Bacterial Proteasomes

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Abstract

Interest in bacterial proteasomes was sparked by the discovery that proteasomal degradation is required for the pathogenesis of *Mycobacterium tuberculosis*, one of the world's deadliest pathogens. Although bacterial proteasomes are structurally similar to their eukaryotic and archaeal homologs, there are key differences in their mechanisms of assembly, activation, and substrate targeting for degradation. In this article, we compare and contrast bacterial proteasomes with their archaeal and eukaryotic counterparts, and we discuss recent advances in our understanding of how bacterial proteasomes function to influence microbial physiology.

Keywords

Mycobacterium; proteasome; Pup; pupylation; regulated proteolysis

Introduction

Regulated protein degradation affects virtually every biological pathway across all domains of life. In eukaryotes, the majority of regulated proteolysis is carried out by proteasomes, which are compartmentalized proteases that primarily degrade proteins posttranslationally modified with the protein ubiquitin (reviewed in 22, 77). The proteolytic component of all proteasomes is the 20S core particle (CP) (Figure 1a), a complex composed of four heptameric rings that are stacked on one another to form a cylinder (27, 32, 46, 76, 78) that can cleave multiple types of substrates (27, 30, 52). The active site of the 20S CP is sequestered, which prevents folded proteins from being inappropriately degraded. Thus, protein degradation is highly dependent on proteasomal cofactors, which recruit specific substrates and unfold them so that they may enter the 20S CP.

A cofactor complex responsible for degrading proteins in eukaryotes is the 19S regulatory particle (RP), which binds to either or both ends of the 20S CP to form the 26S proteasome (18). The 19S RP can be separated into two major components, the base and the lid, each of which is composed of several unique polypeptide subunits. The lid contains numerous components that play important functional roles, including removal of ubiquitin and recognition of proteins that are targeted for degradation (25, 79). The 19S RP base contains a hexameric ring of AAA ATPases (ATPases associated with diverse cellular activities) that

unfolds proteins (4, 45) and pushes them into the active site of the 20S CP, and the base also has at least two receptors that help recruit substrates to the proteasome (33, 64).

In eukaryotes, proteins are marked for destruction by ubiquitin, a 76–amino acid protein that can be covalently linked by its carboxyl (C)-terminal glycine to the lysine of a doomed protein. The addition of ubiquitin to a target protein is carried out by an elegant coordinated pathway involving ubiquitin-activating and -conjugating enzymes (Figure 1b). Using ATP, an E1 ubiquitin-activating enzyme adenylates the C terminus of ubiquitin, allowing it to form a thioester bond with a cysteine in the E1 enzyme. Ubiquitin is then transferred to an E2 conjugating enzyme, which forms a more transient thioester bond with ubiquitin and, with the help of an E3 ligase, positions it for transfer to a lysine on a substrate protein (reviewed in 71). Because ubiquitin contains lysines, it can be ubiquitylated to form polyubiquitin chains that help target a substrate for proteasomal degradation (10). Eukaryotes encode numerous E3 ligases that can each target a specific protein or group of proteins for ubiquitylation. Thus, it is not surprising that the ubiquitin-proteasome system regulates many biological processes, and it is essential for eukaryotic life (reviewed in 60).

Proteasomes are also found in all archaea and in bacteria of the orders *Actinomycetales* and *Nitrospirales*. The mechanisms by which archaea target proteins for proteasomal degradation are still unclear; however, it is postulated that a posttranslational modification related to ubiquitylation called SAMPylation is involved in this process (reviewed in 49). In contrast, proteasome-containing bacteria utilize a system termed pupylation that is functionally analogous to but chemically distinct from ubiquitylation. Unlike eukaryotic proteasomes and ubiquitylation, bacterial proteasomes and pupylation are not absolutely required for viability; however, recent studies have begun to shed light on the important pathways that they regulate.

Bacterial 20S Core Particles

Core Particle Genes and Structure

The genes encoding 20S CP subunits and proteasomal cofactors are generally found in operons on bacterial chromosomes. The bacterial 20S CP is composed of just two subunits, α and β, which are encoded by proteasome core A (*prcA*) and proteasome core B (*prcB*) and are in an operon with *pup* (prokaryotic ubiquitin-like protein), which is described in detail in the eponymous section below (Figure 2a) (38, 76). Several other genes are also found near this locus, including *dop* (deamidase of Pup), *pafA* (proteasome accessory factor A), and *mpa* (mycobacterial proteasome ATPase), which are associated with proteasome function and are described in further detail below. *pafB* and *pafC* are cotranscribed with *pafA* but do not appear to contribute to proteasome function (21). Intriguingly, *Rhodococcus* species have two 20S CP operons, each encoding a distinct set of *prcBA* genes; the biological significance of this remains unknown.

The architecture of a bacterial 20S CP is similar to that of the eukaryotic and archaeal complexes. The core protease is composed of four heptameric rings, which stack on one another to form a barrel of ~150 Å length and ~115 Å diameter (32, 41, 44, 76). Each outer α ring is composed of seven identical PrcA subunits, and each inner β ring is composed of

seven identical PrcB subunits to give an overall organization of $\alpha_7\beta_7\beta_7\alpha_7$ (Figure 1a). An opening of 23 Å in the α ring, which is wider than the 13 Å opening in archaea (46), leads to a pore that runs through the center of the entire complex and connects three large cavities, one at each α-ring/β-ring interface and one between the two β rings that contain the proteolytic active sites.

Assembly

To form a mature protease, the 20S CP must be assembled in a precisely ordered manner. In eukaryotes, this is accomplished with the aid of proteasomal chaperones that bind to 20S CP intermediates and facilitate their assembly (reviewed in 40). Bacterial CPs, however, are capable of self-assembly; coproduction of *Rhodococcus* PrcA and PrcB in *Escherichia coli* results in the formation of active 20S CPs without the need for any other *Rhodococcus*-specific factors (88). Interestingly, any combination of PrcA and PrcB from either *Rhodococcus* proteasome operon is capable of forming a mature 20S CP in vitro (87, 88).

Two intermediates of 20S CP assembly have been identified. The first is a half-proteasome, which consists of an α ring and a β ring, that forms spontaneously on coproduction of PrcA and PrcB. Two half-proteasomes then come together to form a preholoproteasome in which the PrcB subunits must undergo autoprocessing to become proteolytically active. This processing event involves removal of the PrcB amino (N)-terminal propeptides, leaving N-terminal threonines (Thr1) that act as the catalytic nucleophiles of the mature holoproteasome (87).

The PrcB propeptide appears to have several functions that are not always consistent among bacterial species. In *Rhodococcus*, the propeptide promotes 20S CP maturation; production of PrcB subunits lacking the propeptide results in the slow formation of preholoproteasomes from half-proteasomes. In contrast, production of a PrcB subunit that cannot remove its propeptide results in a more rapid formation of preholoproteasomes (87). The propeptide is normally buried within the central pore of a half-proteasome and makes inter-β-ring contacts that promote the apposition of two half-proteasomes (41, 84). These contacts can be provided even in *trans*, as addition of exogenously purified propeptide is sufficient to restore a wild-type maturation rate to a system using PrcB lacking the propeptide (87). Thus, as is observed for eukaryotic 20S CPs, *the Rhodococcus PrcB propeptide promotes 20S CP maturation*.

Interestingly, the exact opposite is true for the *Mycobacterium tuberculosis* 20S CP. This was first suggested by experiments that demonstrate the temperature dependence of 20S CP maturation: When PrcA and PrcB are produced in *Escherichia coli* at 37°C they form mature 20S CPs, but at 30°C they arrest at the half-proteasome state. This temperature dependence is overcome by deletion of the PrcB propeptide, which allows mature 20S CPs to form at 30°C (44). Thus, in *Mycobacterium tuberculosis,* the propeptide is a barrier to core particle maturation. Structural studies provided a mechanistic basis for this finding: The *Mycobacterium tuberculosis* propeptides extend from half-proteasomes (32), which is in contrast to the internal propeptides seen in *Rhodococcus* (41). Thus, it is proposed that these protruding propeptides prevent the apposition of two β rings to inhibit the progression from half-proteasome to preholoproteasome. Interestingly, on forming a preholoproteasome, the

Mycobacterium tuberculosis propeptide retracts into the 20S core, taking a similar position to that which is seen in *Rhodococcus* (42). Thus, despite the contrasting effects of propeptides on 20S CP assembly, a similar structure is assumed for the final steps of maturation.

Catalytic Activities

The active sites of bacterial 20S CPs are similar to those described for archaea and eukaryotes (27, 46, 65). The hydroxyl group of PrcB Thr1 is the nucleophile that is responsible for the proteolytic activity of the proteasome (32, 44, 50). The amino group of PrcB Thr1 (Thr1N) acts as a proton acceptor that allows the side chain oxygen (Thr1γO) to attack an electrophilic center on a substrate. Aspartate 17 (Asp17) forms a salt bridge to a lysine (Lys33), and the Lys33 side chain amino group is thought to be protonated and form a hydrogen bond to Thr1γO that would further promote removal of its proton by Thr1N (27, 32, 46). Thus, Thr1γO, Thr1N, Lys33, and Asp17 form a catalytic tetrad to promote nucleophilic attack by Thr1γO.

To hydrolyze a protein at a specific residue, a protease must accommodate a particular set of amino acids in its active site. In 20S CPs, a binding pocket formed by the β rings provides substrate specificity. Eukaryotes encode multiple types of β subunits with different substrate specificities and can therefore hydrolyze multiple distinct sites including sites after residues that are hydrophobic (chymotrypsin-like activity), basic (tryptic activity), and acidic (caspase-like activity) (27, 30). Because bacteria lack β-subunit diversity, they must rely on a single set of binding pocket residues to accommodate substrates. In *Rhodococcus* and *Thermoplasma*, these residues are hydrophobic and therefore have only chymotrypsin-like activity (12, 76). However, despite encoding only a single β subunit, *Mycobacterium tuberculosis* 20S CPs have all three catalytic activities of the eukaryotic protease (44). This was explained by the crystal structure, which revealed an active site lined by hydrophobic residues on one side and hydrophilic residues on the other (32). Thus, the *Mycobacterium tuberculosis* substrate-binding pocket resembles a hybrid of the three eukaryotic binding pockets.

Gating

In the absence of a proteasome activator, 20S CPs are normally in a closed-gate conformation with the N-terminal residues of each α subunit interacting to occlude the opening to the 20S CP active site. The gating residues of the *Thermoplasma* 20S CP are disordered, which may explain the observation that archaeal proteasomes have relatively high protease activity even in the absence of gate-opening cofactors (Figure 2b, *center*) (46). In contrast, each of the seven α-ring subunits occupies a specific position in the closed gate of the eukaryotic 20S CP (Figure 2b, *left*) (27), rendering it incapable of degrading even small peptides in the absence of a proteasome activator.

The formation of bacterial proteasome gates appears to be distinct from the formation of proteasome gates in other species. In contrast to the *Thermoplasma* complex, the *Mycobacterium tuberculosis* 20S CP has a fully closed gate, which is achieved because each PrcA subunit assumes one of three different conformations. Three form an L shape ("L"),

three form an extended linear shape ("E"), and one projects away from the 20S CP to avoid a steric clash ("V") (Figure 2b, *right*) (42).

ATP-Dependent Proteasome Activators

In eukaryotes and archaea, the binding of proteasomal cofactors to 20S CPs leads to the activation of peptidase activity by repositioning α-ring gating residues into an open conformation. Structural studies of these activators have provided a wealth of mechanistic information that is described in detail elsewhere (58, 68, 69, 83). Like the eukaryotic and archaeal proteasome systems, bacteria possess AAA ATPases that target doomed proteins to 20S CPs for degradation. However, unlike eukaryotic 19S RPs that contain six unique ATPases, bacteria use homohexameric ATPase rings (16, 72). The first bacterial proteasomal ATPase described was a *Rhodococcus* ARC (ATPase forming a ring-shaped complex) (85), which was discovered on the basis of its sequence homology to the eukaryotic Rpt activators and the proximity of the *arc* gene to one of the *Rhodococcus* proteasome operons (Figure 2a). This was followed by the characterization of its *Mycobacterium tuberculosis* ortholog, Mpa (mycobacterial proteasome ATPase; the name *arc* was already in use for another gene in *M. tuberculosis*) (14).

Structurally, ARC/Mpa resembles other proteasomal ATPases. From its N terminus to its C terminus, it contains an α-helical domain followed by two oligonucleotide/oligosaccharide-binding (OB) domains and an AAA ATPase domain (Figure 2c). In an Mpa homohexamer, adjacent α-helical domains form three pairs of coiled coils. The OB domain alone is capable of self-assembly into a hexamer (81, 86), which suggests that this region determines the ringed structure of the mature protein. Like the related bacterial ATPase ClpX, Mpa likely utilizes internal pore loops to unfold and translocate a target protein toward its C-terminal end, as mutagenesis of pore loop residues in Mpa abrogates the degradation of substrates in *Mycobacterium tuberculosis* (48, 81).

Evidence that Mpa plays a direct role in proteasomal degradation came from the discovery of the first proteasome substrates. Two proteins, malonyl CoA-acyl carrier protein transacylase (FabD) and 3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB), specifically accumulate in mutants lacking *mpa* or in wild-type *Mycobacterium tuberculosis* treated with a proteasome inhibitor. Mpa also accumulates upon chemical inhibition of the proteasome, which indicates that it is a proteasome substrate (54). Notably, Mpa has a highly conserved penultimate tyrosine (Tyr, Y), which is also found in the C-terminal HbYX (hydrophobic-tyrosine-any amino acid) motif that is required for several eukaryotic proteasomal cofactors to bind to 20S CPs. This penultimate tyrosine is essential for Mpa to facilitate proteasomal protein degradation, as substrate degradation of *Mycobacterium tuberculosis mpa* mutants could not be complemented by *mpa* alleles where the tyrosine was mutated (54). Notwithstanding a lack of data that show robust binding of Mpa to wild-type 20S CPs, it nonetheless appears that the C terminus of Mpa is an essential part of an interaction required for proteolysis.

Pupylation

Prokaryotic Ubiquitin-Like Protein

Although 20S CPs are conserved across domains of life, ubiquitin is found exclusively in eukaryotes. As a result, it was initially uncertain how substrates were targeted for degradation in bacteria. Pearce et al. (55) performed a bacterial two-hybrid screen to search for proteins that bound to Mpa; the goal was to identify cofactors required for proteolysis. This screen identified Pup, a 64–amino acid protein encoded upstream of *prcBA* that robustly interacts with Mpa. This study also determined that Pup covalently links to a specific lysine of the proteasome substrate FabD, and it showed that mutagenesis of the preferred lysine in FabD stabilizes the protein in vivo (55). Later in vitro degradation assays showed Mpa-, 20S CP–, and Pup-dependent degradation of both FabD and PanB, albeit very slowly (5, 72). Collectively, these data showed that pupylation directly targets proteins for proteasomal degradation in bacteria in a manner functionally analogous to eukaryotic ubiquitylation.

Studies assessing the structural characteristics of Pup elucidated further differences between Pup and ubiquitin. Ubiquitin folds into a characteristic compact structure, whereas Pup is not a compactly folded protein and for the most part lacks secondary structure (11, 43). Although Pup has been categorized as an intrinsically disordered protein, it has a propensity to form helices that are critical for protein-protein interactions necessary for protein degradation. Importantly, unlike ubiquitin, it does not appear that Pup forms poly-Pup chains.

Deamidation and Ligation of Pup to Substrates

Like ubiquitin, the C terminus of Pup forms a covalent bond with a lysine of a doomed protein. Pearce et al. (55) noted that Pup, although encoded with a C-terminal glutamine (Gln), forms an isopeptide bond with its target via a C-terminal glutamate (Glu), which suggests that the C terminus of Pup is deamidated prior to substrate ligation. Pearce et al. also noticed that a mutation in *pafA* prevents pupylation. PafA was identified at the same time as Mpa in a genetic screen for *M. tuberculosis* mutants that were hypersensitive to nitric oxide (NO) (13), but its enzymatic activity had not yet been elucidated. Not long after the discovery of Pup, the Aravind group (36) hypothesized that another protein, Rv2112c, encoded upstream of *pup-prcBA*, might also have a function in pupylation because PafA and Rv2112c share 32% amino acid sequence identity in addition to a predicted similar overall structure (Figure 2d). Both proteins were predicted to resemble carboxylate-amine ligases such as glutamine synthetase, but they have unique C-terminal domains found only in PafA/ Rv2112c homologs.

With these observations in place, the Weber-Ban group (73) made the key prediction that either Rv2112c or PafA was required for Pup$_{Gln}$ deamidation or Pup$_{Glu}$ ligation to substrates. Striebel et al. (73) showed that deamidation by Rv2112c, now called Dop, activates Pup prior to substrate ligation by PafA (Figure 2d, e). Interestingly, Dop requires ATP as a cofactor but does not hydrolyze ATP during the deamidation reaction (73). Although Dop lacked any obvious catalytic sites, the use of a chemical trap showed that Dop

utilizes an aspartate (Asp95 in *Mycobacterium tuberculosis* H37Rv Dop) as a nucleophile for deamidation, a chemically unusual mechanism for this reaction (6). The development of a Pup reporter with a fluorescent moiety on the terminal amino acid side chain confirmed that Dop hydrolyzes this bond. Importantly, this reporter could not be hydrolyzed using mycobacterial lysates or other cellular lysates (including macrophages) lacking Dop (51; K. Burns & K.H. Darwin, unpublished observations), which strongly suggests that the deamidation of Pup is a highly specific reaction that is performed only by Dop (51).

After Pup is deamidated, the γ-carboxylate of Pup$_{Glu}$ is phosphorylated (and not adenylated like ubiquitin) by PafA using ATP, which produces an ideal leaving group for nucleophilic attack by the amino group of a substrate lysine side chain. The result is a complex of the two proteins covalently linked by an isopeptide bond (Figure 2e) (28). Ligation of Pup to a target substrate requires only the C-terminal 26 residues of Pup (7, 67), a finding that was supported by the crystal structure of the PafA-Pup complex. The normally disordered Pup C terminus folds into a helix upon binding to PafA, positioning the terminal Glu of Pup near ATP for phosphorylation (2).

PafA appears to be the only Pup ligase in bacteria; a *pafA* mutant of *Mycobacterium tuberculosis* is completely devoid of pupylated proteins (55). Three independent studies revealed that dozens if not hundreds of proteins are targeted for pupylation in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* (20, 56, 82), and the same appears to be true for *Corynebacterium glutamicum* (39). Despite this wealth of proteomic information, we still do not understand how proteins are selected for pupylation.

Depupylation

Two observations led to the hypothesis that Pup can be removed from pupylated proteins: Approximately 50% of bacterial species that have both *dop* and *pup* encode Pup$_{Glu}$ and not Pup$_{Gln}$, and partial purification of pupylated proteins (the pupylome) from *Mycobacterium tuberculosis* results in the rapid depupylation of these proteins in the presence of ATP (5). This led to the discovery that Dop has a second function as a depupylase. Similar to eukaryotic deubiquitylating enzymes (DUBs) that counteract the action of ubiquitin ligases, Dop can remove Pup from a pupylated protein, yielding Pup$_{Glu}$ (Figure 2e) (5, 34). Unlike several DUBs, Dop cannot hydrolyze Pup from a linear sequence and is thus specific for removing Pup that is part of an isopeptide linkage with its substrate (5, 35).

An important function for Dop appears to be the recycling of Pup. In a *Mycobacterium tuberculosis dop* mutant producing Pup$_{Glu}$ (to circumvent the need for Pup$_{Gln}$ deamidation), free or substrate-conjugated Pup is undetectable (9). This phenotype could be reversed by the addition of the proteasome inhibitor epoxomicin, which suggests that Pup-protein conjugates can be degraded by the proteasome without depupylation. Thus, the failure to remove Pup from substrates prior to proteasomal degradation results in the reduction of the overall Pup pool.

Mpa/Pup-Dependent Proteolysis

Regulated proteolysis requires that protease substrates be distinguished from nonsubstrates. In mycobacteria, this is accomplished through an interaction between Pup and Mpa. The N-terminal coiled coils of an Mpa hexamer serve as a template for the C-terminal half of Pup to fold into a helix, thus providing a mechanistic basis for substrate specificity (80). Although there are three coiled coils in an Mpa hexamer, only one Pup binds per hexamer (11, 75, 80). Pupylation of PanB with an N-terminally truncated Pup variant ('Pup) produces 'Pup-PanB that is capable of binding to Mpa but unable to be degraded by the proteasome in vitro (72). Similarly, production of an N-terminally truncated Pup in *Mycobacterium smegmatis* results in the modification of proteins with a truncated Pup that cannot facilitate protein degradation (7). We presume that, like in eukaryotes (57), the bacterial proteasome requires an unstructured region to initiate degradation and that this region is supplied by the N terminus of Pup. Finally, as in eukaryotes, where the removal of ubiquitin chains is coupled to degradation (79), it appears that Mpa and Dop function synergistically to unfold and depupylate a target protein (5, 72). There is currently no evidence that depupylation is required for substrate degradation. However, the observation that depupylation occurs prior to or concurrent with substrate degradation in *Mycobacterium tuberculosis* leaves us with an unsolved mystery: How does Pup avoid degradation after translocation through Mpa? Ubiquitin is not usually translocated through the proteasomal ATPase ring in eukaryotes, whereas several lines of evidence suggest Pup must be threaded through Mpa to target substrates for degradation. We speculate that Pup escapes through a gap between the Mpa hexamer and the 20S CP while a doomed substrate proceeds into the proteasome chamber.

ATP-Independent Proteasome Activators

Several in vivo phenotypes suggested that the 20S CP had Mpa/Pup-independent functions in mycobacteria. For one, disruption of *mpa* in *Mycobacterium tuberculosis* produces only a mild growth defect, whereas mutagenesis (23, 24) or chemical inhibition (13) of the 20S CP dramatically affects bacterial growth. In eukaryotes, the 20S CP interfaces with several activators in addition to the 19S RP, which allows it to degrade peptides and proteins in a ubiquitin- and ATP-independent manner (reviewed in 70); we thus hypothesized that the *Mycobacterium tuberculosis* 20S CP might work similarly with a pupylation-independent proteasome activator.

In an effort to identify such an activator, we looked for proteins that interacted with 20S CPs in *Mycobacterium tuberculosis* and identified PafE, a functional homolog of the eukaryotic 11S ATP-independent proteasome activators (37). Although PafE has no sequence homology to eukaryotic activators, it forms multimeric rings, caps the ends of 20S CPs, and enhances proteasomal degradation of peptides and a model unfolded protein β-casein in an ATP-independent manner. Interestingly, *pafE* is not found near the Pup-proteasome system (PPS) genes. Furthermore, PafE has C-terminal residues identical to those of Mpa (GQYL). Gly, Tyr, and Leu, but not Gln, are required for PafE and Mpa function (37) and thus constitute an essential motif for proteasomal activation; consistent with this observation, the three required residues are absolutely conserved in both proteins among proteasome-bearing

bacteria. Furthermore, this motif serves as a functional homolog of the HbYX motif found in many eukaryotic cofactors.

In another study, Delley et al. (15) also reported the identification of PafE, which they named bacterial proteasome activator (Bpa). This study also showed the requirement of its GQYL motif for the degradation of β-casein and suggested that PafE/Bpa forms hexamers based on size-exclusion chromatography analysis. However, using size-exclusion chromatography coupled with multiangle light scattering, we found that PafE forms dodecamers (37). Because dodecameric proteasomal activators are unprecedented, it will be interesting to see the mechanistic basis of 20S CP activation by PafE.

Proteasomes and Bacterial Physiology

Nitric Oxide Resistance of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis primarily resides in macrophages, a first line of defense against numerous infectious agents (62). In mice, macrophages produce NO, which can form a variety of reactive nitrogen intermediates leading to the damage of proteins, nucleic acids, and lipids (3). Several studies suggest that NO is an important component of the immune response to *Mycobacterium tuberculosis*, as mice lacking inducible nitric oxide synthase (iNOS), which catalyzes the production of NO in macrophages, are highly susceptible to infection (47). As discussed earlier, a screen identified *pafA* and *mpa* as essential for NO resistance in *Mycobacterium tuberculosis* (13). Moreover, although these strains are attenuated for growth in animals, the growth defect of an *mpa* mutant is partially ameliorated by genetic disruption of iNOS in mice (13, 14). This result suggested that part of the effect of Mpa on virulence is due to its contribution to NO resistance, but it must also affect bacterial pathogenesis in other ways.

The first link between the PPS and NO resistance was established through the use of a genetic suppressor screen. Operating with the hypothesis that NO sensitivity was due to the accumulation of a specific PPS substrate, Samanovic et al. (63) mutagenized a *Mycobacterium tuberculosis mpa* mutant and screened for secondary mutations that suppressed its NO-sensitive phenotype. Remarkably, genetic disruption of Rv1205 restores NO resistance to an *mpa* mutant. Rv1205 was previously uncharacterized, but a structural homology search indicated that it has high similarity to the plant enzyme LONELY GUY (LOG), which catalyzes the final step in the synthesis of cytokinins, a family of plant hormones. In vitro studies demonstrated that Rv1205 indeed has LOG activity, and it therefore was named Log in *Mycobacterium tuberculosis*. Supporting the original hypothesis of this study, Samanovic et al. (63) determined that Log is a pupylated proteasome substrate, the accumulation of which sensitizes *Mycobacterium tuberculosis* to NO. Importantly, Samanovic et al. (63) showed that *Mycobacterium tuberculosis* synthesizes and secretes several cytokinins. An *mpa* mutant accumulates Log and therefore accumulates cytokinins, whereas *log* mutants have dramatically reduced cytokinin production (63). Metabolomic studies of these strains demonstrated that it is likely the breakdown of accumulated cytokinins into aldehydes that kills *M. tuberculosis* in the presence of NO. Thus, the PPS is required to posttranslationally regulate Log levels to prevent sensitizing mycobacteria to an antimicrobial agent (Figure 3a).

Copper Resistance of *Mycobacterium tuberculosis*

Proteasomes in eukaryotes are intimately associated with transcriptional regulation (reviewed in 29). It was thus a logical step to determine if proteasomal degradation was important for gene expression in *Mycobacterium tuberculosis*. Microarray analysis identified a novel copper-responsive regulon that is repressed in both *mpa* and *pafA* mutants when compared with wild-type *Mycobacterium tuberculosis*. One of these genes, *ricR* (regulated in copper repressor), encodes a transcriptional repressor of copper homeostasis genes (19). Under low copper concentrations, RicR represses transcription by binding to a palindromic element in the promoters of five loci: its own gene, *mymT* (mycobacterial metallothionein), the putative membrane protein genes Rv2963 and *lpqS, mmcO* (mycobacterium multi-copper oxidase), and two small open reading frames, *socA* and *socB* (small ORF induced by copper A and B), which are encoded together in an operon (Figure 3b) (19). Metallothioneins are small, usually cysteine-rich proteins that bind metals to protect against toxicity (reviewed in 1). Thus, it is not surprising that disruption of *mymT* results in copper sensitivity in vitro (26). MmcO is a membrane-anchored multi-copper oxidase, and like a *mymT* mutant, an MmcO-deficient strain is sensitive to copper killing (61, 66). Although the functions of the other RicR-regulated genes are currently unknown, Shi et al. (66) determined that disruption of any single RicR-regulated gene was not sufficient to attenuate bacterial growth in mice. Only upon the construction of a *Mycobacterium tuberculosis* strain producing a mutant RicR protein that constitutively represses all genes in the RicR regulon can one observe an attenuated phenotype in a mouse infection model (66).

How the proteasome is linked to copper resistance is not known. RicR does not appear to be a substrate of the proteasome under any conditions tested so far. Interestingly, several genes that are associated with iron and zinc metabolism are expressed at increased levels in *mpa* and *pafA*-deficient *Mycobacterium tuberculosis* strains (19), which suggests that metal homeostasis in general may be affected by proteasomal degradation.

Survival Under Nutrient-Limiting Conditions of *Mycobacterium smegmatis*

In eukaryotes, the proteasome is required for recycling amino acids (74). Thus, studies were undertaken to determine if the proteasome played a similar role in *Mycobacterium smegmatis,* a nonpathogenic, environmental saprophyte and distant relative of *Mycobacterium tuberculosis*. Gur and coworkers (17) determined that proteasome-associated genes are induced and that pupylated proteins accumulate during the transition from logarithmic to stationary phase growth; it was thus hypothesized that the *Mycobacterium smegmatis* proteasome was required for survival under starvation conditions. A *Mycobacterium smegmatis Δpup ΔprcBA* mutant has a survival defect under nitrogen starvation conditions, which suggests that the proteasome is required for amino acid recycling. However, mitigation of this defect by supplementation with amino acids was not shown, and it is possible that the proteasome is instead required to regulate other pathways that are necessary to survive under these conditions. Intriguingly, complementation of the *Δpup ΔprcBA* strain with *pup*, but not with *prcBA*, restores resistance to the starvation conditions, which suggests that another protease is capable of degrading pupylated proteins (Figure 3c).

Heat Shock Protein Repressor Regulation in *Mycobacterium tuberculosis*

A *pafE* strain has a growth defect on solid and in liquid media, and wild-type *pafE,* but not an allele with a mutated GQYL motif, can complement these phenotypes (37). Importantly, this mutant is attenuated for growth in mice, which indicates a role in tuberculosis pathogenesis. We performed several proteomic studies to identify putative PafE-dependent substrates and identified a set of proteins that are completely distinct from known Mpa-dependent substrates; the most highly accumulated protein was heat shock protein repressor (HspR). We found that PafE promotes the robust proteasomal degradation of HspR in an ATP-independent manner in vitro. Consistent with a role for PafE in promoting the degradation of unfolded proteins and HspR, a *pafE* mutant is sensitive to heat shock (Figure 3d). Thus, PafE contributes to a distinct proteasomal degradation pathway that has a significant role in *Mycobacterium tuberculosis* biology.

Unanswered Questions

What Additional Cofactors Are Needed for Robust Pup-Dependent Proteasomal Degradation?

In sharp contrast to the eukaryotic proteasome system, no group has yet recapitulated the robust in vitro degradation of an endogenous, pupylated bacterial proteasome substrate using wild-type proteasomes. Although the 20S CP and Mpa could in theory provide all of the activity necessary for degradation of a pupylated protein, the best attempts at reconstitution using wild-type 20S CPs achieve substrate degradation on a timescale of ∼12–24 h (5, 72). A possibility is that an additional protein is needed to facilitate the entry of a protein or the exit of peptides, as is proposed for the eukaryotic ATP-independent activator PA28α/β (59). Alternatively, the weak interaction between 20S CPs and Mpa may require an adaptor protein to enhance binding for efficient degradation.

How Is the Pup-Proteasome System Regulated?

Hundreds of proteins are potentially pupylated in *Mycobacterium* species. Remarkably, expression of *Mycobacterium tuberculosis pafA* and pup_{Glu} in *Escherichia coli*, which does not have a PPS, results in the pupylation of an almost equal number of proteins in *Escherichia coli* as are modified in mycobacteria (8). However, although a crystal structure of PafA has now been solved both alone (53) and in complex with Pup (2), these structures provide only clues to the structural constraints of Pup binding and phosphorylation and no information about how PafA chooses one substrate over another.

Levels of Mpa, PafA, Dop, and 20S CP proteins in *Mycobacterium tuberculosis* do not significantly change between logarithmic and stationary phase growth (N. Bode & K.H. Darwin, unpublished observations), which suggests the regulation of Pup-dependent degradation is not necessarily dependent on protein levels. We currently have data that show Ino1 (inositol-1-phosphate synthase) is degraded only in deep stationary phase in both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* (K.E. Burns & K.H. Darwin, unpublished observations). It is possible that Ino1 is pupylated only during stationary phase or that Pup~Ino1 is not delivered to the proteasome until stationary phase. Both scenarios could be due to an intrinsic change in Ino1 or due to the presence of one or more growth

phase–specific adaptor proteins that promote pupylation or delivery to the proteasome. Determination of whether or not this occurs will be a key advance toward understanding how the PPS is regulated.

How Is PafE-Dependent Degradation Regulated?

Although several putative PafE-dependent substrates have been identified in *Mycobacterium tuberculosis*, we do not yet know how they are targeted for degradation. We speculate that PafE enhances the degradation of small, somewhat unstructured proteins. This is supported by the observation that β-casein and HspR are robust substrates of PafE-proteasome mediated proteolysis. PafE does not further enhance the degradation of HspR when using an open-gate 20S CP, which suggests that it is unlikely to provide substrate specificity by binding and recruiting individual substrates (37). The discovery of additional substrates may help us to understand how substrates are targeted for PafE-dependent degradation as well as reveal the pathways to which PafE contributes. This may also provide clues to conditions that may regulate its levels or activity.

Another question that remains to be answered: Do Mpa and PafE compete for access to 20S CPs? PafE was shown to block Mpa-mediated protein degradation in vitro (15), whereas Mpa was unable to inhibit PafE-dependent activation of peptide degradation (37), suggesting that PafE binds to the 20S CP with greater affinity than Mpa. However, neither activator binds to 20S CPs with high affinity, unlike their eukaryotic counterparts. This might suggest that the CPs only interact with the relevant activator when absolutely needed. To date, we do not know with any certainty the relative amounts of 20S CPs, PafE, or Mpa/ARC in any bacterial species.

Can Pupylation and the 20S Core Particle Serve Different Functions in Different Species?

Accumulating evidence suggests proteasomes play distinct roles in the biology of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. This is most evident when comparing the phenotypes of 20S CP–deficient strains. Genetic disruption of *prcBA* in *Mycobacterium smegmatis* or *Streptomyces* does not affect normal axenic growth (31, 38), whereas deletion of the *Mycobacterium tuberculosis* 20S CP produces a severe growth defect under most conditions tested (23, 24). Interestingly, these phenotypic data correlate with an effect on the degradation of pupylated proteins: Known Pup-proteasome substrates accumulate only mildly in a *Mycobacterium smegmatis* strain deficient in the 20S CP, whereas deletion of *mpa* leads to substantial substrate accumulation, which suggests that the stability of pupylated proteins is only partially proteasome dependent in *Mycobacterium smegmatis* (Figure 3e). In contrast, pupylated proteins accumulate to a similar degree in PafA-, Dop-, Mpa-, and PrcBA-deficient strains of *Mycobacterium tuberculosis*. Together, these data indicate that *Mycobacterium smegmatis* may be able to use additional proteases to degrade pupylated proteins, whereas *Mycobacterium tuberculosis* cannot. Thus, it appears that pupylation and the 20S CP play distinct roles in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* physiology. Going forward, it will be critical to avoid the assumption that proteasome data acquired from one species can necessarily be extended to that of others.

Finally, not all bacteria that encode a pupylation system have proteasomes (39). It is therefore possible that Pup also acts as an inhibitor or facilitator of protein activity or localization. Alternatively, Pup may direct proteins to a different protease for degradation, as hinted at by *Mycobacterium smegmatis* data from our lab and the Gur group (17); the intrinsically disordered nature of Pup may make it a target for other compartmentalized proteases such as ClpP, HslUV, or Lon. It is curious that *Corynebacterium* also encodes ARC/Mpa homologs in the absence of proteasomes; this might be explained by the observation that Mpa facilitates depupylation (7, 72), or it might imply that Mpa is capable of interfacing with one or more other proteases in these organisms.

Future Directions

The characterization of Mpa/Pup- and PafE-dependent proteasomal degradation has finally begun to reveal how bacterial 20S CPs that are highly similar to eukaryotic 20S CPs carry out regulated proteolysis in the absence of ubiquitin. We still do not understand how the bacterial systems select particular substrates; elucidation of the basis of PafA substrate selection will undoubtedly be essential to fully appreciate proteasomal degradation in bacteria. In addition, structural studies of how ARC/Mpa and PafE interact with 20S CPs will be critical to reveal the mechanisms of gate opening, which may also contribute to a better understanding of gate activation in eukaryotes and archaea. Finally, much remains to be determined about the biological roles of pupylation, with or without proteasomes. Careful studies of various bacterial species will ultimately reveal what are likely to be numerous functions of pupylation and proteasomal degradation in bacterial physiology.

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Glossary

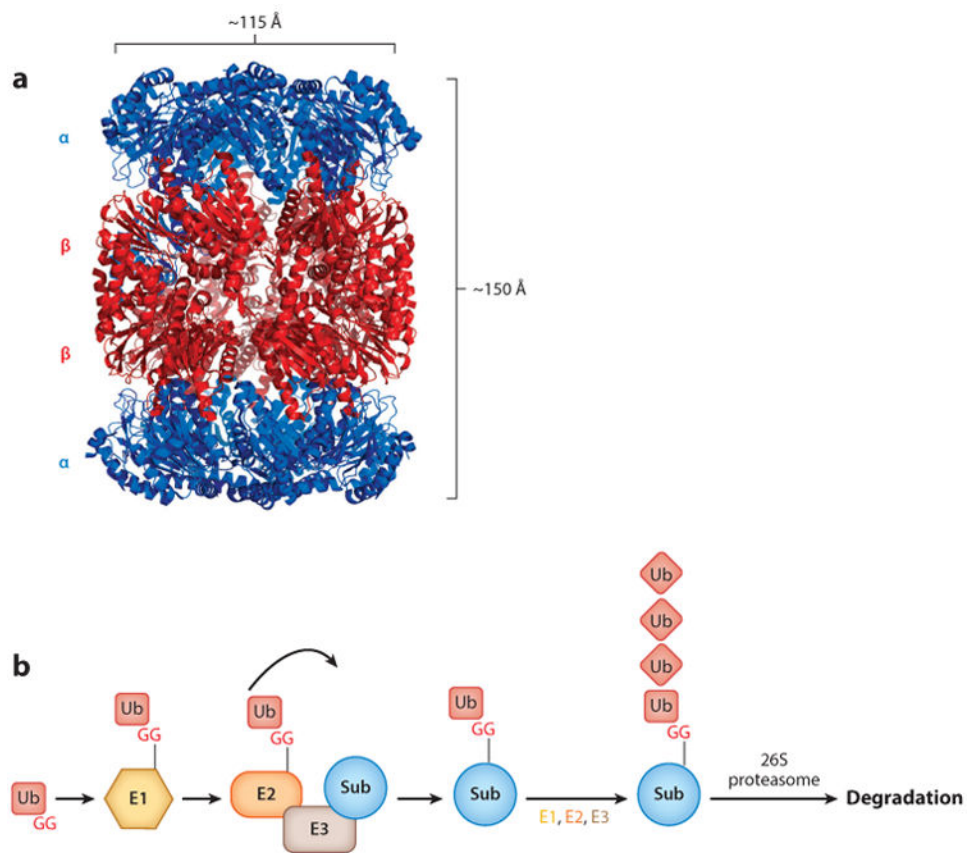|	
Pup	prokaryotic ubiquitin-like protein
Dop	deamidase of Pup
PafA,-B, -C, -E	proteasome accessory factor A, B, C, E
Mpa	mycobacterial proteasome ATPase
PPS	Pup-proteasome system

Figure 1.

Structure of a proteasome 20S CP and schematic of the Ub-proteasome system. (*a*) Crystal structure of the 20S CP from *Mycobacterium tuberculosis* [Protein Data Bank (PDB) ID: 3MI0] (32). (*b*) Eukaryotic Ub-proteasome system. An E1 enzyme activates Ub by adenylating the C-terminal G of Ub and then forming a thioester bond. Ub is then transferred to an E2 Ub-conjugating enzyme, which then transfers Ub to an E3 ligase that conjugates Ub to a lysine on a target substrate. Additional rounds of ubiquitylation on Ub are generally required for receptors on the 26S proteasome to recognize the doomed protein for degradation. Abbreviations: CP, core particle; G, glycine; Sub, substrate; Ub, ubiquitin.
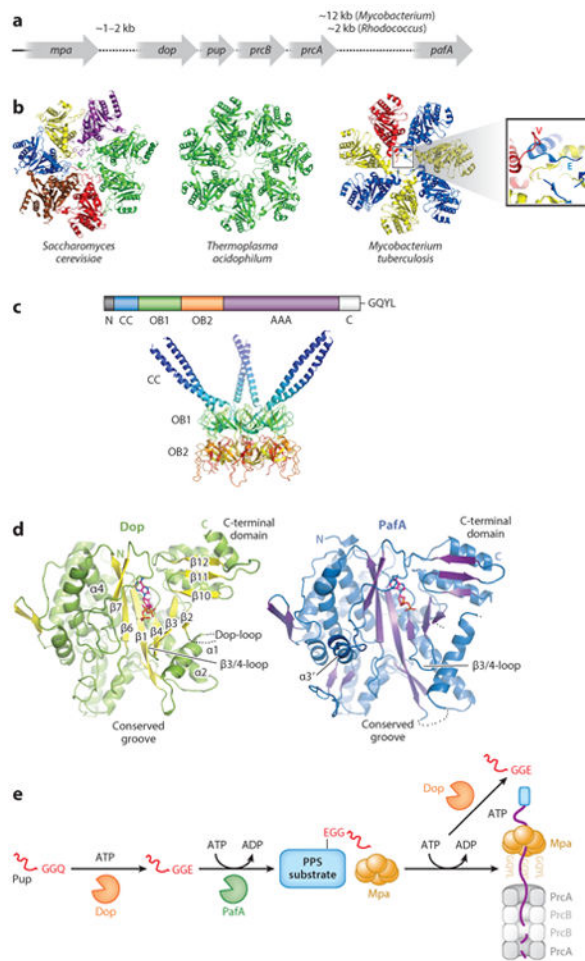
Figure 2.

The Pup-proteasome system (PPS). (*a*) Genomic organization of bacterial proteasome operons in *Mycobacterium tuberculosis* and *Rhodococcus erythropolis.* (*b*) Proteasome gate structures differ among domains of life. Top-view comparison of the 20S CP α ring from *Saccharomyces cerevisiae* [Protein Data Bank (PDB) ID: 2F16] (27), *Thermoplasma acidophilum* (PDB: 1PMA) (65), and *Mycobacterium tuberculosis* (*inset* view is rotated and magnified) (32). Subunits that assume equivalent structures are colored similarly. (*c*) Mpa/ARC is a bacterial proteasomal ATPase. (*Bottom*) X-ray crystal structure of the coiled coil (CC)- and oligosaccharide/oligonucleotide-binding (OB) domains of *Mycobacterium tuberculosis* Mpa (PDB: 3M9B) (adapted from 80). (*d*) PafA and Dop have highly similar structures. X-ray crystal structure of PafA from *Corynebacterium glutamicum* (*left*) and Dop from *Acidophilus cellulolyticus* (*right*) (adapted from 53). (*e*) Schematic representation of the PPS.
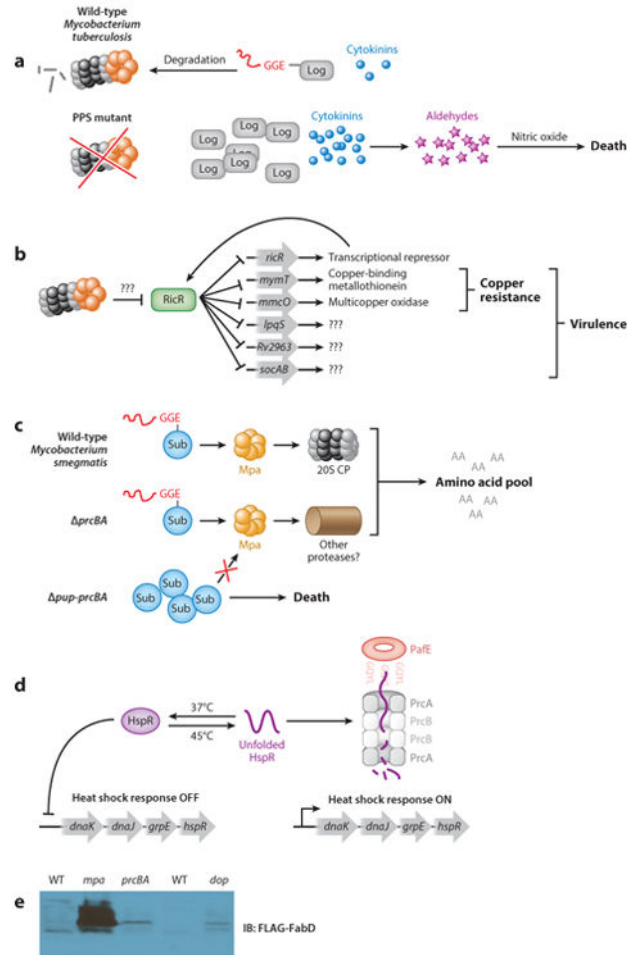
Figure 3.
Biological roles of bacterial proteasomes. (*a*) Accumulation of Log sensitizes PPS-deficient *Mycobacterium tuberculosis* to nitric oxide. (*b*) The RicR regulon mediates copper resistance of *Mycobacterium tuberculosis*. (*c*) The PPS is required to survive nitrogen starvation in *Mycobacterium smegmatis*. (*d*) PafE-dependent proteasomal degradation of HspR contributes to the heat shock response of *Mycobacterium tuberculosis*. (*e*) Mpa, Dop, and the 20S CP contribute differently to the degradation of pupylated substrates in *Mycobacterium smegmatis*. Ectopically produced FLAG-FabD-His$_6$ detected in *Mycobacterium smegmatis* strains mc^2 155 and in isogenic *mpa∷kan* and *prcBA* strains, as well as in SMR5 and isogenic *dop∷kan* strains. Panel *e* is reproduced with permission. Abbreviations: AA, amino acid; CP, core particle; HspR, heat shock protein repressor; IB, immunoblot; PPS, Pup-proteasome system; Sub, substrate; WT, wild-type.