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The proteasome: a macromolecular assembly designed for controlled proteolysis

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In eukaryotic cells, the vast majority of proteins in the cytosol and nucleus are degraded via the proteasome–ubiquitin pathway. The 26S proteasome is a huge protein degradation machine of 2.5 MDa, built of approximately 35 different subunits. It contains a proteolytic core complex, the 20S proteasome and one or two 19S regulatory complexes which associate with the termini of the barrel-shaped 20S core. The 19S regulatory complex serves to recognize ubiquitylated target proteins and is implicated to have a role in their unfolding and translocation into the interior of the 20S complex where they are degraded into oligopeptides. While much progress has been made in recent years in elucidating the structure, assembly and enzymatic mechanism of the 20S complex, our knowledge of the functional organization of the 19S regulator is rather limited. Most of its subunits have been identified, but specific functions can be assigned to only a few of them.

Keywords: proteasome; protein degradation; proteolysis; ubiquitin

1. INTRODUCTION

Protein degradation serves multifunctional tasks. As cellular structures are continually rebuilt, homeostasis between anabolic and catabolic pathways must be maintained. Misfolded and malfunctioning proteins must be scavenged and degraded, because they are prone to aggregation. In addition to these 'housekeeping' functions, protein degradation has a key regulatory role in many cellular pathways.

To avoid havoc, degradation of proteins *in vivo* must be subject to spatial and temporal control. A central stratagem in controlling proteolysis, in one form or another, is compartmentalization. Proteolytic action can be confined to specialized membrane-bounded compartments such as the lysosome, where control is exercised by vesicle sorting. Once proteins are internalized, their proteinaceous cargo is degraded by a non-selective bulk process.

Another form of compartmentalization, that emerged from structural studies of the 20S proteasome, is self-compartmentalization (Lupas *et al*. 1997b; Baumeister *et al*. 1998). In fact, several multisubunit proteases, unrelated in sequence, have converged towards a common barrel-shaped architecture which allows the formation of inner compartments, several nanometres in size, harbouring the active sites. Access to these proteolytic nanocompartments is restricted to unfolded proteins. Hence, these proteases must be linked to a machinery capable of recognizing, binding and unfolding target proteins that present appropriate signals. These tasks are performed by accessory or regulatory complexes, which invariably contain ATPase subunits rendering protein degradation energy dependent (Gottesman *et al*. 1997a; Larsen & Finley

1997). The ATPases found in association with proteases have different evolutionary origins; in several cases, close homologues exist which do not function in a proteolytic context, but instead are involved in a broad spectrum of cellular activities (Schirmer *et al*. 1996; Beyer 1997; Gottesman *et al*. 1997b).

The necessity of cooperating has driven these very different molecular complexes towards a common architecture (Lupas *et al*. 1997b). In eukaryotes, whole modules of subunits have been recruited from precursor complexes and added to the 20S proteasome–ATPase complex (Glickman *et al*. 1998a); together with the ATPases these additional subunits form the regulatory 19S complex. The resulting 26S complex is a giant protein degradation machine of 2.5 MDa composed of *ca*. 35 different subunits. Most of the additional subunits serve to link the proteasome to the ubiquitin pathway, the pathway which confers specificity to proteasomal protein degradation (Jentsch 1992; Hochstrasser 1996; Varshavsky 1997; Hershko & Ciechanover 1998). Proteins destined for degradation via this pathway are marked by covalent attachment of (multi)ubiquitin which mediates recognition by the 26S proteasome.

2. THE 20S PROTEASOME

(a) *Occurrence and subunit composition of 20S proteasomes*

The 20S proteasome is a protease complex of 700 kDa which is ubiquitously distributed in all three domains of life (Zwickl *et al*. 1999). In archaea, the 20S proteasome was first isolated from the thermoacidophilic species *Thermoplasma acidophilum* (Dahlmann *et al*. 1989; Zwickl *et al*. 1992a). It is composed of two different types of subunits only, α and β (figure 1a,b), which assemble into a cylinder-shaped complex indistinguishable from eukaryotic 20S
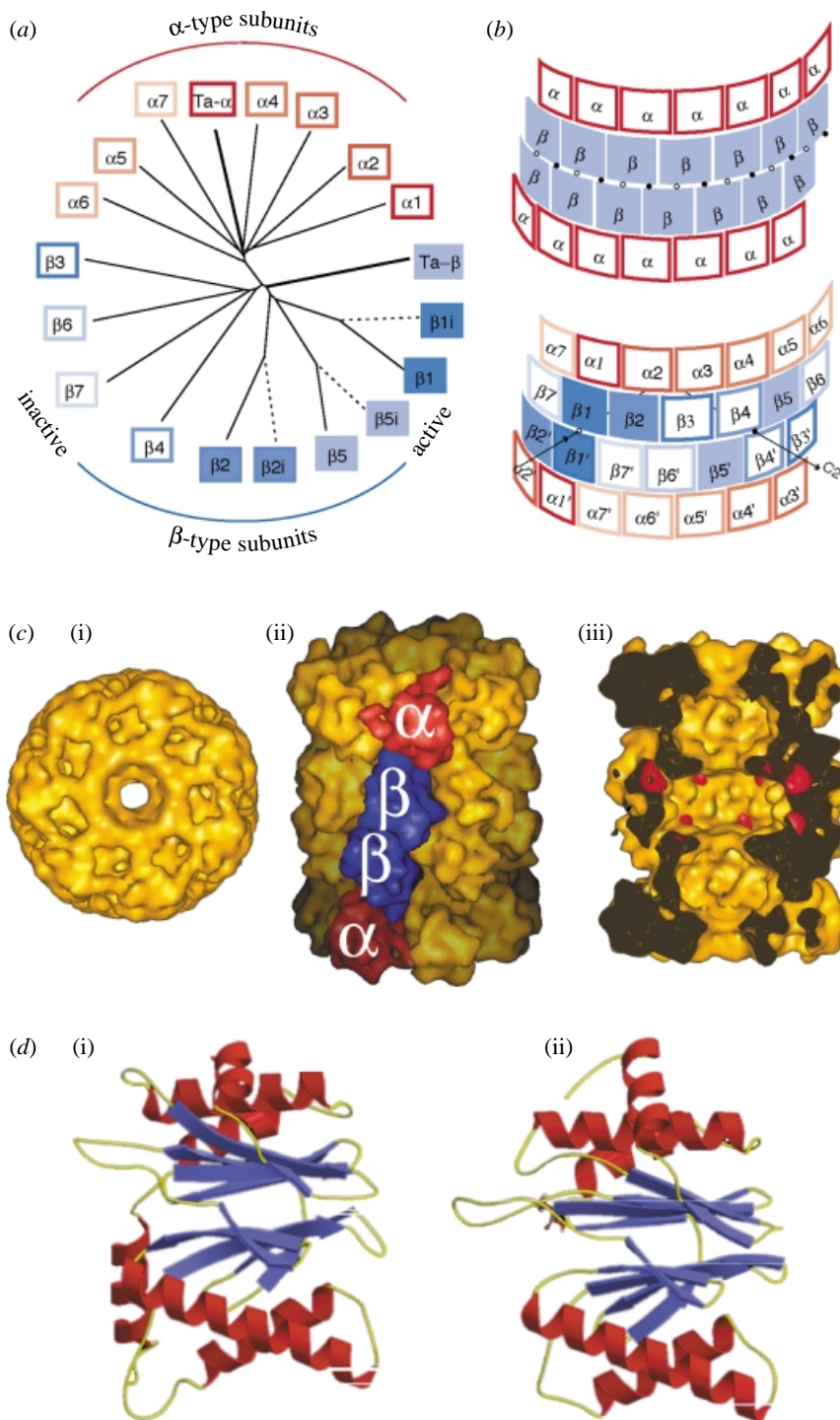
Figure 1. Evolution and structure of the 20S proteasome. (*a*) The dendrogram shows the classification of *Thermoplasma* and human proteasome subunits into α-type (red) and β-type (blue) subunits and the division of β-subunits into active (closed boxes) and inactive (open boxes) branches. The terms active and inactive refer to the presence or absence of Thr1 as the N-terminal nucleophile. Thick lines indicate *Thermoplasma* subunits and dashed lines indicate the human γ-interferon-inducible, β-type subunits. The nomenclature of the human subunits is according to the location of the homologous yeast subunits within the seven-membered rings (Groll *et al.* 1997). (*b*) Localization of the 28 subunits within proteasomes. The diagram represents partially unrolled cylinders of the *Thermoplasma* (top) and yeast (bottom) 20S proteasomes showing the positions of α-type and β-type subunits. The single C2 symmetry axis in the yeast 20S proteasome is shown and the respective positions of the seven C2 symmetry axes in the *Thermoplasma* 20S proteasome are indicated by filled and open circles. (*c*) Structure of the *Thermoplasma* 20S proteasome (Löwe *et al.* 1995) in surface representation low-pass filtered to 1 nm resolution. Top view along the sevenfold axis (i), slightly tilted side view with coloured α-type and β-type subunits (ii), and side view cut open to allow the view into the central cavity with the active sites coloured in red (iii). (*d*) Similar structural fold of the α- (i) and β-subunits (ii) of the *Thermoplasma* 20S proteasome (Löwe *et al.* 1995). Both subunits contain a sandwich of two five-stranded, antiparallel β-sheets flanked by helices on both sides.

proteasomes on electron micrographs. Subsequently, 20S proteasomes were purified and proteasomal genes were cloned from several other species, indicating a ubiquitous distribution in archaea (Zwickl *et al.* 1999).

In eukaryotes, the 20S proteasome is present in the cytoplasm and nucleus and constitutes *ca.* 1% of the cellular protein (Coux *et al.* 1996). The main difference between prokaryotic and eukaryotic proteasomes is one of complexity; the yeast 20S proteasome is built of seven different but related α-type and seven different but related β-type subunits, of which two copies of each are arranged in a stack of four seven-membered rings (figure 1*a,b*) (Groll *et al.* 1997). In higher eukaryotes, three additional β-type subunits are synthesized after induction by γ-interferon and replace their related, constitutively expressed β-type subunits in newly formed 'immuno-proteasomes' (figure 1*a,b*) (Monaco & Nandi 1995).

The first 20S proteasomes found in bacteria were purified from the actinomycete *Rhodococcus erythropolis* (Tamura *et al.* 1995). The *Rhodococcus* proteasome is composed of two distinct but related α-type and two distinct but related β-type subunits, which assemble into the same 20S particle (Zühl *et al.* 1997*b*). The existence of four distinct subunits in *Rhodococcus* 20S proteasomes seems to be an exception, since 20S proteasomes purified subsequently from other actinomycetales (*Mycobacterium smegmatis* and *Streptomyces coelicolor*) contained only one α-type and one β-type subunit (Knipfer & Shrader 1997; Nagy *et al.* 1998). Proteasome α-type and β-type genes could not be indentified in the currently completely sequenced genomes of bacteria, with the exception of the actinomycete *Mycobacterium tuberculosis* (Cole *et al.* 1998). So far, in bacteria 20S proteasomes seem to be restricted to the actinomycetales (De Mot *et al.* 1999).

However, single genes with sequence similarity to proteasomal β-type subunits have been identified in the genomes of some bacteria (Lupas *et al.* 1994). The respective gene from *Escherichia coli*, called *hsl*V, encodes for a protein, which assembles into a complex formed by a stack of two six-membered rings, which is called the HslV protease (Rohrwild *et al.* 1997). 20S proteasomes and the HslV protease seem to be mutually exclusive in their occurrence, since either one or neither of both complexes are present in the same bacterium (Zwickl *et al.* 1999).

(b) *Structure and catalytic mechanism of 20S proteasomes*

In electron micrographs, 20S proteasomes from prokaryotes and eukaryotes appear as barrel-shaped complexes composed of four stacked rings of subunits (Baumeister *et al.* 1988; Pühler *et al.* 1992; Tamura *et al.* 1995).

Immunoelectron microscopy of the *Thermoplasma* 20S proteasomes has shown that the two outer rings are formed by the α-subunits and the two inner rings by the β-subunits (figure 1*c*) (Grziwa *et al.* 1991). Collectively, the four rings form a cylinder 14.8 nm in length and 11.3 nm in width. Electron microscopic analysis of 20S proteasomes incubated with Nanogold™-labelled insulin showed that substrate uptake occurs via the narrow orifices in the terminal α-rings (Wenzel & Baumeister 1995). X-ray analysis has shown that the cylinder is traversed by a channel which widens into three cavities *ca.* 5 nm in diameter. Substrates to be degraded must wind their way

to the central cavity formed by the β-subunits which harbour the active sites (Löwe *et al.* 1995).

Site-directed mutagenesis has shown that the N-terminal threonine residue, which is exposed after removal of the pro-peptide, is essential for the proteolytic activity of the 20S proteasome (Seemüller *et al.* 1995). This was confirmed by crystal structure analysis, which demonstrated a specific binding of the inhibitor *N*-acetyl-Leu-Leu-norleucinal in close proximity of the N-terminal threonine residue (Löwe *et al.* 1995). Both the α- and β-subunits have the same three-dimensional fold (figure 1*d*) and belong to the family of Ntn-hydrolases, which possess a terminal residue (Thr, Ser or Cys) which acts as catalytic nucleophile (Brannigan *et al.* 1995; Dodson & Wlodawer 1998).

The crystal structure of the yeast 20S proteasome is remarkably similar to that of the *Thermoplasma* particle. The most conspicuous difference is the lack of an orifice in the terminal α-rings, which is occluded by the inter-digitating N-terminal segments of the α-type subunits (Groll *et al.* 1997). This structural difference provides an explanation for the finding that 20S proteasomes can be purified from eukaryotic cells in a latent form. Isolated latent proteasomes have reduced proteolytic activity, but can be activated by treatment with sodium dodecyl sulphate (SDS) or heat. It is assumed that the 'activation' opens the channel in the terminal α-rings allowing the uptake of substrate proteins into and subsequent degradation by the 20S proteasome. *In vivo* regulation of substrate access is achieved by proteasome activators, e.g. PA700 and PA28, which interact with the 20S proteasome via the terminal α-rings. Thereby, they possibly destabilize the interaction of the N-terminal segments resulting in an opening of the channel and, thus, facilitate substrate degradation.

As predicted from sequence comparisons (Seemüller *et al.* 1995), site-directed mutagenesis has demonstrated that only three of the seven different β-type subunits in the yeast 20S proteasome are proteolytically active (Heinemeyer *et al.* 1997). This was confirmed by X-ray analysis of the yeast 20S proteasome, showing that the inhibitor *N*-acetyl-Leu-Leu-norleucinal only binds to the N-terminal threonines of the three active β-type subunits, but not to any residue of the remaining four inactive subunits (Groll *et al.* 1997). Thus, a single 20S proteasome particle from yeast has only six active sites as compared to 14 active sites in a single proteasome particle from *Thermoplasma* or *Rhodococcus*.

The *Rhodococcus* 20S proteasomes have been studied by electron microscopy, which revealed the same typical barrel-shaped appearance as seen in eukaryotic and archaeal 20S proteasomes (Tamura *et al.* 1995). From coexpression and *in vitro* assembly studies it was concluded that the two different α-type and two different β-type subunits, which are present in a single *Rhodococcus* 20S proteasome, most probably assemble randomly in the α- and β-rings, respectively (Zühl *et al.* 1997*b*).

According to electron microscopy in conjunction with image analysis, the HslVU protease from *E. coli* forms a stack of two six-membered HslV rings, which are sandwiched by two rings of the ATPase HslU (Rohrwild *et al.* 1997). Inhibitor studies, site-directed mutagenesis and X-ray analysis have demonstrated that the HslV protein

has the same Ntn-hydrolase fold with an N-terminal active site threonine, as found for the β-type subunits of the eukaryotic and archaeal 20S proteasomes (Missiakas *et al.* 1996; Rohrwild *et al.* 1996; Bochtler *et al.* 1997; Yoo *et al.* 1997).

(c) *Small molecule inhibitors of proteasomes*

Recently, several selective low molecular weight inhibitors of the proteasome, which are substrate analogues, have been identified (Lee & Goldberg 1998). Most are synthetic compounds such as peptide aldehydes (Rock *et al.* 1994), peptide vinyl sulphones (Bogyo *et al.* 1997) and peptide boronates (Adams *et al.* 1998), whereas lactacystin (Fenteany *et al.* 1995; Dick *et al.* 1997) is a metabolite originally isolated from *Streptomyces* (Omura *et al.* 1991). These inhibitors are useful tools for analysing the role of the proteasome in cellular protein degradation.

In vivo studies with proteasome inhibitors have shown that the proteasome degrades not only misfolded and short-lived proteins, but also many long-lived proteins. Thus, the proteasome is responsible for 80–90% of cellular protein breakdown; the remainder is degraded by proteases in the lysosome (Rock *et al.* 1994). Short-lived regulatory proteins degraded by the proteasome are transcriptional regulators, cell-cycle regulators, oncogenes and tumour suppressors (Lee & Goldberg 1998). Interestingly, the use of proteasome inhibitors has shown that the proteasome is also responsible for the degradation of many membrane or secretory proteins (Brodsky & McCracken 1997; Sommer & Wolf 1997; Mayer *et al.* 1998) and for the generation of the majority of peptides presented by major histocompatibility complex class I molecules in higher vertebrates (Rock *et al.* 1994; Craiu *et al.* 1997).

(d) *Size distribution of 20S proteasome products*

It is intriguing to observe that, in spite of cleaving protein substrates in an apparently non-specific manner, the peptide products fall into a relatively narrow size range, averaging around seven to nine residues. It is this feature which predisposed the proteasome for a role it assumed in the course of evolution, namely the generation of immunocompetent peptides (Goldberg *et al.* 1995; Heemels & Ploegh 1995). The observation that peptide products have a restricted range of sizes led to the proposal that proteasomes may possess an intrinsic molecular ruler. One of the options considered at the time was that the distance between active sites acting in concert could provide the mechanistic basis for such a ruler (Wenzel *et al.* 1994). Indeed, the crystal structure of the *Thermoplasma* proteasome revealed a distance of 2.8 nm between neighbouring active sites, which corresponds to a hepta- or octapeptide in an extended conformation (Löwe *et al.* 1995) and it confirmed a proposal for the spatial arrangement of active sites made in the context of the molecular ruler hypothesis (Wenzel *et al.* 1994). Thus, the crystal structure seemed to provide strong evidence in support of this hypothesis (Löwe *et al.* 1995). On the other hand, recent more quantitative analyses of product length, while in agreement with an average length of eight (plus or minus one) residues, showed larger size variations, which are difficult to reconcile with a purely geometry-based ruler which should yield products more

focused in length (Kisselev *et al.* 1998). Moreover, a reduction in the number of active sites to four or two in mutant yeast proteasomes had little effect on the size of the peptides that were generated (Dick *et al.* 1998). It is therefore improbable that the distance between active sites is a major determinant of the product size.

Recent studies with synthetic peptides varying in length but displaying the same pattern of cleavage sites have indicated that, below a certain threshold in length (< 12–14 residues), degradation products have a high probability of exiting the proteolytic nanocompartment formed by the proteasome (Dolenc *et al.* 1998). Although they might re-enter and be degraded further, this appears to be a slow and inefficient process and therefore products smaller than 12–14 residues will accumulate. It should be noted here that a lower 'affinity' of the proteasome for shorter peptides was considered as another option when the molecular ruler hypothesis was originally put forward (Wenzel *et al.* 1994). There is evidence that, in *Thermoplasma*, the tricorn protease and its interacting aminopeptidases further degrade proteasome products to amino acids in order to complete the turnover of cellular proteins (Tamura *et al.* 1998).

(e) *Processing and assembly of 20S proteasomes*

The mature active state of the 20S proteasome is reached via a folding and assembly pathway, which in the case of the eukaryotic proteasome must be able to orchestrate the correct positioning of two copies each of 14 different but related subunits. Moreover, in the course of the assembly the β-type subunits are processed by an autocatalytic mechanism removing the pro-peptide and exposing the catalytic nucleophile, i.e. the N-terminal threonine. Archaeal, bacterial and eukaryotic proteasomes do not seem to follow exactly the same assembly pathways, which is not surprising in view of the increase in subunit complexity.

The *Thermoplasma* β-subunit is synthesized as a precursor with an N-terminal pro-peptide of eight amino-acid residues (Zwickl *et al.* 1994), which is autocatalytically cleaved off during proteasome assembly (Seemüller *et al.* 1996). When expressed alone, the *Thermoplasma* α-subunits assemble into seven-membered rings, whereas β-precursors and processed β-subunits, lacking pro-peptides, do not form an ordered structure (Zwickl *et al.* 1994). Coexpression of α- and β-genes yields fully assembled and proteolytically active proteasomes, independent of the presence or absence of the pro-peptide of the β-precursors (Zwickl *et al.* 1992b). *In vitro*, *Thermoplasma* proteasomes can also be reassembled after complete dissociation, re-emphasizing that the pro-petide is not essential for assembly (Grziwa *et al.* 1994).

The assembly of the *Rhodococcus* proteasome is particularly well characterized. The four different subunits (α1, α2, β1 and β2) of isolated *Rhodococcus* proteasomes assemble into proteolytically active particles both *in vivo* and *in vitro* in any combination of α-type and β-type subunits (Zühl *et al.* 1997b). Contrary to the *Thermoplasma* α-subunits, the *Rhodococcus* α-type subunits by themselves do not form rings and remain monomeric. The *Rhodococcus* β1- and β2-subunits are translated as precursor proteins with relatively long pro-peptides of 65 and 59 residues, respectively. The β-type precursors by themselves do not

form complexes and remain unprocessed and, thus, inactive (Zühl *et al.* 1997*b*). When *Rhodococcus* α-type and β-type subunits are mixed *in vitro*, they most probably form α/β-precursor heterodimers, which quickly assemble into proteolytically inactive half proteasomes (Zühl *et al.* 1997*a*). After dimerization of half proteasomes and concomitant processing of the β-type precursors, the resulting fully assembled proteasomes are proteolytically active. The pro-peptides of the *Rhodococcus* β1- and β2-subunit precursors are not essential for the incorporation of β-type subunits into proteasomes, but in their absence the rate of proteasome complex formation is strongly retarded. The pro-peptides of the β1- and β2-subunits are suggested to act in a chaperone-like manner in the folding of β-subunits and the assembly of proteasomes from two half proteasomes. The pro-peptides can exert their function either in *cis* (covalently linked) or in *trans* (added as a separate peptide) (Zühl *et al.* 1997*a*). When α-type and β-type subunits rendered incompetent for self-processing by mutation of lysine 33 of the β-type subunits are coexpressed, they assemble into pre-holoproteasomes, which are fully assembled particles accommodating the uncleaved pro-peptides in their central cavities and antechambers. It appears that pre-holoproteasome formation triggers the autocatalytic cleavage of the pro-peptides and their degradation (Mayr *et al.* 1998). Such a self-triggering mechanism is fundamentally different from the activation mechanism of other proteases where inhibitory pro-peptides are either cleaved off by means of enzymatic cofactors or where external signals, such as pH changes, trigger the autocatalytic conversion of zymogens to active proteases (Khan & James 1998). The timing of the self-triggering dictated by the proteasome assembly pathway is sufficient to ensure that activation follows sequestration, thus avoiding the risk of uncontrolled proteolysis.

For eukaryotic proteasomes it has been reported that their assembly requires additional factors which probably act as chaperones and are only transiently associated with the nascent complex (Schmidtke *et al.* 1997; Ramos *et al.* 1998). The first detected intermediate of proteasome assembly contains all α-type subunits and a subset of β-type subunits (β2, β3 and β4) in a 300 kDa or 13S complex (Nandi *et al.* 1997; Schmidtke *et al.* 1997). The subsequent incorporation of the residual β-type subunits (β1 and β5–β7) triggers fast dimerization of these precursor complexes into processing competent complexes (Nandi *et al.* 1997). Then the pro-peptides of the β-type subunits are cleaved off in autocatalytic reactions completing the assembly of proteasomes. Again, as seen in *Rhodococcus*, the pro-peptides of the β-type subunits, some of which are quite long and are not conserved in sequence, have a chaperone-like function and promote the efficient incorporation of β-type subunits, as has been shown with the yeast β5-subunit (Doa3) (Chen & Hochstrasser 1996).

(f) *Proteasome activator PA28*

PA28 (or 11S regulator) is an ATP-independent activator of the 20S proteasome, which greatly stimulates the hydrolysis of small peptides, but not the degradation of denatured or ubiquitylated proteins (Dubiel *et al.* 1992*b*; Ma *et al.* 1992). PA28, which is only found in organisms with an adaptive immune system, is a complex formed by

equal amounts of the two different but related 28 kDa subunits, PA28 α and PA28 β, which are suggested to form a heterohexamer (Ahn *et al.* 1996; Kuehn & Dahlmann 1996; Song *et al.* 1996). This 200 kDa PA28 complex can bind to both ends of the 20S proteasome, as shown by electron microscopy (Gray *et al.* 1994; Koster *et al.* 1995). When expressed in *E. coli* in the absence of PA28 β-subunits, PA28 α-subunits assemble into a heptameric particle whose crystal structure is known (Knowlton *et al.* 1997). Currently, the discrepancy in symmetry of the native heterohexameric PA28 activator and the recombinant heptameric PA28 α complex is not understood and awaits clarification by further structural studies of the native PA28 activator. The particle formed by the PA28 α-subunits is a barrel traversed by a central channel, which has a 3 nm opening on the side proximal to the proteasome and a 2 nm opening on the distal side. Structural analysis has not revealed the mechanism of 20S proteasome activation by PA28 but conformational changes in proteasomal α-type and β-type subunits upon binding of PA28 have been implicated in activation (Knowlton *et al.* 1997).

(g) *Proteasome inhibitor PI31*

Several protein inhibitors of the proteasome have been identified previously (Murakami & Etlinger 1986; Li *et al.* 1991; Chu-Ping *et al.* 1992). One of those, PI31, which inhibits the hydrolysis of proteins and peptides by the 20S proteasome, was purified from bovine red blood cells (Chu-Ping *et al.* 1992; G. N. DeMartino, personal communication). The bovine PI31 inhibitor and its human homologue are homodimers of a 30 kDa protein which exert their inhibitory activity by complex formation with the 20S proteasome. After complex formation, the stimulation of 20S proteasomes by the proteasome activators PA28 and PA700 is also inhibited, indicating that PI31 affects the interaction of PA28 and PA700 with the 20S proteasome. Analysis of truncation mutants has demonstrated that the proteasome inhibition is conferred by the C-terminal proline-rich domain of PI31. Database searches have revealed sequence homologues of PI31 in the mouse and rat, but no homologues have been found in the currently completely sequenced genomes of yeast, bacteria or archaea.

3. THE 19S REGULATORY COMPLEX

(a) *Occurrence and function of the 26S proteasome*

In eukaryotes 20S proteasomes associate with one or two 19S regulatory complexes to form the 26S proteasome, which degrades the bulk of cellular proteins. The 19S regulatory complex provides the link for proteasome-mediated proteolysis with the ubiquitin pathway of protein degradation (Coux *et al.* 1996; Hochstrasser 1996; Hershko & Ciechanover 1998; Rechsteiner 1998; Voges *et al.* 1999). The 26S proteasome degrades not only abnormal and damaged proteins, but also cell-cycle regulators, oncogenes and tumour suppressors and is implicated in the processing of antigens, the activation or degradation of transcription factors and the degradation of misfolded or damaged secretory proteins. In higher eukaryotes the subcellular location of the proteasome is mainly cytoplasmic and nuclear (Schauer *et al.* 1993;

Palmer *et al.* 1996; Reits *et al.* 1997). In yeast cells, however, the nuclear envelope–endoplasmic reticulum network and the nuclear periphery seem to be predominant sites of 26S proteasome action (Enenkel *et al.* 1998; Wilkinson *et al.* 1998).

(b) ATP-dependent proteolysis

The degradation of target proteins by the proteasome–ubiquitin system is dependent on the presence of ATP at several stages (Etlinger & Goldberg 1977; Jentsch 1992; Hochstrasser 1996; Varshavsky 1997; Hershko & Ciechanover 1998). ATP is hydrolysed to AMP in the process of conjugating UbGly76 to lysine residues of the target protein, which involves activating enzymes (E1), carrier proteins (E2) and ligating enzymes (E3). Here energy is consumed during linkage of ubiquitin via a high-energy thiolester bond to E1, from which ubiquitin is transferred to E2 and finally ligated to the target protein by E3. In addition, subsequent synthesis of ubiquitin chains on the target protein (where UbGly76 is linked primarily to UbLys48) requires ATP hydrolysis. These ubiquitin chains then function as recognition signals for the degradation by the 26S proteasome (Wilkinson *et al.* 1980; Hershko & Heller 1985; Chau *et al.* 1989; Haas *et al.* 1990; Varshavsky 1992; Finley *et al.* 1994). The second ATP-requiring step is the assembly of the 26S proteasome, which proceeds via association of 20S proteasomes with 19S regulators, also called balls (Hoffman *et al.* 1992), μ-particles (Udvardy 1993), PA700 (Chu-Ping *et al.* 1994) or 19S cap complexes (Peters *et al.* 1994). However, it is not clear whether this assembly is coupled to ATP hydrolysis. The last ATP-dependent step is the degradation of the target protein conjugated to ubiquitin chains by the 26S proteasome (Hough *et al.* 1986, 1987) in which ATP is hydrolysed to ADP. The recognition of the ubiquitin conjugate does not seem to require ATP; similarly the cleavage of the peptide bond itself is exergonic. Since the 20S proteolytic core degrades only unfolded proteins, it is probable that ATP is consumed during unfolding and translocation of the target protein mediated by the 19S regulatory complex.

(c) Subunit composition and organization of the 19S complex

The entire eukaryotic 26S complex formed by the 20S and 19S complex(es) is a huge protein degradation machine of 2.5 MDa. It is still unknown how many subunits the 19S complexes from different organisms contain (Udvardy 1993; DeMartino *et al.* 1994; Dubiel *et al.* 1995) since weakly bound components might dissociate depending on the purification procedure applied. For instance, enzymes for the de-ubiquitylating activities observed remain to be found and the stoichiometric association of other proteins is controversial. In *Saccharomyces cerevisiae*, 17 subunits have been identified and sequenced (Glickman *et al.* 1998b) and it is believed that the total number of subunits in 19S complexes will not exceed 20 (H. Hölzl, unpublished results). The 19S subunits are designated by the mammalian 'S' (subunit) (Dubiel *et al.* 1995) or *S. cerevisiae* 'Rp' (regulatory particle) (Finley *et al.* 1998) nomenclatures. Although a
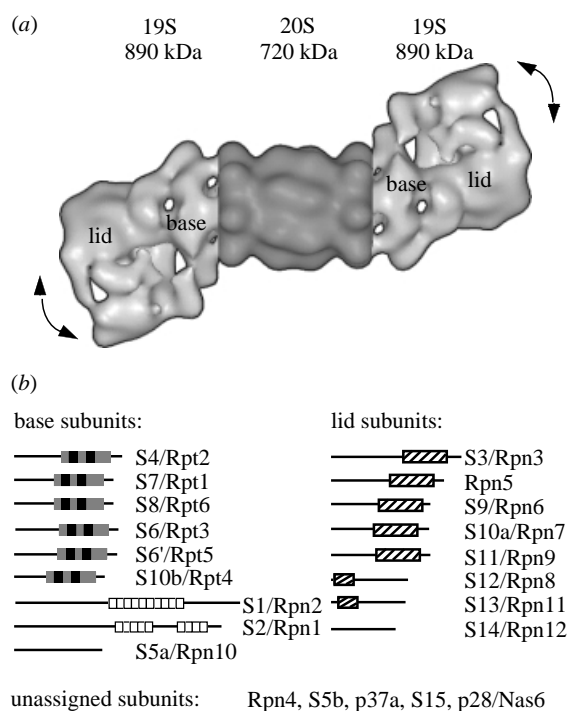

Figure 2. Structure of the 26S proteasome and subunit composition of the 19S regulatory complex. (*a*) Composite model of the three-dimensional structure of the 26S proteasome from *Drosophila* as based on electron microscopy and using the crystal structure of the 20S proteasome from *Thermoplasma* (Walz *et al.* 1998). The indicated masses of the 19S and 20S complexes from *Drosophila* were determined by scanning transmission electron microscopy (STEM) (H. Hölzl, unpublished results). An observed wagging-type motion is indicated by arrows and the approximate location of the base and lid subcomplexes of the 19S complex is designated. (*b*) Subunits of the base and lid subcomplexes of the 19S regulator. Sequence motifs are indicated as boxes (see the text for details).

large number of subunits have been studied genetically, the functions of most of the subunits in the 19S complex are still unknown (Voges *et al.* 1999).

The subunits of the 19S complex are organized into two subcomplexes, the 'base' and the 'lid', which form the portions proximal and distal to the proteolytic 20S core, respectively (figure 2) (Glickman *et al.* 1998a). The base complex contains all six ATPases (S7/Rpt1, S4/Rpt2, S6/Rpt3, S10b/Rpt4, S6'/Rpt5 and S8/Rpt6) and the two largest subunits (S1/Rpn2 and S2/Rpn1), as well as S5a/Rpn10 (figure 2b). The association of the base with the 20S proteasome is sufficient for the ATP-dependent degradation of non-ubiquitylated proteins.

The six distinct ATPases contain an AAA (ATPases associated with a variety of activities) domain (figure 2b) (Dubiel *et al.* 1992a), are all essential, and confer ATP dependence on protein degradation by the 26S proteasome (Rubin *et al.* 1998). Although prokaryotes do not contain 19S complexes, their 20S proteasomes most probably also interact with ATPases of the AAA superfamily (Zwickl *et al.* 1999). The recombinant *Methanococcus* protein PAN (proteasome-activating nucleotidase) (P. Zwickl, unpublished results) forms a high molecular weight complex and stimulates the degradation of protein substrates by the *Thermoplasma* 20S proteasomes in a

nucleotide-dependent manner. The related AAA ATPase ARC (ATPase-forming, ring-shaped complexes) from *R. erythropolis* forms a hexameric complex (Wolf *et al.* 1998). However, the formation of a distinct complex of PAN or ARC with the 20S proteasome has not yet been shown. In both archaea and bacteria, which seem to lack ubiquitin as a degradation signal, the ATPases may not only unfold and translocate, but also recognize substrate proteins (Gottesman 1996). In the eukaryotic 19S complex, the ATPases are believed to unfold target proteins and have therefore been termed 'reverse chaperones' or 'unfoldases' (Lupas *et al.* 1993). They possibly also recognize and bind partially unfolded or aberrant proteins (Rechsteiner *et al.* 1993) and may assist in their translocation into the proteolytic chamber of the 20S proteasome (Larsen & Finley 1997). Specific interactions between different 19S ATPases suggest that the six ATPases assemble into a ring resembling other AAA ATPase complexes. The ATPases also interact with 20S α-type subunits (Gerlinger *et al.* 1997) and may form the interface of the 19S complex with the 20S core particle (Baumeister *et al.* 1998; Voges *et al.* 1999) similar to other protease–ATPase complexes (Gottesman *et al.* 1997*a*). The N-terminal regions of the 19S ATPases, which are predicted to form coiled coils (Rechsteiner *et al.* 1993; Russell *et al.* 1996), are possibly involved in the binding of substrate proteins (Wang *et al.* 1996) or in interactions between neighbouring ATPases (Richmond *et al.* 1997). The monomers in other AAA ATPase complexes have been shown to associate via their AAA domains (Babst *et al.* 1998; Lenzen *et al.* 1998; Yu *et al.* 1998); however, the coiled-coil domains might have a role in the initial recognition and positioning of the six 19S ATPases.

The two largest subunits of the base complex are related by sequence and contain leucine-rich-like repeats (figure 2*b*), which are also found in BimE, the largest subunit of the anaphase-promoting complex (APC) or cyclosome (Lupas *et al.* 1997*a*). These repeats were predicted to fold into a β/α-structure resembling the horseshoe motif in the ribonuclease inhibitor (Kobe & Deisenhofer 1995); the β-strands may serve as a binding surface for unfolded proteins.

S5a/Rpn10 is the only subunit in the 19S complex yet identified which binds Lys-linked ubiquitin chains *in vitro* (Deveraux *et al.* 1994). Ubiquitin binding sites have been localized in the C-terminal part of the protein involving the regions GVDP and LAL/MALRV/LSM, with alternating large and small hydrophobic residues (Haracska & Udvardy 1997; Fu *et al.* 1998; Young *et al.* 1998). S5a interacts with the hydrophobic stretch UbLeu8, UbIle44, UbVal70 present on the surface of ubiquitin chains (Beal *et al.* 1996, 1998). It is still controversial whether the ubiquitin binding properties of free S5a and the 26S proteasome are identical. Furthermore, S5a is not essential in yeast (Van Nocker *et al.* 1996; Rubin *et al.* 1997) suggesting that there are other ubiquitin recognition components in the 19S complex. The N-terminal part of S5a/Rpn10 seems to be involved in interactions between the lid and the base complexes (Glickman *et al.* 1998*a*).

For degradation of ubiquitylated target proteins, the whole 26S complex including the lid complex is necessary. The lid comprises eight subunits: S3/Rpn3, Rpn5, S9/ Rpn6, S10a/Rpn7, S11/Rpn9, S12/Rpn8, S13/Rpn11 and S14/Rpn12. Five of these subunits (S3/Rpn3, Rpn5, S9/ Rpn6, S10a/Rpn7 and S11/Rpn9) contain a PINT/PCI domain in their C-terminal parts and S12/Rpn8 and S13/ Rpn11 contain an MPN domain in their N-terminal parts (Aravind & Ponting 1998; Hofmann & Bucher 1998) (figure 2*b*). These motifs are found in subunits of other large protein complexes, such as the COP9–signalosome complex (Seeger *et al.* 1998; Wei *et al.* 1998) and the eukaryotic initiation factor 3 (eIF3) complex (Asano *et al.* 1997). There is possibly a one-to-one correspondence between 19S and COP9 signalosome subunits which may indicate a common evolutionary origin of the complexes. The functions of the lid subunits is unknown, but some may be involved in the recognition of ubiquitin conjugates. In addition, the de-ubiquitylating activity of the 26S proteasome (Eytan *et al.* 1993; Lam *et al.* 1997) is believed to reside in the lid complex (H. Hölzl, unpublished results) or to be transiently bound to the 26S complex (Hegde *et al.* 1997). By consecutive removal of distal ubiquitin moieties, the 26S complex-associated 'PA700 isopeptidase' could perform an 'editing function' on poorly ubiquitylated target proteins in order to rescue them from degradation (Lam *et al.* 1997). However, it is not known which of the yet sequenced 19S subunits is the PA700 isopeptidase.

(d) *Structural features of the 19S regulatory complex*

26S proteasomes from different organisms with two 19S caps attached to the 20S core have an overall length of 45 nm and a width of 20 nm on electron micrographs (Peters *et al.* 1993; Yoshimura *et al.* 1993; Fujinami *et al.* 1994). 20S proteasomes are also observed with only one 19S cap attached and both species can be separated on native gels (Glickman *et al.* 1998*b*). A three-dimensional electron microscopic study of 26S proteasomes has revealed that the 19S complex is flexibly linked to the 20S core. The relative continuous movement of the 19S caps with respect to the 20S core occurs in the adsorption plane (figure 2*a*) (Walz *et al.* 1998). However, it is currently unknown whether the observed structural flexibilities and variations have functional relevance.

The proposed association of the ATPases with the α-rings of the 20S proteasome would introduce a symmetry mismatch between the rings. Symmetry mismatches have been suggested to favour the relative rotation of adjacent rings, since rotation by a small angle increment (8.6° in the case of adjacent six- and sevenfold rings) would bring them into an energetically equivalent position (Beuron *et al.* 1998). In the case of the proteasome, one may speculate that such a rotation could facilitate unfolding or translocation of target proteins; however, no direct experimental evidence for such a scenario exists.

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Discussion

A. Hershko (*Technion—Israel for Technology, Haifa, Israel*). Where do you think the site of entry of ubiquitinated proteins into the proteasome is? I understand that the crystallographic data on the yeast proteasome shows that the site is closed.

W. Baumeister. The entry is definitely where the channel is located in the *Thermoplasma* proteasome. The fact that in yeast this is partly occluded is not an argument that it should be elsewhere. I think the regulatory subunits must somehow be involved in the gating of the channel. How this works awaits further structural studies.

R. T. Hunt (*Clare Hall Laboratories, Hertfordshire, UK*). Could I follow that up? I've never understood why the proteasome is a 'two-headed monster'. I take it you think the substrate enters from one end, but do the products then come out of the middle or the other end?

W. Baumeister. In the *Thermoplasma* proteasome there is no opening which is large enough in the middle of the complex. Yeast has these 'side windows'. Some mutagenesis might help to determine whether these windows are important. The structure of the 26S complex looks symmetrical, but this is a little misleading. This is partly because symmetrical complexes were selected for the structural analysis. A large fraction of the complexes have regulatory complexes on only one end.

R. T. Hay (*University of St Andrews, Fife, UK*). In *Thermoplasma* where there is no 19S regulatory complex, how do you achieve specificity of degradation?

W. Baumeister. The specificity really resides in the ubiquitin system and not in the proteasome itself. Of course we don't know what targets proteins for degradation in prokaryotes. In all bacteria, there is an enigmatic open reading frame (ORF) called ORF7 which produces a protein of about 7 kDa with two glycine residues at the end. In all cases where we have found proteasomes, this ORF is part of the proteasome operon. Still, there has been no demonstration of conjugates of any kind. However, if you knock it out it has a similar phenotype to knocking out the proteasome.