

A Tale of Two Giant Proteases

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Abstract. The 26S proteasome and tripeptidyl peptidase II (TPPII) are two exceptionally large eukaryotic protein complexes involved in intracellular proteolysis, where they exert their function sequentially: the proteasome, a multisubunit complex of 2.5 MDa, acts at the downstream end of the ubiquitin pathway and degrades ubiquitinated proteins into small oligopeptides. Such oligopeptides are substrates for TPPII, a 6-MDa homooligomer, which releases tripeptides from their free N-terminus. Both 26S and TPPII are very fragile complexes refractory to crystallization and in their fully assembled native form have been visualized only by electron microscopy. Here, we will discuss the structural features of the two complexes and their functional implications.

1 Intracellular Proteolysis

Maintenance of cellular homeostasis relies upon the spatial and temporal control of protein degradation: regulatory proteins such as transcription factors or components of signal transduction chains need to be degraded at specific moments of their life spans. Misfolded or damaged and, as a consequence, dysfunctional proteins are prone to aggregation and must be removed from the cytoplasm; the immune system relies on the availability of immuno-competent peptides such as obtained by degradation of foreign proteins. Major sites of proteolysis are the cytoplasm and the lysosome, a membrane-bound compartment housing several small proteases. Cytosolic protein degradation in eukaryotes is mainly effected via the ubiquitin pathway: substrates destined for degradation are modified with ubiquitin chains by a cascade of (1) ubiquitin activating, (2) ubiquitin conjugating, and (3) ubiquitin ligating, and these steps are mediated by the enzymes E1, E2, and E3, respectively. Proteins tagged with multiubiquitin chains are then selected by the 26S proteasome and subsequently degraded in an ATP-dependent process (see Ciechanover 2005 for a review). The products of the proteasome's action are peptides of a length of 8–12 amino acids (Kisselev et al. 1999), which subsequently can both be trimmed and presented to the immune system or be degraded into amino acids. The ATP-independent proteases involved in these processes are often referred to as “downstream proteases” and some of them occur as homooligomeric complexes with a size exceeding that of the 26S proteasome. Examples of such “giant proteases beyond the proteasome” (Yao and Cohen 1999) are the tricorn protease (TRI) of the Archaeon *Thermoplasma acidophilum* (Tamura et al. 1996; Walz et al. 1997) and its functional equivalent in eukaryotes, Tripeptidyl peptidase II (TPPII), (Geier et al. 1999; Rockel et al. 2002). The 20S proteasome and TRI have been crystallized (Löwe et al. 1995; Groll et al. 1997; Bosch et al. 2001; Brandstetter et al. 2001), but the holocomplexes of 26S, TRI, and TPPII in their fully assembled and fully functional oligomeric form have thus far only been studied via electron microscopy (Walz et al. 1997, 1998; Rockel et al. 2002; Nickell et al. 2007a) (Fig. 1).

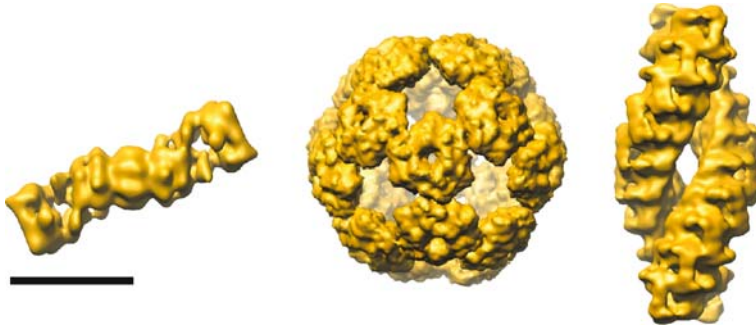


Fig. 1. Structures of three giant proteases obtained by electron cryo-microscopy. *Left* 26S proteasome (Nickell et al. 2007a); *center* Tricorn protease (Walz et al. 1999); *right* Tripeptidyl peptidase II (Rockel et al. 2005). *Scale bar*, 25 nm

2 The 26S Proteasome

The 26S proteasome links the ubiquitin-pathway with protein degradation and hence is involved in many cellular processes (for reviews see Baumeister et al. 1998; Voges et al. 1999; Pickart and Cohen 2004). In contrast to the cellular function of the 26S proteasome, the better part of its functional mechanism is still only dimly understood, partly due to the lack of a solid structural framework. Aside from its complexity (it consists of more than 30 different subunits) and its fragility, also the plasticity of the 26S proteasome presumably contributes to these difficulties: whereas previously the proteasome population in a cell has been viewed as uniform, it now becomes apparent that a whole array of functionally and structurally distinct complexes might exist and, moreover, that the subunit composition is subject to regulation.

The 26S proteasomes contained in a normal cell can be separated into two subcomplexes; the 20S proteasome—the 700-kDa proteolytic core—and the 19S particle, a 900-kDa regulatory complex required for the recognition of ubiquitinated proteins and their preparation for degradation. To mediate its diverse cellular functions, the 20S proteasome also associates with other specific adaptor complexes, like the PA28/11S complex or the B110/PA200 complex, which function as ac-

tivators of the proteolytic core (for reviews see Glickman and Raveh 2005; Hanna and Finley 2007).

2.1 The 20S Proteasome

The 20S proteasome is a barrel-shaped complex consisting of four seven-membered rings. These rings are composed of two distinct but related proteins termed α - and β -subunits with molecular masses of approximately 25 kDa, which are arranged in α -rings and β -rings, respectively. The two β -rings enclose the central proteolytic chamber of this barrel-shaped complex, and one α - and one β -ring jointly form the outer (ante-) chambers. The three chambers of the 20S proteasome are interconnected by a narrow channel. The quaternary structure of 20S proteasomes is the same in all kingdoms, but their level of complexity varies: the simplest 20S proteasomes are found in prokaryotes, which contain only one or two types of α - and β -subunits, respectively. In eukaryotes, the two subunits have developed into seven different subunits of each type, resulting in seven paralogous α -subunits and seven paralogous β -subunits (reviewed in Voges et al. 1999; Zwickl et al. 2001).

20S proteasomes from eukaryotes, Archaea, and bacteria have been crystallized; the proteasomal α - and β -subunits have the fold of Ntn-hydrolases: a pair of five-stranded β -sheets is flanked on both sides by α -helices (Löwe et al. 1995; Groll et al. 1997; Unno et al. 2002; Groll et al. 2003; Kwon et al. 2004) (Fig. 2). The β -subunits are catalytically active threonine hydrolases in which the N-terminal threonine of the β -subunit of the *Thermoplasma* proteasome acts as both the catalytic nucleophile and the primary proton acceptor. The peptidolytic activity of the *Thermoplasma* proteasome is chymotrypsin-like (Löwe et al. 1995; Seemüller et al. 1995a, 1995b). In eukaryotes, four β -subunits lack the N-terminal threonine residue and, consequently, only three out of the seven β -subunits are proteolytically active. The eukaryotic proteasome possesses three different peptidolytic activities: tryptic, chymotryptic, and postacidic (Kisselev et al. 2006). In higher eukaryotes with an adaptive immune system, γ -interferon excites the expression of three additional active β -subunits. These β -subunits replace the related, constitutively expressed active subunits and the re-

sulting immunoproteasomes are characterized by a modulation of proteolytic specificity (Niedermann 2002).

Prior to assembly, the proteolytically active β -subunits contain propeptides. Formation of the active sites requires post-translational removal of these propeptides, which occurs autocatalytically and only after the 20S complex is fully assembled. This delay ensures that the active sites are sequestered within the central chamber, which is only accessible via the antechambers. These again are accessible only through narrow orifices at both ends of the 20S complex. The exact function of the antechambers is currently unknown. Since it has been demonstrated that they can store substrate, their function might be to retain proteins in a partially folded state and—once the previous substrate has been degraded and enough space has become available—to translocate them into the catalytic chamber (Pickart and Cohen 2004; Sharon et al. 2006b).

2.2 The Regulatory Complexes

The ports leading into the 20S proteasome are constricted by an annulus built from turn-forming segments of the seven α -subunits. In the basal state they are shut, but even in their open conformation they are too narrow for folded polypeptides to enter, a feature ensuring that randomly encountered native proteins are denied admission. Thus 20S proteasomes in isolation generally show negligible protease activity. Only by association with adaptor complexes are they transformed into efficient and, dependent of the nature of the particular adaptor complexes, ubiquitin-dependent proteases. The adaptor complexes interact with the terminal α -rings of the proteasome by mechanisms that open the gate for substrate uptake (Pickart and VanDemark 2000).

2.2.1 The 19S Regulatory Complex

In vivo, most eukaryotic 20S proteasomes are flanked on one or both sides by the 19S regulatory complex, which associates with the 20S proteasome in an ATP-dependent manner, and the resulting 2.5-MDa complex is the canonical 26S proteasome (Babbitt et al. 2005). The 19S complex, which comprises approximately 20 different subunits with

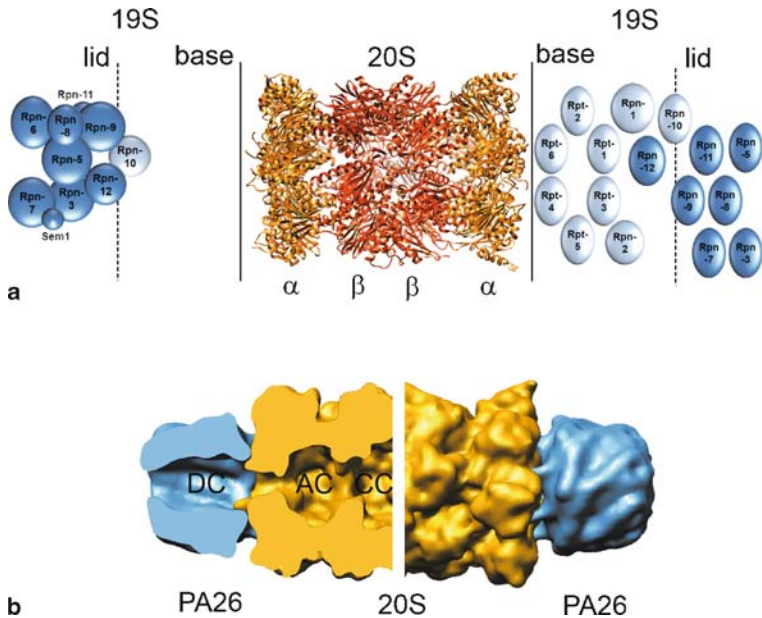


Fig. 2a,b. The 20S proteasome and its adaptor complexes. **a** 20S and 19S; *left* structural organization of the *Saccharomyces cerevisiae* 19S lid, obtained by mass spectrometry and chemical cross-linking (modified from Sharon et al. 2006a); *center* crystal structure of mammalian 20S (PDB-entry 1IRU); *right* interaction map for the 19S lid of *C. elegans*, obtained by two-hybrid screening (Davy et al. 2001). Lid subunits are *dark blue*, base subunits are *light blue*. **b** Crystal structure of the PA26–20S–PA26 complex (PDB-entry 1fnt), low-pass filtered to 1 nm. *Left* cut open view, *right* surface view. DC dome-shaped cavity, AC antechamber, CC central chamber

a combined mass of 900 kDa, is probably the most important and at the same time the most complicated of the adaptor complexes. It bridges the sites of recognition and degradation, since it contains the recognition sites for the ubiquitinated target proteins as well as chaperone complexes that unfold the substrates and translocate the now unfolded polypeptide chain through the entry ports of the 20S proteasome. The 19S complexes from various organisms have been studied, and differ-

ent nomenclatures exist for the names of their subunits in humans, fruit flies, and yeast (Ferrell et al. 2000). Using biochemical methods, the regulatory particle can be divided into two subcomplexes, the base and the lid (Glickman et al. 1998), which are located proximally and distally in relation to the 20S core, respectively. Among the subunits of the base are the six paralogous AAA-ATPases Rpt1-Rpt6. Like other members of the AAA-family, they exhibit chaperone activity (Braun et al. 1999; Liu et al. 2005) and are thought to form a ring and to be involved in substrate unfolding and translocation. Since only the 26S holoenzyme but not the 20S-base complex is capable of degrading ubiquitinated proteins, recognition and binding, as well as deubiquitinylation of ubiquitin-tagged substrates appears to be mediated by the subunits of the lid complex (Glickman et al. 1998). Until now, no high-resolution structure of the 19S regulatory complex has been available and only very few atomic structures of individual subunits exist (Wang et al. 2005; Nakamura et al. 2007; Sanches et al. 2007; Schreiner et al. 2008). Still, by yeast two-hybrid studies (Davy et al. 2001), mass spectrometry (Sharon et al. 2006a) and GST-pulldowns (Chen et al. 2008), a wealth of information on interaction of proteasome subunits has been obtained and topology maps have been constructed (Fig. 2a).

2.2.2 Alternative Cap Complexes

Aside from the 19S regulatory particle, alternate structures can also cap the 20S proteasome; all of them are ATP-independent and less complex in composition. Organisms with an adaptive immune system contain the PA28 activator, a 200-kDa cap protein that is induced by interferon and consists of two related subunits of a mass of approximately 28 kDa (Rechsteiner et al. 2000). These α - and β -subunits assemble into a heteroheptamer and form a dome-shaped structure built of a bundle of alpha-helices. Like the 19S complex, this structure can bind to both ends of the proteasome and also hybrid PA28–20S–19S complexes exist (Cascio et al. 2002). PA26, a PA28-related protein in *Trypanosoma brucei* that also stimulates peptidolytic activity of the 20S proteasome, has been crystallized in complex with 20S, and the interactions that lead to gate opening in the 20S proteasome have been visualized: the N-terminal tails of the alpha subunits are straightened and thus are

moved away from the pore, thereby opening the entrance port (Whitby et al. 2000) (Fig. 2b). PA200/Blm10 is another non-ATPase proteasome cap. It consists of a single polypeptide of 250 kDa, which likewise induces gate opening in the 20S proteasome, as was shown by electron cryomicroscopy (Ortega et al. 2005; Iwanczyk et al. 2006).

2.3 The 26S Proteasome from *Drosophila Melanogaster*

In general, structural studies of 26S proteasomes are hampered by their low intrinsic stability, leading to dissociation into various subcomplexes. The 26S complexes from various sources have been isolated and examined by electron microscopy and it turned out that embryos of *Drosophila melanogaster* provide relatively stable 26S particles, comprising a well-defined complement of subunits (Yoshimura et al. 1993; Walz et al. 1998; Hölzl et al. 2000). Still, electron micrographs of *Drosophila*-26S display a degree of structural heterogeneity that complicates image analysis and three-dimensional reconstruction. Two-dimensional averages of negatively stained 26S proteasomes feature the characteristic dragon head, where the 19S complexes in the double-capped particles face in opposite directions, apparently reflecting the C2-symmetry of the eukaryotic 20S proteasome. While negatively stained 26S complexes adsorbed on carbon film are still acceptably intact, cryo-preparation can trigger their disassembly, and in the self-supporting layer of vitrified ice the number of dissociated particles is often relatively high. Despite all these adversities, a structure of the 26S proteasome from *Drosophila melanogaster* has been obtained by cryoelectron microscopy. Double-capped 26S proteasomes were “purified” in silico. Their 3D reconstruction depicts the linear assembly 19S-20S-19S, the regulatory complexes facing opposite directions (Nickell et al. 2007a) (Fig. 3). Most likely, the part of the 19S complex attached to the α -rings of the 20S proteasome represents the base complex and contains the six paralogous AAA-ATPases. The base complex and the proteasomal α -rings enclose a dome-shaped compartment next to the antechamber, as is also seen in the ClpAP complex (Ishikawa et al. 2004), and according to current understanding, attachment of the base complex should open the gate to the interior of the 20S proteasome (see Sect. 2.2). How this is carried out mechanically is still an open question. A recent study with PAN

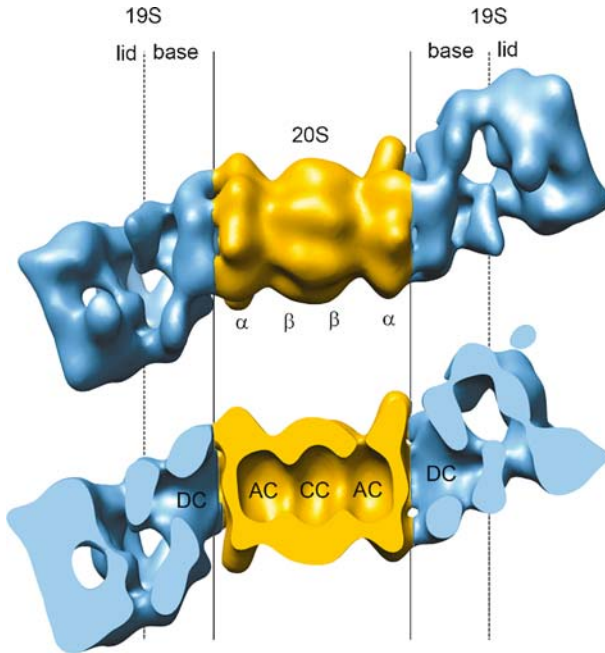


Fig. 3. Structure of the 26S proteasome from *Drosophila melanogaster* 26S obtained by single particle electron cryomicroscopy (Nickell et al. 2007a). *Top* surface representation; *bottom* cut-open view. Note the potential substrate entry/exit sites at the 19S–20S interface. *DC* dome-shaped cavity, *AC* antechamber, *CC* central chamber

(proteasome-activating nuclease), an archaeobacterial adaptor complex with homology to the AAA-ATPases of the 19S base, suggests that the C-terminal domains of the ATPases insert into binding-pockets at the 20S α -rings and thereby trigger gate-opening (Smith et al. 2007).

An interesting feature visible in the present map but also in an earlier reconstruction obtained by electron cryotomography is a sideward channel at the 20S-base interface. This channel connects the cavity underneath the base with the outside and possibly provides an entry or exit site for substrates (Nickell et al. 2007a,b). While features visible in such medium-resolution structures of the 26S proteasome can hint

at the function of certain subcomplexes or building blocks, a detailed understanding of its mechanism will require the atomic structure of the holocomplex. Given the complexity and fragility of 26S proteasomes, it is unlikely that they can be crystallized *in toto*. Furthermore, their dynamics represent an additional problem: besides the set of canonical subunits, there are several variable subunits that modulate proteasome function (Glickman and Raveh 2005). Thus, obtaining a detailed structural model of the 26S proteasome will require a multidisciplinary approach integrating electron microscopic reconstructions of the holocomplex, atomic structures, and interaction maps of its building blocks with all available information from other sources (Robinson et al. 2007).

3 Tripeptidyl Peptidase II

In eukaryotes, the proteasome is essential. Its inhibition leads to cell death, although some cells can adapt to proteasome inhibitors. In those cells, the induction of an alternative protease with the capability of substituting for some metabolic functions of the proteasome has been reported (Glas et al. 1998; Geier et al. 1999; Wang et al. 2000; Princiotta et al. 2001). This protease has been identified as Tripeptidyl peptidase II (TPPII) and its basic cellular activity is the removal of tripeptides from the free N-terminus of oligopeptides such as produced by the 26S proteasome (Balow et al. 1983; Tomkinson 1999). In addition to this exopeptidase activity, a much lower endopeptidase activity of the trypsin type was also detected (Geier et al. 1999). TPPII has broad substrate specificity. It has a preference for cleaving after hydrophobic residues but cannot cleave before or after proline residues. As an exopeptidase, it preferentially cleaves after lysine residues but as an endopeptidase, can in fact cleave after proline residues (Geier et al. 1999; Seifert et al. 2003). The endopeptidase activity can create a specific epitope (Nef73-82) of the human immunodeficiency virus (HIV) independently of the proteasome (Seifert et al. 2003).

Like the proteasome, TPPII is involved in the generation of antigenic peptides for presentation by the MHC class I complex (Levy et al. 2002). Here, it appears to trim the N-terminus of some peptides generated by the proteasome and apparently it is the only downstream pro-

tease that can degrade peptides of more than 15 amino acids in length (Reits et al. 2004; York et al. 2006). However, there is some controversy regarding the degree to which TPPII is involved in this process (see van Endert 2008 for a recent review) and its role in MHC class I processing might be that of another cytosolic peptidase mainly destroying epitopes (Firat et al. 2007; Marcilla et al. 2007).

Generally, TPPII is upregulated in diseases that are based on increased or uncontrolled proteolysis such as in septic muscles (Hasselgren et al. 2002; Chand et al. 2005; Stavropoulou et al. 2005) or in malignant cells (Stavropoulou et al. 2006), and inhibition of TPP leads to radiation sensitivity in cancer cells (Hong et al. 2007). Being implicated in tumor cell survival and proliferation, TPPII is discussed as a target for tumor therapy. However, TPPII is also necessary for normal cell survival, since although TPPII knock-out mice are viable, lack of TPPII results in the activation of cell death programs (Huai et al. 2008).

A more specialized task is carried out by a membrane-bound TPPII variant, which inactivates the cerebral neurotransmitter cholecystokinin-(26–33) octapeptide (CCK-8), an endogenous satiety agent (Rose et al. 1996). Its involvement in obesity makes TPPII an interesting target for drug design. A specific inhibitor, butabindide, has been designed and shown to influence the feeding behavior of mice (Rose et al. 1996). Based on the similarity of TPPII to subtilisin, a homology model of its active site has been published and is used for the design of additional inhibitors (De Winter et al. 2005).

3.1 TPPII Structure

TPPII is found in most eukaryotic organisms. The molecular weight of the TPPII monomer ranges from 138 kDa for the mammalian variant to 150 kDa for the plant, worm, and insect homologs. The N-terminal half of the sequence contains the subtilisin core; the catalytic triad in human TPPII has been mapped to Asp-44, His-264, Ser-449, by site-directed mutagenesis (Hilbi et al. 2002). An insert of approximately 200 amino acids interrupts the first two residues of the catalytic triad and has been suggested to be necessary for complex formation (Tomkinson et al. 2002). In contrast to the 26S proteasome, which is a multisubunit complex, TPPII is a large homo-oligomer of 5–6 MDa. TPPII par-

ticles isolated from human red blood cells as well as from *Drosophila melanogaster* embryos have been visualized in the electron microscope, and the only 3D structure available to date is the density map of *Drosophila* TPPII (Macpherson et al. 1987; Geier et al. 1999; Rockel et al. 2002). TPPII complexes are spindle-shaped 28×60 -nm particles consisting of two segmented and twisted strands. Each of the two strands is composed of a linear assembly of ten interdigitated segments. These segments are dimers, wherein the globular domains of the comma-shaped monomers are connected by a “handle” formed by the tails (Fig. 4). Whereas intact TPPII complexes isolated from mammals and *Drosophila* are of defined length (Geier et al. 1999; Rockel et al. 2002), TPPII particles heterologously expressed in *Escherichia coli* often possess extensions beyond their spindle poles or occur as single strands of variable lengths. This is presumably a consequence of the comparatively high TPPII concentration in cells overexpressing the protein. Treatment of such extended spindles and single strands with destabilizing agents leads to trimming of extensions and causes disassembly of single strands, and it demonstrates that the spindles observed in native preparations are the thermodynamically favored conformation. This stabilization of the spindles probably results from a double-clamp structure at their poles, where the terminal dimer of one strand locks the two terminal dimers of its neighboring strand (Rockel et al. 2005).

3.2 Size–Activity Relationship

TPPII exhibits its highest activity only when assembled into strands; its dissociation (e.g., upon dialysis) results in loss of activity. When human TPPII dissociates into dimers, the specific activity decreases to approximately one-tenth and this activity loss can be reversed by reassociation (Tomkinson 2000). The relationship between size and activity has been studied in more detail with *Drosophila* TPPII (Seyit et al. 2006). Here, assembly studies in conjunction with cross-linking revealed (1) that strand-elongation proceeds by addition of dimers, (2) that the specific activity of TPPII increases with strand length, and (3) that the length distribution of the TPPII strands at equilibrium is dependent on the protein concentration and that high protein concentrations lead to polymorphism. Under conditions favoring dissociation, tetramers are the most

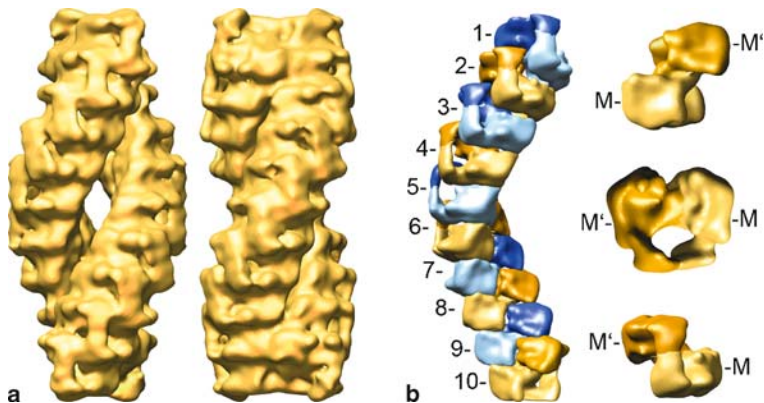


Fig. 4a,b. Architecture of TPPII from *Drosophila melanogaster*: **a** Two perpendicular views of the TPPII complex; **b** computationally extracted TPPII dimers; *left* arrangement of the ten dimers of a strand (constituting monomers) are color-coded; *right* TPPII dimer in different orientation (monomers are denoted *M* and *M'*)

stable disassembly product of *Drosophila* TPPII. Whereas dimers of *Drosophila* TPPII have a specific activity of approximately 8% of that of spindles, the activity of a tetramer is as high as 50%. The activation is thought to be triggered by the formation of new monomer–monomer interfaces upon addition of a dimer, which would induce a conformational change at or near the active site. According to the activation model proposed, the increase in specific activity upon strand elongation at equilibrium is described by the equation $P = ((N - 2) * 100 + 16)/N$, with P the specific activity and N the number of monomers assembled in a strand (Fig. 5) (Seyit et al. 2006).

3.3 Structure–Function Relationship

While the linear arrangement of the subcomplexes in the 26S proteasome mirrors the sequence of events during its functional cycle (binding, unfolding, translocation, degradation) the functional reason for linearly stacking the subunits of TPPII into strands remains obscure. The 20S proteasome is a self-compartmentalizing protease. Whether this

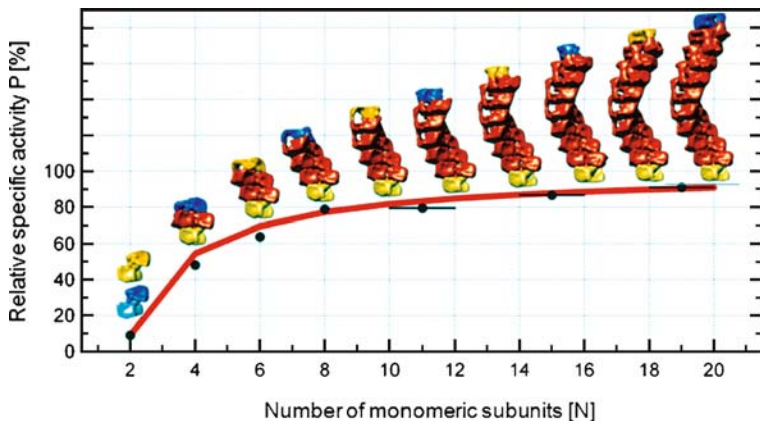


Fig. 5. Size-activity relationship of TPPII. Dependence of the specific activity on the number of subunits assembled. *Black lines* experimental data; *red line* $P = ((N - 2) * 100 + 16)/N$, P specific activity, N number of monomers assembled in a strand. Surface representations show TPPII strands of different lengths, activated dimers are colored in *red*. (Adapted from Seyit et al. 2006)

also applies to TPPII remains to be demonstrated and can only be definitely proven when a crystal structure becomes available or when the locations of the active sites within the complex have been determined otherwise. Indeed, the stacking of dimers leads to the formation of a cavity system traversing the strands (Fig. 6). This system includes chambers that result from the stacking of the dimers, where each dimer provides a cavity as well as a cap that seals off the cavity of the subjacent dimer (Fig. 7). Provided the active sites are indeed located within this cavity system, it is not obvious how the relatively small substrates of TPPII should be channeled through the strands. A longitudinal substrate flow appears inefficient with respect to access to the active sites and release of products: while active sites close to the ends of the strands would be easily accessible, at the center of the strands they might be undersaturated because of the diffusion limit. In contrast, a lateral substrate flow through the arcade would lead to equal saturation of all active sites and at the same time protect the substrates from their complete hydrolysis

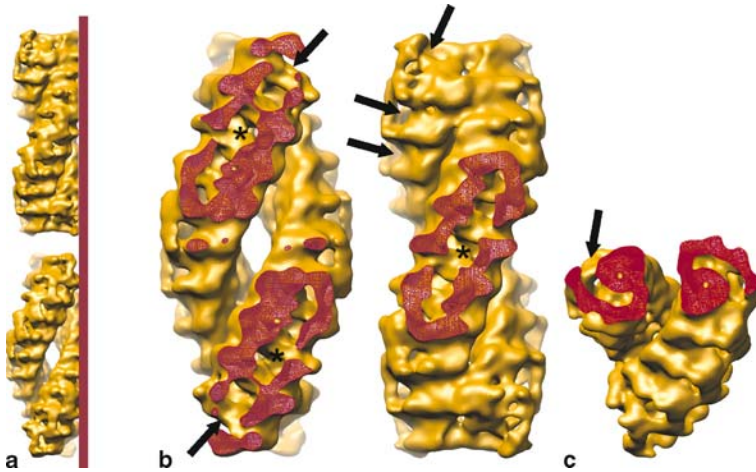


Fig. 6a-c. Channel system of TPPII. **a** Section planes; **b** two perpendicular cut-open views of TPPII; **c** cross-section through TPPII. *Arrows* mark the entry ports to the channel system (labeled with an *asterisk*)

in the cytosol. Such a feature might be important for the role ascribed to human TPPII in peptide trimming (Burri et al. 2002).

Recently, a role in fat metabolism was ascribed to TPPII, and its involvement in adipogenesis appears to be independent of its peptidolytic activity. Both peptidolytically inactive mutants HsTPPIIAsp44Ala and Hs Δ NTTPPII, where the N-terminal aspartic-acid-containing protease domain was deleted, stimulated adipogenesis in mammalian cell culture to the same extent as wild-type TPPII. As opposed to these mutants, mutants lacking either the N-terminal or the C-terminal domain were not functional (McKay et al. 2007). Since mutations in the TPPII sequence often lead to altered assembly behavior (Tomkinson et al. 2002; Rockel et al. 2005; Seyit et al. 2006), it is tempting to speculate that the loss in function of the latter two TPPII mutations was caused by the loss of their native quaternary structure. If for certain cellular functions of TPPII only its intact structure but not its peptidolytic activity were required, this would allude to a functional relevance of the

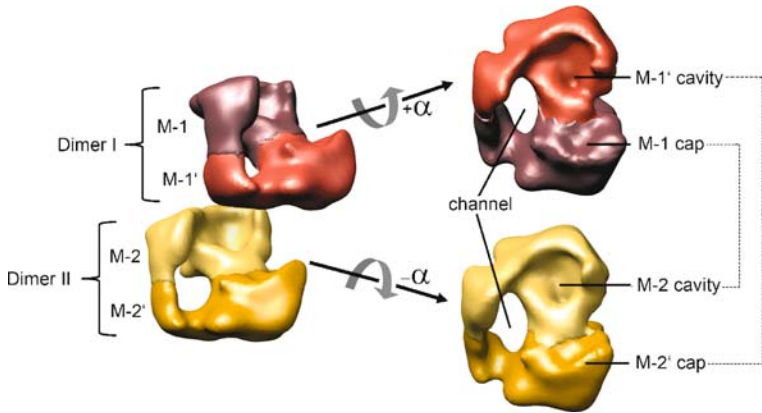


Fig. 7. Stacked dimers enclose a double chamber. *Left* two dimers (I and II) with color-coded monomers (*M-1*, *M-1'* and *M-2*, *M-2'*). *Right* composition of the double chamber: dimer I was rotated counterclockwise in order to visualize cavity *M-1'* and cap *M-1*, which in their original position are oriented toward the back of the image plane. In the original orientation of the two dimers, cavity *M-1'* is sealed off by the cap *M-2'* and cavity *M-2* by cap *M-1*. Figures were created with the Chimera software package (Pettersen et al. 2004)

spindle architecture of TPPII and imply that the spindle might serve as interaction scaffold.

4 Conclusions

The 26S proteasome and TPPII are two major players in eukaryotic cytosolic proteolysis and exert their proteolytic activity mostly consecutively: the 19S caps of the 26S proteasome bind and unfold proteins destined for degradation and feed them into the proteolytic chamber. The products of the 20S proteasome are short peptides of a length suitable for degradation into tripeptides by TPPII. Macromolecular complexes with functions corresponding to 26S and TPPII also occur in other kingdoms of life. In bacteria, the proteases ClpP or ClpQ associate linearly with the AAA-ATPases ClpX, ClpY, or ClpA; adaptor complexes for 20S proteasomes in Archaea are PAN or VAT, which presumably are

associated with the 20S core only transiently. Tricorn is a downstream peptidase with a molecular mass comparable to that of TPPII. Its 121-kDa subunits assemble into a hexamer, which *in vivo* forms an icosahedral capsid of 14.5 MDa. This capsid is thought to be necessary for the docking of the tricorn interacting factors F1, F2, and F3 (Tamura et al. 1998). Most of these large protease complexes are labile, which complicates their isolation in the large amounts and high purity and homogeneity necessary for crystallization. Nevertheless, in some cases it was possible to construct (partial) hybrid models by docking high-resolution crystal structures into the densities obtained by electron microscopy.

Typically, molecular complexes are purified either from their native source or an expression system and subsequently studied *in vitro*. Their functional interplay with other molecules is investigated by interaction studies and the regions of structural interactions are narrowed down by chemical cross-linking. The size of such gargantuan molecules such as 26S proteasomes or TPPII permits a type of functional investigation that is not applicable to smaller molecules: with sizes larger than 30 nm, giant proteases should be easily visible in cellular tomograms and could thus be depicted directly within their functional cellular environment. Admittedly, in the average eukaryotic cell such protease complexes are much less abundant than, for example, ribosomal complexes, but their localization and visualization can be facilitated by the current developments of structure recognition in tomograms of cells and cell sections in combination with correlative microscopy.

Acknowledgements. We thank J. Peters for critically reading the manuscript.

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