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The *Mycobacterium tuberculosis* Proteasome: More Than Just a Barrel-shaped Protease

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Abstract

In eukaryotes the proteasome is responsible for the degradation of many proteins that are targeted for turnover by post-translational modification with ubiquitin. A similar system was identified in *Mycobacterium tuberculosis* and has shown to be essential for the pathogenesis of this bacterium. Here, we overview the current information of the *Mtb* proteasome and discuss the role of this protease in pathogenesis.

Keywords

Proteasome; prokaryotic ubiquitin-like protein; Pup; tuberculosis

Introduction

Mycobacterium tuberculosis (Mtb) is among the leading causes of death in the world and as a consequence, it is a priority for investigators to find new targets for chemotherapy of increasingly drug-resistant strains. In order to identify cellular pathways that could be targeted for drug development, a screen of *Mtb* transposon mutants for genes encoding proteins that provide defense against nitric oxide (NO) and other reactive nitrogen intermediates (RNI) was performed [1]. RNI are produced by macrophages to slow the growth of invading pathogens and are thought to inhibit microbial growth by damaging nucleic acids, proteins, and lipids (reviewed in [2]). Importantly, the production of NO by the inducible nitric oxide synthase (iNOS) in macrophages is essential to control *Mtb* growth in mice [3]. Although wild type mice survive much longer than iNOS-deficient mice after *Mtb* infection, bacteria are rarely sterilized from these immunocompetent animals. This observation suggested that *Mtb* contains defenses to resist eradication by NO. The screen for NO sensitive *Mtb* mutants identified two proteins putatively associated with proteasome function: Mpa (*Mycobacterium* proteasome ATPase) and PafA (proteasome accessory factor A). Significantly, several groups also determined that mutants of the proteasome pathway are severely attenuated in mice [1,4–7]. These results provided evidence that the bacterial proteasome is essential for resisting the toxic effects of NO and causing disease in mice, making the mycobacterial proteasome a plausible candidate for the rational development of drugs targeting tuberculosis.

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In this review we discuss the most recent literature concerning the *Mtb* proteasome system, and speculate how regulated proteolysis by this highly conserved protease may be required for *Mtb* pathogenesis.

Proteasomes: chambered proteases found in all domains of life

Proteasomes are found in all eukaryotes and Archaea but only in some bacteria of the order Actinomycetales. In eukaryotes the 26S proteasome is composed of two functionally distinct sub-complexes: the 20S core particle (CP), where proteins are degraded, and the 19S regulatory particle (RP) that binds ubiquitylated substrates to be degraded by the 20S CP [8,9] The 20S CP is a self-compartmentalized broad-spectrum protease that has a cylindrical structure [9]. It is composed of four stacked rings with catalytic activity located within the center of the cylinder. The two inner rings are composed of seven distinct catalytic β-subunits while the outer two rings are composed of seven distinct α-subunits[9] The β-subunits have post-acidic, tryptic, and chymotryptic activities, imparting the capacity of the proteasome to cleave most types of peptide bonds. The main function of the α rings is to form a gated channel that controls the passage of unfolded substrates and cleaved peptides in and out of the proteolytic cylinder. The α rings also serve as a docking surface for protein complexes such as the 19S RP, which interacts with either entrance into the 20S CP [9,10]

The 19S RP is responsible for recruiting substrates and regulating the opening of the 20S CP. It is composed of multiple ATPase (Rpt) and non-ATPase (Rpn) subunits. The ATPase subunit is formed by six distinct ATPases associated with different cellular activities (AAA^+) that form a ring and interacts with the α-subunits of the 20S CP [8]. Most importantly, the 19S RP contains numerous proteins that bind and remove Ub from substrates prior to degradation by the 20S CP.

20S CPs in Archaea and Actinomycetes are similar in sequence and structure to those in eukaryotes, but are composed of homo-heptameric β-subunit rings stacked between homo-heptameric rings of α-subunits [reviewed in [11,12]]. In bacteria, 20S CPs have been characterized for the genera *Frankia* [13], *Rhodococcus* [14], *Streptomyces* [15], and *Mycobacterium* [16]. With one exception to be discussed, the presence of only one type of β-subunit limits the 20S CP to chymotryptic activity.

The best-characterized prokaryotic AAA^+ ATPases is PAN (proteasome activating nucleotidase) from the Archaeon *Methanococcus janaschii* [17]. PAN can associate with *Thermoplasma* 20S CP *in vitro* and induce gate opening, unfolding and translocation of substrates to the catalytic domains of the proteasome [17–19]. In contrast, no bacterial proteasome has ever been shown to interact with its cognate ATPase or promote the degradation of a polypeptide. The mechanism by which substrates are recognized for degradation is still unknown because regulatory complexes that associate with PAN or other prokaryotic proteasome-associated ATPases have not been identified.

The structure of the *Mtb* 20S CP has been solved and, like other prokaryotic proteasomes, is composed of only one type of α-subunit encoded by *prcA* and one type of β-subunit encoded by *prcB* [20]. These subunits interact with each other to form a canonical 20S CP [16,20]. Using different peptide substrates Lin and colleagues demonstrated that the *Mtb* proteasome has post-acidic, tryptic and chymotryptic protease activity [16]. This broad specificity resembles that of eukaryotic proteasomes rather than the limited chymotryptic specificity present in other prokaryotic proteasomes. However, it is not yet known how a single type of β-subunit is able to confer such a broad spectrum of protease activity. Importantly, proteolytic activity was best observed upon deletion of the N-terminal eight amino acids of the α-subunit [16]. This suggested that the α-ring effectively blocks access of even small peptide substrates

to the 20S CP. This observation strongly supports the hypothesis that an activating protein or proteins are needed to open the α-ring for substrate degradation.

Proteasomes are essential for eukaryotic life, but its requirement in bacterial functions and/or survival appears to be varied. For example, it has been reported that deletion of the *prcBA* genes from *Mycobacterium smegmatis (M. smegmatis)*, a non-pathogenic relative of *Mtb*, did not severely impact bacterial growth [21,22]. In contrast, chemical or genetic silencing of *Mtb* proteasome activity dramatically slowed bacterial growth *in vitro* and *in vivo* [1,6]. Interestingly, unlike *M. smegmatis*, *Mtb* lacks Lon, another ATP-dependent chambered protease, which may explain the non-essentiality of the *M. smegmatis* proteasome.

Proteins associated with *Mtb* proteasome function

The *mpa* and *pafA* genes were inferred as proteasome associated given their close vicinity to the 20S CP *prcBA* genes, and that they are present only in bacteria containing proteasomes [23]. Furthermore, mutations in either gene resulted in identical NO-sensitive and *in vivo* attenuated phenotypes, suggesting that both of them participate in the same pathway.

Mycobacterium proteasome ATPase (Mpa)

Mpa is an ATPase similar to ATPases present in the eukaryotic 19S RPs [4]. Studies using cryo-electron microscopy revealed that Mpa forms hexameric rings similar to other AAA ATPases [4,24]. Additionally, Mpa contains Walker A and B motifs involved in the binding and hydrolysis of ATP [24,25]. Mutations within these motifs or S-nitrosylation of Mpa inhibited the ability of Mpa to hydrolyze ATP *in vitro* [4,26]. Most importantly, it has been shown that the ATPase activity is essential for RNI resistance [4].

Although structural analysis predicts that Mpa physically interacts with the proteasome 20S CP and plays a role in binding, unfolding and translocating substrates into the 20S CP, this has not yet been experimentally shown. Interestingly, *mpa*607, an *mpa* mutant identified in the NO screen with a transposon insertion in its penultimate codon, had the same phenotype as the *mpa* null strain; it was sensitive to RNI *in vitro* and highly attenuated in the mouse model of infection [1,4]. The *mpa607* mutant lacks two terminal amino acids, tyrosine (Tyr) and leucine [1]. Further analysis revealed that the penultimate Tyr is also found in several ATPase subunits of the eukaryotic 19S RP, most Archaeal proteasome-associated ATPases and all bacterial proteasome-associated ATPases [5]. In Archaea, the penultimate Tyr of PAN was shown to be essential for activating the opening of the 20S CP [27]. When the penultimate Tyr of Mpa was mutagenized to either phenylalanine or glutamate (Glu), *Mtb* was sensitized *Mtb* to RNI *in vitro* to a similar degree as the *mpa* null mutant [5]. Taken together, these studies suggest that the C-terminus of Mpa may interact with the 20 CP in order to open and deliver proteins into the catalytic core for degradation.

Prokaryotic ubiquitin-like protein (Pup)

Until very recently it was a mystery how proteins were targeted to prokaryotic proteasomes since ubiquitin (Ub) or ubiquitin-like modifiers (Ubl) had not been conclusively found in prokaryotes. Most proteins degraded by the eukaryotic proteasome are first modified at one or more lysines through the covalent attachment of Ub, a 76 amino acid protein that tags proteins for selective turnover. Ub is ligated to substrates in a tightly regulated, multi-step ATP-dependent cascade involving activation (E1) and conjugation (E2, E3) enzymes (reviewed in [28]). But attachment of a single Ub to a substrate Lys is not generally sufficient to initiate degradation; a number of Ubs need to be added, therefore, after attachment of an initial Ub moiety to a substrate, additional activated Ubs are ligated to lysines (usually Lys48) of the

previously conjugated Ub molecule. These polyUb chains serve as an unambiguous trigger for proteolysis by the 26S proteasome [8].

Using an *E. coli* bacterial two-hybrid screen of an *Mtb* genomic DNA library searching for potential binding partners of Mpa, Pearce and colleagues discovered the first prokaryotic ubiquitin-like protein, Pup, a 64 amino acid protein that modifies and targets mycobacterial proteins to the proteasome for degradation [29]. BLASTP analysis [30] identified Pup homologues only in Actinobacteria. Furthermore, *pup* is part of a putative operon together with the proteasomal genes *prcBA*, supporting its association with proteasome function. Although Pup and Ub are similar in size, they lack sequence and structural homology [31,32]. Furthermore, the last three C-terminal residues of Pup are Gly-Gly-glutamine (Gln), while the last two C-terminal residues of proteolytically activated ubiquitin and other ubiquitin-like modifiers is Gly-Gly. Pearce and colleagues showed that Pup interacts both non-covalently and covalently with Mpa (which is also a degradation substrate), and covalently with the degradation substrate FabD. Mass spectrometry analysis revealed that Pup formed an isopeptide bond between its C-terminus and the ε-amino group of a specific lysine residue (Lys173) of FabD. Surprisingly, the C-terminal residue of Pup participating in the linkage was glutamate (Glu), indicating that the C-terminal Gln was deamidated before or during conjugation to FabD. Purification of recombinant Pup from mycobacteria and *E. coli* revealed that the C-terminal Gln of Pup was specifically deamidated in mycobacteria, suggesting the presence of a specific Pup deamidase [29].

Further studies showed that multiple “pupylated” proteins accumulated in the *Mtb mpa* mutant strain, indicating that Pup-dependent proteasomal degradation was not limited to FabD. In a parallel study, Burns and colleagues showed that *M. smegmatis* Pup was conjugated to superoxide dismutase (SodA) and myoinositol-1-phosphate synthase (Ino1) using the same Gly-Gly-Glu~Lys linkage, and that pupylated proteins also accumulated in a *prcBA* mutant compared to wild type *M. smegmatis* [21]. Deletion of the Pup C-terminal Gln inhibited “pupylation”, confirming the importance of this amino acid in Pup conjugation [21].

These exciting findings showed that pupylation is functionally similar to ubiquitylation in that substrates are covalently conjugated with a small protein modifier at lysines in order to target them for degradation. However, this process differs from ubiquitylation in that Pup is not proteolytically activated to reveal a C-terminal Gly-Gly motif, but is rather activated and conjugated to target proteins via a C-terminal glutamate.

Proteasome accessory factor A (PafA) and de-amidase of Pup (Dop)

Enzymes similar to those involved in the activation and conjugation of Ub to proteasome substrates have not been described, suggesting that different enzymatic activities participate in pupylation. Pup-conjugated substrates could not be detected in an *Mtb pafA* mutant, demonstrating that PafA is essential for pupylation [29]. A bioinformatic analysis found that PafA is likely to be structurally related to γ-glutamyl-cysteine synthetase-2, suggesting that it is member of the carboxylate-amine ligase superfamily [33,34]. Based on this observation it was proposed that PafA phosphorylates the γ-carboxyl group of the C-terminal Glu of de-amidated Pup in an ATP-dependent manner; this “activated” form of Pup would then react with the amino group of the target Lys to form an isopeptide bond [33]. However, it is not known whether the last Glu of Pup attaches by its α- or γ-carboxylate. In addition, the mechanism of Glu~Lys bond formation is not yet understood.

The observation that most Pup molecules were deamidated at the C-terminus when purified from mycobacteria but not *E. coli* suggested the presence of a specific enzyme required for this process [29]. Using Pup as bait for *in vitro* pull down experiments with *Mycobacterium bovis* lysates, Striebel and colleagues identified Dop (deamidase of Pup) that converted the C-

terminal Gln of Pup to Glu [35]. Dop was predicted to be involved in proteasomal protein degradation because it is highly similar to PafA and encoded immediately upstream of *pup* and the 20S CP genes (reviewed in [34]). Interestingly Dop required the presence of ATP for the deamidation of Pup, but ATP hydrolysis was not detected [35]. This was supported by the observation that ATPγS, a non-hydrolyzable ATP analogue, also allowed the deamidation to proceed, but at a slower rate. Similar to PafA, the catalytic mechanism of Pup deamidation by Dop remains to be characterized.

A model for proteasomal degradation in *Mtb*

Based on the current data, we propose the following model for pupylation: Pup is synthesized with a Gln residue at the C-terminus, which is deamidated by Dop, resulting in a Glu residue at the C-terminus of Pup (Fig. 1). Subsequently, PafA phosphorylates the γ-carboxylate of the Glu residue, forming an intermediate that would favor nucleophilic attack by the amino group of a Lys residue present in the substrate destined to be degraded. The active site amino acids in Dop and PafA involved in catalysis remain unknown. Pup then interacts with Mpa, facilitating the delivery of substrate to the 20S CP. It is not yet known if Pup, like Ub, is removed prior to degradation or is also degraded.

Striebel and colleagues noted that Dop is present in bacteria encoding Pup that naturally terminates in Glu [35]. This suggests that Dop has another function in addition to Pup deamidation. Perhaps Dop is required, along with PafA, to ligate Pup to substrates. *In vitro* data suggest that this is not the case because PafA was sufficient to conjugate recombinant Pup terminating with Glu to FabD [35]. Analysis of a *dop* mutant will be required to ultimately determine if it indeed has functions in addition Pup deamidation. Finally, several bacterial species encode PafA and Dop but do not have conspicuous Pup or proteasome homologues [34]. It is not known if *pafA* and *dop* are expressed in these bacteria, or if they have pupylation or proteasome independent functions.

The link to pathogenesis?

It is likely that the proteasome is required for the virulence of *Mtb* for numerous reasons. One possibility is that the bacterial proteasome is required to degrade one or many proteins that are toxic after nitrosative or oxidative damage. Another possibility is that certain proteasomal substrates must be degraded in order to allow other proteins to participate in NO detoxification or to repair macromolecules damaged by NO. These may include transcriptional repressors of genes that synthesize DNA repair enzymes or other proteins that must replace essential proteins damaged by RNI.

It is important to note that RNI are not the only stresses that *Mtb* encounters *in vivo*. Macrophage acidification [36], the production of reactive oxygen species [37] and even eukaryotic Ub peptides [38] can participate in the control of *Mtb* growth. The *Mtb* proteasome may regulate the expression of genes that counteract these host defenses. *Mtb* has also long been believed to suppress the host immune response to favor its persistence [39,40]. It would not be surprising if the *Mtb* proteasome transcriptionally or post-translationally regulated the production of secreted or surface exposed proteins that modulate host immunity. Because all ATP-dependent proteases, including ClpP, Lon, FtsH, and HslV, degrade transcription factors in numerous non-pathogenic and pathogenic bacteria (reviewed in [12,41]), it is possible that the *Mtb* proteasome is involved in the regulation of several pathways. Transcriptome and proteome analysis of proteasome-deficient strains will begin to test this hypothesis.

It is notable that only a handful of pupylated substrates have been identified so far, and there is no obvious link between them and *Mtb* virulence. The identification of the “pupylome” using epitope tagged Pup and high-resolution mass spectrometry should reveal how many proteins

rely on Pup for regulation or turnover. The identity of these proteins may ultimately begin to explain why defects in protein degradation attenuate *Mtb* virulence *in vivo*.

Conclusions

The discovery of proteasomal degradation as a requirement for *Mtb* virulence has spawned several exciting areas of research, in particular, the understanding of how this activity participates in pathogenesis and the characterization of a novel post-translational modification system. It is likely that the disruption of proteasome function has pleiotropic effects and it will take time to determine which are responsible for the attenuation of *Mtb* in mice. In contrast, there has been remarkable progress in defining the biochemistry of the Pup-proteasome system (PPS). Yet, there is much more to learn about this system. Although it is functionally similar to the eukaryotic Ub-proteasome system (UPS), the biochemistry that targets proteins for degradation appears quite different. As a result, many important questions associated with crucial steps in the path to the proteasome have arisen. For one, how does PafA select proteins for pupylation? Higher eukaryotes have hundreds if not over 1,000 E3 ligases that provide specificity to the UPS, how is this achieved in *Mtb*? PafA could possibly form dimers or hetero-dimers with Dop [33,42]. If this were true, we could speculate that these proteins form a complex that binds both the substrate and Pup for conjugation, and additional proteins may bind to impart an E3-like specificity to the PPS.

It is also unknown if Pup chains are formed on certain substrates in a manner akin to Ub chains. To date, there is no evidence that Pup conjugates to other Pup molecules or onto multiple sites on a single substrate. Another question of interest is whether or not “de-pupylating” enzymes exist. Ub is removed for recycling before the degradation of a substrate, is Pup recycled as well? If so, is a DUB-like protease involved, or are we facing a new enzymatic reaction as seen for the attachment of Pup to substrates?

Now that several components involved in protein degradation are known, it is expected that rapid progress will be made in characterizing these proteins, and in the identification of new players as well. Elucidation of the PPS will not only give us numerous fascinating challenges to ponder, but it will also provide critical data for ultimately developing therapeutics to combat one of the world’s deadliest diseases.

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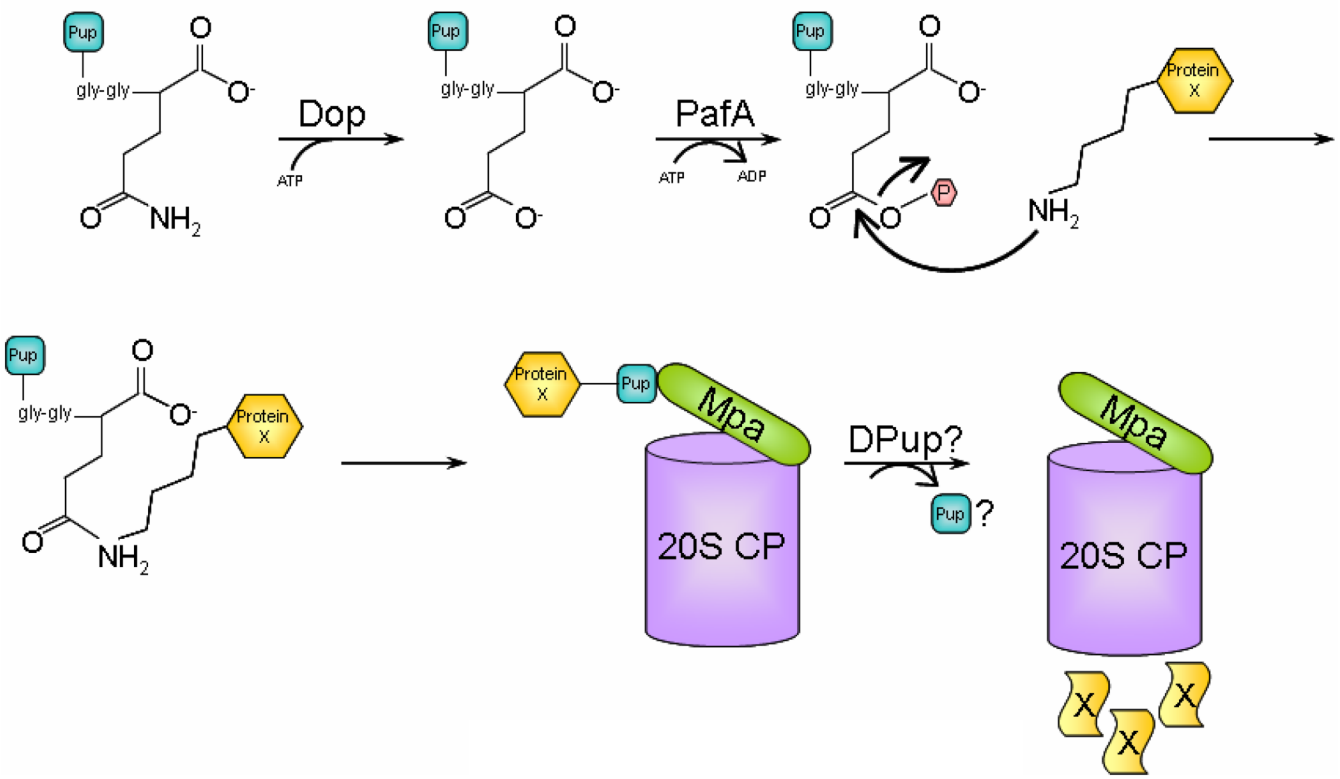

Fig. 1. Model for protein degradation by the Pup-proteasome system (PPS)

Tagging of a proteasome substrate, such as Protein X, is initiated with deamidation of Pup by Dop and ATP binding. Deamidated Pup then binds to PafA that with ATP hydrolysis catalyzes conjugation of a Pup monomer to Protein X. PafA facilitates the nucleophilic attack by an amino group present in Protein X to the γ-carboxylate of the C-terminal Glu residue in Pup. As a result, Protein X gets mono-pupylated; it is unknown if poly-Pup chains can form. Subsequently, Pup binds to Mpa, which might promote unfolding and translocation of Protein X into the 20S CP. During translocation, Pup might be removed by de-pupylating (DPUP) enzymes. Finally, Protein X is degraded to small peptides by the proteolytic activity in the central chamber of the 20S CP.