New insights into proteasome function: from archaebacteria to drug development

The proteasome is not simply a 'garbage disposal unit' but also has functions in the control of the cell cycle and immune responses. The structure of an archaebacterial proteasome has recently been determined to high resolution, and provides insight into the unusual mechanism of proteolytic cleavage by the proteasome.

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Since the introduction of isotopic tracers into biochemistry over 50 years ago, it has been known that intracellular proteins, once synthesized, are continually being degraded back to their constituent amino acids. But clear ideas about the degradative pathways responsible, the proteolytic machinery involved, the mode of action of the enzymes required, and the physiological significance of the process have only recently emerged. It is now known that the bulk of cell proteins are hydrolyzed by a soluble ATP-dependent system that is present in both the nucleus and cytosol [1]. Protein substrates are first marked for degradation by covalent conjugation to multiple molecules of a small protein, ubiquitin [1]. This process involves the activation of ubiquitin by the formation of a thiol-ester at its carboxyl terminus, which is then transferred to the ϵ-amino group on a lysine residue on the protein (Fig. 1). Other ubiquitin molecules are processively linked to the first, forming long chains of ubiquitin on the substrate. This triggers the rapid hydrolysis of the substrate by a very large ATP-dependent proteolytic complex, termed the 26S proteasome (for useful reviews, see [2–8]). The proteolytic core of this 2000 kDa (26–30S) structure is the 20S proteasome, a 700-kDa particle containing multiple peptidase activities [2–8].

The 20S proteasome is a major cellular constituent; it comprises ~1 % of the protein in most cells. It was discovered independently by several labs, and had received as many as 22 different names in the literature; many functions were proposed for it that were unrelated to protein turnover [4]. It was eventually named the 'proteasome' to indicate a particle with proteolytic function [4]. Since then, antibody studies *in vitro*, reconstitution, and finally mutant studies in yeast have shown that the 20S particle functions in the ubiquitin-dependent pathway as the proteolytic core of the 26S complex [9,10]. Formation of this larger structure involves an ATP-dependent association of the 20S proteasome with a 19S (600 kDa) particle [11,12], which appears to function as the 'mouth' for the 20S particle (Fig. 2); it binds substrates, probably unfolds them, and injects the polypeptides into the 20S proteasome for digestion [2,8]. The 19S particle contains five distinct ATPases, a binding site for ubiquitin chains [13], and at least one isopeptidase [14], which disassembles the ubiquitin chain during protein hydrolysis. Despite this exciting recent progress, many aspects of the function of the 26S proteasome complex remain mysterious. For example, the function of ATP hydrolysis is not known. We do not understand why degradation of some polypeptides (e.g. the enzyme ornithine decarboxylase) does not require ubiquitination [15], nor do we know whether the 20S particle ever breaks down proteins by itself *in vivo*.

Structure of the 20S particle

The mammalian 700 kDa proteasome is a barrel-shaped particle composed of four stacked oligomeric rings, two

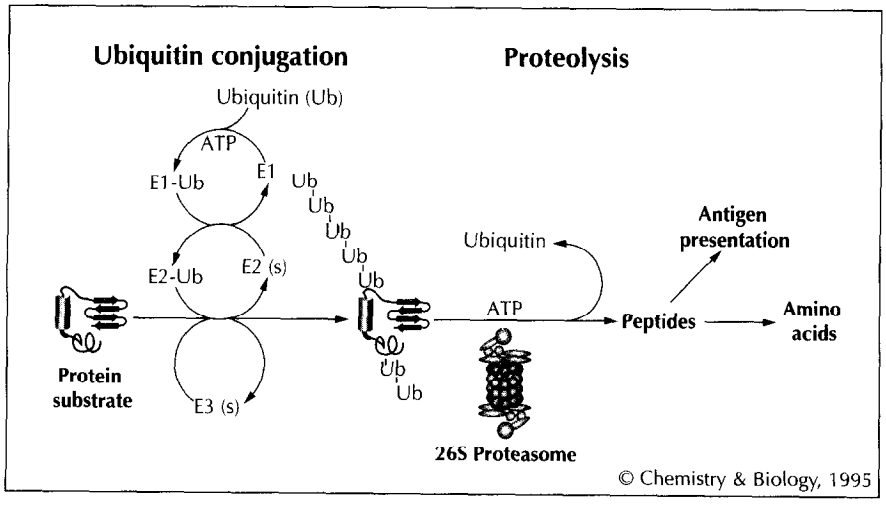

Fig. 1. The ubiquitin–proteasome pathway for protein degradation. Ubiquitin is first activated by an enzyme complex (E1–E3), which generates a thiol-ester at the carboxyl-terminus of the protein. This is then used to form an isopeptide bond to the ϵ-amino group on a lysine residue on the substrate. Other ubiquitin molecules are processively linked to the first, forming long chains of ubiquitin on the substrate. This leads to rapid, ATP-dependent hydrolysis of the substrate by the 26S proteasome.

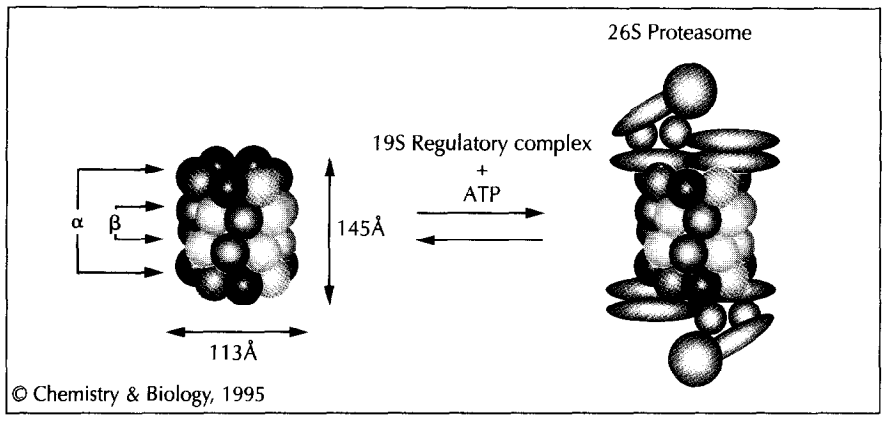

Fig. 2. Relationship between the 20S proteasome (left) and 26S proteasome (right). The 20S proteasome is composed of two central β-subunit rings, with two outer α-subunit rings, each with seven members. Addition of the 19S regulatory complex to the top and bottom of the 20S proteasome forms the 26S proteasome. The 19S particle contains a binding site for ubiquitin chains and several ATPase activities. The 26S proteasome is responsible for the degradation of ubiquitin–protein conjugates; degradation is ATP-dependent.

outer (α) rings and two inner (β) rings . Each ring contains seven distinct 20–30 kDa subunits [5–7]. The structure was first revealed by electron microscopic studies; more recently, electron microscopic tomography [16,17] and X-ray diffraction analyses [18] have provided a more detailed structure. These important advances have been made possible by studies of the proteasome from the archaebacterium *Thermoplasma acidophilum*, which contains only two types of subunit, α and β, arranged in four rings of seven ($\alpha_7\beta_7\beta_7\alpha_7$) [16,18]. The eukaryotic proteasome has a very similar quaternary structure [5–7,16] even though it contains seven different, but related, α-subunits and seven different, but related β-subunits, which are encoded by distinct genes [19] (see below). The *Thermoplasma* proteasome thus offers the advantage of increased symmetry, and is also more stable. The detailed structure of the archaebacterial proteasome has provided insight into issues such as the mechanism of proteolysis and the site at which proteolytic cleavage occurs.

The tertiary structures of the α- and β-subunits are remarkably similar [18]. Each subunit is a sandwich of five-stranded anti-parallel β-sheets, flanked by α-helices on the top and bottom. There is tight contact between the α- and β-subunits in adjacent rings, and together they help define three distinct chambers within the proteasome, which are connected by a central pore (Fig. 3). The minor outer chambers are defined by the space between the α- and β-rings, and a larger central chamber is defined by the inner walls of the β-subunits. Lowe *et al.* [18] analyzed proteasome crystals containing a peptide aldehyde inhibitor, and found that each of the 14 β-subunits contains a site for inhibitor binding, which presumably corresponds to the enzyme's active site. The active sites are positioned in the central chamber.

Intracellular proteolysis must be an extremely selective process for the integrity of the cell to be maintained. One would therefore assume that structural safeguards might have evolved to prevent nonspecific digestion of cell constituents. The isolation of the proteasome's active sites within the central chamber of the particle probably helps to reduce unselective proteolysis. Polypeptide entry into the 20S *Thermoplasma* proteasome requires that the substrate be unfolded [17]. The recent X-ray diffraction studies [18] confirmed the existence of a small central pore in the α-rings, 13 Å in diameter, through which a polypeptide must pass to enter the central cavity. Presumably, this entry point is also a site of regulation, and factors that activate peptide hydrolysis by the 20S particle (e.g. the 19S complex [12]) may do so by enlarging this opening and facilitating substrate entry. In the 26S complex, chemical free energy derived from ATP hydrolysis may be transduced into mechanical energy and used to catalyze unwinding of the polypeptide substrate and injection of the polypeptide into the proteolytic core. The requirement for ubiquitination of substrates for degradation in eukaryotes [1,4] probably evolved to ensure that polypeptide entry into this 20S proteasome is highly selective.

It has previously been noted that protein degradation by the proteasome generally results in oligopeptides of about 6–10 amino acids [20]. There is some reason to believe that the size of these peptides is important, particularly for the function of the proteasome in antigen presentation. Lowe *et al.* [18] hypothesized that polypeptides undergoing degradation are attached to the wall of the central chamber in an extended conformation, and thus are susceptible to cleavage by these symmetrically distributed active sites. The distance between the active sites appears to be approximately the length of a 7–8 amino acid peptide, when extended.

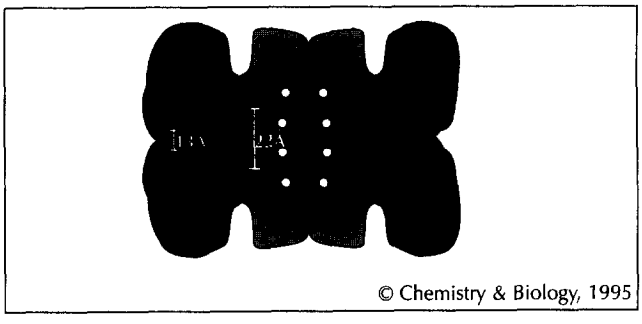

Fig. 3. Arrangement of the chambers within the proteasome. The space between the α-subunit and β-subunit rings defines the small outer chambers, while the space between the two β-subunit rings defines the large central chamber. Proteolysis takes place within the large central chamber; white dots indicate peptidase active sites. The target protein must enter the chamber through a small pore in the center of the α-subunit ring. The 19S complex may enlarge this opening, and may also unfold the protein to allow it to enter the pore more easily.

A novel proteolytic mechanism

The mechanism of peptide bond hydrolysis by the 20S proteasome has long been a puzzle. The sequences of the α- and β-subunits of the proteasome show no homology to those of other proteases, although they are closely related to one another and have been highly conserved during evolution [6,7,19,21]. Studies using protease inhibitors indicated that the proteasome resembled a serine protease in certain properties, but not in others [5]. But mutagenesis in *Thermoplasma* indicated that no serine or cysteine residue was essential for activity [21]. The recent information on the structure of the particle, together with mutagenesis studies, have revealed that the proteasome uses a new type of catalytic mechanism. In the crystal structure, the hydroxyl group of the amino-terminal threonine of the β-subunit is close to the aldehyde carboxyl of the inhibitor Ac-Leu-Leu-Norleunal, and probably forms a hemiacyl intermediate with it. Thus, this threonine appears to function like the active site serine in the catalytic triad of serine proteases. Seemuller *et al.* [21] also showed that mutagenesis of this threonine to alanine prevented peptidase activity, while replacement with a serine did not. The terminal threonine residue has been highly conserved during evolution from archaebacteria to man. It is so far unclear why threonine is used as the catalytic residue instead of serine.

For peptide bond hydrolysis, there must be a basic group to accept a proton from the active site threonine hydroxyl in the catalytic transition state. One likely candidate is the α-amino group of the same threonine. Similarly, it has recently been suggested that the α-amino group of the catalytic serine residue in a penicillin acylase acts as a base during catalysis [22]. In both cases a lysine residue is also found near the active site, and this is an alternative candidate for the catalytic base [18,21]. The proteasome clearly lacks the traditional catalytic triad characteristic of serine or cysteine proteases, however. The lack of a reactive site histidine may explain why proteasomes are not covalently modified by the classic chloromethyl-ketone inhibitors.

Additional chemical evidence supporting this mechanism and its generality has come from the unexpected discovery by Schreiber and coworkers [23] of a natural product inhibitor of proteasome activity, the antibiotic lactacystin. This agent covalently reacts with the terminal threonine residue on a specific β-subunit of the mammalian proteasome. Another inhibitor of the proteasome, dichloroisocoumarin, also modifies this threonine residue (T. Akopian and A. L. G., unpublished data). Thus, the catalytic mechanism of the eukaryotic proteasome appears to be similar to that of the archaebacterial proteasome.

Mammalian proteasomes have least five distinct peptidase activities, as defined by their preferences for different types of residues preceding the scissile bond and their sensitivities to different inhibitors, and therefore can cleave a wide variety of peptide bonds. The most extensively studied of these are the chymotrypsin-like activity (which prefers to cleave on the carboxyl side of aromatic residues), the tryptic-like activity (which prefers to cleave after basic residues), and the peptidyl-glutamyl activity (which cleaves after acidic residues). In addition, proteasomes exhibit activities that cleave preferentially after branched-chain amino acids or after small neutral residues. It appears very likely that most of these five different activities are associated with the different β-subunits that are present in eukaryotic proteasomes. However, terminal threonine residues appear to be present on only four of the seven β-subunits in eukaryotic proteasomes [21]. Therefore, the three remaining β-subunits, though clearly important for proteasome structure, do not appear to contain catalytic sites for protein breakdown. The specific subunits responsible for each peptidase activity remain unclear, but should be resolved by systematic mutagenesis of these catalytic threonine residues.

Physiological functions of the proteasome

The ubiquitin–proteasome pathway has long been known to be important in degradation of damaged or mutated cellular proteins [4], but this is not its only function. Recent work [1] has shown that it is also essential for the degradation of various regulatory proteins. Rapid removal of these proteins is necessary for the control of cell growth and metabolism. For example, the orderly progression of cells through the mitotic or meiotic cycle requires the programmed ubiquitination and destruction of the various cyclins [24,25]. In many cases, ubiquitin-mediated degradation of regulatory proteins is signalled by their phosphorylation (e.g. the mitotic cyclin Cln 3 [25], or IκBα (see below)). The proteasome is also responsible for the slow turnover of the bulk of cell proteins, which is also precisely regulated in many cells. For example, in starvation or disease states (e.g. infections, cancer or cachexia), mammals mobilize essential amino acids from muscle proteins by a hormone-induced activation of the ubiquitin–proteasome pathway [26–28]. Under these conditions, the overall rate of proteolysis exceeds rates of synthesis, and muscle atrophy results.

This continual turnover of cellular proteins by the ubiquitin–proteasome pathway is also used by the immune system to screen for the presence of abnormal intracellular proteins (for review see [29]). In this process, lymphocytes continually monitor small fragments of cell protein that are presented on class I major histocompatibility complex (MHC) molecules. Proteasomes initially degrade proteins to small peptides, most of which are rapidly hydrolyzed to amino acids by cytosolic exopeptidases. But some of these peptides are transported into the endoplasmic reticulum where they bind to MHC molecules and are then transported to the cell surface [29,30] in a process known as antigen presentation. If the peptides are abnormal (for example, if they are derived from viral proteins), they elicit cell destruction by cytotoxic T cells. Inhibitors that prevent

proteasome function have been shown to block the generation of most of the peptides presented on MHC class I molecules [30].

One of the most interesting findings in this area has been the discovery that, in certain pathological states, cytokines induce structural alterations in the proteasome that enhance the efficiency of antigen presentation [31–33]. For example, interferon-γ induces the expression of two small MHC-encoded proteins, LMP2 and LMP7 [31–35]. These proteins are alternative β-subunits of the proteasome; in response to interferon they are expressed and incorporated into proteasomes in place of two of the the normal β-subunits [34,35]. The overall activity of the proteasome is thus subtly altered, so that these particles ('immunoproteasomes') cleave more rapidly after hydrophobic and basic residues, and less rapidly after acidic residues [31–34]. The overall rate of protein breakdown is unchanged, but the number of peptides produced with hydrophobic or basic carboxyl termini is increased. Such peptides are more likely to be transported into the endoplasmic reticulum [36], where they have access to class I MHC, and bind better to the peptide groove in class I MHC. It has recently been shown that mouse mutants lacking the LMP 2 [38]or LMP 7 [37] genes have a reduced capacity to present viral antigens.

Our knowledge about the physiological importance of the ubiquitin–proteasome pathway has been limited by the lack of selective reagents that can inhibit its function *in vivo*. Recently, several such compounds have been identified, and have already proven very useful [23,30,39,40]. For example, several peptide aldehyde inhibitors of the proteasome reversibly block the degradation of abnormal polypeptides, short-lived normal proteins, and also the bulk of long-lived cell proteins [30]. Aside from their value as investigative tools, such inhibitors may have therapeutic applications; one area of particular interest is the possibility that they may suppress the inflammatory response.

The ubiquitin–proteasome pathway in the onset of the inflammatory response

The ubiquitin–proteasome pathway has recently been shown to regulate the activation of the transcriptional factor, NF-κB, which is critical in both acute and chronic inflammatory responses. NF-κB is a heterodimer composed of p50 and p65 subunits, both of which are members of the rel family of transcription factors [41,42]. NF-κB was originally discovered as an activator of the transcription of the immunoglobulin κ light chain, and has since been found to be important in the expression of genes for several cytokines (IL-1, IL-2, IL-6, and TNF-α) and cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) that are important in inflammatory responses. Thus, activation of NF-κB is a key event in a wide variety of disease states.

There are two separate pathways for NF-κB activation ([41,42], Fig. 4), both of which require the ubiquitin–proteasome pathway. In quiescent cells, the inhibitory protein IκBα normally sequesters the p50/65 heterodimer (NF-κB) in the cytoplasm, probably by masking its nuclear localization sequence. To activate the inflammatory response, IκBα must be degraded. Triggers such as viral infection, free-radical oxygen species, or inflammatory cytokines [41] cause phosphorylation of IκBα via still-unidentified signaling pathways. Phosphorylation of IκBα occurs at two specific serine residues, leading to ubiquitination and rapid hydrolysis by the 26S proteasome complex [43]. The liberated p50/65 heterodimer can then translocate into the nucleus and bind to the promoter regions of NF-κB-responsive genes.

The second, slower pathway for generation of active NF-κB requires limited proteolysis of the inactive NF-κB precursor p105/65 heterodimer, which is also sequestered in the cytoplasm [39]. Palombella *et al.* [39] showed that the conversion of p105 to the active p50 requires ubiquitin conjugation to p105 and the 26S proteasome. The carboxy-terminal half of p105 is then completely

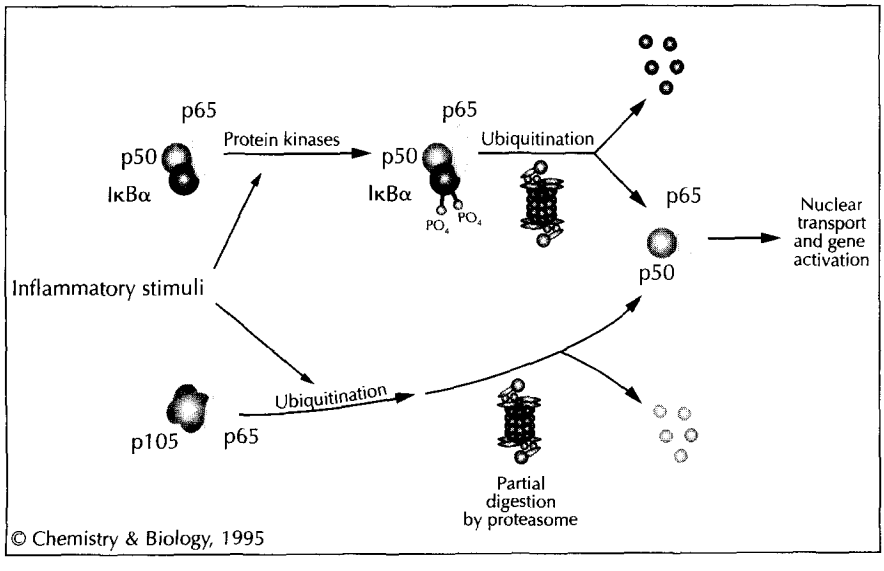

Fig. 4. Activation of NF-κB occurs via two pathways, both of which require the proteasome. NF-κB (p65/p50) is generally held in the cytoplasm of quiescent cells either in complex with the inhibitory factor, IκBα, or as the precursor complex (p105/p65). A variety of inflammatory signals can lead to the phosphorylation of IκBα, which in turn leads to its ubiquination and degradation by the proteasome. In the alternative pathway, controlled degradation of the carboxy-terminal half of p105 by the proteasome produces active p65/p50 dimer. Active NF-κB produced by either pathway then translocates into the nucleus and activates NF-κB-responsive genes.

degraded, leaving the amino-terminal p50 intact. This is the first instance in which the proteasome performs regulated proteolytic processing instead of completely degrading a protein; presumably other examples will be found. The mechanism for preventing the hydrolysis of the amino terminus of NF-κB is still unclear, however.

NF-κB functions both as the initiator and as an amplifier of the inflammatory response. Not only does it promote the transcription of genes for inflammatory cytokines which can activate leukocytes, but it also directs the expression of cell adhesion molecules which lead to the recruitment of leukocytes to inflammatory sites. Furthermore, NF-κB stimulates the synthesis of more p105 and IκBα, and thus provides more substrate, generating a positive feedback loop. NF-κB is believed to be the main regulator initiating and sustaining the inflammatory state in both acute disease states (e.g. sepsis, ARDs, and acute transplant rejection) and chronic conditions (e.g. rheumatoid arthritis, inflammatory bowel disease, and asthma).

Prospects for anti-inflammatory proteasome inhibitors

Inhibitors of the 26S proteasome have been shown to stabilize IκBα and to block the processing of p105 [39,40], and might therefore suppress inflammation by inhibiting expression of NF-κB-responsive genes. Both the tripeptide aldehyde, MG 132 (Cbz-Leu-Leu-Leucinal), which reversibly binds to the active sites of the proteasome [30] and the natural product, lactacystin [23], which irreversibly acylates the active site threonines, can penetrate living cells, block the activation of NF-κB, and thus prevent the expression of IL-2, TNF-α, ICAM-1, VCAM-1 and E-selectin [44]. This ability of inhibitors of the 26S proteasome [39,40,44] to block activation of NF-κB has been demonstrated in multiple cell types. At concentrations of the drug that half-maximally inhibit proteasome-dependent NF-κB activation (ED_{50}), the expression of genes encoding cytokines and cell adhesion molecules is almost completely suppressed (J. Chen, V.J. Palombella, S. Tagerud & S. Brand, unpublished data). The ability of proteasome inhibitors to block the inflammatory response has also recently been demonstrated *in vivo* in animal models of human disease, including delayed-type hypersensitivity reactions and the gastropathy induced by non-steroidal anti-inflammatory drugs. But, as noted above, the proteasome has many other important functions, and blocking its activity may therefore have some untoward effects. It remains to be seen whether our exciting new knowledge about the ubiquitin–proteasome pathway can be translated into useful therapies for debilitating inflammatory diseases in humans.

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