

Docking of the Proteasomal ATPases' Carboxyl Termini in the 20S Proteasome's α Ring Opens the Gate for Substrate Entry

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SUMMARY

The 20S proteasome functions in protein degradation in eukaryotes together with the 19S ATPases or in archaea with the homologous PAN ATPase complex. These ATPases contain a conserved C-terminal hydrophobic-tyrosine-X motif (HbYX). We show that these residues are essential for PAN to associate with the 20S and open its gated channel for substrate entry. Upon ATP binding, these C-terminal residues bind to pockets between the 20S's α subunits. Seven-residue or longer peptides from PAN's C terminus containing the HbYX motif also bind to these sites and induce gate opening in the 20S. Gate opening could be induced by C-terminal peptides from the 19S ATPase subunits, Rpt2, and Rpt5, but not by ones from PA28/26, which lack the HbYX motif and cause gate opening by distinct mechanisms. C-terminal residues in the 19S ATPases were also shown to be critical for gating and stability of 26S proteasomes. Thus, the C termini of the proteasomal ATPases function like a "key in a lock" to induce gate opening and allow substrate entry.

INTRODUCTION

The 26S proteasome is a large ATP-dependent proteolytic complex that catalyzes the degradation of most cellular proteins, generally after linkage to a ubiquitin chain (Glickman and Ciechanover, 2002; Goldberg, 2005; Voges et al., 1999). Proteasomes are also found in archaea and certain eubacteria, where ubiquitin is not present, but proteolysis is still ATP dependent (Goldberg, 2005). The active forms of proteasomes in archaea and eukaryotes are composed of an ATPase complex bound to one or both ends of the 20S core proteasome (DeMartino and Slaughter, 1999; Voges et al., 1999). This hollow cylinder is composed of four stacked rings (Groll et al., 1997; Lowe et al., 1995).

Its outer rings contain seven α subunits that surround a narrow channel, through which polypeptides enter the 20S's central chamber, where its multiple proteolytic sites are located. The passage of proteins and peptides through this channel is restricted by the N termini of the α subunits, which function as a gate (Groll et al., 2000; Groll and Huber, 2003). PAN and the 19S ATPase ring complexes open this gate and thus regulate proteolysis by the 20S particle (Benaroudj et al., 2003; Kohler et al., 2001; Smith et al., 2005). In eukaryotes, the base of the 19S regulatory complex contains six different ATPases (Rpt1–6) (Fu et al., 2001).

In archaea, the proteasome-regulatory complex, PAN, is composed of six identical subunits that are close homologs of the 26S ATPases (Benaroudj et al., 2003; Ogura and Tanaka, 2003; Smith et al., 2006; Zwickl et al., 1999). In electron micrographs, PAN resembles a top-hat structure capping the 20S cylinder and appears similar to densities in the base of the 19S complex (Smith et al., 2005). These ATPase rings associate with the outer α ring of the 20S, where in the presence of ATP they unfold globular proteins and promote their translocation through the gated entry channel into the 20S particle (Benaroudj et al., 2005; Kohler et al., 2001; Smith et al., 2005; Voges et al., 1999).

The simpler PAN-20S complex offers major advantages for exploring proteasome function (Benaroudj et al., 2003; Ogura and Tanaka, 2003; Smith et al., 2005, 2006). For example, it does not require ubiquitin conjugation for proteolysis, the archaeal 20S contains only one type of α and β subunits, and these complexes assemble when expressed in *E. coli*. Our recent studies have established that upon binding of ATP or nonhydrolyzable ATP analogs, PAN associates with the 20S particle and triggers gate opening in the α ring. This gate opening by PAN, even without ATP hydrolysis, facilitates the diffusion of peptides and unfolded proteins into the 20S. Similarly, ATP binding in the eukaryotic 26S complex also causes gate opening and entry of unfolded proteins into the 20S (Smith et al., 2005).

The present studies were undertaken to investigate the mechanism by which these ATPases induce gate opening in the 20S proteasome. One attractive model for gate

opening comes from studies by Hill and colleagues on a very different type of proteasomal regulator, PA26, the 11S invertebrate homolog of the mammalian PA28 γ complex and the PA28 $\alpha\beta$ complex that stimulates antigen presentation (Goldberg et al., 2002). Though the biological functions of PA26 and PA28 γ are not clear, these heptameric complexes associate with the ends of the 20S particle and facilitate entry of short peptides (DeMartino and Slaughter, 1999). The association of PA28/26 with the 20S requires their extreme C termini (Ma et al., 1993), and by X-ray crystallography, Hill and coworkers demonstrated that these C termini dock into pockets between adjacent α subunits, providing binding energy for the PA26-20S complex (Forster et al., 2005; Whitby et al., 2000). However, this binding of PA26's C termini to the α -intersubunit pockets is not sufficient to induce 20S gate opening (Zhang et al., 1998), which instead requires an additional domain distant from its C termini. This "activation domain" is presented by PA26 in a 7-fold symmetric circular array that interacts with the base of the N-terminal gating residues of the seven α subunits. This interaction destabilizes the asymmetrical closed gate conformation of the 20S proteasome and stabilizes its symmetric open gate conformation (Forster et al., 2003).

While it is not known how the proteasomal ATPases induce gate opening, two groups have suggested that these ATPases use a mechanism like that of PA26 (Forster et al., 2005; Kohler et al., 2001), which requires an "activation loop." However, PA28/26 and the proteasomal ATPases are very different protein complexes and do not share any sequence homology. While PA28/26 are heptameric rings, like the 20S proteasome with which they form a matched 7:7 subunit interface, the proteasome-regulatory ATPases, PAN, and those in the base of the 19S (Rpt1–6) are hexameric and thus form an asymmetrical complex with the 20S with a mismatched 6:7 subunit interface. Because PA26 applies its symmetry to the 20S α ring to induce gate opening (Forster et al., 2003, 2005), the asymmetrical proteasomal ATPase complexes should not be able to induce gate opening in the same fashion. Furthermore, PA28/26 appear to be static complexes, whereas the proteasomal ATPases, like other AAA ATPases, are highly dynamic structures that undergo large conformational changes upon binding and hydrolysis of ATP (DeLaBarre and Brunger, 2005; Wang et al., 2001). These major differences suggest that the proteasomal ATPases and PA28/26 probably utilize different mechanisms to stabilize the open-gate conformation of the α subunits' N termini.

To investigate how proteasomal ATPases induce gate opening, we first tested whether PAN's C termini are important for its ATP-dependent association with the 20S and its ability to induce gate opening in the 20S proteasome. We show here (1) that PAN's C termini contain a conserved short domain that binds to the 20S and acts like a "key in a lock" to cause gate opening, and (2) that a similar mechanism functions in gating in the more complex eukaryotic 26S proteasome.

RESULTS

The Proteasomal ATPases' C Termini Contain a Conserved HbYX Motif

If the extreme C termini of these ATPases are important for its association with the 20S, then the critical residues may be conserved, because the requirements for complex formation and gate opening by PAN and the 19S ATPases appear similar (Smith et al., 2005). We therefore compared their C-terminal sequences in various archaea and eukaryotes (Figure 1A). While the terminal arginine residue in PAN is not conserved, the penultimate residue is a tyrosine in PAN from nine different archaeal species (see Table S1 in the Supplemental Data available with this article online), although in five archaeal species, it is a phenylalanine. A penultimate tyrosine is also found in four ATPases, Rpt1, Rpt2, Rpt3, and Rpt5 (but not Rpt4 and Rpt6), in 26S proteasomes from humans, rats, mice, *Drosophila*, *Arabidopsis*, nematodes (except Rpt1), and yeast. A hydrophobic residue precedes the penultimate tyrosine in PAN from all 14 archaea species and also in Rpt2, Rpt3, Rpt5, and Rpt6 of these eukaryotes. Thus the conserved C-terminal motif, HbYX, where Hb is a hydrophobic residue, Y is a tyrosine (or in some archaea, a phenylalanine), and X can vary widely, appears in nearly all known proteasomal ATPases. Interestingly, no such sequence is found in the C termini of PA26 and PA28 α , β , or γ .

PAN's C-Terminal Residues Are Required to Stimulate 20S Gate Opening

To determine whether PAN requires its C-terminal arginine to associate with the 20S proteasome, PAN and the 20S were pretreated with carboxypeptidase B (CpB), which specifically removes basic C-terminal residues, and after addition of ATP γ S, complex formation, and gate opening in the 20S were assayed (Smith et al., 2005). The stimulation of gate opening was monitored by measuring the hydrolysis of the quenched nine-residue fluorogenic substrate, LFP, whose entry into the 20S is very slow when the gate is closed (Smith et al., 2005). Treatment with CpB did not affect the ability of the 20S proteasome to hydrolyze LFP and did not alter PAN's ATPase activity or its ability to unfold GFP-ssrA (Figures S1A and S1B). However, after CpB treatment, which should remove the arginine but not the penultimate tyrosine, PAN completely lost its ability to stimulate peptide entry (Figure 1C). By contrast, carboxypeptidase A, which cleaves primarily after hydrophobic residues, had no effect on the stimulation of peptide entry. Thus, PAN's C-terminal arginine is required for gate opening, which can account for the recent finding that this residue is also required for the stimulation of casein degradation by PAN (Forster et al., 2005).

Interestingly, if the PAN-20S complex was formed first by addition of ATP γ S before the treatment with CpB, then PAN retained some ability to stimulate peptide hydrolysis (Figure 1C). Therefore, PAN's C-terminal residues are essential for activation of gate opening and are accessible to carboxypeptidases when the ATPase complex is not

ATPase	C Termini	
	<i>S. cerevisiae</i>	<i>H. sapiens</i>
Rpt1	SSTSRMQY YN -COOH	SATPRYMT YN -COOH
Rpt2	VEENLEGL LYL -COOH	QEGTPEG LYL -COOH
Rpt3	NTVDKFD FYK -COOH	KDEQEHE FYK -COOH
Rpt4	LEGTIEYQ KL -COOH	LESKLDYK PV -COOH
Rpt5	RKSKSVS FYA -COOH	KKKANLQ YYA -COOH
Rpt6	TAISVAKL FK -COOH	KNMSIKKL WK -COOH
PAN	EPAHLDV LYR -COOH	EPAHLDV LYR -COOH

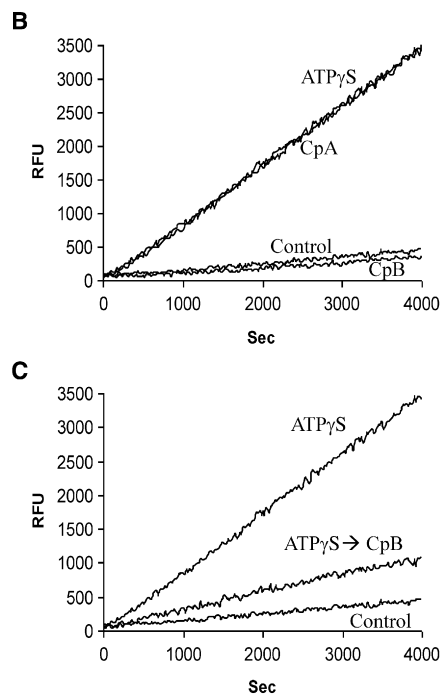


Figure 1. Carboxypeptidase Treatment of the Proteasomal ATPase Regulatory Complexes Eliminates Their Ability to Stimulate the 20S for Peptide Hydrolysis

(A) Two residues preceding the C-terminal arginine in PAN are conserved in the eukaryotic 19S proteasome-regulatory ATPases.

(B) A solution of PAN, 20S, and the fluorescent peptide LFP was preincubated with carboxypeptidase A (CpA) or B (CpB) or without either carboxypeptidase (control and ATP γ S) for 5 min followed by addition of a general carboxypeptidase inhibitor from potato tuber, (0.01 mg/ml). H₂O (control) or ATP γ S (ATP γ S, CpA, CpB) was added, and the rate of LFP hydrolysis was monitored in real time. Both CpA and CpB were used at a final concentration of 0.08 units/ml.

(C) Same as in (A) except ATP γ S was added prior to addition of CpB. All of these experiments were performed at least three times with similar results.

associated with the 20S, but become less accessible once the ATPase-20S complex is formed. (It is noteworthy that this protection from carboxypeptidase inactivation is only partial, probably because the association between PAN and the 20S is transitory [Smith et al., 2005]). Thus, the C terminus is likely to be located in the interface of the ATPase-20S complex, as shown below.

To investigate further the importance of PAN's C-terminal arginine in gate opening, we performed site-directed mutagenesis to remove it, replace it with other amino

Table 1. Mutagenesis to Remove PAN's C-Terminal Arginine Destroys Its Ability to Stimulate Gate Opening, but Its Replacement by Certain Other Residues Allows Wild-Type Activity

PAN Sequence	Peptide Hydrolysis (% WT)
AHLDVLYR ⁴³⁰ (WT)	100
AHLDVLY-	4
AHLDVLYD	5
AHLDVLYA	100
AHLDVLYW	106
AHLDVLYL	13
AHLDVLYG	77
AHLDVLYRA	2
AAAAAHLDVLYR	74
No PAN	5

PAN's C-terminal residue was mutated, the indicated PAN mutant (1 μ g) was incubated with 20S (0.25 μ g) in a 100 μ l reaction buffer, and the rate of LFP hydrolysis was monitored in the presence or absence of ATP γ S (20 μ M). Shown are the mean values of three or more experiments where the \pm SD was less than 5%.

acids, or extend the C terminus. When this arginine was deleted, PAN lost its ability to stimulate gate opening in the presence of ATP γ S (as was found after treatment with CpB). However, mutating this arginine (R430) to alanine or tryptophan did not reduce PAN's ability to stimulate the 20S, and mutation to a glycine (R430G) decreased it only slightly (Table 1). Because the R430G mutation still permitted gate opening, a side chain on the ultimate residue is not essential for 20S activation. Although many amino acids can replace this terminal arginine, surprisingly, replacement by a leucine markedly decreased PAN's ability to stimulate the 20S, and replacement by an aspartate completely prevented activation. Furthermore, addition of an alanine C terminal to the arginine blocked PAN's ability to stimulate gate opening, even though an alanine in place of the arginine did not influence this activity. Thus, the binding site for the C-terminal sequences has some specificity, and while the ultimate residue in PAN does not require any specific side chain, acidic side chains in this position do not allow function.

The Penultimate HbY Residues Are Essential for PAN to Stimulate Gate Opening

To determine whether the conserved hydrophobic and tyrosine residues in the HbYX motif are important for the association with the 20S and stimulation of gate opening, we systematically mutated these residues. Y429 was found to be absolutely essential for PAN's activity (Table 2). When it was mutated to any of eight other residues, including hydrophobic, aromatic, or charged, none stimulated LFP hydrolysis. This lack of stimulation with a phenylalanine

Table 2. The Penultimate Y429 and Preceding Hydrophobic Residue L428 in PAN Are Required for 20S Gate Opening

Peptide Hydrolysis (% WT)		
"X"	PAHLDVLR ⁴³⁰	PAHLDVXR ⁴³⁰
A	4	18
V	2	20
I	3	64
L	3	100 (WT)
Y	100 (WT)	60
F	4	20
W	6	57
R	3	7
D	5	4
C	-	5
P	-	7
No PAN	5	5

Experiment was performed as described in Table 1.

in this position was surprising because the penultimate residue in PAN from five archaeal species is a phenylalanine. Mutating the leucine preceding the tyrosine to any of ten other amino acids confirmed that only hydrophobic (Hb) residues supported activity, and replacement by an arginine, aspartate, cysteine or proline prevented the stimulation of gate opening.

The HbYX Motif Is Required for PAN-20S Complex Formation

Mutations in the HbYX motif might block gate opening either by preventing the formation of the PAN-20S complex or by allowing its formation but interfering with the gate-opening mechanism. We used electron microscopy as described previously (Smith et al., 2005) to test whether the mutations that prevent activation also prevented complex formation. Five different PAN mutants were analyzed by EM. We observed extensive complex formation with those variants that stimulated gate opening, but did not observe any PAN-20S complexes with the PAN mutants that failed to stimulate gate opening (Table S2). Thus, the failure of these PAN mutants to stimulate gate opening is due to a failure to associate with the 20S.

Residues Preceding the HbYX Motif Are Not Essential in Gating

Although the residues preceding the HbYX motif were not conserved in other proteasomal ATPases, we mutated each of the four residues preceding the HbYX motif to alanines to determine their importance for activation of the 20S. None of these residues were absolutely essential for PAN's stimulation of peptide hydrolysis, but some alanine replacements did reduce PAN's activity. Potentially,

this C-terminal sequence may adopt a helical conformation as was suggested for the C termini of PA26 in the PA26-20S complex (Whitby et al., 2000). To test this possibility, we inserted prolines at two positions in the C termini to prevent helix formation or mutated the proline (P422) to an alanine (Table S3). None of these mutations significantly reduced the stimulatory activity of PAN. Therefore, a C-terminal helix is probably not essential for formation of the PAN-20S complex or activation of gate opening. Also, insertion of four alanines before the seven C-terminal residues had little effect (Table 1), suggesting that these terminal residues are flexible or function relatively independently and fit into a constrained pocket that permits only certain residues.

PAN's C Terminus Moves from an Aqueous to a Hydrophobic Environment upon Complex Formation

The susceptibility to CpB (Figure 1C) suggested that PAN's C-terminal residue is exposed in PAN, but not after association with the 20S. To analyze further the environment surrounding the C-terminal residues, we monitored tryptophan fluorescence. Because wild-type PAN and the *Thermoplasma* 20S proteasome both lack tryptophans, we analyzed the fluorescence spectra of mutants where a tryptophan was inserted in the C-terminal position (R430) or in place of the conserved hydrophobic residue (L428). Both mutations allowed wild-type activity (Tables 1 and 2). The maximum emission wavelength of the C-terminal tryptophan (W430) was 357 nm (Figure 2A), which resembles that of free tryptophan in an aqueous solution. Accordingly, this fluorescence was quenched upon addition of acrylamide (data not shown). Therefore, the ultimate C-terminal residue of PAN is indeed exposed to the solvent, where it appears available for docking with the 20S. However, when a tryptophan was placed two residues upstream (W427), it showed a $\lambda_{em\ max}$ of 341 nm, suggesting that this residue is located in a more hydrophobic environment.

ATP binding to PAN is essential for complex formation with the 20S (Smith et al., 2005). One attractive model to explain this activation by ATP is that the C-terminal residues assume a more exposed conformation upon binding ATP, which promotes PAN-20S association, but not upon binding ADP, which inhibits complexation. Therefore, we tested whether the tryptophan at the 430 or 428 positions become more or less exposed to solvent upon binding ATP or ADP. However, we found no such change in the spectrum of PAN with either the solvent-exposed R430W or the more hydrophobically exposed L428W after addition of ATP, ATP γ S, or ADP.

If the C termini of PAN, like those of PA26 (Forster et al., 2005), dock into pockets in the 20S's α ring upon complex formation, then the terminal tryptophan in the R430W PAN mutant should move from an aqueous to a more hydrophobic environment. After adding ATP γ S to a mixture of PAN and proteasomes, the $\lambda_{em\ max}$ of this terminal tryptophan shifted from 357 nm to 352 nm, and the fluorescence

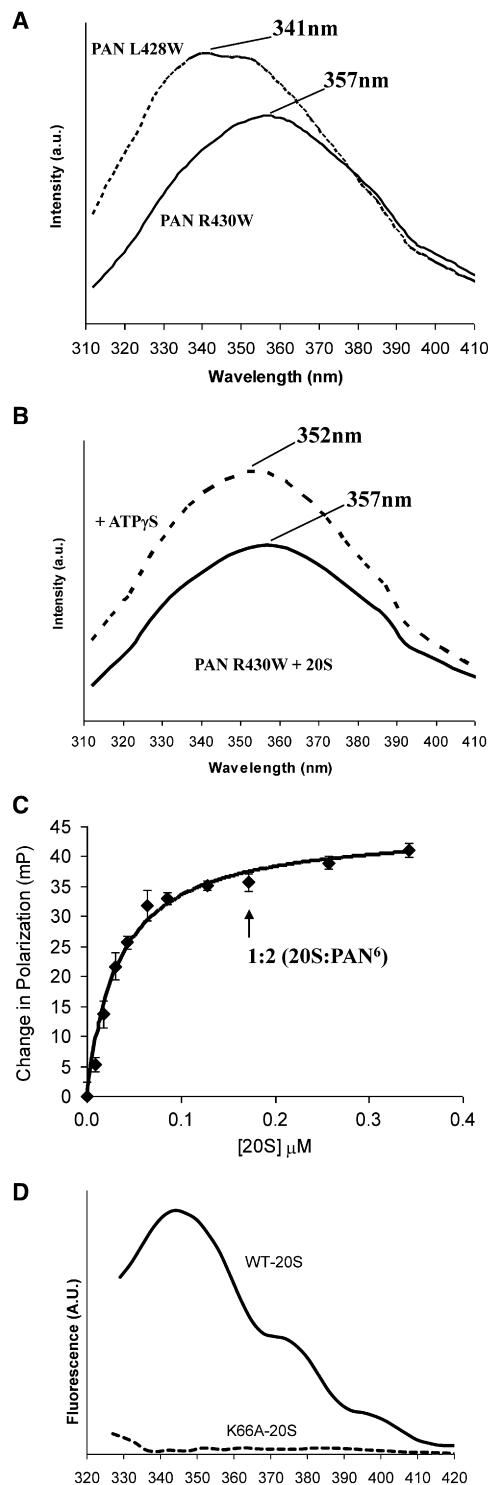


Figure 2. The Fluorescent Spectrum of PAN R430W and PAN L428W Mutants in Solution and upon Binding the 20S

(A) Fluorescent emission spectra of the various PAN mutants. (B) Fluorescent emission spectra of PAN R430W in the presence of 20S proteasomes without and with ATP γ S. $\lambda_{em\ max}$ is indicated. (C) Polarization of PAN's (R430W) tryptophan in the presence of ATP γ S upon addition of increasing amount of 20S proteasomes.

intensity increased by over 50% (Figure 2B), as is characteristic of a tryptophan that shifts from an aqueous environment to a more hydrophobic one. By contrast, the addition of ADP did not change the $\lambda_{em\ max}$. Also, addition of ATP γ S to the 20S (which lacks tryptophans) did not change its background tyrosine fluorescence, and there was no change in the spectrum of the PAN L428W mutant upon association, because the $\lambda_{em\ max}$ of this tryptophan is already blue shifted (Figure 2A). At high concentrations, ADP competes with ATP and promotes PAN-20S dissociation (Smith et al., 2005). Accordingly, if excess ADP was added after formation of the PAN-20S complex with ATP γ S, then ADP shifted the $\lambda_{em\ max}$ to the red, and the fluorescence intensity decreased (data not shown). Therefore, upon ATP-dependent association of PAN with the 20S, the C termini move from an aqueous environment to a more hydrophobic one, presumably the pockets in the α ring (see below).

PAN's C Termini Become Polarized upon Association with the 20S

Formation of the PAN-20S complex has been difficult to demonstrate because of its transient and relatively weak association (Smith et al., 2005). To quantitate the association of PAN with the 20S in solution, we analyzed the polarization of the C-terminal tryptophan in PAN R430W. Fluorescence polarization can be used to measure the rate of rotation of a fluorescent molecule, which can decrease upon association with another protein. When ATP γ S (but not ADP) was added to a solution of PAN and the 20S, a 40 mP change in polarization was detected. Also, without the 20S present, W430 did not become more polarized upon addition of ATP, ATP γ S, or ADP. Thus, the terminal tryptophan becomes polarized upon association with the 20S. To determine what molar ratio of 20S to PAN gave maximal polarization, we added increasing amounts of 20S to a solution of PAN R430W and ATP γ S. This assay is more quantitative than biochemical or EM approaches used previously to show complex formation, and because high concentrations of PAN and 20S could be used, stoichiometric binding and saturation were demonstrated. Polarization approached maximal with two hexameric PAN complexes per 20S proteasome (Figure 2C), i.e., when most of the 20S was doubly capped with PAN, and most of the PAN was associated with a proteasome.

PAN's MW was taken as 585 kDa (two hexameric rings) and 20S as 674 kDa. The final concentration of PAN⁶ (the hexamer) was 340 nM, and the 20S concentration is shown. The fit line was calculated from regression analysis of the simple ligand binding equation with one site saturation, the K_d was calculated to be 34 ± 12 nM, and the maximum change in polarization (B_{max}) was 45 mP.

(D) A difference plot showing the changes that occur in the fluorescent spectrum of the tryptophan in PAN R430W that is induced by addition of wild-type 20S or by addition of K66A-20S. The plot was generated by subtracting the spectrum of PAN R430W with the 20S from its plot without the 20S; a flat line indicates no change in the spectrum. These experiments were performed at least three times with similar results.

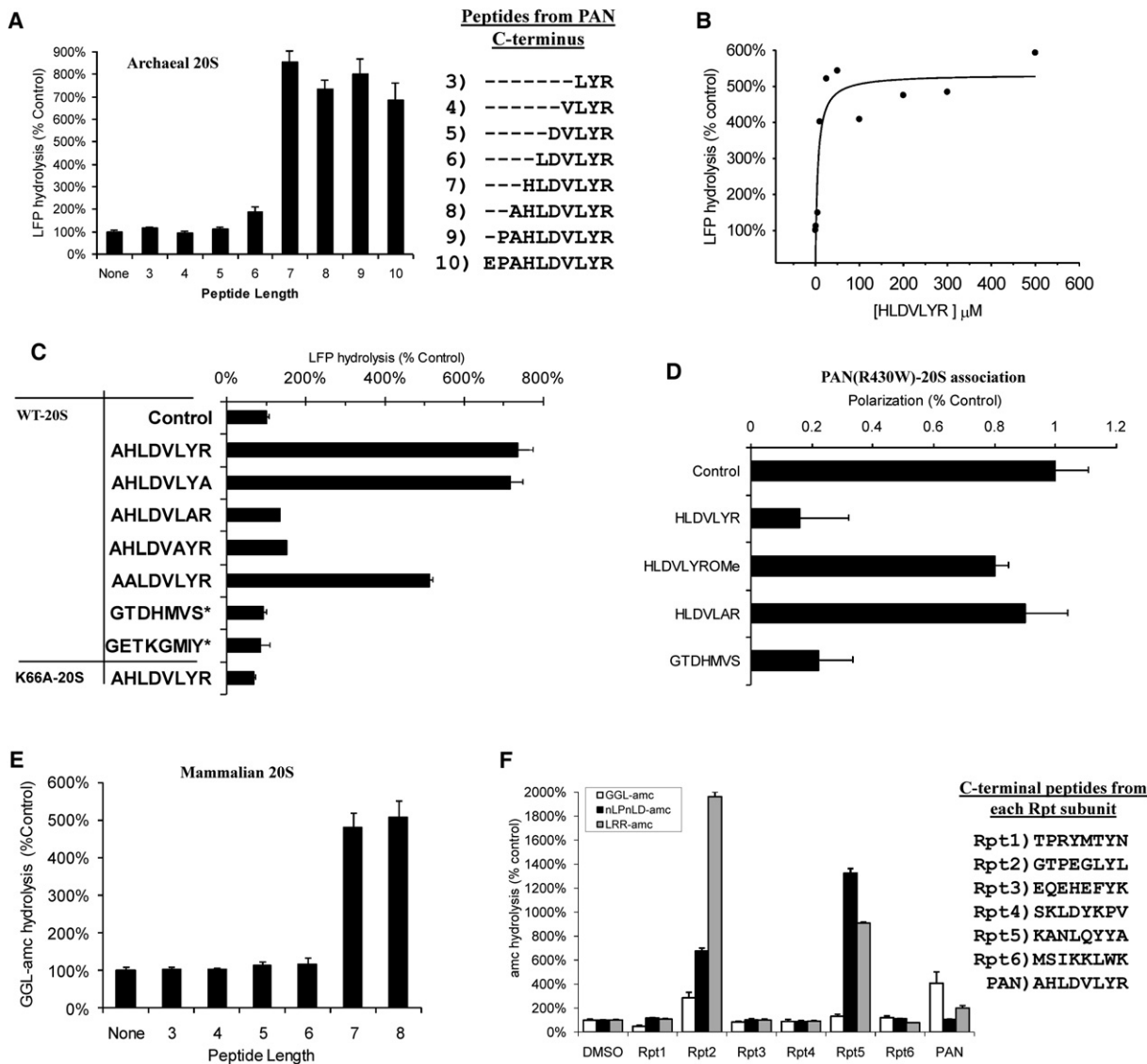


Figure 3. Peptides Derived from PAN's Extreme C-Terminal Sequence Induce Gate Opening in Archaeal and Mammalian Proteasomes and Inhibit PAN-20S Complex Assembly

(A) Ability of peptides of different lengths to stimulate gate opening. Peptides (250 μ M) were incubated with 0.2 μ g 20S proteasomes and LFP. In each case, a correction was made because the added peptide, in addition to causing gate opening, competed with LFP at the active sites. To determine the actual percentage stimulation of LFP hydrolysis due to gate opening, the ability of the various peptides to inhibit LFP hydrolysis by the gateless ($\Delta\alpha$ 2-12) 20S proteasome was also measured, and the values were used to normalize data on LFP hydrolysis by wild-type 20S proteasomes. Without normalization, seven- to eight-residue peptides still stimulated 20S proteasomes 3- to 4-fold (see also [B]). Values in (A) and (C)–(E) are the means of three independent experiments \pm SD.

(B) The seven-residue peptide from PAN's C terminus (HLDVLYR) induces gate opening in the *Thermoplasma* 20S at much lower concentrations than the ten-residue peptide from PAN's C terminus (EPAHLDVLYR). This experiment was performed as in (A) using the concentrations of the peptide shown, except that no adjustment for competition with the fluorogenic substrate was calculated, because such corrections become unreliable at the high peptide concentrations (>500 μ M). The peptide HLDVLYR inhibits cleavage of LFP by $\Delta\alpha$ (2-12)20S at \geq 50 μ M.

(C) Replacement of the Hb (hydrophobic) and Y residues in the eight-residue peptides prevented gate opening. Also shown is the inability of the wild-type peptide (250 μ M) to induce gate opening in the 20S K66A, and the inability of the peptides (*), corresponding to the C termini of PA26 (GTDHMVS) or PA28 (GETYGMIY), to induce gate opening in wild-type 20S.

(D) Peptides that activate gate opening prevent association of the 20S and PAN, as does the peptide corresponding to the C terminus of PA26. Each peptide was preincubated with 20S and PAN for 5 min followed by addition of ATP γ S. Polarization was measured before and after nucleotide addition. No peptide (control) was taken as 100% polarization and was similar to that shown in Figure 2. The values are means \pm SD from at least three experiments. Peptide concentrations were used as in (C).

(E) A similar experiment as in (A) (using peptides corresponding to PAN's C terminus) was carried out using purified rabbit muscle 20S proteasomes and GGL-amc as the fluorogenic substrate (Smith et al., 2005).

PAN Binding to the 20S Requires K66 in Its α Ring

K66 is located in the intersubunit pockets in the 20S's α ring, and when PA26 complexes with the Thermoplasma 20S, the C-terminal carboxyl group of PA26 forms a hydrogen bond with this lysine (Forster et al., 2005). Accordingly, we found that PAN was unable to stimulate gate opening in K66A-20S mutant proteasomes, as assayed by measuring LFP hydrolysis (data not shown). Forster et al. (2005) had shown that this lysine is also required for the stimulation of casein degradation by PAN, which could be due to the requirement of K66 for gate opening or for PAN-20S association. We therefore tested whether K66 is also essential for the association of PAN and the 20S. In the presence of ATP γ S, the K66A-20S, unlike wild-type proteasomes, did not alter the fluorescence spectrum of the terminal tryptophan in PAN-R430W (Figure 2D) and did not cause its polarization (data not shown). These results indicate that PAN cannot stimulate gate opening in K66A-20S because it cannot associate with this particle, and strongly suggest that PAN's C termini dock into the same intersubunit pockets as the C termini of PA26.

Short Peptides Corresponding to PAN's C Terminus Induce 20S Gate Opening

Two possible models can explain the above results: (1) that insertion of PAN's C termini into the hydrophobic binding sites in the α ring functions like a key in a lock to induce gate opening. (2) Alternatively, this interaction is necessary for PAN-20S complexation, but some other domain(s) in PAN (e.g., like the activation loop in PA28/26) are also necessary to induce the open-gate conformation. To test whether docking of PAN's C termini is sufficient to cause gate opening, we synthesized different-length peptides that corresponded to PAN's C-terminal sequence (Figure 3A), incubated them with proteasomes, and monitored gate opening by LFP hydrolysis. Surprisingly, peptides 7–10 residues long by themselves stimulated peptide degradation by 20S proteasomes by up to 9-fold. This large stimulation, which resembled the stimulation seen with PAN and ATP, was seen at the rather high peptide concentrations of 250 μ M (Figure 3A). However, with the seven-residue peptide, near-maximal stimulation of peptide hydrolysis was evident at 10 μ M (see Figure S2). These findings indicate rather high affinity binding of the seven-residue peptide, especially because in PAN, these C termini are likely to function in a multimeric fashion and with a specific steric relationship. However, accurate determination of K_{AS} is unreliable by this approach, because these C-terminal peptides (especially above 50 μ M), not only cause gate opening but also enter the 20S particle and compete at

the active sites with LFP (whose hydrolysis is used to assay gate opening).

The seven-residue peptide, HLDVLYR, stimulated gate opening much better than the six-residue peptide, LDVLYR, and smaller peptides were ineffective. The small stimulation with the six-residue peptide (up to 2-fold) reflects a requirement for peptide length rather than for an N-terminal histidine, because when this histidine was changed to an alanine in the peptide, it showed a similar ability to stimulate gate opening. Accordingly, the corresponding mutation in full-length PAN, A424H, stimulated gate opening like wild-type PAN. Surprisingly, the efficacy of the C-terminal peptides decreased at greater length, and the ten-residue peptide (EPAHLDVLYR) showed activity only above 200 μ M (data not shown). These findings indicate a steep dependence on peptide length. Presumably, a length of seven residues is necessary for the peptide to occupy and assume a similar conformation as the C termini of PAN in the intersubunit pockets.

Peptide-Induced Gate Opening Requires the HbYX Motif and K66 in the α Ring

Gate opening in the 20S can be activated nonspecifically by detergents, high temperature, and some hydrophobic peptides (Coux et al., 1996). To determine whether the seven-residue peptides corresponding to PAN's C terminus cause gate opening by the same mechanism as PAN, we tested whether the peptide must also contain the HbYX motif. When the Hb or the Y residue was replaced by an alanine, the peptide was not able to stimulate gate opening, as was found with PAN (Table 2). Also, replacement of the C-terminal arginine with an alanine did not reduce its activity, as was found with the corresponding mutation in PAN (Table 1). Furthermore, the seven-residue peptide from PAN's C terminus could not stimulate gate opening in proteasomes expressing the K66A mutation in the α subunits (Figure 3B). Thus, gate opening by the C-terminal peptides and PAN shows the same requirements for an HbYX motif and a lysine 66 in the intersubunit pockets, indicating that they function by very similar mechanism.

Peptides from PAN's C Terminus Inhibit PAN-20S Association

If these peptides induce gate opening by docking into the same sites in the α ring as PAN's C termini, then these peptides should act as competitive inhibitors of the PAN-20S association. We therefore preincubated these peptides (250 μ M) with 20S proteasomes and R430W PAN (350 nM) for 5 min prior to the addition of ATP γ S, and then monitored complex formation by measuring polarization of PAN's terminal tryptophan. The wild-type

(F) Peptides from C termini of certain mammalian 19S ATPase subunits cause gate opening in 20S. A similar experiment as in Figure 3C was carried out using purified rabbit muscle 20S but with peptides that corresponded to the C termini of the mammalian 19S ATPases: Rpt1, Rpt2, Rpt3, Rpt4, Rpt5, and Rpt6. The cleavage rates of fluorogenic substrates specific to its three peptidase sites were monitored (see text). The peptide corresponding to Rpt1 stimulated gate opening weakly under some conditions (e.g., no glycerol but with 60 mM KCl). The sequences for each of the C-terminal peptides are shown. All values are the means \pm SD of three independent experiments.

peptide inhibited complex formation nearly completely throughout the course of the experiment, while the inactive peptide containing an alanine in place of the conserved tyrosine did not inhibit (Figure 3C).

Because an additional alanine on PAN's C terminus prevents complex formation (Table S2), it seemed likely that a free carboxyl group in this position is important for binding to the 20S. To test this possibility, we blocked the C-terminal carboxyl group on HLDVLYR by esterification with methanolic acid. After carboxyesterification, the peptide completely lost its ability to inhibit polarization (Figure 3C). Thus a carboxyl group is essential for docking in the α ring. Together, these observations establish that gate opening induced by peptides from PAN's C terminus is a specific response that requires a length of seven residues, the conserved HbYX motif, a free carboxyl group, and lysine 66 in the 20S's α ring. In related studies, we have used cryo-electron microscopy to confirm that the gate-opening peptides bind to the intersubunit pockets in the 20S's α ring adjacent to K66 (D.M.S., A.L.G., J. Rabl, and Y.C., unpublished data).

C Termini of PA26 Bind, but Do Not Cause Gate Opening

Because the C termini of PAN and PA26 both require lysine 66 in the α subunits to associate with the 20S and activate gate opening, they are likely to bind to or act through the same sites (Forster et al., 2005), even though PA28/26 lack the HbYX motif. Therefore, we studied under these same conditions the effects on gating of the seven-residue peptide corresponding to the C terminus of PA26, GTPHMVS, or eight-residue peptides from the C terminus of PA28 α (which by itself forms a heptameric complex that induces gate opening in eukaryotic 20S proteasomes [Cascio et al., 2002]). However, in contrast to the seven- or eight-residue peptides from PAN's C terminus, these peptides could not by themselves induce gate opening at 250 μ M (Figure 3A) or even at 1 mM (data not shown). The failure of this PA26 peptide to stimulate gate opening is consistent with the prior findings that the C-terminal residues of PA26 are required for its association with the 20S, but that this binding does not induce gate opening, which requires the distant activation domain of PA26 (Zhang et al., 1998).

To determine whether the heptapeptide from PA26 actually binds to the 20S, we measured fluorescence polarization of PAN R430W to test whether this C-terminal peptide could competitively inhibit PAN's association with the 20S. After preincubation with the 20S, this peptide, like those from PAN's C terminus, was found to inhibit the polarization that was associated with PAN-20S complex formation (Figure 3D). However, this inhibition of polarization, unlike that by the C-terminal peptides from PAN, was temporary and lasted for only several minutes, presumably because the PA26-derived peptide had much lower affinity for the 20S than PAN and the PAN-derived C-terminal peptide.

C-Terminal Peptides from PAN and Certain 19S ATPases Induce Gate Opening in the Mammalian 20S

Because the HbYX motif is also found in the C termini of three of the ATPases in the 26S proteasome, it seemed likely that these C termini regulate gating in the eukaryotic 20S in a similar fashion as PAN. We therefore tested whether similar mechanisms function in the 26S particle, and whether short peptides from PAN's C terminus could induce gate opening in proteasomes from rabbit muscle. In these particles, gate opening was monitored with the tripeptide substrate GGL-amc, which is excluded by the closed gate conformation. The seven- and eight-residue peptides from PAN were indeed able to cause a 5- to 6-fold increase in peptide entry, but smaller ones had no effect (Figure 3E). Thus, the mammalian 20S showed the same length requirement for gate opening as did the archaeal 20S (Figures 3A and 3E).

To determine whether the ATPases in the 26S proteasome function similarly to PAN, we synthesized eight-residue peptides corresponding to the C terminus of each of the mammalian 19S ATPases, Rpt1–6. Because such C-terminal peptides may also enter the 20S particle and compete with the fluorogenic substrates for binding to the active sites, we monitored the induction of gate opening with substrates specific for its different catalytic sites (i.e., chymotrypsin-like site: GGL-amc; caspase-like site: nLPnLD-amc; and trypsin-like site: LRR-amc) to reduce the chances that substrate competition would mask the effects on gate opening. The C-terminal peptides from Rpt2 and Rpt5, which contains the HbYX motif, strongly stimulated gate opening in the rabbit 20S proteasome and did so to a greater extent than the C-terminal peptide from PAN (Figure 3F). Prior genetic studies had also implicated Rpt2 in regulation of gate opening (Kohler et al., 2001). As expected, Rpt4 and Rpt6, which do not contain the HbYX motif, could not induce gate opening. Rpt1 was found to induce gate opening weakly but only under some conditions, possibly because the residue preceding its penultimate tyrosine is a threonine (not a hydrophobic residue) and thus does not conform to the HbYX motif. Surprisingly, Rpt3, whose last three residues (...FYK) do contain the HbYX motif, was never found to induce gate opening in mammalian 20S particles. Related observations indicated that lysines in the C-terminal (X) position do not support gate opening in the mammalian 20S in contrast to the HbYX motif in PAN, which functions with basic (but not acidic) residues in this position (D.M.S. and A.L.G., unpublished data). Thus, gate opening by the HbYX motif appears to function in 26S proteasomes, although gating seems to be regulated directly by only C termini of Rpt2 and Rpt5. However, other C termini may contribute indirectly (e.g., by stabilizing the 26S complex) (see below).

The Mammalian 19S ATPases' C Termini Appear to Be Essential for Gate Opening

To determine whether the C termini of the 19S ATPases, Rpt1–6, are also required for gate opening in mammalian

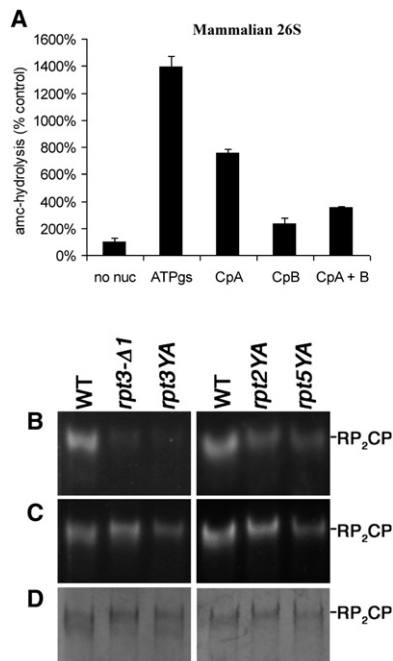


Figure 4. Carboxypeptidase Induced Truncation, and Mutations within the HbYX Motif Result in 26S Instability and Gating Defects

(A) Carboxypeptidase treatment of free mammalian 19S/PA700 inhibits its ability to stimulate peptide hydrolysis by the mammalian 20S in the presence of ATP γ S. The 19S particles (1 μ g/reaction) were preincubated with or without 0.1 units of carboxypeptidase A or B for 10 min prior to addition of the 20S (0.1 μ g/reaction) and ATP γ S (0.1 mM). The assembly reaction mix was diluted 5-fold in the reaction buffer with Ac-nLPnLD-amc, and the rate of hydrolysis was monitored. The values are means \pm SDs from at least three experiments.

(B) Purified proteasomes (3 μ g) from wild-type (WT) and *rpt* mutant strains were resolved on 3.5% nondenaturing gels, and proteasome activity was visualized by staining with the fluorogenic substrate suc-LLVY-AMC. The YA mutants are single-residue substitutions of the penultimate Tyr of the indicated Rpt protein. *rpt3-Δ1* is a deletion of the C-terminal lysine of Rpt3.

(C) After the initial peptidase assay in (A), the assay was repeated in the presence of 0.02% SDS to cause gate opening.

(D) The native gels were stained with Coomassie brilliant blue to assess the amount of protein in each sample.

26S, we studied the effects of carboxypeptidase treatment of purified mammalian 19S/PA700 complex (kindly provided by George DeMartino) on its ability to induce gate opening in purified rabbit 20S proteasomes, in an experiment similar to that done with PAN (Figure 1C). The C-terminal residues of the ATPases Rpt3 and Rpt6 are lysines that should be removed by CpB, while those on Rpt2 (Leu), Rpt4 (Val), and possibly Rpt5 (Ala) should be removed by carboxypeptidase A. Treatment of the 19S complex with either carboxypeptidase A or B prior to addition of the 20S and ATP γ S significantly reduced the ability of the 19S to stimulate peptide hydrolysis in the presence of ATP γ S (Figure 4A). Thus, both classes of 19S C termini seem important for the stimulation of gate

opening. (In these experiments, carboxypeptidase B seemed more effective than A, but these effects cannot be compared quantitatively because the extent of removal of the different C-terminal residues by the two enzyme preparations may well differ.)

By contrast, treatment of the isolated 26S complex with carboxypeptidase A or B did not reduce the stimulation of peptide hydrolysis by ATP γ S (data not shown), presumably because the critical C-terminal residues in the 19S ATPases are also buried upon complex formation, as was found with the PAN-20S complex. These latter observations also indicate that the carboxypeptidase treatments did not destroy the integrity of the 19S complex or act indirectly by affecting subunits outside the 19S-20S interacting surfaces.

The C-Terminal YX Residues in the Yeast 26S ATPases Are Required for Gate Opening

These findings suggest that the C termini of multiple ATPase subunits are important in 26S formation and gating. To further test whether the terminal YX residues are necessary for 26S stability or gate opening, we examined the effects of mutagenesis of the YX residues in the C termini of the four ATPase subunits in *S. cerevisiae* proteasomes. We substituted an alanine for the conserved penultimate tyrosine in the four ATPase subunits that contain this residue, Rpt1, Rpt2, Rpt3, and Rpt5, and purified the 26S proteasomes from these mutant strains using affinity tags (Leggett et al., 2002). It is noteworthy that each of these YA replacements in the four ATPase C termini caused highly complex and distinct phenotypes related to protein degradation, but all allowed sufficient proteasome function for growth of the yeast (S.P. and D.F., unpublished data). Analysis of these phenotypic defects is beyond the scope of this study and will be published elsewhere. The activity of each mutant proteasome was assessed in nondenaturing gels, which were incubated in the presence of a fluorogenic substrate to assess the extent of complexation and gate opening (Figure 4B). Replacement of the conserved tyrosine in Rpt1 by an alanine appeared to cause a major defect in the stability of the 26S complex, because we were not able to isolate these particles by standard affinity purification methods. By contrast, the *rpt2YA*, *rpt3YA*, and *rpt5YA* mutant proteasomes were successfully purified by these approaches, but they all exhibited reduced suc-LLVY-amc hydrolysis (Figure 4). These defects reflect primarily decreased substrate entry and not reduced 26S proteasome levels, because equivalent proteasome loading was evident when the gels were stained, and the 26S proteasomes did not disassemble during electrophoresis or during incubation with substrate (see Supplemental Data and Figure S2). In addition, to test the capacity for gate opening, this assay was repeated in the presence of 0.02% SDS, which artificially induces gate opening (Figure 4C). The *rpt3YA* and *rpt2YA* mutant proteasomes showed a significant increase in peptide hydrolysis with SDS, but the *rpt5YA* mutant showed only a limited response. Thus, the conserved tyrosines in

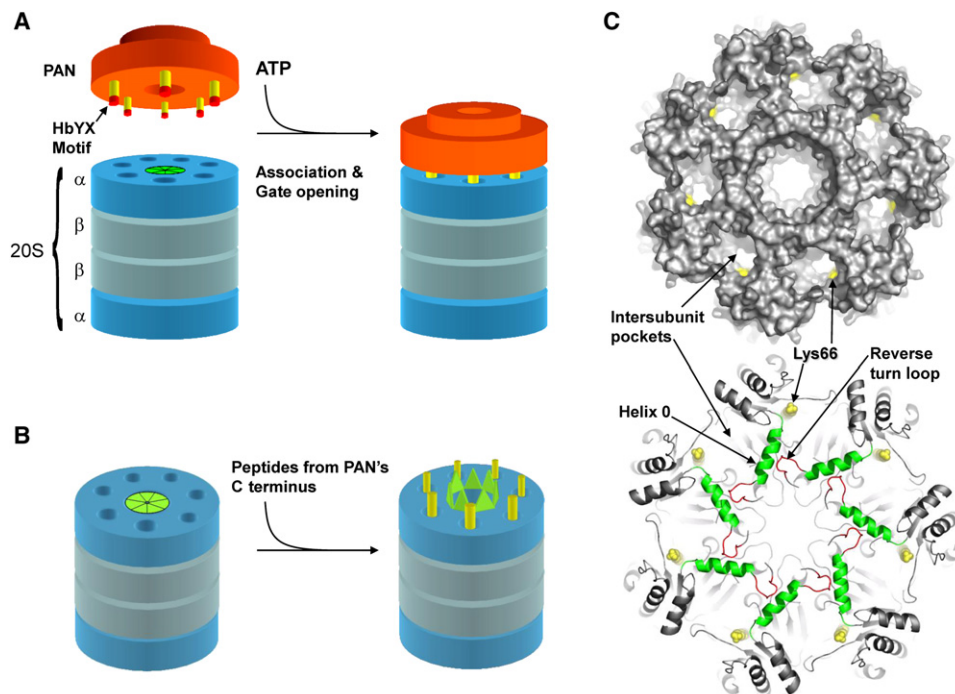


Figure 5. Model Depicting the Association of PAN with the α Ring of the 20S Proteasome

(A) The C termini (yellow) of PAN (orange) dock into the intersubunit pockets in the top of the 20S. (B) Schematic model for gate opening in the 20S upon binding of peptides derived from PAN's C terminus to the intersubunit pockets in the 20S. (C) (Top) Top view of the 20S α ring with a surface rendering to demonstrate the intersubunit pockets and location of lys66 (yellow). (Bottom) Ribbon representation of the 20S α ring: Lys66 (yellow), helix 0 (green), reverse turn loop (red). The N-terminal gating residues are not resolved in this crystal structure (1PMA). Images in (C) were rendered with PyMOL.

each of the four ATPases appear important for regulating gate opening in the 20S particle, but their specific roles appear to differ.

A similar approach was then used to delete the C-terminal residues on the six 19S ATPases. Each of these mutants also had phenotypic defects, related to proteolysis, which will be analyzed elsewhere. The C-terminal deletion in Rpt3 resulted in a clear gating defect similar to that seen with the Tyr to Ala substitutions in Rpt2, Rpt3, and Rpt5 (Figure 4C). When the C termini of the other ATPases Rpt4, Rpt5, and Rpt6 were truncated by a single C-terminal residue, there appeared to be stability problems in the 26S complex that reduced recovery (S.P. and D.F., unpublished data). Thus, the terminal YX residues are necessary for proper gating in the yeast 26S proteasome, and some appear necessary for its stability. While the terminal HbYX motifs in the 19S ATPases play important functions in gating and complex formation that are consistent with the findings on PAN, the specific roles of the individual ATPases' C termini clearly differ from one another.

DISCUSSION

The ATPases' Mechanism for Activation of the 20S

The present studies have uncovered an essential new ATP-dependent step in proteasome function and have

clarified how the regulatory ATPases facilitate substrate entry into the 20S proteasome. When ATP binds to the proteasomal ATPases, the conserved HbYX motifs in their C termini dock into pockets in the 20S α ring and function like a key in a lock to open the gate that limits substrate entry (Figure 5A). Because seven-residue peptides corresponding to the C termini of either PAN, Rpt2, or Rpt5 by themselves bind and trigger gate opening in both archaeal and mammalian proteasomes (Figure 5B), this novel reaction does not require other parts of the ATPase molecule and appears to represent a general mechanism regulating proteasome function. The HbYX motif is present in proteasomal ATPases in both archaea and eukaryotes, and it is noteworthy that similar C-terminal sequences are also present in the presumed proteasome-regulatory ATPases in rhodococcus and mycobacteria. In PAN from several archaeal species, there is an HbFX in place of the conserved HbYX (Table S1). Presumably, in these species, the structure of the intersubunit pockets in the 20S α rings allows selective binding and gate opening by this alternative HbFX motif.

No HbYX or related C-terminal motif is present in the bacterial ATPase complexes (ClpA, ClpX, ClpC, and HslU) that activate the ClpP or HslIV peptidase complexes, and their modes of activation of proteolysis differ from that demonstrated here. Unlike PAN, the regulatory Clp

ATPases associate with ClpP via an internal loop, instead of their C-terminal sequences (Guo et al., 2002). A more similar mechanism for ATP-dependent activation occurs with HslU, which is more homologous to PAN and the 19S ATPases. Upon ATP binding to HslU, its six C termini dock into the six crevices in the HslV peptidase complex and induce conformational changes that enhance its proteolytic activity (Seong et al., 2002). However, removal of up to five residues from the C terminus of HslU does not block its ability to associate with and stimulate HslV (Seong et al., 2002). Thus HslU does not require a specific C-terminal motif for its function. Furthermore, both ClpP and HslV are two-ring complexes that lack an outer ring or a gated pore formed by the subunits' N termini, and thus their ATP-dependent mechanisms of activation, which occur through alteration in the proteolytic sites, are fundamentally different from gate opening the 20S proteasome.

Exactly how binding of ATP or ATP γ S to PAN allows its C termini to dock into the intersubunit pockets is unclear. The simplest mechanism would be that PAN's C termini are buried in the ADP-bound state, but become exposed upon binding of ATP or ATP γ S as has been shown for HslU (Wang et al., 2001). However, our measurements of tryptophan fluorescence and susceptibility to CpB indicate that upon binding of ATP or ADP, PAN's C termini do not shift between buried and exposed states but instead remain in an exposed, aqueous environment until they associate with the intersubunit pockets in the 20S. Nevertheless, ATP-driven conformational changes in PAN's C termini within the aqueous environment may promote association by altering their spacing to allow a better fit with the docking sites on the 20S.

Because of its key role in allowing proteolysis, the binding of the HbYX motif to these intersubunit pockets for gate opening represents an attractive drug target. Proteasome inhibitors, which block some of the peptidase sites within the 20S's central chamber, have found wide application as research tools and in the treatment of certain cancers (Adams, 2004; Kisselev and Goldberg, 2001). Inhibiting the initial binding of the C termini to the α ring should inhibit 26S assembly, substrate entry into the 20S proteasome, and protein degradation.

The Gate-Opening Mechanisms of the ATPases and PA28/26 Are Distinct

Although gate opening by the ATPases and PA28/26 has been proposed to occur by similar mechanisms (Forster et al., 2003, 2005; Kohler et al., 2001), this study demonstrates fundamental differences. Unlike the peptides from PAN's C terminus, the C-terminal seven-residue peptide from PA26 or the eight-residue peptide from PA28 α could not induce gate opening (Figures 3B and 3C) even at 1 mM concentrations. Their inability to stimulate was expected, because gate opening by PA28/26 requires an activation loop, distant from its C termini (Zhang et al., 1998). When this activation domain is mutated, PA28 can still stably associate with the 20S, and its C ter-

mini can dock into the intersubunit pockets in the α ring, but it cannot activate gate opening. Thus, for PA28 (unlike PAN) binding to the α ring and gate opening can be dissociated. Because PAN's C-terminal sequence alone induces gate opening, it clearly does not require such an activation domain and can work at relatively low concentrations (≤ 10 μ M). The gate-opening mechanism of PA26 requires seven equilateral C-terminal residues and a distant activation domain in a specific rigid three-dimensional framework. However, a 7-residue peptide does not have the structural capacity to function by such a mechanism, and it is too small to interact with additional domains outside the intersubunit pockets. In addition, PAN's ability to induce gate opening was preserved even with a four-alanine extension preceding the last seven residues of PAN, and such an insertion should prevent a direct interaction between any upstream activation loops and the surface of the α ring. Our related cryo-EM studies (D.M.S., A.L.G., J. Rabl, and Y.C., unpublished data) demonstrate that upon binding, these HbYX-containing peptides (unlike C-terminal peptides from PA26) induce a novel conformational change in each α subunit, which results in a displacement of the Pro17 reverse turn loop in a manner that stabilizes the open-gate conformation.

Despite having different consequences, the binding of PAN's and PA26's C termini appears to occur in the same pockets, because the peptides from both can inhibit the binding of PAN to the 20S complex. Also, gate opening by both requires lysine 66 in the α subunits (Figure 5C, top). PA26's C-terminal carboxyl group forms a hydrogen bond with lysine 66, and we have confirmed the prior suggestion (Forster et al., 2005) that this lysine is important for the PAN-20S interaction. Accordingly, gate opening by the octapeptide from PAN's C terminus required a free C-terminal carboxyl group. Therefore, though the C termini of PAN and PA28/26 share a common binding site on the 20S, the consequences of binding depend on the presence of the HbYX motif.

Distinct mechanisms for PA28/26 and the proteasomal ATPases should not be surprising because they lack sequence homology throughout, especially in their critical C termini, and differ in their quaternary structures and their *in vivo* functions. Furthermore, the imposing of PA28/26's 7-fold symmetry onto the 20S's α ring is of fundamental importance to its mechanism of gate opening (Forster et al., 2003; Whitby et al., 2000). By contrast, the proteasomal ATPases have a 6-fold symmetry, which implies that only a fraction of the 20S seven-intersubunit pockets can be occupied by the C termini of the ATPases, and because of geometric constraints, it is likely that only a few of the ATPases C termini can possibly bind at any one time. Thus, PAN and the 19S complex cannot impose a 7-fold symmetry on the α subunits. A longstanding fundamental puzzle is how a six-subunit ATPase ring complex can associate with the 20S and stabilize its 7-fold symmetric open-gate conformation. The only solution is that gate opening by the ATPases requires docking of only a subset

of C termini to the α -intersubunit pockets, probably only two to four termini, depending on the extent of C-terminal flexibility.

Such asymmetrical contact between the ATPases and the 20S α ring is supported by electron microscopy of both the 26S (Walz et al., 1998) and the PAN-20S complex (Smith et al., 2005), which demonstrates that often only single small sections of the ATPase ring closely contact the 20S α ring, unlike PA28/26, which makes flush contact with the 20S. This apparent “wobbling” of the ATPase on the 20S may reflect the docking of only a subset of the C termini to the 20S α ring at any time. In addition, several homologous hexameric ATPases (e.g., p97, HslU, and ClpX) have been shown to bind ATP to only two to four subunits at any time (Bochtler et al., 2000; DeLaBarre and Brunger, 2005; Hersch et al., 2005; Singleton et al., 2000), and PAN behaves similarly (D.M.S., C. Reis, and A.L.G., unpublished data). Because ATP binding is required for PAN-20S association, it seems likely that only the C termini from the ATP-bound subunits are docked at any instant, and that the interacting subunits alternate as the different subunits are in their ATP- or ADP-bound forms (Smith et al., 2005). Like other members of the AAA family of ATPases, PAN and the 19S ATPases must undergo extensive ATP/ADP-dependent conformational changes, and are thus highly dynamic structures (DeLaBarre and Brunger, 2005; Rouiller et al., 2002; Wang et al., 2001). By contrast, PA28/26 do not exhibit such dynamics or flexibility, which may help explain their different gate-opening mechanisms. In fact, the function of the activation loop requires that PA28/26 be a rigid structure with a symmetric, multivalent, and flush interaction involving a 7:7 matched interface with the α ring. By contrast, the highly dynamic structure of the ATPases, whose conformational changes drive protein unfolding and translocation, probably requires the C-terminal key in a lock mechanism described here and a flexibly tethered 6:7 mismatched interface, which is probably the only type of mechanism by which an ATPase complex can undergo major conformational changes and simultaneously stabilize the open-gate conformation of the α subunits.

The HbYX Motif Functions in Complex Formation and Gating in the 26S Proteasome

Asymmetric binding of the ATPases' C termini to only some of the intersubunit pockets clearly must be functioning in the 26S complex, because the six ATPases in the base of the 19S complex are not identical molecules, and two lack a penultimate tyrosine and are thus likely to interact quite differently with the 20S. In addition, the conformational arrangement of the gate in the eukaryotic 20S is highly asymmetric. Only three of the α subunits' N termini (i.e., α 2, α 3, and α 4) stabilize the closed gate conformation with α 3 being the central player (Groll et al., 2000), and removal of only one N terminus from the 20S's α subunits (α 3) is sufficient to open the gate (Kohler et al., 2001). In addition, mutation in the ATP-binding domain of a single ATPase subunit, Rpt2, blocks gate opening in the yeast

26S (Kohler et al., 2001), and we show here that the C-terminal peptides only from Rpt2 or Rpt5 are able to stimulate gate opening in the mammalian 20S proteasome. Although we do not know how many pockets these peptides bind to, the docking of only one C-terminal HbYX motif into one specific intersubunit pocket may be sufficient to alter the conformation of the α 3 subunit's N terminus and to induce gate opening. This finding that docking by one (or two) HbYX motifs is sufficient for gate opening is in agreement with the steric restriction that only a limited number of the six C termini can interact with the seven intersubunit pockets. Moreover, because single point mutations in the C-terminal YX of Rpt2, Rpt3, or Rpt5 can inhibit gate opening, while still permitting 26S complex formation, the ATPases also must not contain distinct activation domains, like that in PA28/26.

The processes of 19S-20S association and gating are clearly much more complex than in the PAN-20S complex, due to the presence of six different ATPases with distinct C termini and seven distinct intersubunit pockets. Nevertheless, the key in a lock mechanism seems to be conserved from archaea to mammalian proteasomes as shown by carboxypeptidase treatments and mutagenesis, and the C-terminal YX residues appear to be critical for 26S formation and gating.

It is also evident from these results that the individual 19S ATPase's C termini have distinct functions and may be important in complexation, in gating, in both or in neither. For example, the loss of the penultimate tyrosine interferes markedly with 26S stability in the case of Rpt1, while this mutation in Rpt2, Rpt3, and Rpt5 generates fairly stable complexes but with significant gating defects. Furthermore, only the C-terminal octapeptides from Rpt2 and Rpt5 appear capable of triggering gate opening by themselves. While a mutation in PAN's C terminus alters all six of its C termini similarly, it is quite surprising and impressive that a single substitution in only one of the six different ATPases in the 19S still causes gating defects and can destabilize the complex. Also, it is noteworthy that point mutations in Rpt3's C terminus prevented gate opening in yeast proteasomes even though a C-terminal peptide from Rpt3 could not induce gate opening by itself. These observations suggest that while some C termini play specific roles (e.g., rpt2 and rpt5 in gating), these actions may require the function of other neighboring C termini (e.g., rpt3's association with an intersubunit pocket).

The extensive conservation of these ATPase subunits and the similar roles of ATP binding and hydrolysis in the eukaryotic and archaeal complexes make it likely that these Rpt subunits undergo similar coordinated conformational changes as does PAN in driving gate opening, substrate unfolding, and translocation. One obvious difference is that the 26S proteasome is a much more stable structure than the transitory PAN-20S complex, where association and gate opening appear to be linked events, while in the 26S complex, association and gating can be uncoupled by single targeted point mutations, because its different C termini seem to play different roles in gating

and association. Because of its homogeneity and ease of isolation and mutagenesis, PAN clearly offers major advantages for elucidating the gating mechanisms, and elsewhere we shall report the structural changes in the α subunits that underlie gate opening by PAN (D.M.S., A.L.G., J. Rabl, and Y.C., unpublished data). Similar detailed understanding of this key in a lock mechanism in the 26S proteasome will require precise knowledge about the sites of docking of each of the six ATPases' C termini in the core particle's seven intersubunit pockets and the functional consequences of each binding event.

EXPERIMENTAL PROCEDURES

Enzyme Assays

To measure gate opening in the archaeal 20S, LFP (Mca-AKVY-PYPME-Dpa[Dnp]-amide) in DMSO was used as a reporter substrate (Smith et al., 2005) at a final concentration of 10 μ M, and for rabbit 20S, the indicated peptide in DMF was used at 100 μ M. The amount of DMSO or DMF did not exceed 1% of the total volume. To assay gate opening induced by wild-type PAN or the PAN variants, the indicated PAN (1 μ g) and archaeal 20S (0.2 μ g) were incubated in the reaction buffer (50 mM Tris [pH 7.5], 1 mM DTT, and 10 mM $MgCl_2$) with or without ATP γ S (0.1 mM) in a 100 μ l reaction volume. To monitor stimulation of gate opening by peptides for the archaeal or mammalian 20S proteasomes, the indicated peptides were added to the reaction buffer (100 μ l) at 45°C (archaeal 20S; 0.2 μ g) or at 37°C (mammalian 20S; 0.1 μ g), and the rate of peptide hydrolysis was monitored.

Gate opening in rabbit muscle 20S proteasome either by the C-terminal peptides or by the 19S/PA700 complex was assayed similarly but with 3% glycerol in the reaction buffer and at 37°C with the indicated substrate. Assembly reactions for the mammalian 26S proteasome were performed as described (DeMartino, 2005). The 19S preparations contained a background activity that was not proteasomal (i.e., it cleaved nLpNLD-amc, but not LLVY-amc, and was not stimulated by ATP or ATP γ S) and was thus subtracted out. Purified yeast proteasomes were subjected to nondenaturing PAGE and in-gel activity assay using LLVY-AMC as described (Elsasser et al., 2005). LFP hydrolysis was monitored at λ_{ex} 340 nm and λ_{em} 405 nm (Smith et al., 2005), and amc hydrolysis was monitored at λ_{ex} 380 nm and λ_{em} 460 nm. To follow unfolding of GFP-ssrA by carboxypeptidase-treated PAN (1 μ g), its fluorescence was monitored in the reaction buffer at 45°C at λ_{ex} 400 nm and λ_{em} 510 nm. ATP hydrolysis by carboxypeptidase-treated PAN was monitored as previously described (Ames, 1966). Experimental details regarding the tryptophan fluorescence experiments can be found in the [Supplemental Data](#).

Generation of PAN and Yeast Rpt Mutations

PAN mutations were constructed by PCR as described in the [Supplemental Data](#). Yeast mutations were constructed by integrative recombination and expressed from the appropriate endogenous chromosomal locus. While all mutant yeast strains were viable, most mutations cause various and highly complex phenotypes that will be presented elsewhere after further study. Details of strain constructions and genotypes of yeast strains used in this study are found in the [Supplemental Data](#).

Supplemental Data

Supplemental Data include two figures, four tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.molecule.org/cgi/content/full/27/5/731/DC1/>.

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REFERENCES

- Adams, J. (2004). *Proteasome Inhibitors in Cancer Therapy* (Totowa, N.J.: Humana Press).
- Ames, B.N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8, 115–118.
- Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W., and Goldberg, A.L. (2003). ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol. Cell* 11, 69–78.
- Benaroudj, N., Smith, D.M., and Goldberg, A.L. (2005). What the archaeal PAN-proteasome complex and bacterial ATP-dependent proteases can teach us about the 26S proteasome. In *The Ubiquitin-Proteasome System*, R.J. Mayer, A.J. Ciechanover, and M. Rechsteiner, eds. *Volume 2 of Protein Degradation* (Weinheim, Germany: Wiley-VCH).
- Bochtler, M., Hartmann, C., Song, H.K., Bourenkov, G.P., Bartunik, H.D., and Huber, R. (2000). The structures of HslU and the ATP-dependent protease HslU-HslV. *Nature* 403, 800–805.
- Cascio, P., Call, M., Petre, B.M., Walz, T., and Goldberg, A.L. (2002). Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes. *EMBO J.* 21, 2636–2645.
- Coux, O., Tanaka, K., and Goldberg, A.L. (1996). Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* 65, 801–847.
- DeLaBarre, B., and Brunger, A.T. (2005). Nucleotide dependent motion and mechanism of action of p97/VCP. *J. Mol. Biol.* 347, 437–452.
- DeMartino, G.N. (2005). Purification of PA700, the 19S regulatory complex of the 26S proteasome. *Methods Enzymol.* 398, 295–306.
- DeMartino, G.N., and Slaughter, C.A. (1999). The proteasome, a novel protease regulated by multiple mechanisms. *J. Biol. Chem.* 274, 22123–22126.
- Elsasser, S., Schmidt, M., and Finley, D. (2005). Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol.* 398, 353–363.
- Forster, A., Whitby, F.G., and Hill, C.P. (2003). The pore of activated 20S proteasomes has an ordered 7-fold symmetric conformation. *EMBO J.* 22, 4356–4364.
- Forster, A., Masters, E.I., Whitby, F.G., Robinson, H., and Hill, C.P. (2005). The 1.9 Å structure of a proteasome-11S activator complex and implications for proteasome-PAN/PA700 interactions. *Mol. Cell* 18, 589–599.
- Fu, H., Reis, N., Lee, Y., Glickman, M.H., and Vierstra, R.D. (2001). Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J.* 20, 7096–7107.

- Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82**, 373–428.
- Goldberg, A.L. (2005). Nobel committee tags ubiquitin for distinction. *Neuron* **45**, 339–344.
- Goldberg, A.L., Cascio, P., Saric, T., and Rock, K.L. (2002). The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Mol. Immunol.* **39**, 147–164.
- Groll, M., and Huber, R. (2003). Substrate access and processing by the 20S proteasome core particle. *Int. J. Biochem. Cell Biol.* **35**, 606–616.
- Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H.D., and Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463–471.
- Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D.M., Huber, R., Glickman, M.H., and Finley, D. (2000). A gated channel into the proteasome core particle. *Nat. Struct. Biol.* **7**, 1062–1067.
- Guo, F., Maurizi, M.R., Esser, L., and Xia, D. (2002). Crystal structure of ClpA, an Hsp100 chaperone and regulator of ClpAP protease. *J. Biol. Chem.* **277**, 46743–46752.
- Hersch, G.L., Burton, R.E., Bolon, D.N., Baker, T.A., and Sauer, R.T. (2005). Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine. *Cell* **121**, 1017–1027.
- Kisselev, A.F., and Goldberg, A.L. (2001). Proteasome inhibitors: from research tools to drug candidates. *Chem. Biol.* **8**, 739–758.
- Kohler, A., Cascio, P., Leggett, D.S., Woo, K.M., Goldberg, A.L., and Finley, D. (2001). The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. *Mol. Cell* **7**, 1143–1152.
- Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H., and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. *Mol. Cell* **10**, 495–507.
- Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* **268**, 533–539.
- Ma, C.P., Willy, P.J., Slaughter, C.A., and DeMartino, G.N. (1993). PA28, an activator of the 20 S proteasome, is inactivated by proteolytic modification at its carboxyl terminus. *J. Biol. Chem.* **268**, 22514–22519.
- Ogura, T., and Tanaka, K. (2003). Dissecting various ATP-dependent steps involved in proteasomal degradation. *Mol. Cell* **11**, 3–5.
- Rouiller, I., DeLaBarre, B., May, A.P., Weis, W.I., Brunger, A.T., Milligan, R.A., and Wilson-Kubalek, E.M. (2002). Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. *Nat. Struct. Biol.* **9**, 950–957.
- Seong, I.S., Kang, M.S., Choi, M.K., Lee, J.W., Koh, O.J., Wang, J., Eom, S.H., and Chung, C.H. (2002). The C-terminal tails of HslU ATPase act as a molecular switch for activation of HslV peptidase. *J. Biol. Chem.* **277**, 25976–25982.
- Singleton, M.R., Sawaya, M.R., Ellenberger, T., and Wigley, D.B. (2000). Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell* **101**, 589–600.
- Smith, D.M., Kafri, G., Cheng, Y., Ng, D., Walz, T., and Goldberg, A.L. (2005). ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins. *Mol. Cell* **20**, 687–698.
- Smith, D.M., Benaroudj, N., and Goldberg, A. (2006). Proteasomes and their associated ATPases: a destructive combination. *J. Struct. Biol.* **156**, 72–83.
- Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68**, 1015–1068.
- Walz, J., Erdmann, A., Kania, M., Typke, D., Koster, A.J., and Baumeister, W. (1998). 26S proteasome structure revealed by three-dimensional electron microscopy. *J. Struct. Biol.* **121**, 19–29.
- Wang, J., Song, J.J., Seong, I.S., Franklin, M.C., Kamtekar, S., Eom, S.H., and Chung, C.H. (2001). Nucleotide-dependent conformational changes in a protease-associated ATPase HslU. *Structure* **9**, 1107–1116.
- Whitby, F.G., Masters, E.I., Kramer, L., Knowlton, J.R., Yao, Y., Wang, C.C., and Hill, C.P. (2000). Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* **408**, 115–120.
- Zhang, Z., Clawson, A., Realini, C., Jensen, C.C., Knowlton, J.R., Hill, C.P., and Rechsteiner, M. (1998). Identification of an activation region in the proteasome activator REGalpha. *Proc. Natl. Acad. Sci. USA* **95**, 2807–2811.
- Zwickl, P., Ng, D., Woo, K.M., Klenk, H.P., and Goldberg, A.L. (1999). An archaeobacterial ATPase, homologous to ATPases in the eukaryotic 26 S proteasome, activates protein breakdown by 20 S proteasomes. *J. Biol. Chem.* **274**, 26008–26014.