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Review

Proteasomal AAA-ATPases: Structure and function ☆

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ABSTRACT

The 26S proteasome is a chambered protease in which the majority of selective cellular protein degradation takes place. Throughout evolution, access of protein substrates to chambered proteases is restricted and depends on AAA-ATPases. Mechanical force generated through cycles of ATP binding and hydrolysis is used to unfold substrates, open the gated proteolytic chamber and translocate the substrate into the active proteases within the cavity. Six distinct AAA-ATPases (Rpt1–6) at the ring base of the 19S regulatory particle of the proteasome are responsible for these three functions while interacting with the 20S catalytic chamber. Although high resolution structures of the eukaryotic 26S proteasome are not yet available, exciting recent studies shed light on the assembly of the hetero-hexameric Rpt ring and its consequent spatial arrangement, on the role of Rpt C-termini in opening the 20S 'gate', and on the contribution of each individual Rpt subunit to various cellular processes. These studies are illuminated by paradigms generated through studying PAN, the simpler homo-hexameric AAA-ATPase of the archaeal proteasome. The similarities between PAN and Rpts highlight the evolutionary conserved role of AAA-ATPase in protein degradation, whereas unique properties of divergent Rpts reflect the increased complexity and tighter regulation attributed to the eukaryotic proteasome. This article is part of a Special Issue entitled: AAA ATPases: structure and function.

1. Introduction

The AAA (ATPases associated with various cellular activities) family is a large group of ATPases found in all biological kingdoms and characterized by the presence of one or two conserved ATP-binding domains of a type called the AAA motif. This 200–250 amino-acid domain is defined by sequence and structural properties. Each AAA domain contains Walker A and Walker B motifs, and a SRH (second region of homology) motif located C-terminally to the Walker B motif. A broader classification, termed the AAA+ superfamily (includes but not limited to AAA family), is ring-shaped P-loop NTPases, yet some lack the SRH [1–5]. The AAA subclass of ATPases forms rings, usually hexameric, with a central pore and it generates mechanical force by undergoing conformational changes during cycles of ATP binding and hydrolysis [6–10]. This mechanical force is used to induce conformational remodeling of a wide range of substrates, including proteins and polynucleotides, thus engage these AAA-ATPases in diverse cellular processes. In all organisms, AAA-ATPases are involved in essential processes of protein degradation and DNA replication. In eukaryotes they also participate in membrane fusion and movement of microtubule motors; their involvement in thermotolerance is restricted to bacteria, fungi and plants [11–14]. The ability of AAA+ machines to perform mechanical work is exemplified by the ClpX of the ATP-dependent bacterial protease ClpXP. Direct observations demonstrate the capacity of ClpX to exert energy-driven conformational changes onto its substrates. Very fast and highly cooperative unfolding of individual substrate domains suggests a force-dependent translocation step-size of 5–8 amino acids or 1 nm and threading is interrupted by pauses that are off the main translocation pathway. The data support a power-stroke model of denaturation in which successful enzyme-mediated unfolding of stable domains requires coincidence between mechanical pulling by the enzyme and a transient stochastic reduction in protein stability [15,16].

2. Evolutionary perspective: lessons from the archaeal AAA-ATPase PAN

2.1. AAA-ATPases in protein degradation

The AAA-ATPases play a universal role in protein degradation because the majority of intracellular degradation in all organisms is carried out by macromolecular assemblies that confine their proteolytic activity to an

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inner nanocompartment that is accessible only to unfolded protein substrates. Therefore, these proteolytic chambers must be functionally linked to unfolding machinery. This function is exemplified by several AAA-containing proteolytic systems in bacteria, archaea, eukaryotic organelles and cytosol. The principles of how AAA-ATPases unfold protein substrates for degradation appear to be preserved throughout evolution and explain why protein degradation requires ATP hydrolysis [17,18]. In all organisms, the energy-dependent protein degradation is carried out by bipartite assemblies of conserved molecular architecture: AAA-ATPase rings cap hollow proteolytic cylinders, thus control access to the active sites, translate ATP hydrolysis into a force that unfolds the substrates, and translocate them into the protease within the enclosed cavity [19]. In bacteria this function is simultaneously carried out by a number of unrelated AAA activators, each activating a dedicated protease complex. Examples include hexameric ring complexes such as ClpA, ClpC and ClpX that compete for the ClpP protease, HslU that similarly activates the HslV protease, ATP-dependent protease complexes like Lon and FtsH, as well as ATPase activator rings such as *Rhodococcus erythropolis* ARC and *Mycobacterium tuberculosis* Mpa that allow proteolysis by bacterial 20S proteasome complexes [18,20,21].

2.2. The archaeal AAA-ATPase PAN

An 'unfoldase' activity, which was initially shown for the bacterial AAA-ATPase complexes ClpA and ClpX that allow proteolysis by the ClpP complex [22–26], was postulated for the AAA-ATPases associated with the proteasome. The relative complexity of the eukaryotic 19S regulatory particle, also known as PA700, with its six distinct AAA-ATPases (Rpt1–6), multiple non-ATPase subunits (Rpn1–3 and Rpn5–13), a slew of transient auxiliary factors and the requirement for ubiquitin as a targeting signal [27], pose significant hurdles to mechanistic analysis and to deciphering the role of the AAA-ATPase subunits. Therefore, much of what is known regarding proteasomal Rpt subunits is modeled on the relatively simpler archaeal PAN (proteasome-activating nucleotidase) — a ring made up of six copies of a single nucleotidase with no requirement for ubiquitin [28]. Hence, studies in archaea, where AAA-ATPase unfoldase activity associated with proteasome function was first demonstrated, provided the breakthrough in understanding the role of ATP-dependent regulatory complexes in proteasome function. Many archaea, as well as the Actinomycetales bacteria, share homologs of the 20S proteasome whose architecture and proteolytic mechanism resemble those of the eukaryotic 20S particles albeit a bit simpler and made up of fewer gene products [29,30]. In addition to genes encoding for subunits of the proteolytic 20S chamber, the first complete archaeal genome from *Methanococcus jannaschii* revealed one gene (S4), orthologs for which are also found in many other archaeal genomes, that is highly homologous to the six AAA-ATPase Rpt subunits in the 19S particle of eukaryotic 26S proteasome [31]. The product of this gene was named PAN for 'proteasome-activating nucleotidase' because, when expressed in bacteria, it was purified as a 650-kDa complex that showed ATPase activity. Moreover, when mixed with hydrolyzable ATP and purified 20S proteasomes from various sources, this complex stimulated the degradation of several unfolded proteins and unfolded globular ones [32,33]. The obvious advantage in studying PAN is its simple structural organization as a homo-hexamer composed of six identical AAA-ATPases, unlike the 19S AAA-ATPases that form a hetero-hexameric structure made of six different Rpt subunits. However, PAN is the closest known homolog of the eukaryotic 19S AAA-ATPases, sharing 41–45% sequence similarity with all six, which is greater than the similarity between the individual Rpts [34]. Moreover, PAN facilitates ubiquitin-independent degradation and therefore it is easier to monitor its activity, as shown with PAN from the hyperthermophile archaeon *M. jannaschii* that catalyzed unfolding of green fluorescent protein-ssrA (GFP-ssrA).

Substrate unfolding by PAN required ATP hydrolysis and was linked to degradation if 20S proteasomes were also present [35]. The same PAN was used to demonstrate that proteasomal AAA-ATPases are required to mediate the energy-dependent hydrolysis of unfolded casein or folded globular GFP-ssrA by 20S proteasomes [33,36,37]. Threading GFP-ssrA through the PAN AAA-ATPase in a C-to-N direction was required for translocation and degradation, while translocation did not cause but followed ATP-dependent unfolding that occurred on the surface of the PAN ring [38]. PAN's ATPase activity was stimulated similarly by globular GFP-ssrA or unfolded casein and degradation of denatured GFP-ssrA still required PAN and ATP. It was estimated that degradation of one molecule of globular or unfolded substrates consumed 300–400 ATP molecules whose hydrolysis promoted substrate unfolding, 20S gate opening and protein translocation [39]. Electron micrographs revealed association of PAN with the ends of the 20S proteasome cylinder as a two-ring structure capping the 20S at either end [40]. However, efficiency of complex formation was very low in this assay. Surface Plasmon Resonance also demonstrated a positive PAN–20S interaction, but no quantitative assessment of affinity has been provided so far [41]. Interestingly, ATP binding alone to PAN was sufficient to support the transient PAN–20S association, 20S gate opening, translocation and degradation of unfolded and denatured proteins, whereas unfolding and degradation of globular proteins required ATP hydrolysis [35,39–42].

3. Historic perspective: early days of the eukaryotic 26S proteasome, the 19S regulatory particle and its AAA-ATPase base

3.1. The proteasome discovery

Initially, protein degradation in cells was assigned to lysosomes, but in 1977 Alfred Goldberg found ATP-dependent protein degradation in reticulocytes, which lack lysosomes, and suggested a second, soluble ATP-dependent proteolytic system [43]. This discovery was followed by the demonstration of energy-dependent proteolytic systems also in bacteria [44]. Despite the discovery of the eukaryotic proteasome as early as 1977 and the realization that the 20S proteasome resembles other chambered proteases in that it depends on ATPases to unfold its substrates, we could begin to appreciate the actual structure and function of the 19S Rpt AAA-ATPases only recently. In 1986 electron microscopy data revealed the stacked-ring structure of the 20S proteasome from rat skeletal muscle [45]. In 1995, the first crystal structure at 3.4 Å resolution of the 20S proteasome from the archaeon *Thermoplasma acidophilum* was reported [46], and the structure at 2.4 Å resolution of the yeast *Saccharomyces cerevisiae* and mammals shortly followed [47,48]. Yet the structure of the core particle in a complex with the regulatory cap lagged behind.

In 1979 the eukaryotic proteasome was described as a 700-kDa ATP-activated, neutral protease with a critical role in ATP-dependent degradation [49]. A large "multicatalytic protease" complex with chymotryptic and tryptic-like activities was first isolated in 1983 [50] and the same particles were independently isolated as ribonucleoprotein particles, or 'pro-somes', assuming a role in mRNA translation [51]. The renaming of the 'prosome' as 'proteasome' was based on studies of proteolytic systems in mammalian tissues, which indicated that it was identical to a 700-kDa protease complex, also known as LAMP (large alkaline multifunctional protease). The latter contained three distinct endoproteolytic sites and could be activated by ATP [52,53]. This ATP-dependent proteolytic complex, which has been designated the 26S proteasome, is responsible for ubiquitin-dependent protein degradation [17,54,55].

3.2. The 20S core particle

The 20S core particle appears to be more ancient than the ubiquitin system, as it operates in both prokaryotic and archaeal ancestors. Crystal

structures of the 20S proteasomes from Actinomycetes eubacteria *Rhodococcus* [56] or *M. tuberculosis* [57], from the archaeon *T. acidophilum* [46], from yeast *S. cerevisiae* [47] and from mammals [48,58] revealed cylindrical particles with active sites within a large central cavity. The minimal prokaryotic prototype is a homo-dodecamer made of two hexameric rings stacked head to head. The 20S catalytic core in archaea and eukaryota is made of four stacked heptameric rings. The two inner rings that contain the six protease active sites are made of seven catalytic β subunits each that are sandwiched between two outer rings, each of which is made of seven structural α subunits. The N-termini of the α subunits of the outer rings form a 'gate' that blocks unregulated access to the barrel, a function that is controlled by docking of 'cap' structures or regulatory particles [59,60]. In archaea, all the α and all the β subunits are identical, leading to partial obstruction of entry. On the other hand, in the 20S proteasome found in animals, yeast and plants these subunits have diverged into seven distinct types of each subunit, each of which occupies a defined position forming a tight lattice of interactions that plugs the entry pore [61]. Since mammalian and yeast 20S proteasomes are sufficiently homologous, experimental results can be compared and extrapolated, assigning the three major proteolytic activities to distinct β subunits, the peptidyl-glutamyl-hydrolyzing or caspase-like activity to β1 (Pre3), the trypsin-like activity to β2 (Pup1), and the chymotrypsin-like activity to β5 (Pre2) [62,63].

3.3. The 19S regulatory particle

Regulated protein degradation in eukaryotes is carried out by the 26S proteasome holoenzyme, a 2.5–3 MDa molecular machine in which the 20S barrel-shaped proteolytic core complex is capped at one or both ends by AAA-ATPase-containing 19S regulatory complexes. The 19S appears to serve multiple roles in regulating proteasomal activity: identifying substrates, unfolding the substrates for degradation, translocating them into the 20S catalytic particle, and possibly even influencing the nature of products generated by proteolysis [64]. The structure, assembly and enzymatic mechanism of the 20S complex have been elucidated in fine detail. Yet, although 19S subunits have been identified and specific functions have been assigned to several of them, and the general architecture of the entire 26S proteasome has been outlined by electron microscopy, the functional organization of the 19S complex remains much of an enigma to date [65–67]. Only very recently have we started to understand how the 19S associates with and regulates the 20S core particle [60,68–71]. This information is proving instrumental to delineate the precise arrangement of the Rpt subunits in the 19S complex, and to appreciate their individual contribution and mode of action, as detailed below.

The eukaryotic 19S regulatory particle consists of 19 individual proteins divided into a 10-protein 'base' subassembly that binds directly to the 20S α-ring activating proteolysis, and a 9-protein 'lid' subparticle that processes the polyubiquitin [27,72–74]. Six of the ten base proteins (Rpt1–6) are AAA-ATPase subunits that are evolutionary homologs of the AAA-ATPases of the archaeal PAN [32,75]. The base AAA-ATPases interact directly with the 20S α-ring and their activity as a molecular chaperone independently of the proteasome appears to be responsible for protein unfolding by the base subcomplex [61,76,77]. The four other subunits within the base subcomplex are all non-ATPases, and are prefixed "Rpn" for "Regulatory Particle Non ATPase". Of these, Rpn10 and Rpn13 can bind polyubiquitin chains, with Rpn13 also exhibiting surprisingly high affinity for monoubiquitin [78,79]. The remaining base components, Rpn1 and Rpn2, are the largest proteasomal subunits and have been reported to interact with several other subunits [78–82], auxiliary factors [83,84] and even the 20S [71].

The lid consists of nine subunits arranged in a disc-like shape and can detach from the 19S base to form a separate entity and then reattach itself to the base, thereby reforming the 19S regulatory particle [73,85,86]. The best established function of the lid is deubiquitination, or removal of the ubiquitin tag from substrates. This enzymatic function is performed by Rpn11 — a specialized metalloprotease that tightly couples deubiquitination and degradation of substrates [87–89]. To date, no catalytic activity has been associated with any other lid subunit. It should be noted that even in *S. cerevisiae*, the base has deubiquitination activity assigned to Ubp6 [89]. In mammals this activity is probably shared by the ortholog of Ubp6, Usp14, and by the unrelated UCH37/Uch2 [90,91]. Interestingly, UCH37 that is activated by binding to Rpn13 is an exception to the conservation of many 26S subunits, since its homolog Uch2 was discovered in fission yeast but not in budding yeast [90,91]. A comprehensive interaction map of lid subunits was constructed from cumulative studies, culminating with mass spectra of intact lid complex from *S. cerevisiae* [92–95]. Several additional components, not formally considered to be integral subunits of either lid or base, have been characterized in proteasomes either purified under specific conditions or belonging to certain organisms; most of these are likely to be substoichiometric or loosely associated components. At any rate, in budding yeast none of these peripheral subunits appears to be strictly essential [74,96,97].

4. Recent developments in assembly and ring arrangement of the 19S regulatory particle and its AAA-ATPase Rpt subunits base

4.1. Lessons from the assembly of the 20S core particle

The assembly of the proteasome from its 33 subunits is a complex process that is accompanied by proteasome-dedicated chaperones and maturation factors [98,99]. One of the most puzzling aspects of the 26S proteasome holoenzyme is the apparent symmetry mismatch between the hexameric AAA-ATPase ring and the heptameric 20S barrel to which it attaches. Therefore, insight into 20S assembly may illuminate how the 19S is formed and how the two particles attach. Fortunately, while we are only starting to figure out the assembly of the lid and base of the 19S regulatory particle, the assembly of the 20S core particle has been largely deciphered. It is assembled from two half-proteasomes composed of one heptameric α-ring and one heptameric ring of unprocessed β subunits. The catalytic β subunits and non-catalytic β6 and β7 are synthesized with N-terminal propeptides, which are removed at the final assembly step to expose the catalytic Thr residues in β1, β2 and β5. The α-ring initiates the assembly of the half-proteasome and serves as a scaffold for the assembly of each of the β subunits in a defined order, starting with β2 followed by β3 and β4. The propeptides autocatalytic processing requires the association of the β-rings from two half-proteasomes, reflecting an assembly-dependent activation [100–103]. The N-terminal propeptides facilitate β subunit incorporation and prevent premature activation. For example, the propeptide of β5 facilitates its incorporation into the 20S proteasome in yeast and is required for β6 recruitment in human cells. In β1 and β2, the propeptides protect their N-terminal catalytic Thr residue, and β2 propeptide is required for β3 recruitment. The C-terminal tails of β subunits also provide specific interactions within or between β-rings. The C-terminal tail of β2 wraps around β3 in the same β-ring, and incorporation of β7 into half-mers and its C-terminal insertion into a groove between β1 and β2 in the opposing β-ring triggers dimerization of the half-proteasomes [98,99].

Until the discovery of Ump1, the first identified extrinsic assembly factor for 20S proteasomes [104], the 20S proteasome was presumed to assemble autonomously. Ump1 is specifically associated with 20S proteasome assembly intermediates and coordinates the processing of β subunits and the dimerization of half-proteasomes. Following dimerization, Ump1 is encapsulated and degraded within the newly formed 20S particle. Similarly, the human orthologue of Ump1, UMP1/proteassemblin/POMP, is required for the initiation of β-ring formation, is included in precursor proteasomes with unprocessed β subunits, and is degraded upon completion of proteasome assembly. Additional complexes dedicated to early steps in proteasome

assembly were identified in yeast and human cells. They assist formation and prevent aberrant dimerization of α-rings and bind to proteasome precursors until the 20S proteasome is completely assembled and are then degraded by it [96,98,99,105]. In particular, proteasome assembling chaperone-1 (PAC1) and PAC2 are involved in mammalian 20S proteasome maturation, providing a scaffold for α-ring formation and keeping it competent for the subsequent formation of half-proteasomes [106]. In budding yeast, the proteasome-specific chaperones Dmp1 and Dmp2, which structurally resemble the mammalian proteasome-assembling chaperone PAC3, form a complex that binds directly to α5 to facilitate α-ring formation and dissociates before β-rings assembly and half-proteasomes formation. In *Δdmp1* cells, α-rings lack α4 and 20S proteasome formation decreases [107]. Two additional yeast proteins, Pba3 and Pba4, form a 20S proteasome-assembly chaperone that interacts genetically and physically with specific α subunits. Loss of Pba3–Pba4 causes reduction and remodeling of proteasomes, where a second copy of α4 subunit replaces α3. Importantly, these defects in 20S assembly are associated with altered 19S assembly, suggesting that the 20S proteasome can function as a 19S-assembly factor in vivo [108].

4.2. Assembly and spatial arrangement of the 19S base

Until very recently not much was known about the assembly and maturation of the 19S regulatory particle or its two subcomplexes, the AAA-ATPase-containing base and the ubiquitin-recognizing lid. It has long been known that in vitro, the six AAA-ATPases form specific pairs [109]. Dimerization of coiled-coils within their variable N-terminal regions directs their proper placement within the base [110]. The mapped interactions within the human regulatory complex agree with findings on the base and the lid subcomplexes of the regulatory particle from budding yeast [73].

4.2.1. Contribution of the 20S–19S interaction

The contribution of dynamic 20S–19S interactions to 19S assembly or spatial arrangement is beginning to be revealed. As mentioned above, the 19S base is composed of two units, one consisting of the six AAA-ATPases (Rpt1–6), and the other is made up of four non-ATPase subunits, Rpn10, Rpn13 and Rpn1 and Rpn2 [71,78]. Both units maintain contacts with the 20S, the AAA-ATPases via the protruding carboxy-tail residues of Rpt2, Rpt3, and Rpt5 [60,68,70] and the non-ATPases via Rpn2. Independent studies on proteasome configurations from mammals, frogs and yeast have demonstrated that Rpn2 can attach to the α-surface of the 20S in the absence of any Rpt AAA-ATPase, possibly serving as a nucleating agent, with the exposed α-ring serving as a template for the initial assembly of the 19S base [69,71,98,111,112]. Two proteasome-specific auxiliary proteins, Blm10/PA200 and Nob1, are proposed to facilitate 26S assembly. Blm10/PA200 is attached to the 20S α-surface and its role in proteasome assembly is deduced from inefficient 26S assembly and accumulation of incomplete intermediates in a mutant in which a higher concentration of Blm10 is attached to 20S [113]. Blm10 may also act as a coordinator of a late stage of the 20S maturation [105]. Nob1 is proposed to function in 26S assembly and also in the transfer to the nucleus of the 20S proteasome. Nob1 forms a complex with the 19S and is degraded just after the doubly capped 26S proteasome is completed. The use of lid and base mutants reveals that the 20S, the base, and the lid, can be formed and imported into the nucleus independently of each other [114]. The accumulation of 'free' lids in base-assembly mutants (e.g. *rpn2* or *rpn10*) suggests that the base is a limiting factor for the incorporation of the lid into the 19S particle [98].

4.2.2. Assembly of the 19S AAA-ATPase base

A breakthrough in our understanding of the assembly of the 19S regulatory particle came in 2009 upon the discovery of four base-dedicated chaperones that facilitate the assembly of the AAA-ATPase ring of the 19S base [115–121]. This AAA-ATPase ring, a homo-hexamer in archaeal PAN, has diversified in the eukaryotic 19S base into six distinct *RPT* gene products that occupy defined positions around the ring. The assembly of the base and the arrangement of the individual Rpts within the ring are facilitated by a cohort of base-dedicated chaperones. The yeast express Hsm3, Nas2, Nas6 and Rpn14, and their respective mammalian functional homologs are S5b, p27, gankyrin/p28 and Rpn14/PAAF1 (proteasomal ATPase-associated factor 1). These chaperones were previously recognized as proteasome subunits, likely members of assembly intermediates, since Hsm3, Rpn14 and Nas6 bind free 19S particles but only weakly the 19S–20S complete proteasome. Each of these chaperones binds to a specific proteasomal AAA-ATPase by interacting with its C-domain, a four-helix bundle that is characteristic of AAA-type proteins. Hsm3 binds Rpt1, Nas2 binds Rpt5, Nas6 binds Rpt3, and Rpn14 binds Rpt6. No proteins were found in association with Rpt2 or Rpt4 (Fig. 1A). This specificity likely results from unique structural elements in these chaperones: an Armadillo/HEAT repeat in Hsm3, Ankyrin repeats in Nas6, WD40 repeats in Rpn14, and a PDZ domain in Nas2. Structurally, a concave region of Nas6 binds an α-helical C-terminal

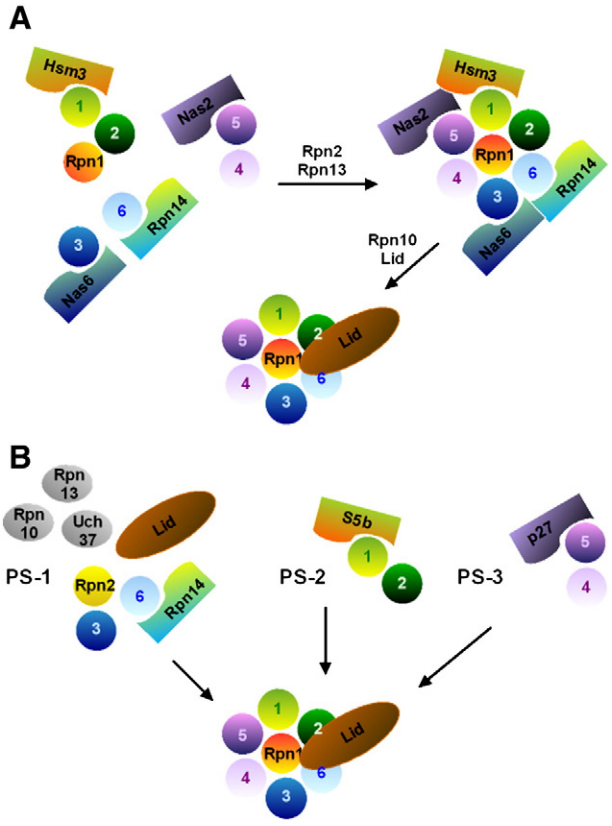

Fig. 1. Assembly pathways of the 19S AAA-ATPases. (A) Assembly of the Rpt1–6 subunits in yeast starts with three pairs of one c-type (dark colors) and one t-type (light colors), each accompanied by specific base-dedicated chaperones (based on [116,117,119,120]). Aided by their chaperones, these three Rpt pairs come together to form the skeleton of the base. A large non-ATPase subunit of the base, Rpn1, is found with the Rpt1·Rpt2 dimer. However, it is unclear at which stage the remaining base subunits, Rpn2 and Rpn13 (which interact with one another), incorporate. Multiple interactions with neighboring subunits are documented for Rpn2, although its location relative to other base subunits is unclear. Lid subunits associate with the base to complete the 19S regulatory particle. Rpn10 stabilizes lid-base association. Intact 19S that includes lid and all base subunits has been isolated, which may then attach to the 20S to form the proteolytically active 26S proteasome holoenzyme [135]. (B) Assembly of the Rpt1–6 subunits in mammalian cells can be reconstituted from three subcomplexes, PS-1, PS-2 and PS-3, made of one c-type (dark colors) and one t-type (light colors) pair of Rpt subunits, specific base-dedicated chaperones and additional base components, and the lid (based on [136]). All subunits are shown schematically as spheres to illustrate their general positions according to documented interactions with neighboring subunits.

domain of Rpt3, an interaction that is mediated by complementary charged patches [122]. Likewise, the complex of gankyrin/p28, the mammalian functional homolog of Nas6, with Rpt3/S6 shows that almost all of the seven ankyrin repeats of gankyrin interact, through its concave region, with the C-terminal domain of Rpt3/S6, again via complementary charged residues [123]. Finally, the crystal structure of yeast Rpn14 at 2.0 Å resolution reveals that this chaperone consists of a unique N-terminal domain with unknown function, and a C-terminal domain that assumes a canonical seven-bladed β-propeller fold. Based on structural comparison with the Nas6–Rpt3-C complex, the predicted Rpt6-binding site on Rpn14 is basic, whereas the top face of Rpn14 is highly acidic. Again, specific residues of Rpn14 and Rpt6 are responsible for complementary charge interactions [124]. Protruding from the C-domain of the six Rpt subunits are C-terminal tails, flexible segments that extend toward the 20S and insert into well-defined pockets formed between the seven α subunits, thus promoting the attachment of the 19S to, and stabilize its association with the 20S core particle. This mechanism and the notion that AAA-ATPase ring formation is templated on pre-assembled 20S are supported by assembly-defective phenotypes in yeast with mutations in the Rpt tail elements and by defects in 19S assembly secondary to primary defects in 20S assembly [115,119,120]. Although the chaperones bind to the C-domains and not to the C-terminal tails, they may compete with the 20S for binding to the proximal C-terminal tails. Consistent with this competition is the release of specific chaperones. Mutations in the Rpt6 tail indicate that tail docking triggers Rpn14 release. Similar findings are reported for Rpt3 and its partner Nas6. Furthermore, Nas6, Hsm3 and Rpn14 can be stripped from purified base by the addition of purified 20S [120,125]. As discussed below, the C-terminal tails of the Rpts also mediate the gate opening of the substrate translocation channel into the 20S [60,68], a function that is conserved between PAN and the eukaryotic Rpt proteins.

4.2.3. The spatial ring arrangement of the 19S AAA-ATPase base

The assembly of the Rpts ring is a highly orchestrated process, with evolutionarily conserved early assembly intermediates that challenge the long-standing ideas as to the arrangement of subunits within the ring [126]. Yeast and mammalian Rpts first form heterodimers, Rpt1·Rpt2, Rpt3·Rpt6 and Rpt4·Rpt5, which are complexed with the base-dedicated chaperones [96]. In the Rpt1·Rpt2 pair, Rpt1 is bound to Hsm3/S5b; in Rpt3·Rpt6 heterodimer, Rpt3 is bound to Nas6/p28/gankyrin and Rpt6 is bound to Rpn14/PAAF1; in the Rpt4·Rpt5 pair, Rpt5 is bound to Nas2/p27 (Fig. 1A) [116–118,127]. This 3-fold rather than 6-fold symmetry and the Rpt·Rpt dimeric assembly intermediates are consistent with the crystal structure of a hexameric fragment of archaeal PAN, which reveals a trimer of dimers. Within each dimer, an α-helix from each neighboring subunit pairs up to form a coiled coil that is slightly offset toward one member of the pair [128,129]. Since PAN is a homo-hexamer, the asymmetry between nearest neighbors is broken at Pro91 in the linker between the coiled-coil and the following OB (oligonucleotide binding) domains, which alternates around the ring between *cis* and *trans* conformations, orienting the preceding α-helix toward one partner. Thus, in each dimeric intermediate, *cis* (c-type) and *trans* (t-type) subunits associate to form a Pro *cis*–*trans* pair of AAA-ATPases with salt bridges between Asp in c-type subunits and Arg in t-type subunits (Fig. 2A). It is fascinating to note that of the six distinct Rpt subunits found in the eukaryotic 19S, three (Rpt2, Rpt3 and Rpt5; i.e., one subunit of each pair) contain a conserved Pro at a position equivalent to Pro91 in PAN. These are considered to be the c-type subunits. The partners in the pairs (Rpt1, Rpt6 and Rpt4, respectively) do not contain this Pro and are considered to be the t-type subunits (Figs. 1 and 2) [125]. It is hypothesized that the Pro is conserved in exactly three of the Rpt subunits of eukaryotic proteasomes in order to preserve the *cis* conformation in one member of each coiled-coiled dimeric intermediate. Based on these intermediates of a pair of one c-type and one t-type Rpt subunit, a new model for the spatial arrangement has been proposed, in which the positions of the c-type and the t-type subunits alternate around the ring (Fig. 2B) [130,131]. Indeed, targeted disulfide crosslinking studies

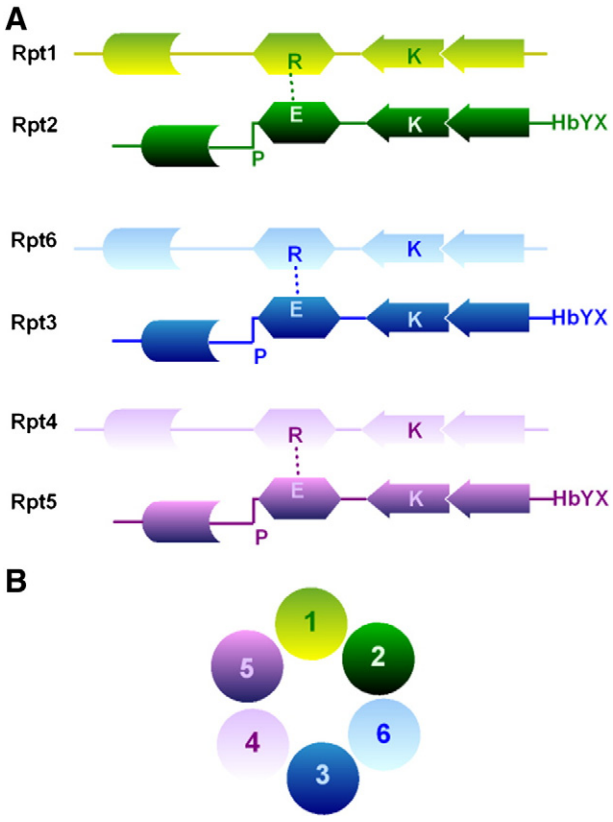

Fig. 2. 19S AAA-ATPase subunits (Rpts), their domains, specific motifs, dimeric assemblies and ring arrangement. (A) Nearest neighbors of Rpt subunits within the AAA ring in the 19S base are stabilized via salt bridges between conserved Asp in a c-type (dark colors) Rpt and conserved Arg in a t-type (light colors) Rpt. These salt bridges are situated within the OB domains depicted by ⬭. Preceding the OB domains are the coiled coil domains depicted by ⊏. The conserved Pro situated between the OB and the coiled coil domains that determines the relative orientation of the two is indicated. The Walker A and B are depicted by ⇦ and the critical Lys in walker B is indicated. The HbYX motif found in c-type Rpts is indicated. (B) The ring arrangement or Rpt1–6, as deduced from the assembly pathway and structural analyses (adopted from [125]).

of *S. cerevisiae* Rpt subunits have confirmed that they occupy unique positions and are ordered as Rpt1–Rpt2–Rpt6–Rpt3–Rpt4–Rpt5 around the ring [132]. The three Rpt pairs (Rpt1·Rpt2, Rpt4·Rpt5 and Rpt3·Rpt6) come together aided by their chaperones to form the skeleton of the base (Fig. 1). The order in which these dimers snap together to forge the AAA ring is unclear. However, Rpt5 was found in complex with the Rpt1·Rpt2–Rpn1–Hsm3 mini complex in a yeast strain mutated at the C-terminus of Rpt4 that interferes with its ability to dimerize with Rpt5. This assembly was termed base-precursor 1 (BP1; [119]) and suggests that under normal conditions, the Rpt1·Rpt2 and Rpt4·Rpt5 dimers (with their chaperones Hsm3 and Nas2 respectively; see Fig. 1A) come together, then recruiting Rpt3·Rpt6, aided by Rpn14 and Nas6, to fill in the final slot and close the Rpt ring. At this stage it is unclear when Rpn2 and Rpn13, which interact one with another [133,134], enter the base precursor. Spatial information supporting ring assembly from the dimer intermediates may be provided by Rpn1 and Rpn2 or rely on scaffolding on the 20S, although no intermediates have been isolated that include Rpn2 or Rpn13 prior to incorporation of lid [125]. Intact 19S that includes lid and all base subunits has been isolated, which may then attach to 20S or detach from it, the difference being the presence of the associated chaperones [135].

A largely compatible model for 19S assembly is based on purification, identification and characterization of three distinct subcomplexes of the 19S from bovine red blood cells [136]. The PS-1 subcomplex contains one of the dimeric ATPase assemblies

(Rpt3 and Rpt6), Rpn2, Rpn10, Rpn13, Uch37, the Rpt6 chaperone Rpn14/PAAF1 with no detection of the Rpt3 chaperone gankyrin/p28, and also all of the lid subunits at similar molar ratio. The PS-2 subcomplex contains Rpt1, Rpt2, the Rpt1 chaperone S5b/Hsm3 and Rpn1. The PS-3 subcomplex contains Rpt4, Rpt5 and the Rpt5 chaperone p27/Nas2 (Fig. 1B). Collectively, these three subcomplexes contain all the 19S subunits with no overlapping components, each with a distinct pair of Rpts and cognate chaperone(s). While these subcomplexes display neither ATPase activity nor proteasome activating activity, both activities are manifested when they are mixed together and undergo very efficient ATP-dependent in vitro reconstitution into 19S particles, with no effect of added 20S on assembly rate. The importance of intact C-termini is shown by carboxypeptidase treatment of any of the subcomplexes that inhibits 26S proteasome assembly and activation but does not affect 19S reconstitution or ATPase activity [136]. Indeed, recent data show that a C-terminal peptide of Rpt3 blocks ATP-dependent in vitro assembly of 26S proteasome from 19S and 20S. In cells, the C-terminus of Rpt3 is required for assembly of this subunit into 26S proteasome but not for its assembly into intact 19S, which can assemble independently of association with 20S proteasome [70]. Pulse-chase analysis in mammalian cells drew a slightly different observation. New labeled 19S assembled on pre-existing unlabelled 20S. Further analysis detected labeling only in Rpn2, Rpn10, Rpn11, Rpn13 and Txnl1, suggesting that these proteins are initially deposited on the 20S and the AAA-ATPases are added at later stages [69]. This possibility generally agrees with an unrelated study that identified mature active 20S core particles in a complex with Rpn2, Rpn1 alongside some additional factors such as Hsp90, yet lacking the Rpt proteasomeal AAA-ATPase subunits [111]. Interestingly, no evidence has yet been put forth for Rpt subunits in complex with 20S core in absence of other 19S subunits, suggesting that in eukaryotes other base subunits provide the 'glue' to stabilize the interaction of the Rpt ring with the 20S. The non-ATPases subunits found with the 20S in this case (Rpn2, Rpn10, Rpn13) are primarily the same factors found in PS-1 with the Rpt3·Rpt6 AAA-ATPases [136] yet unassigned to any specific precursor in yeast experiments (see above). Thus, there may be some flexibility or competition for incorporation of these subunits at various steps of the assembly process. It remains to be determined whether in mammalian cells the free base, detected in yeast, does exist or the lid or its components join the nascent 19S prior to base completion.

5. Recent developments in the structure of the 19S regulatory particle and its AAA-ATPase Rpt subunits base

The 20S core particle, which is highly conserved from archaea to higher eukaryotes, was amenable to structure determination by X-ray crystallography. The crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution was published 15 years ago. This was shortly followed by the structure of 20S proteasome from yeast at 2.4 Å resolution and later structures of the mammalian 20S proteasome were reported at low resolution and at 2.75 Å resolution [46–48,58]. In contrast, the 26S proteasome bearing one or two 19S regulatory particles has so far resisted all crystallization attempts. Binding studies and electron microscopy have demonstrated that the C-terminal region of the AAA-ATPase in the 19S base is adjacent to the proteasome α-ring, and that the central pore of the AAA-ATPase rings is roughly aligned with the 20S entrance pore [40,67,137,138]. Single-particle electron microscopy of the PAN–20S complex provided the first structural information on the proteasomal AAA-ATPases [40] and also clarified electron microscopic structures of the 26S proteasome [65,66]. PAN resembles a "top hat", with a large inner ring and a smaller outer ring capping either or both ends of the 20S particles. Based on its close similarity to PAN, the outer-ring density was proposed to be part of the of Rpt AAA-ATPases ring. Hence, the PAN–20S complex resembles the 26S complex without its lid [34]. Insights into the structure and mechanism of the AAA-ATPase modules were gained from the crystal structures of the major domains of the homo-hexameric archaeal PAN [139]. As discussed above, each PAN monomer consists of coiled coils protruding from an OB fold (PAN-N) and an AAA fold. Both fragments, which were crystallized separately, assemble into hexameric rings with yet unknown spatial relationship [128,129].

An alternative approach to obtain a structural model at near atomic resolution is to use cryo-electron microscopy, which provides medium resolution (1–2 nm) structures. An atomic model for the 19S AAA-ATPase base has been computed from the current structural knowledge on the 19S, which includes structures of the 19S subunits, physical protein–protein interactions based on chemical cross-linking in conjunction with mass spectrometry, and cryo-electron microscopy of the 26S proteasome [140]. In this atomic model, similar arrangement of the AAA-ATPase ring was obtained for proteasomes from *Drosophila melanogaster* embryos [130] and from *Schizosaccharomyces pombe* [137]. This ring arrangement, Rpt1/Rpt2/Rpt6/Rpt3/Rpt4/Rpt5, is in agreement with the dimeric Rpt precursor complexes formed during the assembly of the 19S, as discussed above [141]. This model also suggests that the assembly chaperone Nas6 facilitates 20S–19S association by enhancing the shape complementarity between Rpt3 and its α subunits binding partners in the 20S [131]. Cryo-electron microscopy, in conjunction with advanced image analysis, has outlined the boundaries of the AAA-ATPase module at the base of the 19S complex. These studies have shown that this module can vary in position and orientation relative to the 20S core particle. This variation is consistent with the "wobbling" model that was proposed to explain the role of the regulatory complex in opening the gate in the α-rings of the core particle (discussed below). By correlating the electron microscopy data with quantitative mass spectrometry, a variable mass near the mouth of the AAA-ATPase ring has been identified as containing Rpn10, a polyubiquitin receptor, although it could also be that the variable mass is rearranged due to the absence of Rpn10 that fits elsewhere in the structure [130]. Atomic force microscopy and independently, electron microscopy, have both identified the two large non-ATPase subunits Rpn1 and Rpn2 as solenoid rings that in absence of the Rpt AAA-ATPases can localize to the center of the 20S particle, covering the central channel [71,142]. However, how these toroids come together with the AAA-ATPase ring is yet to be established. A high resolution structural study of *S. pombe* 26S proteasome [137] suggests that both Rpn1 and Rpn2 position outside of the AAA-ATPase ring and surrounding it.

6. Mode of action of the AAA-ATPase Rpt subunits in the 19S base

The proteasomal Rpt subunits comply with the evolutionarily conserved role of AAA-ATPase hexameric rings in generating a mechanical force through conformational changes during cycles of ATP binding and hydrolysis and using this force to induce conformational remodeling of substrate proteins. Indeed, biochemical studies show that the base of 19S contains chaperone-like activity [76,143]. Akin to all AAA-ATPases that are involved in protein degradation, the Rpts in the 19S base or the archaeal PAN stimulate degradation by the 20S proteasome through association with this proteolytic particle. They selectively bind substrates, open the gated entry channel in the 20S, unfold globular or partially folded proteins, and facilitate the translocation of the unfolded substrate through the AAA-ATPase ring into the 20S particle. Although it has been shown that substrates unfolding is the only process that requires ATP hydrolysis, while the other steps depend only on ATP binding [34,40], it is not surprising that the AAA-ATPase domain and the ring arrangement are crucial for proteasome function. However, N-terminal and C-terminal regions, which are unique in the eukaryotic Rpt subunits (Fig. 2A), are also relevant for proteasome function. As discussed below, the C-termini are critical for opening the α-ring gate to allow access of substrates to the

20S proteolytic chamber. The N-terminal regions of the archaeal *Archaeoglobus fulgidus* PAN, and of its actinobacterial homolog, *R. erythropolis* ARC, with their N-terminal coiled coils and C-terminal OB domains, can act as chaperones, preventing protein aggregation in vitro via concerted radial motions of the coiled coils relative to the OB rings [129]. In the *M. tuberculosis* Mpa, the prokaryotic ubiquitin-like protein Pup is recognized by three pairs of tentacle-like N-terminal coiled coils [144].

6.1. The AAA-ATPase domain

The structural complexity, multiple enzymatic activities and involvement of ubiquitin ligation impeded the advance in studying the mechanism(s) of ATP-dependent processes in 26S proteasome functions. The first breakthrough came via genetic approach in yeast. One Lys in the Walker A motif in the consensus sequence GXXXXGK [T/S] is critical for activity and its conservative substitution generally results in complete or partial inhibition of ATP binding and hydrolysis and inactivation of the ATPase function [145]. Systematic mutagenesis of this ATP binding site in each of the six distinct Rpt subunits was achieved by replacing each *RPT* gene by an equivalent mutant version with a conservative substitution of this invariant Lys. This approach reveals unique roles for the individual Rpt subunits in the various proteasomal functions [146,147]. Mutations in the ATP-binding site of Rpt2 exert the most severe effect on global protein degradation and cell cycle progression, while a similar mutation in Rpt1 causes a G1 cell cycle arrest with no effect on protein breakdown [146]. Mutations in the ATP-binding site in the other AAA-ATPase subunits lead to varying degrees of sensitivity to heat shock or growth in the presence of the amino acid analog canavinine. Hence, the individual Rpts facilitate the recognition and degradation of different subsets of substrates and at least one Rpt subunit, Rpt5/S6, interacts with polyubiquitin chains [75]. Binding of the 19S to the surface of the 20S opens the narrow entrance gated by the N-terminal tails of the α subunits, a function that requires an intact ATP binding domain in Rpt2. Indeed, Rpt2 mutant growth defect is rescued by the α3ΔN 20S truncation mutant that cannot seal the gate [59,61,148]. It should be noted that, in the 20S–19S interface, the 7-fold symmetry of the α-ring must align with the 6-fold symmetry of the Rpts ring.

6.2. ATP binding and hydrolysis

The archaeal PAN associates transiently with the 20S proteasome upon binding of ATP or ATPγS, but not ADP. This association stimulates opening of the gate formed by the N-termini of the α subunits, that otherwise prevents entry of long peptides into the 20S. The PAN–20S complex, as well as rabbit 26S proteasomes, translocates and degrades unfolded and denatured proteins in the presence of ATPγS, while degradation of globular proteins requires ATP hydrolysis. Thus, unfolding requires energy from ATP hydrolysis, whereas ATP binding alone supports ATPase–20S association, gate opening, and translocation of unfolded substrates into the proteasome that may occur by facilitated diffusion through the AAA-ATPase in its ATP-bound form [34,40]. Similar requirements for ATP binding and hydrolysis are exhibited by the AAA-ATPase of the eukaryotic 19S particle [42].

6.3. Communication within the hexameric ring

Communication between neighboring subunits within a ring is a well-known feature of AAA-ATPases [4]. In their hexameric form, the nucleotide bound to one subunit is also contacted by a 'sensor' in the α-helical subdomain of the same subunit as well as by a 'sensor' of the adjacent subunit [13]. Crystal structures of bacterial AAA-ATPases and nucleotide titration experiments indicate that the subunits in the hexamer neither simultaneously exist in the same nucleotide state nor adopt the same conformation, as only three to four molecules of ATP bind per hexamer [149–152]. In CDC-48.1, the p97 homolog of *Caenorhabditis elegans*, the ATPase activity of the C-terminal AAA domain is high and displays positive cooperativity. This cooperativity is affected by ATP binding to the N-terminal AAA domain. Moreover, unlike the stochastic model for ClpX, the ATPase activity within the p97 hexamer is highly coordinated and this coordination is generated by a different mechanism than cooperativity. Interestingly, the positive cooperativity of the C-terminal AAA domain is more critical for p97's biological function than its overall ATPase activity [153]. A recent study of nucleotides binding to PAN shows that its six identical subunits bind ATP in pairs. PAN's subunits exhibit three conformational states with high, low, or no affinity for ATP. When PAN binds two ATPγS molecules or two ATPγS plus two ADP molecules, it is maximally active in binding protein substrates, in associating with the 20S, and in promoting 20S gate opening, while binding of four ATPγS molecules reduces these functions. Similar nucleotide dependence is exhibited by the 26S proteasome. These results imply an ordered cyclical mechanism in which two AAA-ATPase subunits bind ATP simultaneously and dock into the 20S, explaining how these hexameric AAA-ATPases interact with and "wobble" on top of the heptameric 20S proteasome [41]. This mechanism nicely agrees with recent findings regarding the assembly and spatial arrangement of the Rpt subunits in the ring base discussed above, where Rpts first form dimers of c-type and t-type subunits that finally assemble into a hexameric ring with alternating c-type and t-type subunits (Figs. 1 and 2), and is also consistent with the trimer of dimers crystal structure of a hexameric fragment of PAN [125,128–131].

How does ATP hydrolysis by the AAA-ATPase rings translate into a mechanical force that unfolds and translocates substrates into the 20S proteolytic chamber? This is a key question. In a current model, which couples unfolding to translocation [149], a conserved loop in the ring's central pore contacts the substrate and cycles through high affinity up-conformations and low affinity down-conformations in response to ATP hydrolysis. The loop movements along the pore-axis apply a pulling force onto the substrate, resulting in its unfolding and translocation. A conserved aromatic–hydrophobic GYVG motif in the loop protrudes from every AAA-ATPase subunit into the ring's central pore. Coupling ATP hydrolysis to loop conformational changes is indicated by loop mutations that are defective in substrate binding and translocation, with either increased or decreased basal ATPase rates. A covalently linked ClpX hexamer with Tyr-to-Ala mutation in the GYVG motif in just a few subunits per hexamer retains unfolding and translocation activities, but dramatically increases the energetic cost to degrade substrates, probably due to inability to grip substrates resulting in 'slippage' [154]. Additional loops positioned at different heights in the ring central pore, larger conformational changes, and domain rotations in response to the nucleotide state have been observed in crystal structures, which could in principle power loop movement or contribute by other means to protein unfolding [19]. The degree to which the hexameric AAA-ATPase ring is 'deformed' to fit the 7-fold symmetry of the 20S is unclear. One possibility with clear implication on the communication between the AAA-ATPase domains is that the Rpts do not form a closed ring because, when assembled with the 20S, their insertion into only six of the seven α cavities might create a gap between two specific Rpt subunits. Then, four subunits would be flanked on either side by other ATPases, whereas one would be flanked only counterclockwise, and another only clockwise. An alternative model, supported by recent studies and discussed below, is that not all the AAA-ATPases in the closed ring insert into the 20S at once, and only a subset of the AAA-ATPases are engaged at any given time. This model suggests that the hexameric AAA-ATPases interact with and "wobble" on top of the heptameric 20S proteasome. An ordered cyclical mechanism ensures that at any given time only two AAA-ATPase subunits bind ATP simultaneously and dock onto the 20S [41].

6.4. Gate opening by the C-terminal tails

The entrance to the 20S is a narrow channel formed by the seven α subunits that allows passage of unfolded polypeptides, whereas globular proteins must first associate with the 19S AAA-ATPases and undergo ATP-dependent unfolding. This entrance is gated by the N-termini of the α subunits whose conformational changes open the gate to allow regulated substrate translocation into the 20S particle. Three different types of proteasomal activators have been identified: the 19S regulatory particle, the 11S activator (PA28/PA26/REG) [155] and the PA200 (or Blm10 in yeast) [156]. All three types of proteasome activators can associate with the 20S and stimulate its peptidase activity by inducing gate opening by similar, yet distinct, mechanisms. It is interesting to note that the two very different families of activators – the asymmetric heterogeneous 19S complex on the one hand and the symmetric toroid PA28 and dome-like PA200 complexes on the other – both compete for attachment to the 20S α-ring surface. Moreover, both types of activators participate in gating the channel to a similar extent, though possibly through a very different mechanism. The 19S is the only activator that contains the AAA-ATPase subunits, which can also unfold globular proteins. As mentioned above, the α subunits N-termini conformational changes that open the gate occur upon direct binding of the 19S AAA-ATPases to the α-ring on the 20S surface. In particular, gate opening requires an intact ATP binding site in Rpt2 and deletion of the N-terminus of α3 suppresses the phenotype of mutations in this Rpt2 site [47,59,61,148,157]. A series of recent reports provide exciting data demonstrating that proteasomal AAA-ATPases trigger gate opening through their C-terminal tails, a function that is conserved between PAN and the eukaryotic Rpt proteins (Fig. 3). Specific interactions between Rpt and α subunits determine 19S–20S binding and gate opening, corroborating the nonequivalent roles of the six different Rpt subunits [60,68]. Treating 19S with carboxypeptidase A selectively cleaves the C-termini of Rpt2 and Rpt5 and renders 19S incompetent for proteasome binding and activation [68]. Moreover, short peptides corresponding to the C-terminus of either Rpt2 or Rpt5, but none of the other Rpts, are sufficient to bind to the 20S and induce gate opening. These peptides bind to distinct sites on the proteasome and generate additive modular gate opening [60,68].

6.4.1. The general role of C-termini in gate opening

The idea that proteasomal AAA-ATPases induce gate opening through their C-termini has been suggested from analogy to the mode of action of the ATP-independent 11S activator PA28/PA26 [59,138]. These heptameric complexes associate with the ends of the 20S via their extreme C-termini and facilitate entry of short peptides [158,159]. The first clue for proteasomal activator-induced 20S gate opening came from X-ray crystallography of 20S in complex with the PA26 from *Trypanosoma brucei* [138,160,161]. PA26/28 is a heptameric 11S activator [162] that is neither an ATPase nor does it unfold globular proteins, but it stimulates degradation of unfolded peptides by inducing 20S gate opening [160]. The atomic details provided by the structure of PA26–20S complex reveal that the PA26 C-termini dock into pockets between adjacent α subunits [138,160]. However, unlike proteasomal AAA-ATPases, the binding of PA26's C-termini to the α-intersubunit pockets is not sufficient to induce 20S gate opening [163] and an additional 'activation domain', distant from the C-termini, is required. This is probably due to a very subtle movement (less than 2 Å) away from the central pore of a reverse-turn loop in the α-subunit with no significant conformational changes in the body of the α-ring [164]. Interaction between the PA26's 7-fold symmetric circular array and the N-terminal gating residues of the seven α subunits destabilizes the asymmetrical closed gate conformation of the 20S and stabilizes its symmetric open gate conformation [161]. Despite this similarity, PA28/26 and the proteasomal AAA-ATPases do not share any sequence homology and they form different protein

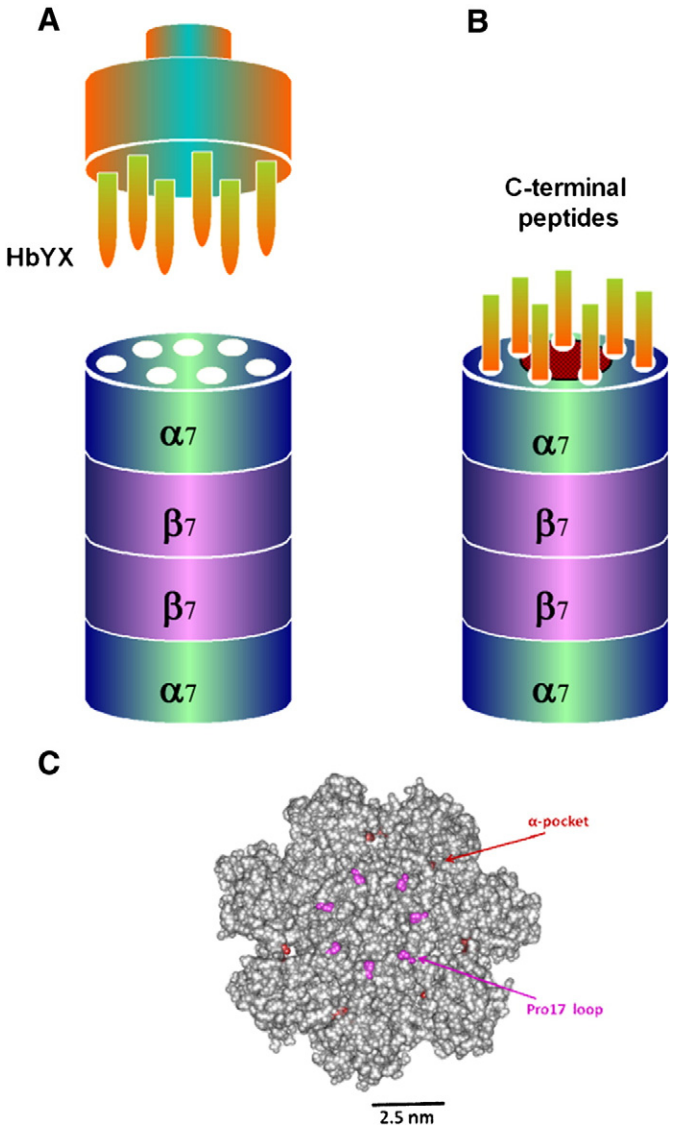

Fig. 3. Model for the association of PAN with the α-ring of the 20S proteasome. (A) The C-termini of the proteasomal AAA-ATPases (PAN in archaea or Rpt subunits in eukaryota) dock into intersubunit pockets in the α-ring at both ends of the 20S. (B) Schematic model for 20S gate opening upon binding of peptides derived from the C-termini of proteasomal AAA-ATPases to the intersubunit pockets in the α-ring (based on [60]). (C) Top view of the 20S α-ring with a surface rendering to demonstrate key residues involved in docking of Rpt C-termini (Lys66; red) and in inducing channel opening (Pro17; pink). The structure of *S. cerevisiae* 20S CP α-surface was modeled with PDB code 1RYP using Viewerlite.

complexes. The PA28/26 are heptameric rings so they form a stable matched 7:7 subunit interface with the 20S, whereas the proteasomal AAA-ATPases PAN and Rpts are hexameric and thus form a mismatched 6:7 subunit interface that 'wobbles' on top the heptameric 20S [40]. Moreover, PA28/26 are static complexes, whereas any AAA-ATPase rings, including the proteasomal ones, are highly dynamic and undergo large conformational changes upon ATP binding and hydrolysis [6,9,10].

6.4.2. The C-terminal gate opening motif HbYX

A key activation mechanism used by the 19S emerges to be very different from that of PA28 [60,68]. Recent studies imply that the Rpt proteins do not use an activation loop to affect gating, nor do they act by directly forcing six-fold symmetry on the α-ring. The 20S α subunits rings were shown to contain seven pockets on their 19S-facing surface, which

are formed at the α–α interfaces. These 'α-pockets' provide binding sites for the C-termini of the 19S Rpt subunits (Fig. 3) [60,68]. These C-terminal regions contain the canonical gate opening motif HbYX, where any hydrophobic residue (Hb) is followed by Tyr and an unspecified C-terminal residue. This motif was originally recognized in PAN and it was shown that synthetic peptides terminating with this motif can open the 20S gate [60,68]. Comparing C-terminal sequences in various archaea and eukaryotes identified conserved penultimate Tyr in PAN from nine different archaeal species (or Phe in five archaeal species) as well as in four eukaryotic AAA-ATPases, Rpt1, Rpt2, Rpt3, and Rpt5 (but not Rpt4 and Rpt6) from humans, rats, mice, *Drosophila*, *Arabidopsis*, nematodes (except Rpt1), and yeast. A hydrophobic residue precedes the penultimate Tyr in PAN from all 14 archaea species and also in Rpt2, Rpt3, Rpt5, and Rpt6 of these eukaryotes [60]. It was further demonstrated that PAN's C-terminal HbY residues, but not residues preceding the HbYX motif, are essential to stimulate 20S gate opening. Also, the HbYX motif is required for PAN–20S complex formation, moving PAN's C-terminus from an aqueous to a hydrophobic environment, a process that requires Lys66 in the α-ring. Finally, short peptides corresponding to PAN's C-terminus induce 20S gate opening, depending on the HbYX motif and Lys66 in the α-ring, and inhibit PAN–20S association (Fig. 3) [60]. Although PAN and PA26 association with 20S and activation of gate opening require Lys66 in the α subunits [138], C-termini of PA26 that lack the HbYX motif bind but do not induce gate opening [163] and consistently, peptides corresponding to the C-termini of PA26 or PA28 do not by themselves induce gate opening. On the other hand, PA200/Blm10, another non-ATPase regulator that can bind to the same 20S surface, has only a single C-terminus that can insert into only a single Lys pocket on the α-ring and, accordingly, it only mildly activates proteasome peptidase activity [105,113].

The eukaryotic Rpts that carry the canonical HbYX motif are the c-type subunits Rpt2 Rpt3 and Rpt5. However, while binding of the 19S to the 20S is mediated by the C-termini of only Rpt2 and Rpt5 that constitute the interaction surface between the subcomplexes and the C-termini of Rpt2 and Rpt5 exert the strongest effect on gating [60], the C-terminal HbYX of Rpt3 binds to the 20S proteasome but does not promote gate opening, yet this Rpt3 motif is essential for 26S proteasome assembly [70]. It is possible that in Rpt3, the C-terminal Lys does not support gate opening in mammalian 20S, although basic residues in the HbYX motif of PAN do not interfere with gate opening of archaeal 20S. The C-terminal YX residues in the yeast 26S AAA-ATPases are also required for gate opening, as shown by substituting with Ala the conserved penultimate Tyr in Rpt1, Rpt2, Rpt3, and Rpt5. These substitutions caused distinct and very complex phenotypes related to protein degradation [60]. Finally, C-terminal synthetic peptides from certain 19S Rpts induce gate opening in the mammalian 20S [60]. C-terminal peptides of either Rpt2 or Rpt5 bind to the 20S proteasome and activate hydrolysis of short peptide substrates. Simultaneous binding of both C-terminal peptides had additive effects on peptide substrate hydrolysis, suggesting that they bind to distinct sites on the proteasome. In contrast, only the Rpt5 peptide activated hydrolysis of protein substrates. Nevertheless, the C-terminal peptide of Rpt2 greatly enhanced this effect, suggesting that proteasome activation is a multistate process [68].

6.4.3. The gate opening mechanism

The emerging mechanism indicates that the C-terminal HbYX motif of the AAA-ATPases dock into pockets between adjacent α subunits and tether the AAA-ATPase ring to the 20S, thus joining substrate unfolding equipment with the 20S degradation machinery. The crystal structure of the archaeal 20S proteasome in complex with the C-terminus of the archaeal PAN unravels the detailed interactions between the HbYX motif and the 20S α subunits and indicates that inter-subunit pocket in the 20S undergoes an induced-fit conformational change upon binding of the HbYX motif. This structure, together with related mutagenesis data, suggests how in eukaryotes certain proteasomal AAA-ATPases bind to specific pockets in an asymmetrical manner to regulate gate opening [165]. Apparently, repositioning a Pro17 reverse turn in the α subunits, described initially for an internal activation loop in PA26 [163], is a mechanism to open the 20S gate that is shared by proteasomal AAA-ATPases [166]. Synthetic peptides of only 7 or 8 residues, corresponding to the C-terminus of proteasomal AAA-ATPases, stimulate the 20S gate opening [60], indicating that binding of the HbYX motif is sufficient to open the 20S gate. Single particle cryo-electron microscopy revealed that these peptides bind to the same α-ring pockets as the C-termini of PA26, where they interact with conserved residues required for gate opening [60,138,166]. However, unlike the PA26/28, the HbYX-containing peptides do not require an internal activation loop and use only interactions from their C-terminal residue to cause gate opening by inducing a rocking motion of the α subunits rather than by directly contacting the Pro17 reverse turn [60,166]. These peptides induce a rotation of the individual α subunits by ~4° and the reverse-turn loop of the 20S α subunit is displaced along such that the closed gate position is disrupted and open gate conformation is stabilized. How are the C-termini of the PAN/19S AAA-ATPases, with their HbYX motif, sufficient to provide both binding and gate opening functions in the absence of an internal activation loop [164,166]? Crystal structures and binding studies indicate that in both PA26 and PAN/19S the C-terminal residues, which bind in a superimposable fashion, induce gate opening by using direct contacts with residues of the Pro17 reverse turn. The penultimate PAN/19S side chains play a role in both binding and stabilizing the proteasome Pro17 residues in an open conformation [28].

6.5. Substrate unfolding and translocation by proteasomal AAA-ATPases

Degradation of native proteins by chambered proteases requires collaboration, and even coupling, between regulatory and catalytic complexes, where the regulatory complex actively unfolds substrates containing structured domains and translocates them into the catalytic complex. The 19S unfolds substrates and translocates them into the 20S for degradation. While it has been debated whether unfolding and translocation are separable events, the most widely accepted model now is that unfolding is driven by translocation. In this model, the proteasome generates an ATP-dependent pulling force on the substrate, first exerted on an 'initiation sequence'. Later on, other segments of the substrate are engaged as it is reeled into the 20S. Owing to the small size of the substrate translocation channel; folded domains of the substrate are impediments to translocation. According to the model, the blockage in translocation is relieved once the pulling force of the proteasome drives unfolding of the substrate. A detailed understanding of protein unfolding and translocation by the proteasome has not been reached, and the major difference between existing models is whether substrate unfolding and translocation steps are dissociable. One model proposes that unfolding occurs on the surface of the proteasome and that translocation is a distinct process that can begin only after unfolding has generated the loose structure needed to thread through the narrow translocation channel [38]. Studies carried out with the PAN protease agree with this model [38]. This model requires multipoint contacts between the substrate and the surface of the AAA-ATPase ring, followed by ATP-driven domain motion within the ring, which mechanically destabilizes the folded state of the substrate. In an alternative model, unfolding of the substrate may be driven by substrate interactions within the translocation channel [17,167–169]. In this model, the motor for translocation is the same as the motor for unfolding, and unfolding occurs as a result of collisions between the substrate and the entry port of the translocation channel. These collisions would be induced by the pulling action of the translocation motor on the substrate.

6.5.1. Substrate recognition

Substrates degraded by the proteasome must include a flexible sequence that can reach the AAA-ATPase pore loops (see below) and affinity for the ATPase, either inherent or added posttranslationally. Proteasome-targeting, analogous to the role of ubiquitin in eukaryotes, is assigned to the archaeal SAMP1/2 proteins [170]. However, the archaeal PAN appear to be an autonomous AAA-ATPase that can process substrates without the assistance of additional factors, unlike the more complex eukaryotic 19S regulatory particle that contains 13 different stoichiometric subunits in addition to the six AAA-ATPases. These subunits assist in the eukaryotic targeting pathways, the most prominent of which involves ubiquitylation [171]. Many proteins function in ubiquitylation and deubiquitylation pathways, some of which are 19S subunits. Several of the 19S non-ATPase subunits recognize ubiquitin conjugates, some are enzymes that can extend or trim polyubiquitin chains to alter substrate affinity for 19S or remove polyubiquitin chains as the AAA-ATPases translocate the substrate into the proteasome. This process is likely regulated by many additional proteins that have been characterized as substoichiometric 19S subunits that presumably associate with the core machinery and finely tune its activity at specific physiological contexts [171].

6.5.2. Substrate unfolding by the proteasomal AAA-ATPases

Substrate unfolding by bacterial ATP-dependent proteases is often rate-limiting for degradation, and increased mechanical stability of substrates slows down degradation [172]. Studies with eukaryotic proteasomes are consistent with this notion [75,167,173–176], suggesting a conserved mechanism for unfolding of globular proteins. Clearly, long sequences of alternating Gly and Ala, such as those found in silk fibroin and in the Epstein–Barr virus-encoded nuclear antigen (EBNA)-1 protein, have been shown to inhibit substrate unfolding and decrease the efficiency of proteasomal degradation, resulting in the release of partially degraded byproducts [174]. In Epstein–Barr virus, such Gly-Ala repeats interrupt EBNA-1 processing by the proteasome, thus help the virus to propagate by preventing antigen presentation by the major histocompatibility complex [177]. Rigorous studies with purified proteasomes and homogeneous substrates of defined structure and stability have recently determined kinetic parameters associated with proteasome action [178]. The test substrates, which consisted of the I27 module of Titin fused to mammalian dihydrofolate reductase, were identical in structure and proteasomal interaction and only differ in their resistance to unfolding. To bypass the requirement for ubiquitin chains, these proteins were directly tethered to the proteasome [171,179–181]. Degradation kinetics were compared for pairs of test proteins whose stability was altered either by point mutations or a tight-binding ligand but were otherwise identical. In both intact cells and in in vitro reactions using purified proteasomes and substrates, increased substrate stability led to an increase in substrate turnover time. The steady-state time for degradation ranged from 5 to 40 min and proteasomes engaged the tightly-folded substrates in multiple iterative rounds of ATP hydrolysis. Yet, ATP turnover was 110/min./proteasome and was not markedly changed by the substrate [178].

6.5.3. Substrate translocation by the proteasomal AAA-ATPases

A crystal structure of a monomeric, unassembled C-terminal ATPase domain of PAN, modeled according to the bacterial AAA-ATPase HslU [182,183], indicates that the AAA-ATPase domain forms a ring with a central pore that displays an aromatic-hydrophobic (Ar-Φ) loop from each of the six subunits [128]. Ar-Φ loops are conserved among ATPase domains of AAA-ATPases and are thought to move upon ATP hydrolysis to drive substrate translocation [184]. Thus, the PAN Ar-Φ loop (Phe244-Ile245-Gly246) likely "paddles" substrates through the pore, with aromatic Phe244 side chain playing a leading role and Gly246 being required to allow conformational changes. Indeed, mutations of equivalent residues in 19S Rpt subunits in *S. cerevisiae* lead to proteolysis defects [185], further supporting the analogous structures and mechanisms adopted by PAN and 19S AAA-ATPases [186]. The paddling model may also explain how "simple" sequences thought to interact weakly with the pore loops [187] allow adjacent stable domains to escape degradation. Several models in various AAA-ATPases address the question of how the coordinated movement of pore loops promotes substrate translocation through the hexameric ring. These include sequential action of each subunit [188,189], stochastic/probabilistic firing of individual subunits [5], and concerted movement of all pore loops [190]. A composite PAN model proposes that the coiled coils sit above a conduit of OB domains through which substrates pass before engaging the translocating pore loops of the ATPase domains [128] and corresponding Rpt assembly has been localized in the yeast 19S activator [137]. This model explains why proteasome substrates must include a flexible segment in order to be processed, because only an unstructured sequence could reach from the top surface to engage the ATPase pore loops and initiate translocation [169,191]. Although substrate translocation promotes unfolding by forcing the substrate through a narrow channel, ATP-independent mechanisms may also contribute to the functions of ATP-dependent activators. The N-terminal coiled coils structurally resemble the chaperone prefoldin and, by virtue of their overall structure, these domains can promote protein unfolding [129]. It also appears that unfolding on the ATPase surface can be stimulated by nucleotide binding and hydrolysis, although the mechanism of coupling between the N- and C-terminal regions of the ATPase subunits is currently unclear [185].

7. The role of proteasomal AAA-ATPases in the endoplasmic reticulum-associated degradation

The endoplasmic reticulum (ER)-associated protein degradation (ERAD) is a quality control mechanism that recognizes misfolded proteins and orphan subunits of oligomeric proteins in the ER, dislocates them back to the cytosol where they are tagged by polyubiquitin and degraded by the 26S proteasome [192]. The evolutionary conservation of ERAD from yeast to man is remarkable, and numerous studies in many species provide ample genetic and biochemical evidence for the different steps and cellular components that function in this pathway. Among the components implicated in ERAD are chaperones that recognize the aberrant proteins, lectins and sugar-modifying enzymes that bind and process their N-glycans, an array of dedicated ubiquitin-conjugating enzymes and E3 ligases, and many subunits of the proteasome, mostly the proteolytic 20S β subunits and the 19S Rpt subunits [193,194]. One of the hallmarks of ERAD is the dislocation, or retrotranslocation, of luminal and membrane proteins from the ER back to the cytosol, where ubiquitylation and degradation take place. This function of extraction is attributed to a ubiquitous, abundant and conserved cytosolic homohexameric AAA-ATPase, known as p97/VCP in higher organisms or Cdc48p in yeast [195–199]. p97/Cdc48, along with its cofactors Ufd1 and Npl4, is essential for ERAD and provides the driving force for the dislocation of ERAD substrates, as demonstrated by the strong dominant negative effect of substitutions in the walker A or Walker B of its AAA-ATPase domains. Such catalytically inactive p97 inhibits the dislocation of the single-pass major histocompatibility complex class I heavy chain [196], or the polytopic HMG-CoA reductase and Insig-1 in mammalian cells [200] and causes the entrapment within the ER lumen of the soluble ERAD substrate CPY*, a mutant form of the vacuolar carboxypeptidase Y in yeast cells [201]. Once again, the ability of p97/Cdc48 to pull ERAD substrates out of the ER demonstrates the ability of AAA-ATPases to generate a mechanical force by undergoing conformational changes during cycles of ATP binding and hydrolysis [6–10], as discussed above.

7.1. The role of the proteasome in dislocation

Another component that may provide the driving force for dislocation of ERAD substrates is the 26S proteasome, in addition to its established function at the proteolytic end step of the ERAD pathway. In fact, both the 20S catalytic particle and the AAA-ATPase-containing 19S regulatory particle have been implicated in dislocation. The proteolytic activity of the 20S β subunits was shown to be required for the extraction of several ERAD substrates from the ER [198], although the proteolytic activity of the proteasome does not constitute a general pulling mechanism. For example, dislocation μs, the heavy chain of secretory IgM and a luminal ERAD substrate in B lymphocytes, was not affected by proteasome inhibitors that effectively blocked μs degradation [201,202]. If dislocation is coupled to proteolysis, the substrate is extracted from the ER membrane while being degraded [203]. However, the pulling force can be either proteolysis itself and/or the AAA-ATPases that couple dislocation to degradation by pulling the substrate from the ER membrane and feeding it to the 20S. The role of the 19S itself as the sole driving force in dislocation of nonglycosylated pro-alpha factor was by cell-free assays that examined the roles of different components during ERAD. Addition of only the 19S catalyzed ATP-dependent dislocation from ER-derived yeast microsomes [204]. Likewise, real time continuous measurements of the efflux rate of fluorescently-labeled ERAD substrate from mammalian microsomes showed that the dislocation kinetics of nonglycosylated pro-alpha factor were not significantly altered by replacing all cytosolic proteins with purified 19S [205].

7.2. The role of individual Rpt AAA-ATPases in ERAD and dislocation

The involvement of the 19S Rpt subunits in ERAD was demonstrated by various types of yeast mutants such as *cim5-1*(*rpt1*), *rpt1S*, *rpt2RF*, *rpt4R*, *rpt5S* and *cim3-1*(*rpt6*) [198,206–208]. Cim5/Rpt1 was involved in extracting membrane ERAD substrates [207], and Rpt4 and Rpt5, but surprisingly not Rpt2, were also implicated in ERAD [198]. In that study, the luminal ERAD substrate CPY* was stabilized in the ATPase mutants *rpt4R* and *rpt5S* but hardly in *rpt2RF*, and because a small but significant proportion of CPY* remained protease-sensitive in *rpt4R*, it was concluded that protein dislocation could occur independently of Rpt4 ATPase [198]. To systematically explore the possibility that distinct sets of proteasomal AAA-ATPases might be engaged in unique processes along the ERAD pathway, a set of six

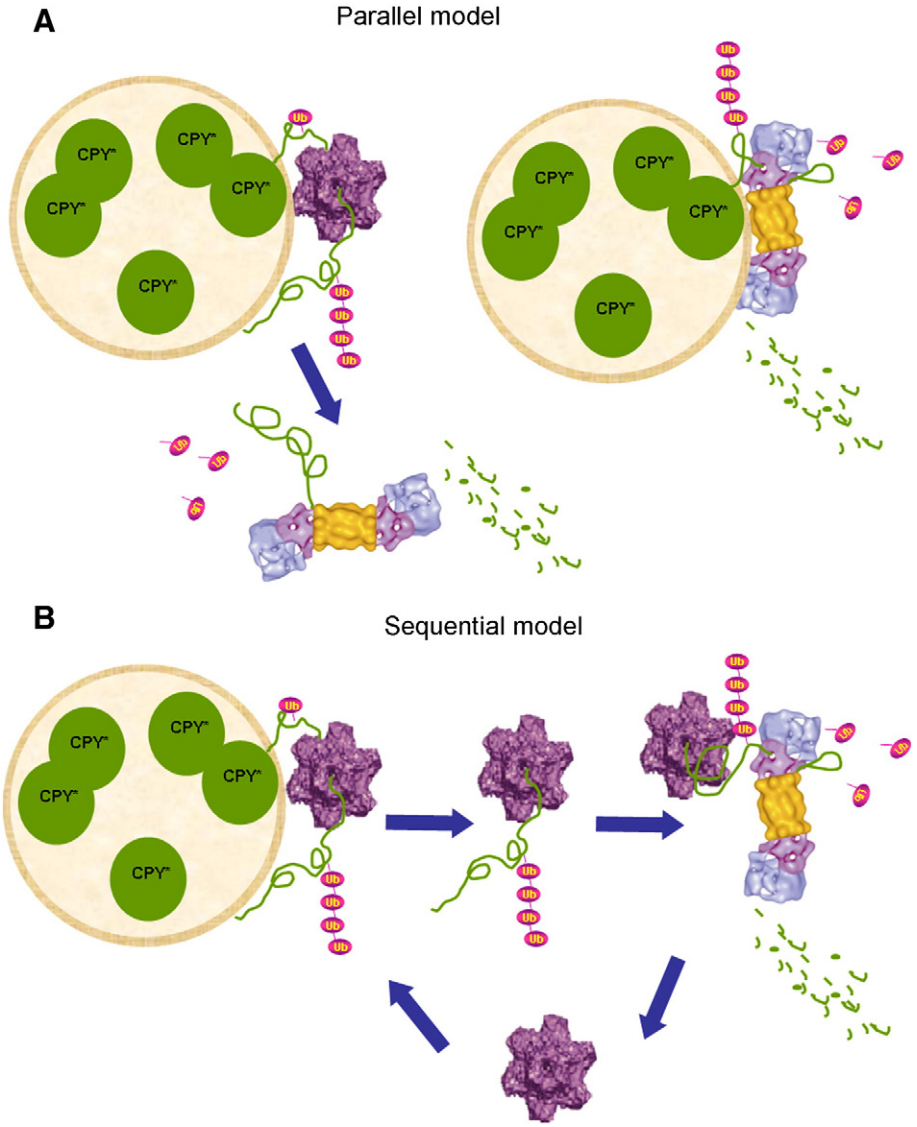

Fig. 4. Models of Rpt4 and Cdcd48 cooperation in ERAD. (A) Parallel model. (B) Sequential model (see text). Common to both models but not shown are Rpt4 and other Rpt subunits, which are part of the proteasome (this figure was originally published in [147]).

equivalent mutant strains was used. In this set, each *RPT* gene was replaced by a mutant version with conservative Lys-to-Arg substitutions of the invariant Lys of the Walker A ATP-binding motif [146]. As discussed above, this substitution generally results in complete or partial inhibition of ATP binding and ATP hydrolysis [145]. These strains exhibit diverse phenotypes with regard to growth sensitivity to temperature and amino acid analogs, protein degradation in vivo and proteolytic activities of purified proteasomes in vitro, with the strongest effect being exerted by the Rpt2 mutant [146].

Also in ERAD, the individual Rpts have unique contributions, as demonstrated by the fate of two ERAD substrates, luminal CPY*-HA and membrane 6myc-Hmg2, in this set of six equivalent mutant strains. Both ERAD substrates are stabilized only in *rpt4R* and *rpt2RF* mutants. Conversely, degradation of a cytosolic substrate, the Δ*ss*CPY*-GFP in vivo and cleavage of a synthetic peptide (Suc-LLVY-AMC) in vitro are hardly affected in *rpt4R* mutant but are strongly inhibited in *rpt2RF* mutant [147]. Hence, equivalent mutations in *RPT4* and *RPT2* result in different phenotypes. As demonstrated in numerous studies, the Rpt2 mutation displays a global arrest in proteasomal activity. In contrast, the Rpt4R-containing proteasomes are structurally stable and proteolytically active. Yet, the Rpt4 mutation is manifested preferentially in ERAD defects. Accordingly, *rpt4R* strain is particularly sensitive to ER stress and exhibits an activated unfolded protein response, whereas *rpt2RF* strain is sensitive to general stress. Further characterization of Rpt4 involvement in ERAD reveals that it participates in CPY*-HA dislocation, a function generally attributed to p97/Cdc48. This latter AAA-ATPase, which is essential for ERAD of CPY*-HA [201], is dispensable for the proteasomal degradation of the cytosolic ΔssCPY*-GFP. Hence, Cdc48 and Rpt4 appear to have overlapping functions. Indeed, overexpression of Cdc48 suppresses the ERAD deficiency in rpt4R mutant but not the impaired proteasomal degradation in *rpt2RF* strain or in mutants in the 20S β subunits [147].

A role for Rpt4 in protein dislocation across the ER membrane has been subsequently demonstrated for the cytotoxic A chain of ricin (RTA). RTA inhibits protein synthesis by depurinating 28S rRNA in the cytosol. In target cells, ricin traffics to the ER lumen so in order to dislocate to the cytosol, RTA pretends to be an ERAD substrate. However, RTA is not an ERAD substrate, as it is poorly polyubiquitylated as a result of lysyl residues scarcity. Once in the cytosol, RTA refolds to a catalytic conformation. When native RTA is compared to RTA_{Δ}, a structurally defective form that is known to be an ERAD substrate, it appears that both polypeptides are dislocated in a mechanism that involves the transmembrane Hrd1 ubiquitin ligase complex, although polyubiquitylation is not a prerequisite. However, the pathways for RTA and RTA_{Δ} diverge: the Cdc48 complex is required only for RTA_{Δ} degradation, which also requires the ATPase activity of Rpt2 but not that of any other Rpt subunits. By contrast, dislocation of native RTA involves Rpt4 and shows no obvious requirement for the other Rpt subunits or the Cdc48 complex [209].

7.3. The interrelations between Rpt4 and Cdc48, two AAA-ATPases that participate in dislocation

Two possible models may explain the cooperation between Cdc48 and Rpt4 in the ERAD-specific dislocation process [147]. In the parallel model (Fig. 4A), both AAA-ATPases pull substrates across ER membranes. This model is supported by the finding that the 19S provides the sole driving force for the dislocation of pro-alpha factor in vitro [204] and by the distinct roles of Cdc48 and Rpt4 in the dislocation of RTA_{Δ} and native RTA, respectively [209]. In the sequential model (Fig. 4B) Cdc48 pulls ERAD substrates across ER membranes while Rpt4 operates in relaying the substrates from Cdc48 to 26S proteasomes. The activity of Rpt4 is required to accept substrates from Cdc48 and release it for additional pulling cycles. This scenario is in agreement with the finding that Cdc48 remains associated with ERAD substrates for extended periods of time in *rpt4R* mutant. The finding that excess Cdc48 partially restores the impaired ERAD when Rpt4 is rendered dysfunctional [147] is compatible with both models: in the parallel model, excess Cdc48 can compensate for the absence of Rpt4 as the pulling device; in the sequential model, excess Cdc48 can compensate for the absence of active free Cdc48 that remains occupied with the undelivered substrate.

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