003). The present studies, however, demonstrate that this ATPase associates directly with the proteasome and have defined the requirements for and consequences of this association.

A variety of observations have suggested that archaeal 20S proteasomes, unlike their eukaryotic counterparts,

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lack a functional gate in the α ring that prevents substrate entry (Groll et al., 2003; Voges et al., 1999). For example, 20S proteasomes alone rapidly cleave tri- or tetrapeptide substrates, and PAN and ATP do not stimulate their hydrolysis (Zwickl et al., 1999). Also, deletion of residues 2–12 in the α subunits, which are homologous to the residues forming the gate in eukaryotic proteasomes, does not enhance the degradation of these short peptides (Benaroudj et al., 2003). However, after deletion of residues 2–12, a central pore in the α rings is evident by EM, and the proteasome shows a dramatic increase in its capacity to degrade proteins with little tertiary structure (e.g., β -casein) (Benaroudj et al., 2003). Therefore, the archaeal 20S may contain an entry channel large enough to allow rapid entry of tetrapeptides but able to impede the entry of proteins and longer peptides. Benaroudj et al. (2003) concluded that PAN with ATP opens this gate in the α ring because PAN stimulates degradation of β -casein. Interestingly, of the 19S ATPase subunits, PAN is most closely homologous to Rpt2, the subunit implicated in regulating the gate in the 20S particle (Kohler et al., 2001). One goal of the present studies was to test whether the gate in the archaeal proteasome excludes peptides larger than four residues. We have identified nine-residue fluorescent peptides whose entry into the 20S requires PAN and ATP and have used them to demonstrate that PAN stimulates gate opening in the 20S even without energy consumption.

Prior studies of PAN (Benaroudj and Goldberg, 2000; Benaroudj et al., 2003) and the *E. coli* ATP-dependent proteases ClpXP (Kenniston et al., 2003) and ClpAP (Weber-Ban et al., 1999) demonstrated that unfolding of globular substrates (e.g., GFPsrA) requires ATP hydrolysis and showed that unfolding, by PAN, can take place on the surface of the ATPase ring in the absence of translocation (Navon and Goldberg, 2001). Thus, unfolding seems to precede and can be dissociated from protein translocation. However, evidence has been presented that proteins are unfolded by energy-dependent translocation of downstream termini through the ATPase ring (Lee et al., 2001; Matouschek, 2003). These studies have assumed that the translocation of an unfolded polypeptide from the ATPase into the 20S is an active process that requires concomitant ATP hydrolysis. To test this important assumption, we have dissociated the sequential steps in ATP-dependant proteolysis and analyzed the requirements for ATP binding and hydrolysis for protein unfolding and translocation into the 20S proteasome.

Results

PAN-20S Complex Formation

Although it seems very likely that PAN directly associates with the 20S proteasome to stimulate protein degradation, prior studies by others and ourselves have failed to observe an association between PAN and the 20S using several different approaches, including native PAGE, gel filtration, ultracentrifugation, and immunoprecipitation (e.g., even in the presence of ATP, ATP analogs, or substrates) (Forster et al. [2003] and our unpublished data). Some therefore suggested that PAN stimulates proteolysis by altering the substrate and does not interact with the 20S (Forster et al., 2003). Alter-

natively, the association between PAN and the 20S may be short lived and thus transient. We therefore used a more sensitive approach to assay complex formation. 20S particles were incubated with ^{35}S -PAN (prepared by growing *E. coli* expressing PAN with ^{35}S -methionine) with or without nucleotides at 25°C, and the 20S was immunoprecipitated with anti-20S proteasome antibodies. After incubation without nucleotide or in the presence of ADP, anti-20S antibodies pulled down very little ^{35}S -PAN, but after incubation with ATP or AMPPNP, at least five times more PAN was coprecipitated (Figure 1A). Because this ATP-dependent association could not be demonstrated by less-sensitive methods (e.g., Western blot), this large 20S-PAN complex must be short lived.

To further analyze this association of PAN with the 20S, we used surface plasmon resonance (SPR). The archaeal 20S particles were immobilized on the chip through a 6-His tag on their β subunits. When buffer containing PAN and the nonhydrolyzable ATP analog ATP γ S (Figure 1B) flowed over the chip, binding of PAN to the 20S was observed and the extent of PAN/20S association depended on the concentration of PAN (Figure 1C). The PAN-20S complex also formed with AMPPNP present but to a lesser extent than with ATP γ S (data not shown). No such binding of PAN was seen in the channels lacking the 20S particles, in the absence of any nucleotide, or with ADP present, in accord with the findings using immunoprecipitation. Presumably, with ATP present, complexes also formed, as shown by coimmunoprecipitation (Figure 1A), but could not be demonstrated by SPR, because ATP also caused non-specific binding of PAN to the channels lacking immobilized 20S (data not shown). After the PAN-20S complex was formed with ATP γ S, we were able to study its dissociation by washing with different nucleotides. Analysis of this dissociation confirms that this complex is transitory in the presence of ATP and that it dissociates more slowly with ATP γ S present (D.M.S., G.K., and A.L.G., unpublished data). Presumably, this interaction is weaker with ATP because it is rapidly hydrolyzed to ADP, which does not support complex formation.

EM of the PAN-20S Complex

Because we could demonstrate nucleotide-dependent formation of the PAN-20S complex, we attempted to visualize its structure by EM. With ATP γ S present, more than 50% of the 20S particles (seen on side view) were complexed with PAN at one (“singly capped”) or both ends (“doubly capped”) of the 20S particles (Figures 2A–2D). As expected from Figure 1, no such complex was observed without any nucleotide or with ADP present. PAN did not appear as simply an additional ring bound to the outer face of the α ring but contained a second smaller outer ring in parallel with the major PAN ring (Figures 2C and 2D, see black arrows). The larger ring has a similar diameter as the 20S, ~ 120 Å, and a height of ~ 80 Å. The smaller outer ring has a diameter of ~ 75 Å and height of 30 Å. Thus, PAN resembled a “top hat” capping the 20S particle. This outer ring probably corresponds to the N termini of PAN based upon a similar two-ring appearance of a related AAA complex, HslU (Rohrwild et al., 1997; Song et al., 2000).

The “class averaged” images of the PAN-20S complex demonstrated that PAN was not always flush

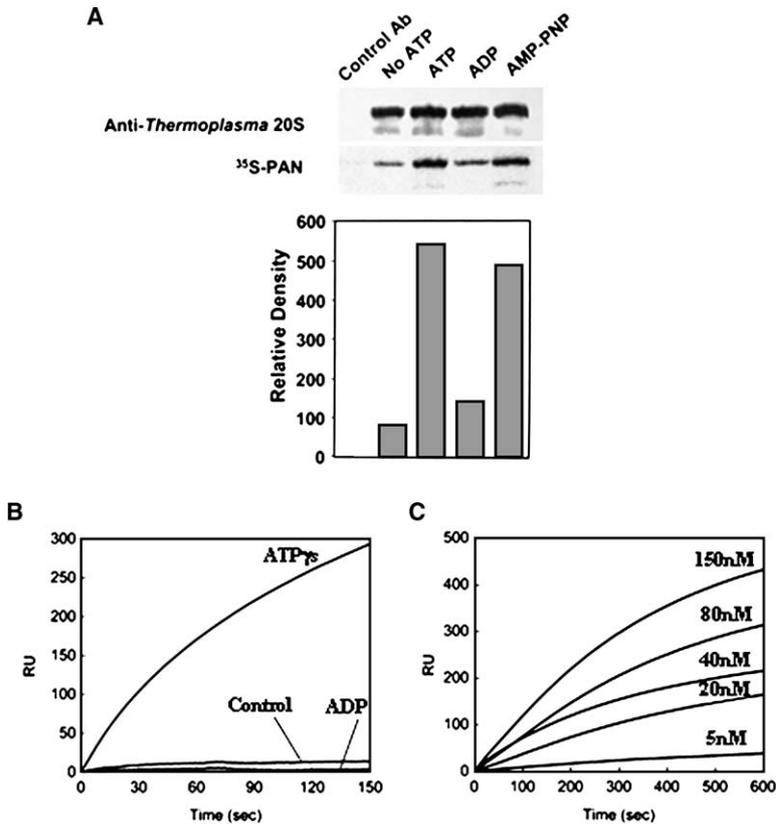


Figure 1. ATP Binding to PAN Promotes Its Association with 20S Proteasomes

(A) Nucleotide requirement for complex formation between ^{35}S -PAN and 20S proteasome analyzed by coimmunoprecipitation with anti-proteasome antibodies. ^{35}S -PAN-proteasome association was determined by phosphorimager scans, and densitometric values were calculated by Image Gauge software. A Western blot for 20S was performed to confirm equal loading. Similar results were obtained in three experiments.

(B) Real-time detection of PAN-20S association by surface plasmon resonance (SPR). 20S was immobilized on the chip, and PAN (lacking His tags) was injected with or without 1 mM of the indicated nucleotide at 20°C. The graph is given as resonance response units (RU) as a function of time. No binding was observed if PAN was injected without nucleotide or in the presence of ADP. PAN binds to the immobilized 20S in the presence of 1 mM ATP γ S. Similar results were obtained in five experiments.

(C) The extent of binding response is proportional to the concentration of PAN that flowed over the immobilized 20S. The experiment was the same as in (B) with 1 mM ATP γ S, but the concentrations of PAN were varied. Only the association phase is shown.

against the α rings and often showed a slight angle between PAN and the α ring. Variations in the class averages suggested that PAN has an upward and downward movement with respect to the 20S particles but maintained contact with the particles at a hinge region at the interface of PAN and the α subunits (Figures 2C and 2D, panels 2–4). Very similar observations were made with 26S proteasomes by Walz et al., who termed this apparent motion between the 19S base and 20S “wagging” (Walz et al., 1998). Moreover, for doubly capped PAN-20S complexes, this wagging at each end of a class average structure was independent and the maximum wagging distance found was about 30 Å.

A remarkably similar two-ring structure can be seen in EM images of 26S proteasomes from rabbit, *Drosophila*, and *Xenopus laevis* oocytes (Figure 2E and Cascio et al. [2002] and Walz et al. [1998]). This top hat structure was also present at the base of the 19S particle adjacent to the 20S’s α ring just as in the PAN-20S complex (Figure 2E). An additional mass is also evident in the base of the 19S particle (see asterisk). Because these two rings appear to represent the Rpt1–Rpt6 ATPases, this mass probably corresponds to the subunit Rpn2, which apparently associates with the ATPases on the opposite side of the ring from the lid contact point (Ferrell et al., 2000).

The Gate in the Archaeal Proteasomes

In the 26S proteasome, the Rpt2 ATPase subunit appears to regulate gate opening, which is necessary for entry of even small peptides (e.g., suc-LLVY-amc) into the 20S (Groll et al., 2000; Kohler et al., 2001). If the N termini from archaeal proteasome’s α subunits also function as a gate, then they must have the capacity to exist

in both closed and open states, and PAN would likely regulate this process. Benaroudj et al. (2003) showed that the N termini of the *Thermoplasma* α subunits impede the entry of proteins having little or no secondary or tertiary structure (e.g., casein and denatured GFPssrA). Although these α N termini cannot impede the entry of tetrapeptide substrates (Groll et al., 2003; Zwickl et al., 1999), they may be able to exclude longer peptides. Therefore, we used a variety of internally quenched fluorogenic peptides ranging in length from 7 to 18 residues to test if any were rapidly degraded by “gateless” mutant $\Delta\alpha(2-12)$ 20S proteasomes but slowly degraded by wild-type (wt) 20S. These peptides contain a fluorescent reporter (MCA) at the N terminus and a quenching group (DNP) at or near the C terminus so that a cleavage within the peptide would lead to fluorescence. The gateless mutant cleaved all of these 7–18 residue peptides at least three times faster than did wt 20S. Thus, the “closed form” of the gate in the *Thermoplasma* 20S allows free entry of tetrapeptides but impedes the entry of a peptide as small as seven residues. In principle, any one of these peptides could be used to monitor gate opening, but the nonapeptide mca-AKVYPYPMEDpa(Dnp)-amide, which we named LFP, was cleaved 15 times faster by the gateless mutant 20S than by wt 20S proteasomes (Figure 3 and Table 1). Therefore, LFP was used in subsequent studies, because its entry, like that of casein (Table 1), is prevented by the N termini of the α subunits.

PAN Regulates Gate Opening

To test if PAN and ATP act directly on the 20S particle to trigger opening of the gate, we monitored LFP

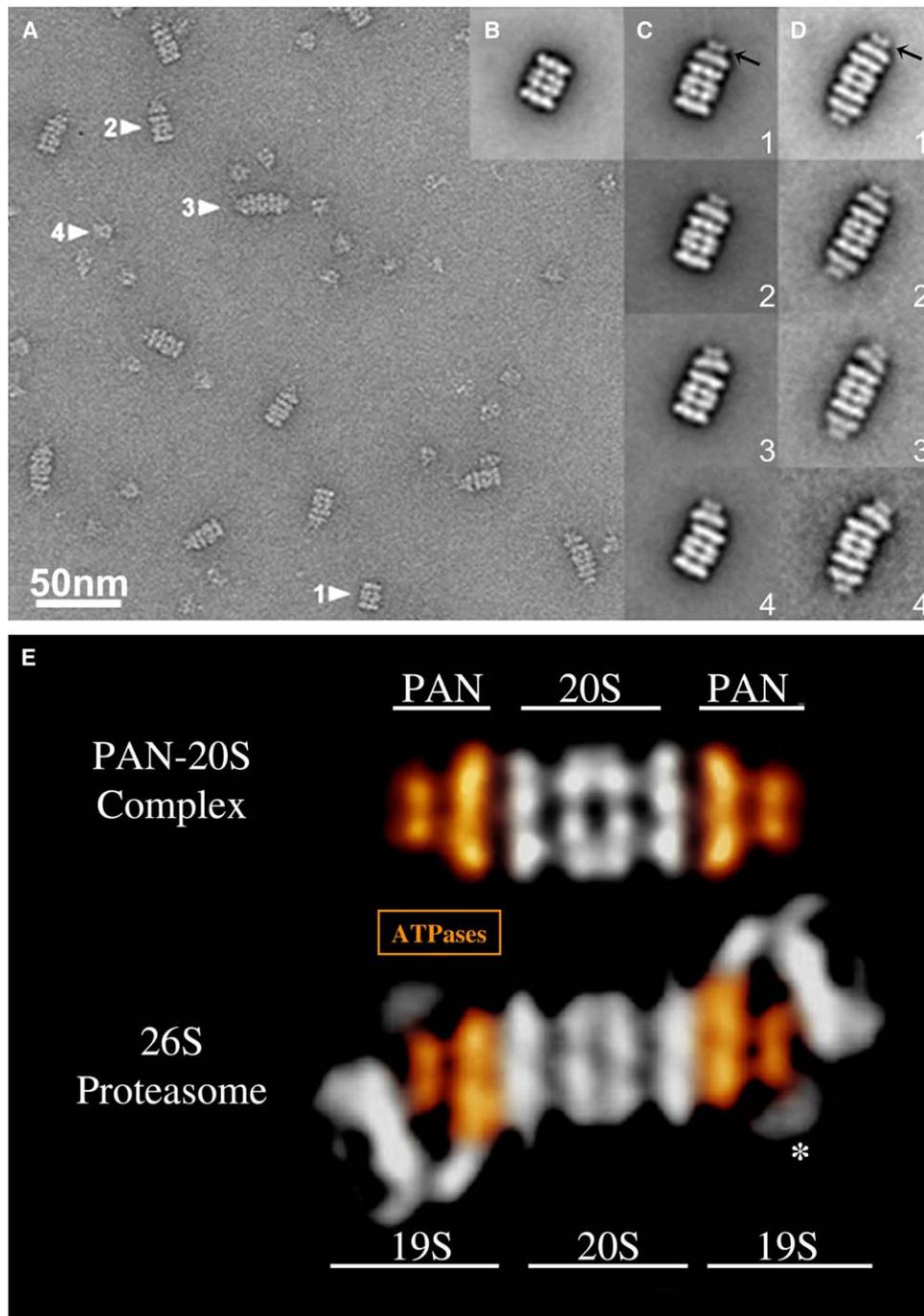


Figure 2. Imaging of the PAN-20S Complex by Electron Microscopy

(A) Electron micrograph of negatively stained complexes of archaeal 20S proteasome with PAN and ATP γ S. The arrows point to: 1, 20S proteasome alone; 2, 20S proteasome singly capped with PAN; 3, 20S proteasome doubly capped with PAN; and 4, top views of unbound PAN. The scale bar of the image is 50 nm.

(B) Class average of 20S proteasome.

(C) Panels 1–4 show different conformations of 20S proteasome singly capped with PAN.

(D) Panels 1–4 show different conformations of 20S proteasome doubly capped with PAN.

In (C) and (D), the image box size is 50 nm \times 50 nm.

(E) The PAN-20S complex compared to a representative EM image of a 26S proteasome from *Xenopus laevis* oocytes (borrowed from Walz et al. [1998]). Both PAN and the corresponding structures in the 26S proteasome are colored orange. The asterisk (*) marks the density likely to be Rpn2.

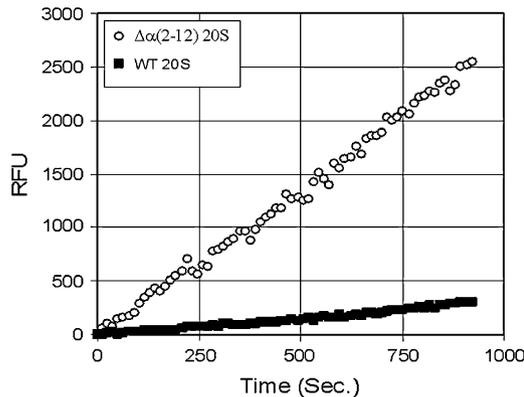


Figure 3. Deletion of Residues 2–12 in the N-Terminal Tails of the α Subunits from *Thermoplasma* Proteasomes Allows Rapid Entry and Degradation of the Internally Quenched Nonapeptide LFP

0.2 μ g of wt or $\Delta\alpha(2-12)$ 20S proteasomes were incubated with 10 μ M LFP at 45°C in 0.1 ml of reaction buffer. Cleavage of the nonapeptide was followed in real time (RFU, relative fluorescence units). Shown is a representative of at least three independent experiments in which the SD was less than 5%.

hydrolysis. When PAN, 20S, and LFP were incubated with ATP, PAN stimulated LFP degradation 3- to 4-fold. Without ATP, no stimulation was seen (Table 1). Thus, entry of LFP correlates with PAN-20S association (Figure 1 and Table 1). A similar stimulation by PAN + ATP was also seen with the other 7–18 residue substrates (data not shown). This effect of ATP cannot be attributed to PAN’s ability to unfold proteins, because these peptides should lack any tertiary structure. In addition, this effect is not due to an allosteric activation of the proteasome’s active sites, because PAN with ATP failed to enhance hydrolysis of LLVY-amc, which freely enters the 20S. Furthermore, PAN with ATP could not stimulate LFP degradation by the gateless 20S proteasome. Therefore, this stimulation must involve alterations in the gate, not the active sites. The simplest explanation of these effects is that PAN with ATP is able to reversibly remove the barrier to peptide entry imposed by the N termini. Interestingly, increase in LFP hydrolysis by 3- to 4-fold with ATP was smaller than the 15-fold activation seen in the gateless mutant 20S, presumably because ATP is continually hydrolyzed to ADP (see below), which may only allow for temporary opening of the barrier to substrate entry, whereas the 2–12 deletion in all seven α subunits completely eliminates this barrier and probably creates a larger entry pore.

Proteins that are degraded by PAN-20S in the presence of ATP bind to PAN and stimulate its ATPase activity several fold as does the 11 residue peptide *ssrA* (Figure 4 and Benaroudj et al. [2003]), which binds tightly to PAN (A. Horwitz, A. Navon, and A.L.G., unpublished

data) and targets proteins for degradation. Thus, PAN by itself exhibits a low ATPase activity, but addition of β -casein or *ssrA* increases ATP hydrolysis by up to 5-fold (Figure 4A). In contrast, LFP did not alter the rate of ATP hydrolysis at even 100 μ M. Thus, unlike a typical protein substrate or a specific “recognition peptide,” LFP does not appear to interact with PAN. Instead, the accelerated degradation of LFP in the presence of PAN and ATP seems to be due simply to gate opening and LFP diffusion into the particle (without alterations in the ATPase or 20S peptidase activities).

The Role of Nucleotide Binding in Gate Opening

Possibly, the binding of PAN to the 20S itself induces gate opening, as occurs when the PA28 or PA26 regulatory complexes associate with the 20S in a process not requiring ATP (Forster et al., 2005; Whitby et al., 2000). We therefore tested if nonhydrolyzable analogs of ATP could support LFP degradation in a similar way as ATP, and because ATP γ S can be hydrolyzed by some ATPases, we used both ATP γ S and AMPPNP. Both analogs stimulated LFP degradation four to six times control (Figure 4B) and had no effect on 20S activity in the absence of PAN (data not shown). Thus, ATP binding to PAN, not its hydrolysis, accounts for the stimulation of nonapeptide degradation. In fact, ATP γ S stimulated LFP degradation nearly twice as strongly as ATP (6-fold versus 3-fold). As shown in Figures 1 and 2, nonhydrolyzable analogs also support PAN-20S complex formation more efficiently than ATP. By contrast, ADP, which failed to support complex formation, did not stimulate LFP degradation. Because PAN rapidly hydrolyzes ATP to ADP, it is not surprising that these nonhydrolyzed analogs are more effective than ATP at stimulating both processes (Figure 4B). Moreover, ADP at high concentrations inhibited the stimulation by ATP γ S or ATP, presumably by competing for binding to PAN (data not shown). Thus, the nucleotide requirements for the stimulation of gate opening (i.e., LFP degradation) appear identical to those for PAN/20S association and differ sharply from those for PAN-induced unfolding of globular proteins (Benaroudj and Goldberg, 2000). Furthermore, complex formation and the stimulation of LFP degradation by PAN have a very similar dependence on the concentrations of these nucleotides (D.M.S., unpublished data). Together, these data strongly suggest that formation of the PAN-20S complex triggers gate opening (as does complex formation between PA-26 and the 20S).

Nonhydrolyzable Analogs Support Degradation of Unfolded Proteins by PAN

Because ATP hydrolysis is essential for unfolding and degradation of globular proteins, it is generally assumed

Table 1. PAN + ATP Like Loss of the Gating Residues Stimulate Degradation of Peptides that Are Larger than Four Residues

	20S	$\Delta\alpha(2-12)$ 20S	20S/PAN	20S/PAN/ATP
suc-LLVY-AMC	100% \pm 3%	103% \pm 4%	96% \pm 3%	98% \pm 10%
Mca-AKVYPYPME-Dpa	100% \pm 21%	1489% \pm 176%	96% \pm 2%	341% \pm 17%
β - ¹⁴ C-Casein	100% \pm 11%	1018% \pm 49%	71% \pm 10%	434% \pm 21%

The rate of hydrolysis of each substrate was normalized to 20S alone (100%) to demonstrate the level of stimulation induced by either gate deletion or PAN and ATP. Values are means of three independent experiments plus or minus their SD. [ATP] = 1 mM with 10 mM MgCl.

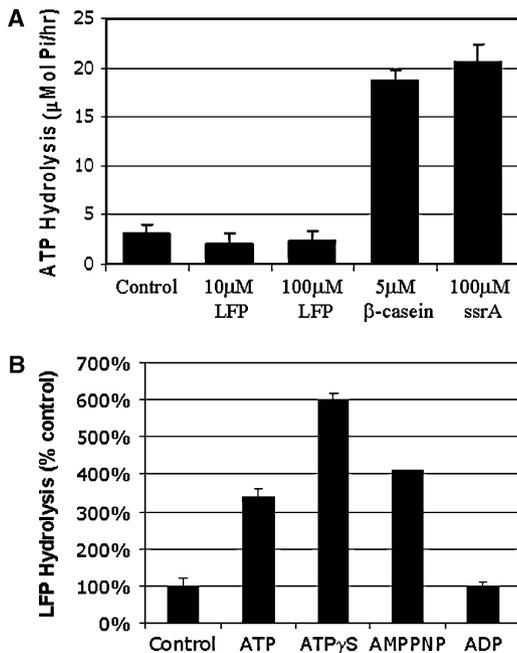


Figure 4. ATP Binding to PAN Stimulates LFP Hydrolysis by 20S Proteasomes, and LFP Does Not Stimulate PAN's ATPase Activity (A) The nonapeptide LFP (unlike casein or *ssrA*) does not stimulate PAN's ATPase activity. LFP, β -casein, or the signal recognition peptide *ssrA* were incubated with 1 mM ATP and PAN at 45°C. Control is PAN alone. ATP hydrolysis was assayed as described (Ames, 1966). All values are means \pm SD of three experiments. (B) Both ATP and nonhydrolyzable analogs (1 mM) stimulate LFP degradation by the PAN-20S complex. All reactions contain PAN (1.0 μ g), and the 20S (.25 μ g) were assayed as in Figure 3 and are normalized to control (no nucleotide). All values are means \pm SD of at least three experiments.

that the translocation of proteins into the 20S is also an active process requiring ATP hydrolysis. Prior studies had indicated that ATP hydrolysis by PAN was important for degradation of unfolded proteins (Benaroudj and Goldberg, 2000). The finding that PAN and ATP γ S induced gate opening and degradation of 7–18 residue peptides led us to reinvestigate whether nucleotide binding may alone support translocation of proteins (i.e., whether proteins may enter the 20S simply by carrier-mediated diffusion after binding to PAN). To traverse the open 13 Å pore in the α ring, a polypeptide must be unfolded and linear (Lowe et al., 1995). We therefore examined whether ATP hydrolysis was necessary for PAN-mediated degradation of the 22 residue insulin B chain and the 24 kDa protein β -casein, which lack defined folded structures. As expected, ATP stimulated degradation of both. Surprisingly, both ATP γ S and AMPPNP also supported their degradation as well as ATP. The rate of β -casein degradation consistently was slightly faster with ATP than with ATP γ S (as assayed either by measuring acid-soluble radioactive products or peptide bond cleavages with fluorescamine), whereas the rate of insulin B chain degradation was slightly greater with ATP γ S. Under these conditions, β -casein degradation went to completion, as judged by the absence of small fragments on SDS PAGE. ADP had no effect, as was expected because it fails to promote complex formation and gate opening.

Thus, formation of the PAN-20S complex and gate opening into the 20S are sufficient to promote translocation of unfolded proteins into the 20S, and ATP hydrolysis is clearly not required for this process.

This stimulation of casein breakdown with AMPPNP was surprising, because in our initial studies, we found that AMPPNP was not able to support 14 C-casein degradation (Zwickl et al., 1999). The prior experiments were performed with a wt PAN construct, which when expressed in *E. coli* formed a heterooligomer that contains both full-length (50 kDa) and some truncated (40 kDa) subunits. This truncation was due to the use of an internal initiation site at Met74 by *E. coli* and is not seen in the native protein in *M. jannaschii* (data not shown and Wilson et al. [2000]). In these experiments, we eliminated potential problems, due to the internal initiation site, by using a PAN construct containing a Met74 to Ala mutation, allowing purification of a homogeneous complex composed of full-length subunits. The absolute stimulation by PAN and ATP of the degradation of casein was similar with the homogeneous M74A mutant as with the heterogeneous oligomer studied previously. However, AMPPNP supported casein degradation far better with PAN-M74A than it did with PAN containing the truncated subunits. ATP γ S was found to stimulate both preparations, although sometimes more strongly with the native enzyme (data not shown). Exactly why the heterocomplex containing truncated subunits did not respond as well to AMPPNP is unclear.

Nucleotide Requirement for Translocation of Unfolded and Globular Proteins

Clearly, upon binding ATP γ S or AMPPNP, the PAN-20S complex forms an open channel through PAN and the α ring that is large enough to allow diffusion of an unfolded protein into the 20S. We therefore reinvestigated whether nucleotide binding could also stimulate entry and degradation of globular proteins. Ovalbumin is a 45 kDa globular protein, whose tertiary structure is stabilized by a disulfide bond. As expected, no significant degradation of native ovalbumin by the PAN-20S complex was detected with either ATP or ATP γ S (Figure 5C), presumably because the disulfide bond prevents unfolding and translocation by PAN. However, if ovalbumin was first oxidized with performic acid and denatured with guanidium HCl, it is degraded by PAN-20S with ATP as well as with ATP γ S (Figure 5C). Thus, even large proteins, if linear and unfolded, require only nucleotide binding, not hydrolysis, to be degraded by the PAN-20S complex.

Our prior studies (Benaroudj and Goldberg, 2000) on PAN and related studies of bacterial ATP-dependent proteases (Kenniston et al., 2003) concluded that ATP hydrolysis is necessary for the degradation of GFP $ssrA$, a tightly folded globular substrate. Accordingly, ATP supported its degradation, but ATP γ S and AMPPNP did not (Figure 5D). However, if the GFP $ssrA$ was first denatured by acid treatment, then ATP γ S could support GFP $ssrA$ degradation. Therefore, gate opening without ATP hydrolysis (ATP γ S) can support efficient translocation of large proteins, once they are unfolded, but PAN-mediated unfolding requires ATP hydrolysis. It is noteworthy that with LFP and to a lesser extent with insulin B chain, degradation was more efficient with ATP γ S

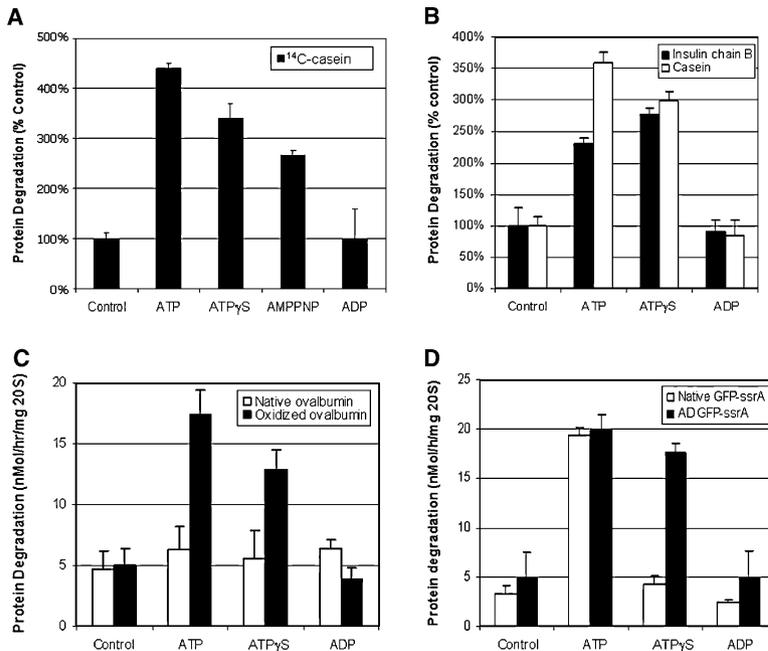


Figure 5. ATP Hydrolysis Is Not Required for Translocation of Unfolded Proteins

(A) ATP and the nonhydrolyzable analogs support degradation of casein. 300 nM ¹⁴C-methy- β -casein were incubated with PAN and 20S without (control) or with 1 mM nucleotides for 30 min prior to addition of trichloroacetic acid. Degradation was assayed by measuring the production of acid-soluble radioactivity.

(B) Both ATP and ATP γ S (1 mM) support degradation of insulin chain B (10 μ M) and β -casein (5 μ M). Degradation products (new amino groups) were measured by using the fluorescamine reaction and expressed as in Table 1.

(C) Both ATP and ATP γ S support degradation of denatured ovalbumin, but not native ovalbumin (2.5 μ M) as assayed in (B).

(D) Degradation of native GFPssrA requires ATP hydrolysis, but degradation of acid-denatured GFPssrA only requires ATP binding. One micromolar native or acid-denatured (AD) GFPssrA was incubated with 1 μ g of 20S and 4 μ g PAN with the nucleotides (1 mM) in a 0.1 ml reaction volume for 15 min as assayed in (B).

All values are means \pm SD of at least three experiments.

than ATP, but the translocation of the larger proteins (casein, ovalbumin, or denatured GFP) proceeded 10%–30% faster with ATP than with ATP γ S, perhaps because they have some residual tertiary structure, which is eliminated when ATP hydrolysis is permitted.

Nucleotide Requirement for Gate Opening and Translocation in 26S Proteasomes

To test if the present findings with PAN also apply to the homologous ATPases in the eukaryotic 26S proteasome, similar studies on the effects of nonhydrolyzable nucleotides were performed with rabbit 26S proteasomes purified to near homogeneity. The 19S ATPases (especially Rpt2) promote gate opening in the 20S in the presence of ATP (Glickman et al., 1999; Rubin et al., 1998). This gate in the eukaryotic 20S proteasomes in its closed form can exclude the tetrapeptide suc-LLVY-amc (unlike that of the archaeal 20S). With the 26S complex, ATP, ATP γ S, and AMPPNP all stimulated degradation of suc-LLVY-amc (Figure 6A). ADP could not do so, and ATP was less effective than ATP γ S in causing gate opening. These observations are in exact agreement with our findings on the PAN-20S complex. Furthermore, with the mammalian 26S, both ATP and ATP γ S stimulated degradation of ¹⁴C- β -casein (Figure 6B) in a similar manner as in the archaeal complex. Therefore, gate opening induced by nucleotide binding without hydrolysis is sufficient to support translocation of an unfolded protein by the 26S proteasome, and the mechanisms of gate opening and substrate translocation appear very similar for the archaeal and mammalian complexes.

Discussion

The PAN-20S Complex

The present studies have uncovered several new features of proteasome activation by the PAN ATPase

that help clarify the structure and function of the 26S proteasome. In contrast to prior reports, we have directly demonstrated that PAN associates with the 20S and that complex formation correlates with gate opening in the α ring. This association required binding of ATP, ATP γ S, or AMPPNP, whereas ADP could not support and even inhibited this association. Similarly, ATP binding without hydrolysis supports and ADP inhibits the association of the ATPase and proteolytic components of the bacterial ATP-dependent proteases ClpAP (Maurizi et al., 1998) and HslUV (Huang and Goldberg, 1997; Yoo et al., 1997). Actually, this association of PAN with the 20S was readily observed only in the presence of ATP γ S but was difficult to demonstrate with ATP. Because ADP inhibits complex formation, it is likely that ATP hydrolysis to ADP weakens this association (Figures 1 and 3) as was also found for the HslUV complex (Yoo et al., 1997). Thus, the PAN-20S association appears to be particularly transient in the presence of ATP and more stable with ATP γ S. Accordingly, we found by SPR that the PAN-20S complex (once formed in the presence of ATP γ S) dissociates five times faster with ATP present than with ATP γ S (D.M.S., G.K., and A.L.G., unpublished data). Although the PAN-20S complex is short lived even with ATP γ S, it clearly is sufficiently stable to allow substrate translocation. Perhaps, protein substrates by binding to PAN increase its affinity for the 20S complex just as they enhance its ATPase activity.

The EM images of this complex provide the first structural information on PAN and also clarify aspects of the structure of the 26S proteasome. In addition to the expected large inner ring, PAN contains a secondary outer ring and thus resembles a top hat that caps either or both ends of the 20S particles. A very similar top hat structure is also evident in EM images of 26S proteasomes from several species. This secondary outer ring in the base of the 19S cap had not been thought to be

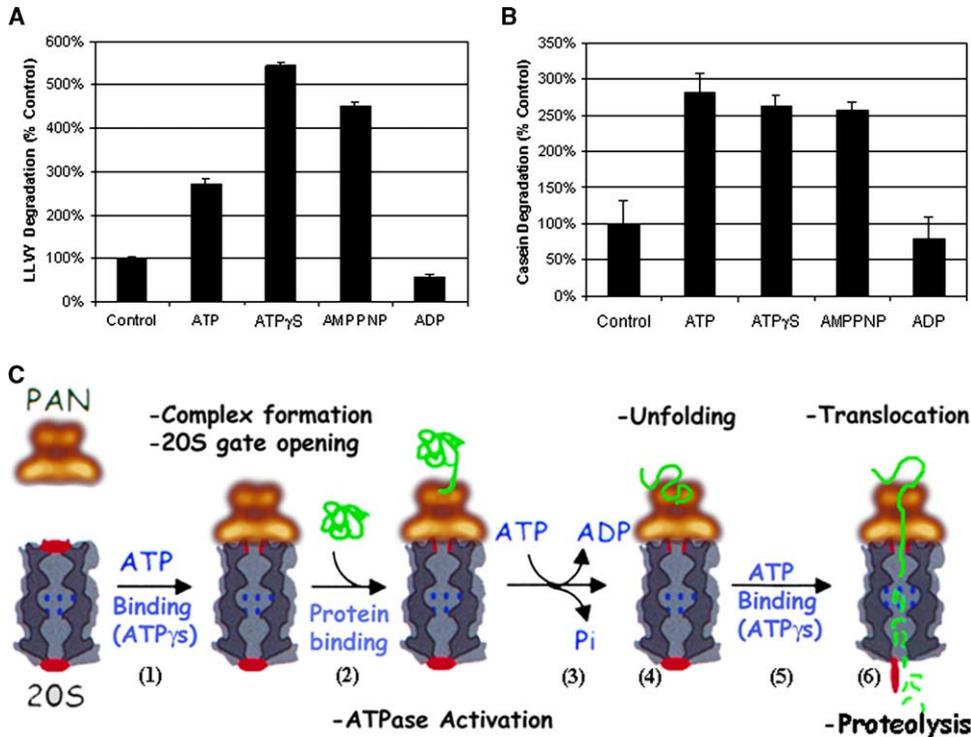


Figure 6. Nonhydrolyzable ATP Analogs Stimulate Rabbit Muscle 26S Proteasomes to Degrade a Tetrapeptide Substrate as Well as ^{14}C -Methyl- β -Casein

(A) ATP γ S (1 mM) stimulates hydrolysis of suc-LLVY-amc (100 μM) upon incubation at 37°C without (control) or with the nucleotides. Experiment was performed as in Figure 3 but contained 0.25 μg of purified 26S proteasomes.

(B) ATP γ S (1 mM) supports degradation of β -casein (5 μM) by 26S proteasomes. After incubation without or with nucleotides, degradation was measured by using fluorescamine.

In (A) and (B), all values are means \pm SD of at least three experiments.

(C) The multiple ATP-dependent steps in protein degradation and the requirement for nucleotide binding and hydrolysis. A cross-section of the 20S particle is shown with the PAN complex. The gating residues of the α subunits N termini are shown in red. (1) ATP binding to PAN promotes its association with the 20S and causes opening of its gate. (2) Protein binds to PAN preferentially in its ATP bound form (Benaroudj and Goldberg, 2000). (3) This binding stimulates ATP hydrolysis. (4) Substrate unfolding is the only step in the pathway that requires ATP hydrolysis. (5) In its ATP bound state, PAN supports facilitated diffusion of the unfolded protein. (6) The 20S architecture and nucleophilic attack on the substrate by the active sites probably retards outward diffusion, thus biasing diffusion inward and allowing processive degradation to small peptides. Therefore, ATP binding without hydrolysis can support complex formation, gate opening, and translocation of unfolded proteins.

part of the ATPases and had been proposed to correspond to Rpn1 and Rpn2 (Kajava, 2002). Based on its close similarity to PAN, we propose a model whereby this density is part of the ring of ATPases Rpt1–Rpt6. The N-terminal regions of these six ATPases, like that of PAN, are predicted to adopt a coiled-coil fold (Gorbea et al., 1999; Zwickl et al., 1999), which may mediate the binding of protein substrates (Wang et al., 1996). A similar coiled-coil region also exists in the homologous ATPase HslU, which forms a structure similar to PAN when complexed with HslV (Rohrwild et al., 1997). The coiled-coil region is found in the outer ring of HslU and is important for substrate degradation (Song et al., 2000). Most likely, these outer rings in PAN and the 19S also correspond to the coiled-coil domain and are important in substrate recognition, because they form the entrance of the axial translocation channel into the 20S.

In addition, the images of the 26S display a weaker density bound to this top hat structure on the opposite side to where the 19S lid joins the base (Figure 2, see asterisk). It had been suggested that this density corresponds to a substrate (Walz et al., 1998), but this proposal seems unlikely because this density is seen

consistently in proteasomes purified by different approaches from several species. The Rpn1 and Rpn2 subunits both associate with the ATPases (apparently at opposite flanks of the ATPase ring), and Rpn1 is known to contact Rpn10 at the joint where the base and lid intersect (Ferrell et al., 2000). Therefore, by exclusion, we propose this density probably corresponds to Rpn2.

The only significant class average variation observed here was in the inclination of PAN with respect to the 20S, which suggests a wagging motion, as was also described for 26S proteasomes by Baumeister and colleagues (Walz et al., 1998). This wagging also suggests that PAN's contact with the 20S is not very tight and occurs at local regions at the interface of the two complexes. Because this apparent wagging is conserved between archaeal and eukaryotic proteasomes, this motion may well be functionally significant and also implies that the 26S proteasome is a highly dynamic structure. Because ATP binding promotes association with the 20S, it is likely that these ATPase subunits tightly associated with the 20S are those with ATP bound, whereas the others have ADP or no nucleotide bound. Such a mechanism could generate a wagging motion if ATP

hydrolysis (and then ATP-ADP exchange) occurs sequentially around the ATPase ring rather than in a concerted manner.

Gating in the Archaeal 20S Proteasome

The N termini of the α subunits in eukaryotic proteasomes can assume either of two ordered structures, an open conformation and a closed one, both of which require the YDR motif for stabilization (Forster et al., 2003; Groll et al., 1997, 2000; Groll and Huber, 2003). Switching the gate from the closed to open state is regulated by at least one ATPase subunit, Rpt2 (Kohler et al., 2001; Rubin et al., 1998), and also occurs in an ATP-independent manner upon association of the 20S with the 11S PA26 (or PA28) complex (Forster et al., 2003, 2005; Whitby et al., 2000). The conserved YDR motif in the N termini is essential in gating in eukaryotic proteasomes but is also present in the N termini of the α subunits of many archaeal strains (Groll and Huber, 2003). These N-terminal residues under certain crystallization conditions form an ordered open structure, which is dependent on the YDR motif (Groll et al., 2003) and is congruent with the eukaryotic open structure (Forster et al., 2003). However, a closed gate structure, like that in eukaryotic proteasomes, has not been observed in archaeal proteasomes, and Forster et al. (2003) concluded that the archaeal 20S N termini are not able to form such a closed gate because they lack sequence asymmetry, which is necessary for gate closure in eukaryotic proteasomes.

Nevertheless, archaeal proteasomes may have a unique closed gate structure that is easily disrupted by the crystallization conditions. The present studies demonstrate that the archaeal 20S has a functional gate that can exist in both open and closed states and thus can act as a barrier to entry of peptides as small as seven residues. When these N termini are deleted, these peptides and nonglobular proteins enter readily, as occurs in the yeast 20S when the α -3-N terminus is deleted (Groll et al., 2000). However, the configuration of the closed archaeal gate clearly differs from that in eukaryotes because it contains an opening large enough for four-residue peptides to traverse readily.

ATP-Dependent Regulation of Gate Opening

Upon association of PAN with the 20S, the barrier to substrate entry is reversibly removed. This reversible stimulation of LFP hydrolysis was smaller than that seen when the gating residues (α 2–12) were completely deleted. Gate opening occurred under the exact conditions where PAN is found complexed with the proteasome (i.e., with ATP, AMPPNP, and ATP γ S present, but not with ADP), and gate opening and complex formation show similar dependence on nucleotide concentrations (D.M.S., G.K., and A.L.G., unpublished data). The mechanism of this activation of 20S function by PAN and ATP is quite different from the activation of the bacterial ATP-dependent proteases HslUV and ClpA(X)P, in which binding of ATP induces an allosteric activation of their peptidase sites (Kim et al., 2001). By contrast, activation by PAN occurs strictly by gate opening without any alteration in the activity of the peptidase sites. Thus, upon binding of ATP, PAN associates with the 20S and induces gate opening, perhaps in a similar fashion as PA26 (Whitby et al., 2000), which interacts with the

base of the α N termini to destabilize the closed conformation and induce the open conformation (Forster et al., 2003; Whitby et al., 2000). However, PA26 does not share any sequence homology with Rpt1–Rpt6 or PAN.

Although the 19S and 20S dissociate slowly in the absence of a nucleotide and reassociate when ATP is added (Coux et al., 1996; Voges et al., 1999), the 26S proteasome is quite stable in the presence of ATP. In the 26S, as with the PAN-20S complex, ADP prevented gate opening and ATP analogs stimulated this process even better than ATP. Because the removal of the N terminus of only the α -3 subunit in yeast 20S causes complete destabilization of the closed structure (Groll et al., 2000), it seems likely that ATP binding to the ATPase subunit adjacent to the α -3 subunit can induce the open gate conformation in all the N termini. Possibly, binding of ATP to any of the 19S ATPases or PAN subunits may induce the open-gate conformation in the adjacent α subunit. This mechanism would suggest that each component of the gate in the 20S could be cycling through open and closed states, as different ATPase subunits bind ATP and hydrolyze it to ADP. However, thus far, only one subunit in the 26S complex, Rpt2, has been shown to regulate gate opening (Kohler et al., 2001).

The Nucleotide Requirement for Substrate Translocation

Protein translocation through a small pore is a critical step in many cellular processes. Investigators have proposed “active” mechanisms that utilize ATP hydrolysis to pull or push a protein in one direction through a pore, as has been proposed for the 26S proteasome (Matouschek, 2003) and bacterial ATP-dependant proteases (Sauer et al., 2004). Alternatively, there may be a “passive” mechanism in which translocation occurs by biased diffusion, in which retrograde movement is blocked by a Brownian ratchet mechanism (Glick, 1995; Pfanner and Meijer, 1995). A growing body of evidence has implicated Brownian ratchets in many translocation processes initially thought to be active, including transcription elongation (Bar-Nahum et al., 2005), collagenase (Saffarian et al., 2004) and kinesin (Nishiyama et al., 2002), and transport across the ER and mitochondrial membranes (Matlack et al., 1999), where the Hsp70 homolog BIP binds the protein to be translocated, blocks retrograde flow, and then dissociates in an ATP-hydrolysis driven cycle. Because the 26S proteasome seems to unfold globular substrates by sequential unraveling, it has been suggested that protein unfolding and translocation are linked processes in which the ATPases pull an unstructured region through the pore in the ATPase into the 20S particle, thus causing unfolding of upstream regions (Lee et al., 2001; Matouschek, 2003; Prakash et al., 2004). Such a mechanism requires concomitant ATP hydrolysis and implies that unfolding cannot occur without translocation.

Although ATP hydrolysis is necessary for degradation of globular proteins, we found that if a globular protein is first denatured, then ATP is no longer necessary for translocation and degradation. Thus, for an unfolded substrate, translocation by PAN can occur by purely passive diffusion. With peptides (e.g., LFP or Insulin chain B), ATP γ S is more effective than ATP in promoting

degradation, whereas with unfolded proteins, ATP is more efficient than ATP γ S, probably because these substrates contain some residual secondary or tertiary structures that slow diffusion in the absence of ATP-dependent unfolding. When translocation proceeds by this “passive” mechanism, unfolding must occur through an independent mechanism requiring ATP hydrolysis. Accordingly, Navon and Goldberg found that if translocation of GFPssrA through the ATPase ring was prevented, unfolding of the GFP still occurred (Navon and Goldberg, 2001). Thus, two very different types of experiments indicate that unfolding and translocation can be dissociated.

The present demonstration that degradation of denatured proteins can occur without ATP hydrolysis contradicts our earlier findings (Zwickl et al., 1999) that used preparations of PAN in which some subunits were truncated, due to an internal initiation site (Wilson et al., 2000). Mutagenesis to eliminate that problem and use of homogenous PAN, as exists naturally, led to the present, more definitive conclusions. Rapid protein degradation in the presence of ATP γ S also implies that unidirectional translocation and processive degradation of proteins can occur without metabolic energy. In fact, these same unfolded proteins are quickly degraded to small peptides by open-gated or gateless 20S proteasomes alone (Akopian et al., 1997; Bajorek et al., 2003; Benaroudj et al., 2003; Cascio et al., 2002; Forster et al., 2003), and the sizes of peptides generated resemble those produced by the 26S complex in the presence of ATP (Cascio et al., 2002; Kisselev et al., 1998). Thus, 20S particles, even without this gate, have an inherent capacity to insure unidirectional translocation.

Most likely, the architecture of the 20S (its large internal chambers and small exit pores) and its proteolytic mechanism act as a ratchet to retard backward diffusion of proteins out of the particle and thus bias diffusion into the central chamber. Several possible mechanisms may favor diffusion inward: (1) the hydrophobic residues exposed upon unfolding may interact with the hydrophobic ring at the mouth of the central antechamber (Lowe et al., 1995) or with the walls of the internal chambers; (2) eventually substrates bind to the many active sites on the β rings; and (3) during peptide bond hydrolysis, a transition state covalent bond forms between the polypeptide and the hydroxyl group on the N-terminal threonine that should prevent retrograde movement of the polypeptide while allowing further inward movement of regions upstream. In fact, the N-terminal portion of a substrate remains covalently attached to these active sites for a significant time before the acyl-enzyme intermediate is hydrolyzed (Vigneron et al., 2004). This unusual property is probably related to the presence of a threonine in the proteasome’s active site, which is more efficient than serine in degradation of proteins, but not short peptides (Kisselev et al., 2000). Because of these structural and catalytic properties, a polypeptide may be bound to multiple active sites at any one moment, allowing cleavage of terminal segments, whereas bound upstream regions prevent retrograde movement. In addition to these possible ratchet mechanisms inherent in the 20S, the repeated cycles of ATP hydrolysis by PAN or the 19S ATPase may trigger continual opening and closing of the gate in the α ring (Groll

et al., 2000), which may function as an additional ratcheting mechanism to ensure unidirectional movement of polypeptides into the 20S. However, some protein backflow is permitted in those special cases where the proteasome carries out endoproteolytic cleavage of a loop (Liu et al., 2003) or partial degradation of one terminus (e.g., NF κ B) while sparing upstream structured regions (Rape and Jentsch, 2002).

These findings indicate also that after a polypeptide binds to PAN or the 19S ATPases and is unfolded, its diffusion potential provides sufficient driving force for translocation into the 20S, because ATP, ATP γ S, and AMPPNP stimulated proteolysis similarly in eukaryotic and archaeal complexes. Protein substrates bind preferentially to PAN in its ATP bound form (Benaroudj and Goldberg, 2000), the same form that facilitates passive diffusion into the 20S. The translocation process thus resembles a type of carrier-mediated diffusion in which the ATPase in its ATP bound form binds tightly the linearized polypeptide, thus facilitating its diffusion through the ATPase ring and the open gate in the 20S. This multicomponent translocation process appears to be the rate-limiting step in the degradation of many (perhaps most) proteins by the PAN-20S complex (Benaroudj et al., 2003).

Together, these findings provide a more complete understanding of the multiple steps in protein degradation by the PAN-20S complex (Figure 6C). This reaction scheme indicates the several ATP-dependent steps that we have dissociated and that presumably also function in the 26S complex after deubiquitination of the substrate. This multistep scheme differs in important ways from the one we proposed previously (Benaroudj et al., 2003): (1) the initial formation of the ATPase-20S complex is coupled to gate-opening (which had been assumed to be a late step triggered by protein substrates or ATP hydrolysis); (2) ATP binding without hydrolysis can activate multiple steps including PAN-20S association, gate-opening, and surprisingly, translocation of unfolded polypeptides; and (3) the only step requiring nucleotide hydrolysis is the unfolding of globular proteins, then translocation can occur by carrier-mediated diffusion through the ATPase and α rings, leading to processive degradation.

Experimental Procedures

Detailed Experimental Procedures and supplemental references are available online with this article.

Enzyme Assays

Hydrolysis of ATP was assayed by following the production of inorganic phosphate at 45°C as described elsewhere (Ames, 1966). To measure peptide hydrolysis, fluorogenic peptides in DMSO were used at a final concentration of 100 μ M for Suc-LLVY-amc and 10 μ M for LFP. For archaeal 20S, peptides were added to the buffer at 45°C in the absence or presence of 1 mM nucleotides. For rabbit 26S proteasomes, the same buffer was used at 37°C plus 40 mM KCl for 30–60 min. 26S proteasomes were stored in 1 mM ATP and diluted 100-fold for experiments. Under these conditions, 26S proteasomes remain intact even without additional ATP. Hydrolysis of suc-LLVY-amc was monitored at λ_{ex} 380 nm and λ_{em} 440 nm, and LFP at λ_{ex} 380 nm and λ_{em} 480 nm.

To follow unfolding of GFPssrA, its fluorescence was measured at 45°C at λ_{ex} 400 nm and λ_{em} 510 nm. GFPssrA was diluted in the reaction buffer, in the presence or absence of PAN, 20S proteasomes, and the indicated nucleotide. GFPssrA was denatured by incubation

with 50 mM HCl (pH 2.0) for 10 min at room temperature until >95% of fluorescence had been lost. Denatured GFPssrA was diluted directly into the reaction mixture at 45°C and used immediately. All bar graphs show the calculated means \pm the standard deviation (SD) of at least three independent experiments.

Degradation of Proteins

[¹⁴C]methyl-casein was prepared by reductive alkylation (Rice and Means, 1971) and stored in 50 mM Tris-HCl (pH 7.5). A 30–60 min reaction of the ¹⁴C labeled protein with 20S proteasomes and PAN, with or without 1 mM nucleotide, at 45°C was done as previously described (Benaroudj et al., 2003). The degradation products from nonradioactive β -casein, insulin chain B, GFPssrA, and ovalbumin were measured by using the fluorescamine reaction, as described previously (Venkatraman et al., 2004). Values were expressed as percent of controls, and the basal rate of hydrolysis for each substrate expressed in amount of 20S was as follows: β -casein, 11 nM/h/mg; insulin chain B, 16 nM/h/mg; native and oxidized ovalbumin, 2–3 nM/h/mg; and native and denatured GFP-ssrA, 1–2 nM/h/mg. All reactions, including those using peptides, had 0.25 μ g of 20S proteasomes and 1 μ g of PAN in 0.1 ml of reaction buffer (sufficient to saturate the 20S particles) unless otherwise noted. The reactions with 26S proteasomes contained 0.25 μ g of proteasome in 0.1 ml reaction buffer plus 40 mM KCl.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and Supplemental References and are available with this article online at <http://www.molecule.org/cgi/content/full/20/5/687/DC1/>.

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