CMLS, Cell. Mol. Life Sci. 61 (2004) 1579–1588
1420-682X/04/131579-10
DOI 10.1007/s00018-004-4131-y

CMLS Cellular and Molecular Life Sciences

Keepers at the final gates: regulatory complexes and gating of the proteasome channel

M. Bajorek and M. H. Glickman*

Department of Biology and the Institute for Catalysis Science and Technology (ICST), Technion – Israel Institute of Technology, Haifa (Israel), Fax: +972 4 8225153, e-mail: glickman@tx.technion.ac.il

Abstract. The proteolytic active sites of the 26S proteasome are sequestered within the central chamber of its 20S catalytic core particle. Access to this chamber is through a narrow channel defined by the outer alpha subunits. Free proteasome 20S core particles are found in an autoinhibited state in which the N-termini of neighboring alpha subunits are anchored by an intricate lattice of interactions blocking access to the channel. Entry of substrates into proteasomes can be enhanced by attachment of activators or regulatory particles. An important part of this activation is channel gating; regulatory particles rearrange the blocking residues to form an open pore and promote substrate entry into the proteolytic chamber. Interestingly, some substrates can open the entrance themselves and thus facilitate their own destruction. In this review, we will discuss the mechanisms proposed for channel gating and the interactions required to maintain stable closed and open conformations.

Key words. Ubiquitin; proteasome; protein degradation; gating.

Introduction

Proteolysis of regulatory proteins allows the organism to adapt to changing environmental and physiological conditions. Proteins are selectively targeted for removal with differing half-lives. Thus, the proteome is in a dynamic state of synthesis and degradation. That the substrates and the components of the system that remove them reside in the same cellular compartments necessitates tightly regulated machinery that uses metabolic energy for control. To attain accurate targeting of such a broad spectrum of substrates, multiple levels of regulation are required. At the broad level, certain conditions can enhance or inhibit bulk protein degradation. At the specific level, processing of individual substrates is tightly controlled at multiple steps, from selection through to degradation.

Degradation of a regulatory protein in eukaryotes normally involves two discrete and successive steps: tagging of the substrate by covalent attachment of multiple ubiquitin molecules ('ubiquitination'), and degradation of the tagged protein by the 26S proteasome concomitant with release of free ubiquitin [1–3]. Regulation of intracellular proteolysis is considered to lie mainly at the level of ubiquitination. Specific E3 ubiquitin ligases each recognize a subset of substrates and tag them by linking the carboxy-terminus of ubiquitin with an amino group on the target protein via an amide bond [2]. Once a substrate is polyubiquitinated, it is either rapidly deubiquitinated by enzymes that remove the ubiquitin label intact, or processed by the 26S proteasome and irreversibly degraded. Naturally, great efforts have focused on understanding substrate selectivity by the E3 ubiquitin ligase enzymes.

The 26S proteasome in eukaryotes is a roughly 2.5-MDa complex made up of at least 32 different subunits arranged in two subcomplexes. Proteolysis takes place within the 20S core particle of the proteasome (CP), a cylindrical structure composed of four stacked heptameric rings engendering a sequestered proteolytic chamber [4–7]. Each of the two identical outer rings is composed of seven structurally similar yet distinct α subunits, and each of the two identical inner rings is formed from seven similar β subunits. The β rings contain the proteolytic active sites, while the outer α rings define a gated channel leading into the internal proteolytic chamber [8–10]. When visualizing the 20S CP barrel from the outside, these subunits are labeled counterclockwise α1 through α7 and β1 through β7, respectively (fig. 1). Thus, the CP barrel exhibits pseu-

* Corresponding author.

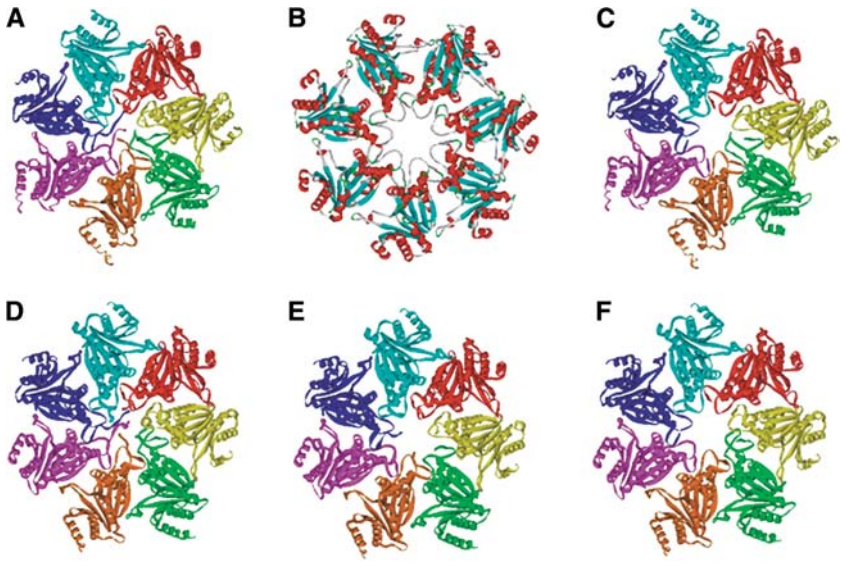

Figure 1. Open and closed conformations of proteasome 20S core particles (CP). Top view of 20S CP from showing ribbon plots the seven-membered α-ring: (*A*) mammalian, (*B*) archaea (*T. acidophilum*), (*C*) mammalian in theoretical open state, (*D*) yeast (*S. cerevisiae*), (*E*) the α3ΔN mutant from yeast, (*F*) yeast in theoretical fully open state. Structures depicted in *A*, *B*, *D*, and *E* are the actual crystal structure determinations extracted from the Protein Data Bank (PDB) and visualized with the Viewerlite program. Structures shown in *C* and *F* are models in which the electron density corresponding to the tail residues of all α subunits described in table 2 were manually deleted to mimic a fully open conformation similar to that found in archaea (*B*). All α subunits are color-coded starting with α1 (light blue) on top, and running counterclockwise to α7 (red). The exception is the archaeal structure that contains a homomeric ring of a single a subunit, and is color-coded for secondary structure. Note that the tertiary structure of all α subunits is remarkably similar, except for a breakdown of symmetry in the center of the ring. The N-terminal tails of the different α subunits adopt different conformations in the closed state and point inwards to block access through the channel (*A*, *D*). Interestingly, these unique conformations are strictly conserved between the two species. Removal of the tail segments unveils a pore in the open state (*C*, *F*). Deletion of the α3 tail alone is pivotal for promoting the open state by causing disordering in neighboring subunits (*C*); however, residual order remains that may explain why proteasomes purified from this mutant do not exhibit maximal activity [15]. Additional details can be found in the original publications [8–10, 36].

do-sevenfold symmetry in the form of an $\alpha_7\beta_7\beta_7\alpha_7$ structure. The 20S CP can hydrolyze short or unstructured polypeptides and, at least in vitro, some proteins with hydrophobic or misfolded patches [11, 12]. To degrade polyubiquitinated substrates, attachment of a 19S regulatory particle (RP) to the surface of the α ring is required. Together, the 19S RP and the 20S CP form the 26S proteasome holoenzyme. The 19S RP recognizes the polyubiquitin signal, binds the substrate and prepares it for proteolysis in the 20S CP by unraveling its tertiary structure and translocating it through the gated channel [13, 14]. Interestingly, the 19S RP also partakes in deubiquitinating (i.e. removing ubiquitin from) its substrates with an outcome that can lead either to release and rescue or to accelerated degradation [15, 16]. As will be detailed below, other regulatory particles can also influence proteolytic activity of the proteasome [4, 17–20]. In archaea and select prokaryotes, a ring of ATPases called the proteasome-activating nucleotidase complex, or PAN, forms a rudimentary regulatory particle that enhances proteolysis by the 20S proteasome. In eukaryotes, a number of non-ATPase activators, such as PA28 or PA26 and other 11S-Reg type complexes, can attach to the outer surface of the 20S CP and enhance rates of peptidase activity, but not proteolysis rates.

At least in vitro, purified eukaryotic 20S CP is usually found in a latent form, for which peptidase activity is repressed and does not reflect the maximal peptidase activity attainable upon activation. Attachment of the 19S regulatory particle activates the peptidase activity by at least an order of magnitude [21, 22]. One manner by which ATPase-containing regulatory particles prepare proteins for proteolysis is by unfolding them, as has been deduced from the chaperone-like activity of the 19S RP and other ATPase-containing regulatory particles such as the base, or in the case of archaeal proteasomes, PAN [14, 23–28]. In this scenario, the ATPase subunits of the regulatory particle use energy released from ATP hydrolysis to unfold the substrate and translocate it into the proteolytic chamber of the 20S where it is hydrolyzed into short peptides. However, the need to unravel globular proteins or extended polypeptides does not adequately explain all modes of 20S CP activation. For instance, 19S regulatory particles with a mutation in one of the ATPases, Rpt2, fail to activate the 20S CP for hydrolysis of short peptides that clearly do not need to be unfolded as they lack any tertiary structure [29]. Furthermore, some proteins with hydrophobic or unfolded patches can enter WT 20S CP without assistance of regulatory particles [30], and constitutively active 20S CP purified from the α3α7ΔN mu-

tant can readily hydrolyze denatured proteins without attachment of any regulatory particle [15]. These observations point to an intrinsic property of the 20S CP that is decisive in switching between the latent and activated forms. This review will focus on the narrow channel that leads substrates into the proteolytic chamber, and on properties that gate this channel to regulate proteolysis.

Gating and regulating proteasome activity

As mentioned, stand-alone eukaryotic 20S CP is found in a repressed state. Structural determination of free 20S CP purified from eukaryotes showed that each of the N-termini of the seven α subunits assumes a unique conformation while pointing inwards to the center of the ring [8–10]. Neighboring tails are anchored by an intricate lattice of interactions blocking access to the channel that leads from the center of the α ring into the proteolytic chamber of the 20S CP (fig. 1). In comparison, structural determination of a 20S CP-PA26 complex depicts the same N-termini pointing upwards and away from the center of the ring in a symmetric open state relieving channel obstruction ([31]; see also fig. 1). The differences in conformations of these N-termini between closed and open states could explain how regulatory particles activate proteolytic activity by rearranging the blocking residues to facilitate substrate entry [27, 31, 32]. Indeed, attachment of regulatory particles such as the 19S RP, the base, and even non-ATPase complexes such as 11S Reg-type activators, enhances peptidase activity (table 1). In light of the structural models, activation of proteolytic properties probably reflects facilitated access of substrates into the proteolytic chamber. However, it should be mentioned that attachment of regulatory particles is not an absolute requirement for proteolytic activation. Free 20S CP can switch between the latent 'closed' form and the activated 'open' conformation spontaneously [33]. Repeated freeze-thaw cycles of purified CP promote the unrepressed conformation [unpublished observations]. Mild chemical treatments, such as exposure to low ionic strength or to levels of sodium dodecylsulfate (SDS) are effective as well. Low ionic strength of solution, small hydrophobic peptides, minutes amount of detergents or specific mutations in channel gating residues can enhance peptide hydrolysis by free-standing 20S CP [9, 15, 22, 31, 32, 34]. It is presumed that all these treatments promote disordering in channel blocking residues, thus facilitating substrate entry. Under physiological conditions, however, it appears that the greater part of free core particles are found naturally in the closed state.

Merely describing the 20S CP in terms of open and closed conformations does not fully explain the diverse properties of proteasome configurations and their relative rates of peptide or protein hydrolysis. Restrictions governing entry of small peptides versus entry of larger proteins appear to be somewhat different. Thus, activation of proteasomes occurs along a continuum reflecting the diverse methods of activation (table 1). For instance, the rate of hydrolysis of small peptides generated by 20S CP upon deletion of N-terminal tail of the α3 subunit (the so-called α3ΔN strain) is enhanced compared to WT [9, 35]. Removal of the first nine residues at the N-ter-

Table 1. Proteasome activation on a sliding scale.

Proteasome	Basal rate peptidase (latent)	Activated for			Reference
		Peptidase (activated)	Protease	Ubiquitin-protein degradation	
26S holoenzyme (α3α7ΔN)*		X	XX(?)	XX(?)	[15]
26S holoenzyme		X	X	X	[16, 21, 22, 59]
20S $CP_{(arch;\ \Delta 1-7\ mut)}$+PAN‡		X	XX		[31, 43]
20S $CP_{(arch)}$+PAN		X	X		[25–27, 37, 38]
20S CP+Base		X	X		[16, 21, 22, 59]
20S CP(α3α7ΔN)		X	X		[15]
20S CP+PA26		X			[31, 43]
20S CP+PA28/11S Reg		X			[46–48, 58, 60]
20S CP+SDS		X			[16, 21, 22, 59]
20S CP(α3ΔN)		X			[9, 29, 35]
20S $CP_{(arch)}$ 20S		X			[25–27, 37, 38]
20S $CP_{(arch;\ YD\ mut)}$+PAN‡		X(?)			[31, 43]
26S holoenzyme *rpt2RF**	X				[9, 29, 35]
20S CP†	X				[16, 21, 22, 59]

* Enhanced ubiquitin-protein degradation is inferred indirectly from in vivo data.

† Latent 20S CP can proteolyze some proteins with hydrophobic or unfolded domains [11, 12, 20, 30, 34].

‡ PAN failed to activate proteolytic activity of archaeal 20S CP that was mutated in the α-subunit YD motif. Peptidase activity is probably similar to basal rate [31].

minus of the α3 subunit causes significant disordering in neighboring tails to open up a channel roughly 13 Å across (fig. 1). This channel opening is sufficient to enhance entry of small peptides, and purified 20S CPs from this strain are found in a constitutively activated state. However, no enhancement of protein degradation rates has been observed with substrates tested so far. Deletion of the equivalent N-terminal tail of α7 does not significantly increase the peptidase activity compared to WT, suggesting that interactions of other subunits (or at least those of α7) do not play a significant role in maintaining the closed conformation [15]. Because of its peripheral location at the α ring surface, truncation of α7 alone may not result in sufficient loss of order in neighboring tails to generate an opening wide enough for entry of small peptides. However, 20S CP from a double truncation of the N-termini of both α3 and α7 subunits (the α3α7ΔN strain) was significantly more efficient in the proteolysis of casein when compared to either of the single truncations [15]. Deletion of tails from two opposing α subunits may act synergistically to relieve hindrance of entry of proteins into the proteolytic chamber. This double mutant is often referred to as the 'open channel' mutant. Thus, gating of the proteolytic channel emerges as a pivotal property in regulating proteolysis rates and possibly selectivity as well.

The behavior of archaeal proteasomes is somewhat different from that of proteasomes from eukaryotes, and may be used to shed light on the importance of gating in proteolysis (table 1). In contrast to the sealed chamber in the eukaryotic complex, crystal structures of 20S proteasomes obtained from archaeal organisms show disordering in the α subunit tail, creating a pore that is aligned with the central channel leading into the proteolytic chamber [36, 37]. This open state accounts for naturally enhanced peptidase rates measured for this complex, rates that cannot be further activated by PAN [25, 37]. Interestingly, even though they are constitutively activated for peptide hydrolysis, archaeal 20S proteasomes do not exhibit maximal protein proteolysis on their own. Proteolysis can be further activated upon attachment of archaeal ATPase-containing regulatory complexes such as PAN [25, 26, 38, 39]. Clearly, unraveling a substrate's tertiary structure in an ATP-dependent manner accounts for a portion of this activation, but there is evidence that PAN also participates in gating the archaeal 20S channel [27, 28, 31]. Despite the appearance of an open pore in archaeal 20S CP, the disordered tails of the α subunits probably introduce a measure of constraint on passage through the channel. This restriction is insufficient to hamper entry of short peptides dramatically, thus PAN has no effect on the intrinsic peptidase rate that is already found at elevated levels. In has been proposed that PAN facilitates entry and translocation of polypeptides by imposing an ordered conformation on tail residues, hence the experimental activation [31]. A similar situation probably occurs upon attachment of the ATPase-containing base subcomplex to eukaryotic core particles, which is sufficient to enhance proteolysis of non-ubiquitinated substrates (table 1).

Mechanisms of gating

What are the mechanisms that are put in place for maintaining distinct open and closed conformations, and how does attachment of regulatory particles promote switching to the open state? In the repressed state, the N-termini of the seven α subunits of the 20S CP from eukaryotes point towards the center of the ring sealing the entrance to the proteolytic channel (fig. 1). The corresponding region, which covers the first 12 residues in the archaeal complex, is naturally disordered, giving the impression of an open channel. The most striking difference between the α ring of eukaryotic and archaeal core particles is that the former is made up of seven (similar yet) different subunits [8, 10], whereas the latter comprises seven copies of a single gene product [33, 36, 37]. The tail region that is N-terminal to Thr13 in the single α subunit of the 20S CP from *Thermoplasma acidophilum* is disordered, accounting for the appearance of an open pore in archaeal proteasomes. In eukaryotes, the paralogous α subunits show structural and sequence similarities over the bulk of the protein, yet diverge at their amino-terminal region in both sequence and relative length (table 2). Interestingly, the heterogeneity in the tail regions and the formation of a well-defined closed configuration in free 20S core particles from eukaryotes involves a dramatic departure from the pseudo-sevenfold symmetry of the CP. Precisely at the center of the ring each tail accepts a unique conformation (fig. 1). The tail of α3 is somewhat distinct from the others in that it points directly across the surface of the α ring towards the center, maintaining close contacts to every other α subunit (fig. 1). The importance of these tail regions is highlighted by their extreme conservation across eukaryotes; while each tail is highly conserved in different species, the corresponding regions are divergent from one subunit to another (table 2). These properties suggest that the N-termini play a critical structural role that has been maintained in core particles in all eukaryotes, and it is precisely the difference between them that is integral to their function.

In order for substrates to enter the proteolytic chamber, and most likely for products to exit as well, the blocking N-terminal residues of the α subunits in the closed state must be rearranged. Rearrangement obviously necessitates breaking the interactions that anchor the tails in the closed conformation, while forming competing interactions to stabilize them in an open conformation. In the following paragraphs, we will discuss the nature of these

Table 2. Conservation of core particle α-subunit amino-termini.

Subunit	Homologs (old names)	N-terminal sequence	% Identity/similarity in tail	% Identity over entire protein
α1	S.c. C7	SAAGYDRHI	78/100	51
	H.s. iota	SSAGFDRHI		
α2	S.c. Y7	MTDR-YSFSL	70/90	57
	H.s. C3	MAERGYSFSL		
α3	S.c. Y13	MGSRRYDSRT	90/90	53
	H.s. C9	M-SRRYDSRT		
α4	S.c. Pre6	MSGYDRAL	75/88	59
	H.s. C6	MS-YDRAI		
α5	S.c. Pup2	SEYDRGV	100/100	62
	H.s. Zeta	SEYDRGV		
α6	S.c. Pre5	MFRNNYDGDT	70/80	52
	H.s. C2	MFRNQYDNDV		
α7	S.c. C1	MTSIGTGYDLSN	83/92	51
	H.s. C8	MSSIGTGYDLSA		

The N-terminal sequence up to the first α-helical structure of each CP α subunit, as determined by the crystal structure of the yeast CP [8, 9] is shown. This region is homologous to the disordered segments in the N-terminal regions of the α subunit from *T. acidophilum* [36, 37]. Dark- or light-grey highlighted amino acids indicate identical or similar residues, respectively, between yeast and human subunits. The YD(R) motif in each tail is underlined and bold. Note that in all subunits from yeast a tyrosine residue is present in the same location. In six of the seven subunits, an aspartate follows, and in three, an arginine completes the YDR motif. The remainder of the tail region is divergent among subunits though, as shown in the table, it is significantly conserved in each subunit among organisms.

interactions, and the possible mechanisms proposed to switch between closed and open conformations.

The YDR motif and the YD-P-Y cluster in closing and opening the channel

Embedded within the N-terminal segments of most α subunits is a short consensus sequence: Tyr8-Asp9-Arg10, or 'the YDR motif'. Conservation of tyrosine at position 8 is absolute among subunits from yeast (and is invariable at this location in most subunits from other organisms as well); aspartate at position 9 is present in the tail of all yeast subunits except for α2, while conservation of arginine as residue number 10 is less strict (table 2). Interestingly, the YDR sequence is found intact in the α subunits from various archaea as well [37]. The remarkable conservation of the YDR motif suggests a key role for this element.

Interactions involving YDR residues could be critical for maintaining distinct open and closed conformations of 20S proteasomes. Indeed, detailed crystallography analysis of yeast 20S CP shows that side chains of YDR residues participate in stabilization of the closed state of the channel. In particular, Asp9 of α3 contacts both Tyr8 and Arg10 in neighboring α4: a salt bridge is formed between the carboxylate group of Asp9 in α3 and the guanidinium group of Arg10 in α4, simultaneous with a hydrogen bond linking Asp9 of α3 with Tyr8 of α4 [9]. Similar bonds probably link the analogous residues in mammalian 20S CP ([10]; see also fig. 2). The direct contacts formed between these residues in adjacent subunits may explain their correlated evolutionary conservation. The strict conservation of the YDR residues in other subunits makes it somewhat puzzling that crystal structure determination did not pick out similar contacts between other neighboring subunits in the pore region. This raises the possibility that the interaction between α3 and α4 plays a unique and central role in maintaining the close conformation of the proteasome. Additionally, the YDR motif in other subunits may be important for other reasons. Support for the pivotal role of α3 in gating the 20S CP channel was provided upon truncation of the tail region of α3. Truncation of the N-terminus of the α3 subunit in yeast (the α3ΔN mutant) resulted in purified 20S CP that was found in the open pore conformation (figs 1, 2). Furthermore, removal of the N-terminus of α3 caused disordering in neighboring subunits concomitant with stimulation of 20S CP peptidase activity. These results indicate that the tail of α3 is important for stabilizing neighboring tails in the closed conformation. Moreover, an aspartate to alanine substitution in YDR of α3 (the α3 D9A mutant) appears to increase peptidase activity of purified 20S CP in vitro, on par with the activation observed upon deletion of the entire tail region in α3ΔN [9]. To conclude, both mutations associated with α3 point to a functional significance of the YDR motif in stabilization of the closed state of the gate.

The existence of the conserved YDR motif in the α subunits of archaeal 20S proteasome suggests that the mechanism of gating may be evolutionarily conserved. Surprisingly, however, studies on archaeal proteasome point to the YDR motif as participating in channel opening rather than stabilizing the closed state. The crystal struc-

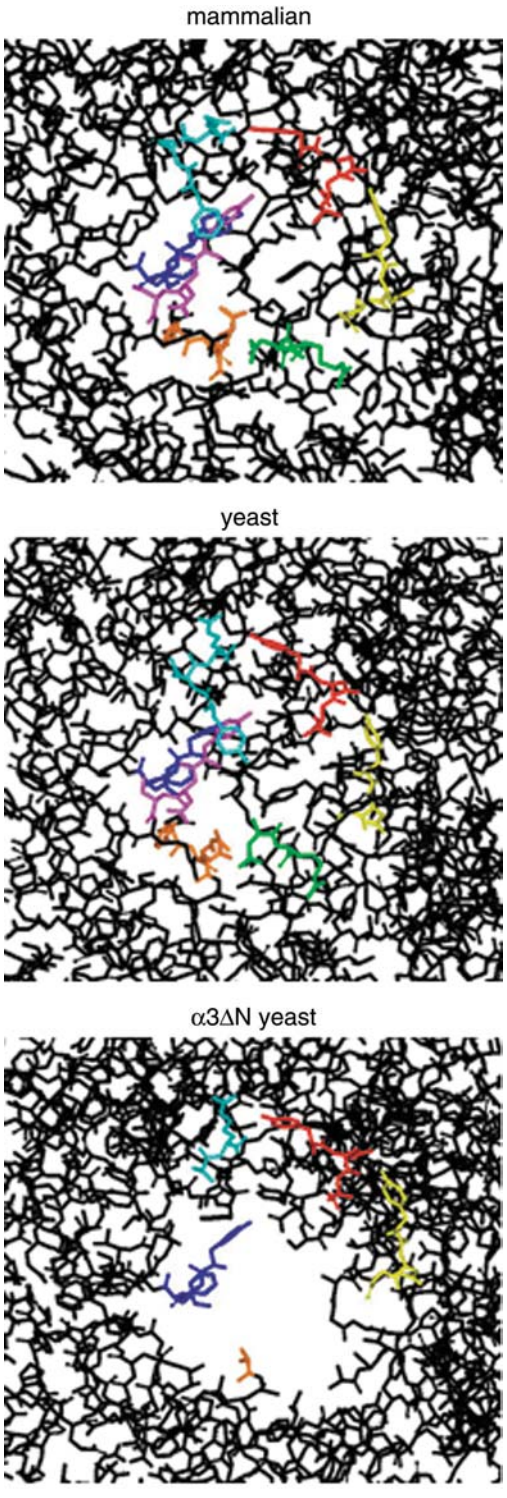

Figure 2. The YD(R) motif in the closed state of proteasome 20S core particles. Close-up of the pore region in the α ring. (*A*) mammalian, (*B*) yeast (wt), (*C*) yeast (α3ΔN). Only residues of the YDR motif (or the replacement amino acid where appropriate; see table 2) are color coded as in figure 1. Note that the configuration of the YDR motif exemplifies the breakdown of symmetry at the center of the ring. In the closed state, the residues that correspond to the YDR motif in each subunit adopt unique conformations and form distinct contacts with neighboring tails. These interactions are strictly conserved in proteasomes from the two species.

ture of 20S from archaea revealed that the N-terminal tails (12 amino acids) of the α subunits are disordered; nevertheless, they occupy the pore region and provide a partial barrier to passage of protein substrates. Only small peptides can enter rapidly. Precisely the region that is disordered encompasses the YDR motif [36, 37]. The lack of defined electron density reflects that under the experimental conditions multiple conformations of these segments are possible between individual molecules in the sample. Apparently, mutual interactions were not strong enough to anchor these tails into a single stable conformation resulting in opening of the channel. Evidence that the YDR motif may play a role in stabilizing the open state was provided when studying an archaeal proteasome assembly intermediate containing only the α subunits: the α_7 ring. During proteasome biogenesis, the seven α subunits form an intermediate homomeric ring, which only then interacts with β subunits and finally dimerizes to yield the mature complex with $\alpha_7\beta_7$ composition. In contrast to mature archaeal 20S CPs, structure determination of this α_7 ring precursor found the N-terminal segments anchored in a stable, open state [37]. In this conformation, the tail regions that contain the YDR adopted a helical structure motif and pointed away from the ring surface. Tyr8 of each subunit made a hydrogen bond with Asp9 of the preceding α subunit, whereas Arg10 pointed inwards towards the central channel and did not partake in anchoring neighboring tails. The region N-terminal to Tyr8 of each subunit was disordered, creating a channel roughly 13 Å in diameter.

The single interaction between the N-termini of the α subunits in the α_7 ring precursor appears to be broken in mature archaeal 20S CPs, causing disordering in a greater portion of the tail regions up to residue number 12 (inclusive). These disordered tails, which are not stably anchored, partially block the pore, and thus while giving the impression of an open channel by X-ray crystallography actually interfere with the passage of proteins through the channel. Attachment of regulatory complexes such as PAN can activate archaeal proteasomes for proteolysis (table 1 and references therein). As mentioned above, activation is due in part to ATP-dependent unraveling of substrates, but also includes participation of PAN in channel gating [27]. Could attachment of PAN promote anchoring of the disordered blocking tails in the stable open conformation, such as that observed for the α_7 ring precursor? Mutagenesis studies support just that. GFP-ssrA is a poor substrate of purified archaeal 20S proteasomes, but is rapidly proteolyzed upon addition of PAN. In an illuminating study, PAN was unable to activate proteolysis of GFP-ssrA by 20S proteasomes from the archaeon *Thermoplasma acidophilum* that were mutated in Tyr8 or Asp9 of their α subunit [31]. Substituting Asp9 to serine or alanine, and likewise mutating Tyr8 to alanine or phenylalanine, dramatically mitigated the ability of PAN

to activate proteolysis. Mutating Arg10 to alanine also slowed down GFP degradation, but to a significantly lesser extent, in agreement with the minor role that Arg10 plays in stabilizing the 'active' open conformation. The suggestion is that PAN imposes order on the α tails, and promotes switching their conformation to the open state. A recent study indicates that stabilizing the N-termini in the open conformation necessitates additional interactions involving residues besides the YDR motif. Crystallography analysis of a PA26-20S CP complex revealed the α ring in an ordered open symmetric conformation [31]. Attachment of PA26 induces all seven of the α-subunit N-terminal tails to adopt an ordered conformation for residues 7–12 away from the center of the ring. A cluster of four highly conserved residues, Tyr8 and Asp9 (from the YDR motif in the tail) together with downstream residues Pro17 and Tyr26 are critical for the open state. Tail residues make only limited interactions with PA26; however, attachment of PA26 to the surface of the α ring repositions Pro17, which in turn induces a conformational change in tail segments. In stark contrast to the closed state, the open state is remarkably symmetric, with all tails conforming to a similar structure held in place by similar interactions. Asp9 forms a hydrogen bond with Tyr26 situated in the first stable α helix (HO), causing the tail to lift up from the surface of the ring and away from the center. Cooperativity between subunits is communicated via an additional hydrogen bond linking Asp9 and Tyr8 of the preceding (clockwise) tail [31]. Consequently, the hydrogen bond that holds Asp9 and Ty8 of α3 and α4, respectively, in the closed state must be broken and rearranged to allow for the open state. Switching between these two conformational regions is the key to proteasome gating. Undoubtedly, a principal role of regulatory particles is to promote channel gating by stabilizing one conformation over the other. However, that the interactions stabilizing the open conformation do not include direct interactions with PA26 may explain how in some instances 20S CPs could spontaneously adopt the activated form [33].

Interestingly, the importance of the Tyr8, Asp9, Pro17 and Tyr26 cluster in channel opening appears to hold true for archaeal and eukaryotic proteasomes alike, even though they probably employ different mechanisms for maintaining the closed state. The interactions occurring in the pore region of eukaryotic PA26-20S complex are quite similar to those found in the open α_7-ring precursor complex from archaea. As discussed above, the interaction of Asp9 in each subunit with Tyr8 of the preceding tail in α_7-rings are reminiscent of the structure observed for the PA26-activated eukaryotic complex. Strong support for this mechanism also comes from in vitro proteolysis assays. Whereas the WT archaeal 20S proteasomes degrade protein substrates rapidly in an assay with PAN, proteasome mutants in any of the cluster residues (Tyr8, Asp9, Pro17 and Tyr26) showed greatly reduced proteolytic activity [31]. This result points to each residue in the conserved YD-P-Y cluster as critical for stabilizing the open conformation. It may be concluded that disordered tail residues impinge on substrate entry, explaining the requirement for a stable open conformation. In agreement, proteasomes lacking residues 2–7 or 2–12 in their α subunits possess higher protease activity when activated by PAN compared to WT. By removing all blocking residues, there is no need to stabilize the open conformation in the Δ2–12 mutant. In contrast, the 2–7 deletion does not cover the conserved tyrosine and aspartate residues (residues eight and nine respectively) that participate in stabilizing the open conformation.

Gating and regulatory complexes

A number of ATP-independent activators are known to attach to the 20S CP and activate its peptidase activity. These include the 11S Reg/PA28, PA26 and PA200 [40–47]. PA28, for example, increases V_{max} for hydrolysis of certain peptides by the 20S CP by up to 100-fold, but as opposed to ATPase-containing activators (such as the 19S RP or PAN) does not promote protein degradation by the 20S CP [40, 48]. In light of the crystal structure documenting how attachment of PA26 promotes channel opening, enhanced proteolytic activity can be attributed to facilitated substrate entry through the open pore. *Saccharomyces cerevisiae* appears to lack homologs of this class of activators; however, a PA26-20S hybrid complex was formed in vitro by introducing 20S from *S. cerevisiae* with PA26 from *Thermoplasma brucei*. Since PA26 is a species-specific complex not found in other organisms, the generality of the implications has to be taken with caution. Nevertheless, PAN appears to drive formation of the open conformation of archaeal proteasomes in much the same manner: the same YD-P-Y cluster is critical for activation of archaeal 20S proteasome by PAN, as for channel opening in yeast 20S CP by PA26.

Evidence linking the 19S RP to gating can be deduced from a substitution mutation in the ATP binding site of a single ATPase (*RPT2*) that severely affects peptidase activity of the proteasome, probably due to hampering the ability of the RP to properly gate the channel into the CP [29, 35]. This indicates that even the entry of small peptides – which do not need to be unfolded – can be controlled by the RP. Furthermore, a constitutively open channel 20S CP generated upon deletions of tail residues in α3 and α7 subunits (α3α7ΔN mutant) exhibits activated peptidase activity similar to that measured for 26S proteasome holoenzymes [15]. This suggests that attachment of the 19S RP realigns the α-subunit tails to facilitate passage of substrates.

Ultimately, the question remains how channel gating in 26S proteasome holoenzymes regulates proteolysis. It is tempting to propose that attachment of the 19S RP in eukaryotes activates the proteasome by promoting channel opening. However, it should be emphasized that the 19S RP is a heterogeneous complex that contains six different ATPases, in contrast to the homomeric ring-of-seven, PAN. Due to the symmetry mismatch, it is not inconceivable that the two regulators induce different conformational changes on the sevenfold symmetry of the α ring. Furthermore, even though it is highly likely, it has not yet been established that the six ATPases form a six-membered ring in the 19S RP, and if so, whether they all interact with the seven-membered α ring. So far, the pair Rpt2-α3 has been shown to be involved in gating the channel into the CP [9, 29, 35], though gating may be controlled by additional Rpt-α subunit interactions. For instance, a limited subset of Rpt subunits have been found to come in direct contact with an α subunit (α2-Rpt4, α2-Rpt5, α4-Rpt4, α7-Rpt4, α1-Rpt6, α2-Rpt6, α4-Rpt2, α6-Rpt4; [49–52]). As there does not appear to be a simple pairing of Rpt with α subunits, the resulting 26S proteasome holoenzyme may not engender the same 'symmetric' open state observed for the PA26-20S CP hybrid.

Substrate-facilitated gating

Although it was generally thought that substrates must be recognized by 19S RP via ubiquitin in order to be degraded, recent papers show that some natively disordered substrates can enter the proteasome without assistance of activators [11, 12]. The ability of latent 20S CP to catalyze cleavage of some unfolded proteins suggests that they possess certain features that promote gating to facilitate their own entry. This mechanism of gate opening is not general; latent 20S CP shows slower proteolysis rates towards many substrates when compared with activated proteasomes (table 1 and references therein). Nevertheless, some substrates with unfolded domains or hydrophobic patches are proteolyzed by latent 20S CP from eukaryotes even faster than by 26S holoenzymes [30]. Presumably, specific sequence motifs in the substrate interact with the channel residues in some – or all – α subunits, and aid in channel opening. These 20S CP recognition/gating sequences are transferable; For example, p21 and α-synuclein facilitate their own degradation, and when fused to stable and hard-to-degrade proteins such as green fluorescent protein, promote their degradation as well. Support for such a mechanism can be deduced from certain peptides that interact with the proteasome in a noncompetitive way to modulate the proteolytic activity of the proteasome [34, 53]. This stimulation was not observed for open channel proteasomes (such as α3ΔN or the PA26-20S CP complex), suggesting that they specifically interact with channel gating residues and promote channel opening. Whether these interactions involve the YDR motif or the YD-P-Y cluster in α subunits similar to the manner by which regulatory particles activate proteasome activity has not yet been elucidated.

Conclusions

It appears that proteasome-dependent proteolysis is a regulated process that can be enhanced or inhibited under certain conditions. This would be especially acute if proteolysis itself is a rate-limiting step. Indeed, enhancement of overall in vivo proteolysis rates observed in the open channel mutant indicates that the proteasome may be partially rate limiting in the overall cascade of ubiquitin-dependent protein degradation [15]. Polyubiquitinated substrates must be stable enough, even if only transiently, to allow for competition between degradation and reversal of fate. Channel gating within 26S holoenzymes may participate in the delicate balance of proteolysis and rescue.
One reason for a gated channel in the CP could be to serve as a transition from one form of inhibition to another during assembly of the mature CP. In the final stage of CP assembly, self-compartmentalization is achieved by the association of two $\alpha_7\beta_7$ half-CPs at the β-β interface. These half CPs are inactive due to pro-peptides in the critical β subunits that mask their active site. As these half-CPs are joined, inhibition by β-subunit N-termini is relieved by autolysis [8, 54], while inhibition by the blocking N-termini of the α subunits is imposed. Binding of the regulatory particles relieves this inhibition by opening the channel, thus activating proteolysis. There is increasing evidence that (at least in yeast) certain stress constitutions such as prolonged starvation or severe heat shock naturally promote proteasome dissociation into separate 20S CP and 19S RP subcomponents [15, 55, 56]. These conditions may require repressed proteasome-dependent degradation for survival. One manner by which proteasome activity could be downregulated is by reinstating autoinhibition of the dissociated 20S CP. Indeed, the open channel mutant that lacks the ability to enter the closed conformation exhibits low viability under conditions that promote proteasome disassembly [15].
An additional reason for a gated channel could be to regulate exit of products from the proteasome. It is possible that under normal conditions product release is slowed down by a gated channel in order to increase processivity or decrease average peptide length. Most of these short peptides are quickly removed from the cytoplasm. Under certain conditions (such as during immune response), it might be beneficial to produce peptides with other lengths or properties. For example, upon interferon-γ induction, attachment of PA28/11S-Reg plays a role in antigen processing by altering the makeup of peptides gener-

ated by the hybrid proteasome 19S RP-20S CP-11S-Reg complexes [46, 47, 57, 58]. In analogy to the distantly related PA26, PA28 probably attaches to the α-ring surface and rearranges the blocking N-termini, promoting the open channel conformation. It is possible that the open state increases the exit rate of peptides generated in the proteolytic chamber and alters their makeup to fit better antigen presentation requirements.

1 Glickman M. H. and Ciechanover A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. **82:** 373–428
2 Pickart C. M. (2001) Mechanisms underlying ubiquitination. Annu. Rev. Biochem. **70:** 503–533
3 Weissman A. M. (2001) Themes and variations on ubiquitylation. Nat. Rev. Cell Mol. Biol. **2:** 169–179
4 Zwickl P. and Seemueller E. (2002) 20S proteasomes. Curr. Topics Microbiol. Immunol. **268:** 23–41
5 Heinemeyer W. and Wolf D. H. (2000) Active sites and assembly of the 20S proteasome. In: Proteasomes: The World of Regulatory Proteolysis, pp. 48–70, Wolf D. H. and Hilt W. (eds), Eurekah.com/Landes Bioscience, Georgetown, TX
6 Bochtler M., Ditzel M., Groll M., Hartmann C. and Huber R. (1999) The Proteasome. Annu. Rev. Biophys. Biomol. Struct. **28:** 295–317
7 Groll M. and Huber R. (2003) Substrate access and processing by the 20S proteasome core particle. Int. J. Biochem. Cell Biol. **35:** 606–616
8 Groll M., Ditzel L., Loewe J., Stock D., Bochtler M., Bartunik H. D. et al. (1997) Structure of 20S proteasome from yeast at a 2.4 Angstrom resolution. Nature **386:** 463–471
9 Groll M., Bajorek M., Koehler A., Moroder L., Rubin D., Huber R. et al. (2000) A gated channel into the core particle of the proteasome. Nat. Struct. Biol. **7:** 1062–1067
10 Unno M., Mizushima R., Morimoto Y., Tomisugi Y., Tanaka K., Yasuoka N. et al. (2002) The structure of the mammalian 20S proteasome at 2.75 A resolution. Structure **10:** 609–618
11 Orlowski M. and Wilk S. (2003) Ubiquitin-independent proteolytic functions of the proteasome. Arch. Biochem. Biophys. **415:** 1–5
12 Forster A. and Hill C. P. (2003) Proteasome degradation: enter the substrate. Trends Cell Biol. **13:** 550–553
13 Hartmann-Petersen R., Seeger M. and Gordon C. (2003) Transferring substrates to the 26S proteasome. Trends Biochem. Sci. **28:** 26–31
14 Liu C.-W., Millen L., Roman T. B., Xiong H., Gilbert H. F., Noiva R. et al. (2002) Conformational remodeling of proteasomal substrates by PA700, the 19S regulatory complex of the 26S Proteasome. J. Biol. Chem. **277:** 26815–26820
15 Bajorek M., Finley D. and Glickman M. H. (2003) Proteasome disassembly and downregulation is correlated with viability during stationary phase. Curr. Biol. **13:** 1140–1144
16 Guterman A. and Glickman M. H. (2003) Complementary roles for Rpn11 and Ubp6 in deubiquitination and proteolysis by the proteasome. J. Biol. Chem. **279:** 1729–1738
17 Gille C., Goede A., Schloetelburg C., Preissner R., Kloetzel P.-M., Gobel U. B. et al. (2003) A comprehensive view on proteasomal sequences: implications for the evolution of the proteasome. J. Mol. Biol. **326:** 1437–1448
18 Volker C. and Lupas A. (2002) Molecular evolution of proteasomes. In: Curr. Top. Microbiol. and Immunol., vol. 268, pp. 1–22, Zwickl P. and Baumeister W. (eds), Springer, Berlin
19 Maupin-Furlow J. A., Wilson H. L., Kaczowka S. J. and Ou M. S. (2000) Proteasomes in the archaea: from structure to function. Front. Biosci. **5:** D837–D865
20 Groll M. and Clausen T. (2003) Molecular shredders: how proteasomes fulfill their role. Curr. Opin. Struct. Biol. **13:** 665–673
21 Adams G. M., Crotchett B., Slaughter C. A., DeMartino G. N. and Gogol E. P. (1998) Formation of proteasome-PA700 complexes directly correlates with activation of peptidase activity. Biochem. **37:** 12927–12932
22 Glickman M. H., Rubin D. M., Fried V. A. and Finley D. (1998) The regulatory particle of the *S. cerevisiae* proteasome. Mol. Cell Biol. **18:** 3149–3162
23 Braun B. C., Glickman M. H., Kraft R., Dahlmann B., Kloetzel P. M., Finely D. et al. (1999) The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. **1:** 221–226
24 Strickland E., Hakala K., Thomas P. J. and DeMartino G. N. (2000) Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26S proteasome. J. Biol. Chem. **275:** 5565–5572
25 Zwickl P., Ng D., Woo K. M., Klenk H. P. and Goldberg A. L. (1999) An archaebacterial ATPase, homologous to ATPases in the eukaryotic 26 S proteasome, activates protein breakdown by 20 S proteasomes. J. Biol. Chem. **274:** 26008–26014
26 Navon A. and Goldberg A. L. (2001) Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. Mol. Cell **8:** 1339–1349
27 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
28 Ogura T. and Tanaka K. (2003) Dissecting various ATP-dependent steps involved in proteasomal degradation. Mol. Cell **11:** 3–5
29 Rubin D. M., Glickman M. H., Larsen C. N., Dhruvakumar S. and Finley D. (1998) Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. EMBO J. **17:** 4909–4919
30 Liu C.-W., Corboy M. J., DeMartino G. N. and Thomas P. J. (2003) Endoproteolytic activity of the proteasome. Science **299:** 408–411
31 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
32 Kohler A., Bajorek M., Groll M., Moroder L., Rubin D. M., Huber R. et al. (2001) The substrate translocation channel of the proteasome. Biochimie **83:** 325– 332
33 Osmulski P. A. and Gaczynska M. (2002) Nanoenzymology of the 20S proteasome: proteasomal actions are controlled by the allosteric transition. Biochemistry **41:** 7047–7053
34 Kisselev A. F., Kaganovich D. and Goldberg A. L. (2002) Binding of hydrophobic peptides to several non-catalytic sites promotes peptide hydrolysis by all active sites of 20 S proteasomes. Evidence for peptide-induced channel opening in the alpha-rings. J. Biol. Chem. **277:** 22260–22270
35 Koehler A., Cascio P., Legget 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
36 Loewe J., Stock D., Jap B., Zwickl P., Baumeister W. and Huber R. (1995) Crystal structure of the 20S proteasome from the archeon *T. acidophilum* at 3.4 Angstrom resolution. Science **268:** 533–539
37 Groll M., Brandstetter H., Bartunik H., Bourenkow G. and Huber R. (2003) Investigations on the maturation and regulation of archaebacterial proteasomes. J. Mol. Biol. **327:** 75–83
38 Wilson H. L., Ou M. S., Aldrich H. C. and Maupin-Furlow J. (2000) Biochemical and physical properties of the *M. jannaschii* 20S proteasome and PAN, a homolog of the ATPase (Rpt) subunits of the eucaryal 26S proteasome. J. Bacteriol. **186:** 1680–1692

39 Benaroudj N. and Goldberg A. L. (2000) PAN, the proteasome-activating nucleotidase from archaebacteria, is a protein-unfolding molecular chaperone. Nat. Cell Biol. **2:** 833–839
40 Ma C. P., Slaughter C. A. and DeMartino G. N. (1992) Identification, purification and characterization of a protein activator (PA28) of the 20S proteasome. J. Biol. Chem. **267:** 10515–10523
41 Realini C., Jensen C. C., Zhang Z., Johnston S. C., Knowlton J. R., Hill C. P. et al. (1997) Characterization of recombinant REGα, REGβ, and REGγ proteasome activators. J. Biol. Chem. **272:** 25483–25492
42 Hendil K. B., Khan S. and Tanaka K. (1998) Simultaneous binding of PA28 and PA700 activators to 20S proteasomes. Biochem. J. **332:** 749–754
43 Yao Y., Huang L., Krutchinsky A., Wong M. L., Standing K. G., Burlingame A. L. et al. (1999) Structural and functional characterizations of the proteasome-activating protein PA26 from *Trypanosoma brucei*. J. Biol. Chem. **274:** 33921–33930
44 McCutchen-Maloney S. L., Matsuda K., Shimbara N., Binns D. D., Tanaka K., Slaughter C. A. et al. (2000) cDNA cloning, expression and functional characterization of PI31, a proline-rich inhibitor of the proteasome. J. Biol. Chem. **275:** 18557–18565
45 Ustrell V., Hoffman L., Pratt G. and Rechsteiner M. (2002) PA200, a nuclear proteasome activator involved in DNA repair. EMBO J. **21:** 3516–3525
46 Rechsteiner M., Realini C. and Ustrell V. (2000) The proteasome activator 11S REG (PA28) and class I antigen presentation. Biochem. J. **345:** 1–15
47 Stohwasser R., Salzmann U., Ruppert T., Kloetzel P. M. and Holzhuetter H. G. (2000) Kinetic evidences for facilitation of peptide channeling by the proteasomal activator PA28. Eur. J. Biochem. **267:** 6221–6230
48 Dubiel W., Pratt G., Ferrell K. and Rechsteiner M. (1992) Purification of an 11S regulator of the multicatalytic protease. J. Biol. Chem. **267:** 22369–22377
49 Coux O. (2003) An interaction map of proteasome subunits. Biochem. Soc. Trans. **31:** 465–469
50 Davy A., Bello P., Thierry-Mieg N., Vagilo P., Hitti J., Doucette-Stamm L. et al. (2001) A protein-protein map of the *C. elegans* 26S proteasome. EMBO Rep. **2:** 821–828
51 Fu H. Y., Reis N., Lee Y., Glickman M. H. and Vierstra R. (2001) Subunit interaction maps for the regulatory particle of the 26s proteasome and the cop9 signalosome reveal a conserved core structure. EMBO J. **20:** 7096–7107
52 Ferrell K., Wilkinson C. R., Dubiel W. and Gordon C. (2000) Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem. Sci. **25:** 83–88
53 Papapostolou D., Coux O. and Reboud-Ravaux M. (2002) Regulation of the 26S proteasome activities by peptides mimicking cleavage products*1. Biochem. Biophys. Res. Commun. **295:** 1090–1095
54 Chen P. and Hochstrasser M. (1996) Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. Cell **86:** 961–972
55 Zhang F., Su K., Yang X., Bowe D. B., Paterson A. J. and Kudlow J. E. (2003) O-GlcNAc modification is an endogenous inhibitor of the proteasome. Cell **115:** 715–725
56 Imai J., Maruya M., Yashiroda H., Yahara I. and Tanaka K. (2003) The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. EMBO J. **22:** 3557–3567
57 Groettrup M., Soza A., Eggers M., Kuehn L., Dick T. P., Schild H. et al. (1996) A role for the proteasome regulator PA28a in antigen presentation. Nature **381:** 166–168
58 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
59 Glickman M. H., Rubin D. M., Coux O., Wefes I., Pfeifer G., Cjeka Z. et al. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9/Signalosome and eIF3. Cell **94:** 615–623
60 Tanahashi N., Murakami Y., Minami Y., Shimbara N., Hendil K. B. and Tanaka K. (2000) Hybrid proteasomes: induction by interferon-gamma and contribution to ATP-dependent proteolysis. J. Biol. Chem. **275:** 14336–14345