Review

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# Pupylation-dependent and -independent proteasomal degradation in mycobacteria

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**Abstract:** Bacteria make use of compartmentalizing protease complexes, similar in architecture but not homologous to the eukaryotic proteasome, for the selective and processive removal of proteins. Mycobacteria as members of the actinobacteria harbor proteasomes in addition to the canonical bacterial degradation complexes. Mycobacterial proteasomal degradation, although not essential during normal growth, becomes critical for survival under particular environmental conditions, like, for example, during persistence of the pathogenic *Mycobacterium tuberculosis* in host macrophages or of environmental mycobacteria under starvation. Recruitment of protein substrates for proteasomal degradation is usually mediated by pupylation, the post-translational modification of lysine side chains with the prokaryotic ubiquitin-like protein Pup. This substrate recruitment strategy is functionally reminiscent of ubiquitination in eukaryotes, but is the result of convergent evolution, relying on chemically and structurally distinct enzymes. Pupylated substrates are recognized by the ATP-dependent proteasomal regulator Mpa that associates with the 20S proteasome core. A pupylation-independent proteasome degradation pathway has recently been discovered that is mediated by the ATP-independent bacterial proteasome activator Bpa (also referred to as PafE), and that appears to play a role under stress conditions. In this review, mechanistic principles of bacterial proteasomal degradation are discussed and compared with functionally related elements of the eukaryotic ubiquitin-proteasome system. Special attention is given to an understanding on the molecular level based on structural and biochemical analysis. Wherever available, discussion of *in vivo* studies is included to highlight the biological significance of this unusual bacterial degradation pathway.

**Keywords:** mycobacteria; mycobacterial proteasomal ATPase Mpa; proteasomal activator Bpa (PafE); proteasome; pupylation.

## Introduction

The genus *Mycobacteria* encompasses >100 different species, which mostly exist as free-living organisms in soil and marshes, where they face rapidly changing conditions in terms of nutrient and oxygen availability (1, 2). The pathogenic members, like, for example, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, have to persist in the hostile intraphagosomal environment of the host (3). As a consequence, mycobacteria have to be highly adaptable and metabolically flexible. Protein degradation plays a critical role in mounting a rapid and effective response to these challenges.

In eukaryotic cells, protein removal can be mediated either by autophagy in combination with lysosomal degradation or by recruitment to the cylinder-shaped protease complex referred to as the proteasome, where compartmentalization is achieved inside a proteinaceous chamber (4, 5).

As bacteria are lacking the option of sorting proteins into separate organelles, they rely exclusively on compartmentalizing protease complexes that are non-homologous yet similar in architecture to the eukaryotic proteasome (6). Among these canonical protease complexes are the Clp proteases, the Lon protease, and the membrane-anchored FtsH protease (Figure 1). Homologs of these typical bacterial proteases can also be found in eukaryotic compartments of endosymbiotic origin, namely mitochondria and chloroplasts (Figure 1). All mycobacteria possess the modular Clp proteases, which are (with the exception of *Mycoplasma*) universal in bacteria. These consist of the ClpP protease cylinder that is associated with either the ClpC or the ClpX ATPase partner to form a fully active

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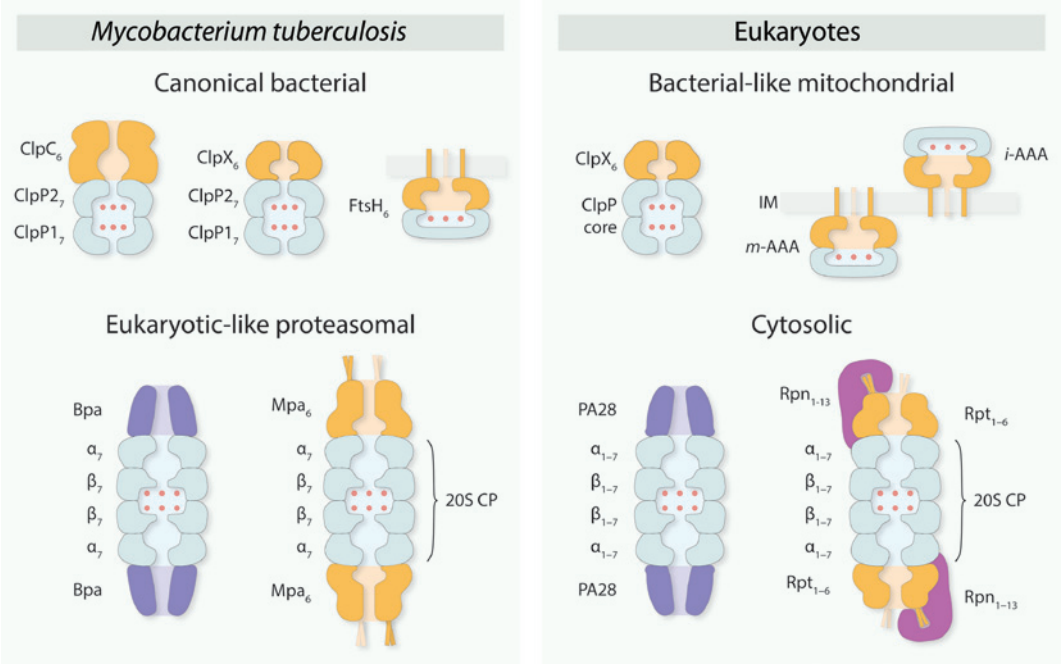


**Figure 1:** Compartmentalizing proteases of *Mycobacterium tuberculosis* and their eukaryotic counterparts.
Compartmentalizing protease complexes comprise a core cylinder (light blue), enclosing the proteolytic active sites (red), that associates with energy-dependent (orange) or energy-independent (violet) regulatory ring-complexes to form the fully active protease. *Mtb* has the canonical bacterial degradation systems, -the cytosolic Clp proteases and the membrane-associated FtsH protease-, homologs of which can also be found in eukaryotic mitochondria. The main eukaryotic degradation machine is the 26S proteasome, built of the 20S core particle (20S CP) and the 19S regulatory particle ATPase (orange) and non-ATPase (pink) subunits. Unusual for the bacterial kingdom, *Mtb* harbors a proteasome complex in addition to the canonical bacterial degradation machines. This bacterial proteasome, found predominantly in the actinobacterial phylum and a few members of nitrospirae and verrucomicrobae, is of simpler subunit composition than its eukaryotic homolog.

protease complex (Figure 1) (6). Like all bacteria, mycobacteria also possess the membrane-associated FtsH protease capable of degrading cytosolic and membrane proteins. Both the Clp proteases and FtsH are essential in mycobacteria (7). Surprisingly, in addition to these canonical bacterial compartmentalizing proteases, mycobacteria and many other actinobacteria possess a proteasome (8), the main cytosolic degradation complex in eukaryotes, which is usually absent in bacteria. Although not essential under standard culture conditions (9), the proteasome is maintained in all mycobacteria (8), indicating that it is important for survival under particular environmental conditions. In cooperation with the ATP-dependent Mpa ring-complex, it recruits proteins that have been covalently modified with prokaryotic, ubiquitin-like protein Pup in a process called pupylation (10, 11). A pupylation-independent proteasome degradation route also exists in these bacteria, which is mediated by the bacterial proteasome activator Bpa (also referred to as PafE) (12, 13).

In this review, we summarize the mechanistic principles of proteasomal protein degradation in mycobacteria and discuss its cellular function in the context of the bacteria's life style. Furthermore, we draw parallels to the eukaryotic proteasome system, including the modes of substrate recruitment, and highlight similarities and fundamental differences.

## The mycobacterial proteasome – an added eukaryotic-like feature

In all three domains of life, nature has evolved a multitude of high-molecular weight, multisubunit proteolytic complexes that accomplish the removal of non-functional, damaged or misfolded proteins, or proteins that are needed in the cell only for a limited amount of time (14). The major player in eukaryotic protein turnover is the 26S proteasome (15) (Figure 1). It comprises two functional elements: the 19S regulatory particle (19S RP) and the 20S core particle (20S CP) (5). The latter is composed of seven distinct α and β subunits that form heptameric rings ($\alpha_{1-7}/\beta_{1-7}$), respectively (16). These rings are stacked in an αββα order, resulting in the barrel-shaped appearance of

the 20S CP. The β-rings contain the proteolytically active subunits of the complex, featuring the N-terminal threonine hydrolase sites, whereas the α-subunits provide the interface for interaction with the 19S RP and alternative regulators of the 20S CP (17). The 19S RP is a multisubunit complex that combines several functions related to the recognition of poly-ubiquitinated substrates, ubiquitin-chain processing, substrate unfolding, and the translocation of unfolded polypeptides to the proteolytic core of the proteasome (18–20).

First indications for the presence of an ATP-dependent proteolytic activity in eukaryotes that is mediated by a complex protease were found nearly four decades ago (21, 22). The efforts of various groups were dedicated to the characterization and isolation of this activity [e.g. refs. (22–26)], finally resulting in the purification of the first eukaryotic 26S proteasome complex (27). Initially thought to be restricted to eukaryotes, the discovery of a 20S CP in the archaeon *Thermoplasma acidophilum* put an end to this notion (28). The earliest indications of a bacterial proteasome derived from a study with nitrogen-fixing *Frankia* (29). A high-molecular-weight protease, partially purified from the bacterium, largely resembled the eukaryotic 20S CP in shape and size as judged by electron microscopy, and exhibited cross-reactivity with an antiserum against eukaryotic proteasome. By comprehensive database searches and by an increasing amount of genome data available, the presence of genes coding for the α- and β-subunits of the 20S CP was confirmed in various bacterial species (9, 30–33). In contrast to eukaryotes, bacterial and archaeal genomes encode only one or two α- and β-subunits. While the archaeal proteasome is generally considered to be the direct evolutionary precursor of the eukaryotic proteasome, the existence of a 20S CP in bacteria remained puzzling. The occurrence of a bacterial 20S CP is predominantly associated with the phylum *Actinobacteria*, a heterogeneous group of high-GC, Gram-positive bacteria (34). Likely adopted by horizontal gene transfer, the reasons why an additional proteolytic machinery was established in these bacteria next to the canonical bacterial ones remained unclear. As the genes coding for the proteasome appear to be mostly dispensable under standard growth conditions (9, 35), evolution most likely selected for it under special conditions, where survival could critically benefit from an additional compartmentalizing protease system (36). The identification of the proteasomal subunit genes – usually referred to as *prcA* and *prcB* – was eventually followed up by the characterization of purified 20S CP from *Rhodococcus erythropolis* (32), *Streptomyces coelicolor* (37), and *Frankia* (38). A few sporadic members outside the actinobacterial phylum also harbor proteasomes, possibly as a result of secondary gene transfer from actinobacteria (33, 39, 40).

Investigations of the mycobacterial proteasomal degradation system were initiated by a study in *Mycobacterium smegmatis* (*Msm*) (9). Expression of a functional proteasome was strongly supported by the observation that lysates of an *Msm* Δ*prcB* strain displayed a 95% decrease in degradation activity for the proteasomal model peptide substrate succinyl-Leu-Leu-Val-Tyr-AMC. Accordingly, turnover of Suc-LLVY-AMC in *Msm* wild-type-derived lysates could be inhibited by the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal. These findings were corroborated by the identification of PrcA and PrcB in the proteome of *Mycobacterium tuberculosis* (*Mtb*) (41) and the presence of a proteasome inhibitor-sensitive proteolytic activity in lysates of the bacterium (42).

## Structure of the Mtb proteasome core particle

The crystal structure of the *Mtb* 20S CP revealed the canonical barrel-shaped overall architecture, highly reminiscent of eukaryotic and archaeal CPs (43, 44). The mycobacterial CP is constituted of a single type of both α- and β-subunits that form homoheptameric rings, which are arranged in the usual $\alpha_7\beta_7\beta_7\alpha_7$ order (Figure 1). This results in the formation of three chambers inside the CP cylinder that are connected with each other by pores. The central chamber, formed by the β-rings, harbors the active-site N-terminal threonine residues, sequestered from the cytosolic environment. Regarding the proteolytic specificity, the 20S CP of *Mtb* differs from CPs found in other actinobacterial species. While the latter merely exhibit chymotryptic activity, the CP in *Mtb* additionally features tryptic and caspase-like activity (43). This broad substrate specificity is striking as it is accomplished by a single type of β-subunit. Eukaryotic 20S CPs harbor three distinct β-subunits, each dedicated to a specific activity (17). Analysis of the *Mtb* 20S CP substrate-binding pocket revealed a unique chimeric structure that joins elements found in the three eukaryotic proteolytically active β-subunits (43).

The α-rings, attached to either end of the central β-double ring, form the entrance pores to the 20S CP. To prevent unregulated degradation, access to the internal proteolytic chamber needs to be restricted. In eukaryotic 20S complexes, the N-termini of the α-subunits interact with each other through a sequence motif containing a conserved tyrosine and aspartate residue, the YD motif,

thereby effectively sealing the 20S CP's axial entrance pores (45). The deletion of seven N-terminal residues from the α-subunits leads to an open-gate CP with deregulated peptidase activity (45). Cryo-electron microscopy and structural analysis showed that the *Mtb* 20S CP is also a gated complex (44, 46). Although a YD-motif is absent, the α-subunit's N-terminal residues are arranged such that the external pores are tightly sealed.

Although homologous to the β-subunits, both the eukaryotic and the bacterial α-subunits feature an additional N-terminal helix referred to as H0, that extends radially outward from the α-ring pore (16, 44, 47). The α-N-termini are connected to this helix either directly (bacterial 20S CP) or via a reverse turn element (eukaryotic 20S CP). In either case, gate closure is achieved by neighboring subunits adopting alternating conformations around the ring with respect to H0. In the *Mtb* 20S CP, six of the seven α-N-termini around the ring alternatingly feature an extended or L-shaped orientation with respect to H0, such that the hydrophobic stretches (Y5-F6-I7) in the L-shaped N-termini of three subunits around the ring stack on top of one another, blocking the gate (44). This is similar to the alternating conformations of the α-N-terminal tails preceding the reverse turn in the eukaryotic 20S CP, where three of the seven α-N-terminal strands point into the α-pore and interact through their YD-motifs, effectively closing the gate (47).

## Assembly and maturation of the Mtb proteasome core particle

In eukaryotes, assembly of the 20S CP depends on structural features of the subunits and involves dedicated chaperones that support the correct arrangement of the heteroheptameric rings (17, 48). Orthologs of eukaryotic assembly factors have been identified in archaea and bacteria (49–51); yet, their exact physiological function needs to be scrutinized. Archaeal and bacterial 20S CPs readily assemble when expressed heterologously in *Escherichia coli* (38, 46, 52–54). However, a prerequisite for *Mtb* and *Rhodococcus* 20S CP formation is the simultaneous expression of *prcA* and *prcB*. When expressed individually, α- and β-subunits fail to form heptameric rings (46, 53). In contrast, α-subunits of *Thermoplasma* also oligomerize in the absence of interacting β-subunits (52).

A common characteristic of bacterial and active eukaryotic β-subunits is an N-terminal pro-peptide that is present upon *de novo* synthesis and is autocatalytically removed to reveal the catalytic Thr1 residue. In the yeast proteasome, the pro-peptide was shown to prevent *Na*-acetylation of the active-site threonine residue until it is sequestered in the interior of the proteasome chamber (55). This protects Thr1 against inactivation, as the N-terminal α-amino group plays an important role in accepting a proton from the Thr hydroxyl to activate it for nucleophilic attack (55). A similar role could be played by the propeptides for the mycobacterial proteasome. CP assembly is finalized by autocatalytic removal of the β-subunit's pro-peptides, which results in the exposure of the threonine residues and renders the complex proteolytically active (56, 57). In eukaryotes and in *Rhodococcus erythropolis*, the β-subunit's pro-peptides were shown to be crucial for the formation of the fully assembled 20S CP (53, 56, 58, 59). In contrast, *Mtb* and archaeal β-subunit pro-peptides are dispensable (46, 52). In fact, assembly of the *Mtb* 20s CP is slowed down by the pro-peptides. Due to their outward-facing conformation in half-proteasomes, they possibly hamper the association of two half-proteasome particles (44). A recent study in *Msm* showed that 20S CP subunits are phosphorylated by the kinases PknA and PknB (60). Phosphorylation of the α-subunits by PknB results in an increase of the proteasome's proteolytic activity. In contrast, PknA activity affects the assembly of the CP, acting on α-subunits as well as the pro-peptide bearing β-subunits in half-proteasomes. This apparently interferes with the apposition of two half-proteasomes and slows down the formation of fully processed mature 20S CP.

## Proteasome activators open the gates

The proteolytic core cylinders of compartmentalizing proteases (the 20S CP or the ClpP cylinder) are lined on their inner walls with the hydrolytic active sites (61–63) (Figure 1). In addition to compartmentalization, however, selectivity of access to the proteolytic chamber is required to prevent random proteolysis of cytosolic proteins. Proteasomal core cylinders are therefore gated and require association with additional interactors that switch the conformation of the α-subunit's N-termini to an open conformation (47, 64–67). In keeping with the principle of ring-stacking architecture, interaction partners of the proteasome are themselves ring-shaped, multisubunit assemblies that associate with the 20S CP α-rings coaxially, thereby roughly aligning their ring pore with the CP's axial entrance pore (18, 64, 68) (Figure 1). Proteasome interactors, also referred to as regulators or activators,

serve not only to open the gate, but they also play important roles in substrate recruitment.

A common principle for the interaction between the 20S CP and the gate-opening regulators has been observed (Figure 2A). The very C-terminal tails of the regulator subunits dock into binding pockets between adjacent α-subunits of the 20S CP, supporting a conformational change in the α-N-termini, which eventually results in gate opening (47, 64, 69, 70). For eukaryotic 20S CPs, this presumably involves direct or indirect contact of the C-terminal tails with the reverse turn following the α-N-terminal strand of the 20S CP containing the YD-motif (71). While the identity of the C-terminal residue in the C-terminal tails of the proteasome interactors is usually not important, the two or three residues preceding the terminal amino acid confer specificity. Many of the regulators (e.g. eukaryotic 19S ATPases, eukaryotic PA200, eukaryotic Cdc48, and archaeal proteasomal ATPases) (72), but not all (PA26) (47), possess a conserved penultimate tyrosine residue (sometimes also phenylalanine) preceded by

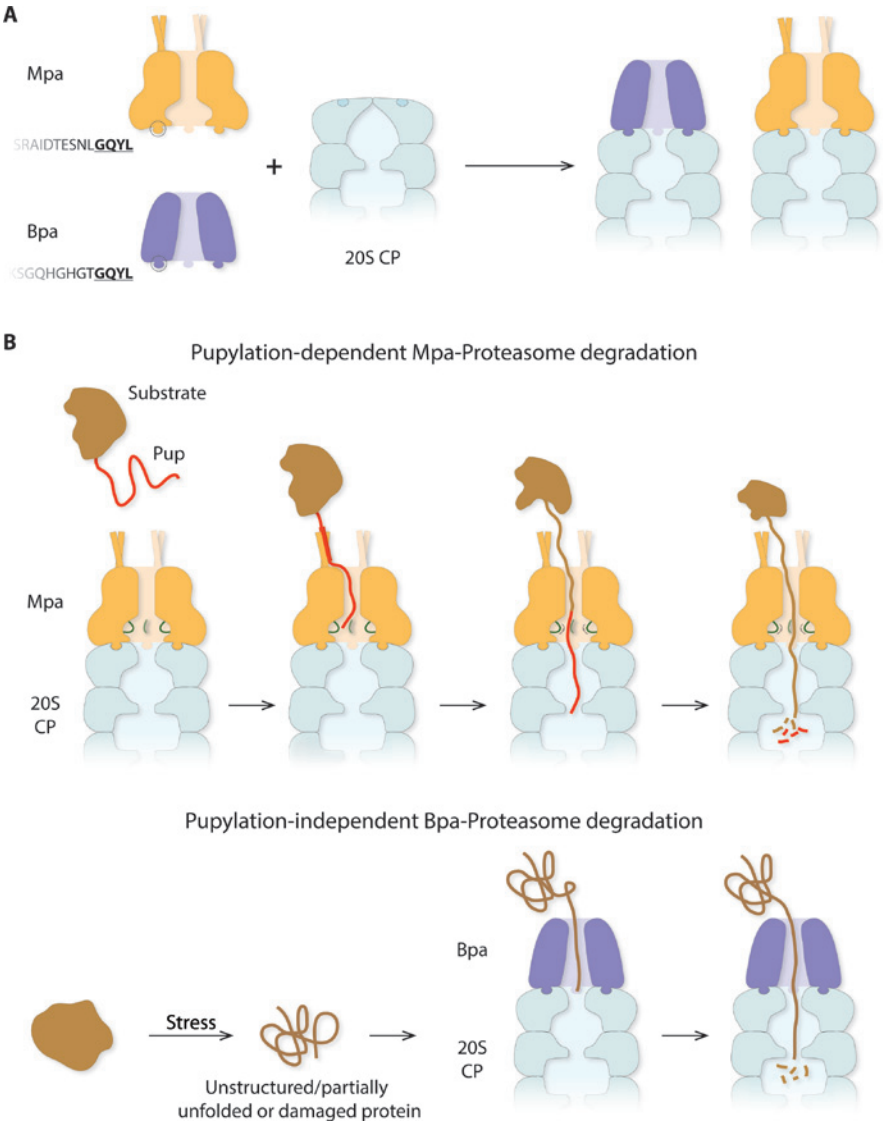


**Figure 2:** Mycobacterial proteasomal activators and their mode of action.
(A) The energy-dependent Mpa and energy-independent Bpa complexes interact with the 20S core particle by docking into binding pockets of the core particle using a shared C-terminal GQYL motif. (B) The ATPase Mpa recognizes pupylated substrates (brown) by binding the covalently attached Pup (red) to its N-terminal coiled-coil domains forming a shared coiled-coil. The N-terminal region of Pup interacts with translocation loops (dark green) inside the Mpa pore. The loops undergo up and down movements in response to ATP hydrolysis leading to translocation through the pore and unfolding. The ATP-independent Bpa possibly only opens the proteasomal gate, allowing damaged, non-native proteins to access the proteasomal core. Once in the core, protein chains are cleaved by the proteolytically active β-subunits.

a hydrophobic residue, together referred to as the HbYX motif (69).

Some activators (e.g. PA26, Cdc48) employ an interaction element in addition to the C-terminal tails to trigger gate opening, a so-called activation loop located on the 20S CP-proximate face of the activator (47, 72). The loops are usually located at a smaller diameter position than the C-terminal tails and help open up the gates by pressing down on certain structural features of the α-N-termini. In case of the eukaryotic 20S CPs, this feature is again the reverse turn following the α-N-terminal strand. Although this reverse turn appears to be absent in bacterial 20S CPs, interactions of proteasome activator loops in a more axial position with the α-ring pore region of the 20S CP might nevertheless contribute to complex stabilization and gate opening here as well.

## The mycobacterial proteasomal ATPase Mpa

Cellular proteins that have reached their native, folded, and fully assembled state do not gain access to the proteolytic inner chamber of the 20S CP without being first processed by ATP-hydrolyzing proteasomal interactors (20). These generally ring-shaped ATP-dependent interactors unfold recruited target proteins under the expense of energy and translocate them through their ring-pore into the proteolytic chamber, where degradation into small peptides takes place (14). In eukaryotes, the 19S regulatory particle (19S RP) associates with the 20S CP to from the 26S proteasome complex (5) (Figure 1). The 19S RP consists of a hexameric ring of ATPase subunits of the AAA type (Rpt1-6) and a number of non-ATPase subunits (Rpn1–13) involved in substrate recruitment and regulation (18).

The mycobacterial 20S CP was shown to interact with an AAA (ATPases associated with various cellular activities) family protein referred to as mycobacterial proteasomal ATPase (Mpa) (73), encoded upstream in the vicinity of the proteasomal α- and β-subunit genes. Mpa shows similarity in domain architecture to eukaryotic ATPase subunits (Rpts) (74). The large C-terminal AAA module forms the main ring structure that stacks to the 20S CP face via a C-terminal GQYL motif (Mpa) (Figure 2A) or the HbYX-motif (Rpts), both featuring the canonical aromatic residue in the penultimate position. This AAA domain is preceded by one (in Rpts) or two (in Mpa) oligonucleotide binding (OB-) domains, each adopting a five-stranded β-barrel fold. The OB-domains sit on top of the AAA ring, forming a single-tiered (Rpts) or two-tiered (Mpa) rigid neck. From the six OB-domains, three coiled-coil structures emerge that are built through the intertwining of N-terminal helical domains of neighboring subunits (Figure 1).

The eukaryotic 19S RP and Mpa also share the mechanistic principle of substrate recognition. Proteasomal substrate proteins in eukaryotes are recruited to the 19S RP by means of a post-translational small protein tag called ubiquitin (Ub) that is attached covalently to lysine side chains of the degradation target (75–78). Likewise, bacterial proteasome degradation targets are modified on lysine side chains with a small protein modifier referred to as Pup (prokaryotic ubiquitin-like protein) for its functional analogy to Ub (10, 11). However, while the eukaryotic and bacterial proteasomes are clearly homologous, the substrate recruitment strategy is a result of convergent evolution. The bacterial Pup and eukaryotic Ub show no sequence or structural homology. Ub adopts a stable globular fold (β-grasp fold), whereas Pup is intrinsically disordered (79–82). As a consequence, the molecular mechanisms of recognition by their respective binding partners are also different. Both proteins are attached via their C-terminal residue to the ε-amino group of a lysine side chain in the target, forming an isopeptide bond (83–85). In case of Ub, the C-terminal residue is a glycine and the bond is formed through the C-terminal carboxylate. The C-terminal residue of Pup that is attached to target lysines is a glutamate, and the bond is formed through the side-chain carboxylate (86). The machinery responsible for attaching the modifier to the target protein has evolved independently for ubiquitination and pupylation. Therefore, the responsible enzymes exhibit no homology. The mechanism of pupylation will be discussed in detail in the subchapter on pupylation.

Both Pup and Ub are recognized by their respective regulatory particles, Mpa or the 19S complex (20, 68, 80, 87). The 19S RP of the eukaryotic 26S proteasome recognizes ubiquitinated substrates via dedicated non-ATPase subunits like Rpn13 and Rpn10 (88, 89). The homohexameric Mpa recognizes Pup directly via the N-terminal coiled-coil domains (Figure 2B) (80). Binding to Mpa induces the formation of a long helix in Pup residues 21–58 (of 64 residues total) and arrangement into a three-helical shared coiled-coil with Pup in antiparallel orientation to the Mpa N-termini (80, 87). The N-terminal region of Pup remains unstructured and plays an important role in the initiation of unfolding by interacting with Mpa translocation loops located deeply in the Mpa pore (90) (Figure 2B). The ATP-driven up and down movement of these loops exerts a pulling force on the pupylated substrate, leading

to directional translocation into the pore and unfolding (Figure 2B). Ub, on the other hand, does not include the translocation initiation module, and the ubiquitinated substrate must feature a loose N- or C-terminal region or extended loop to be successfully pulled into the proteasomal core (91, 92). Ub is not translocated into the 20S CP but is cleaved off and thereby recycled. Pup, on the other hand, serving as a threading handle, is consequently degraded along with the substrate *in vitro* (90). It remains to be seen if additional mechanisms present *in vivo* might act to prevent this co-degradation. It has been hypothesized that the depupylase Dop (discussed later in this review) could play a role in such a scenario *in vivo* (93), but no conclusive evidence is available to date to support this.

## The ATP-independent bacterial proteasome activator Bpa

The modular interaction between a cylinder-shaped proteolytic core and alternative toroidal interaction partners is a common principle observed for both the bacterial Clp proteases and the eukaryotic proteasome (Figure 1) (94). Interestingly, energy-independent proteasome activators (PA complexes) exist in eukaryotes (66), for example the PA28 (11S) (95, 96) and the PA200 complex (97), leading to the formation of ATP-independent PA-20S CP complexes (Figure 1). No such interactors were known for the bacterial proteasome thus far.

Now, a recent report describes the discovery of a novel ring-shaped bacterial proteasome activator termed Bpa that is shown by electron microscopy (EM) to associate coaxially with the 20S CP (12). Top views of Bpa alone present in negative-stain EM micrographs with a ring-shaped topology of roughly 6- or 7-fold symmetry or pseudosymmtery and a central stain-filled opening. While gel filtration analysis suggests a possible hexameric or heptameric assembly state, an independent study also describing the discovery of Bpa (referred to as PafE in that study) suggests a ring with 12 subunits based on size exclusion chromatography with multiangle light-scattering analysis (13). The exact oligomeric state of Bpa and arrangement around the central axis awaits further structural characterization.

Interestingly, Bpa and Mpa share the C-terminal interaction motif (both *Mtb* proteasome interactors end in the sequence GQYL) and are expected to use the same mode of interaction with the 20S CP, inserting the C-termini into binding pockets on the α-rings of the CP, leading to a rearrangement of the α-N-termini and thereby opening of the proteasomal gate (Figure 2A) (12, 13). Bpa can compete with Mpa for binding to the proteasome *in vitro*, efficiently inhibiting the degradation of pupylated substrates by the Mpa-proteasome complex (12). However, *in vivo* competition is unlikely, as quantitative mass spectrometric analysis of the *Mtb* proteome detected protein levels equivalent to roughly 3-fold more 20S CP compared to Mpa ATPase rings under standard culture conditions (98), allowing formation of both Bpa- and Mpa-proteasome complexes simultaneously. The ability of the Bpa-proteasome complex to stimulate the degradation of an unstructured model substrate suggests a role in the removal of non-native, damaged proteins under stress (12). This notion is further supported by the fact that a mutant *Mtb* strain deficient in *bpa* (*pafE*) only exhibits a slight growth defect in liquid standard culture, but under more stringent conditions (solid culture) or in a mouse infection model shows more severe growth phenotypes (13). Interestingly, heat shock repressor HspR accumulates in the *bpa*-deficient strain and serves as a degradation substrate of the Bpa-proteasome complex *in vitro* (13). Stimulating the expression of HspR-repressed chaperones Hsp70 and ClpB by degradation of HspR while at the same time removing damaged, unstructured proteins, would combat proteostasis stress on two fronts.

The discovery of Bpa has provided yet another parallel between the bacterial and the eukaryotic proteasomal degradation systems (12, 13). Both feature two distinct degradation routes to the proteasome; an ATP-dependent route mediated by recruitment via ubiquitination or pupylation, respectively; and an ATP-independent route that is not mediated by a small protein modifier tag (Figure 2B).

## Pupylation – Pup ligase applies the mark of death

Recruitment of substrate proteins for degradation by the Mpa-CP complex relies on their post-translational modification with the small protein Pup (10, 11). The attachment to lysine side chains and the function as a recognition signal for the proteasome complex underscores the similarity to eukaryotic ubiquitination (75). However, pupylation and ubiquitination are biochemically distinct processes that have evolutionarily converged to perform related functional roles. Pupylation is mediated by enzymes unrelated to the E1, E2, and E3 cascade of enzymes involved in ubiquitination (33, 99–101). A single

enzyme, the Pup ligase PafA encoded in the Pup-proteasome gene locus, is responsible for the modification of all pupylation targets, and combines the activating, conjugating, and ligating activities in one active site (101). While wild-type *Mtb* shows a dense ladder of pupylated substrates in polyacrylamide gel electrophoresis followed by blotting with anti-Pup antibody, pupylated substrates are undetectable in a *pafA*-deficient *Mtb* strain (10, 102). Structural analysis (103, 104) showed that PafA is composed of a large N-terminal domain of about 400 residues that is structurally homologous to the carboxylate-amine ligase superfamily. Tightly associated with it is the much smaller C-terminal domain of about 70 residues (Figure 3). This smaller domain is not present in other members of the carboxylate-amine ligase family, but is unique to the pupylation enzymes. The active site is contained mostly in the N-terminal domain. It consists of an antiparallel, concave β-sheet with the ATP-binding site located at the end of the sheet that makes contact with the C-terminal domain, which together with loops emanating from the β-strands effectively closes off the β-sheet cradle from that side. The tri-phosphate chain of ATP extends along the β-strands toward the opposite, accessible end of the sheet. The co-crystal structure of PafA with Pup (103) (Figure 3) shows that the C-terminal glutamate of Pup binds at the accessible end of the sheet with the γ-carboxylate positioned for phosphoryl transfer from ATP (Figures 3 and 4A). Leading away from the β-sheet cradle is a roughly 50 Å long groove lined by conserved residues. The C-terminal half of Pup binds into the groove by adopting a structure of two orthogonal helices connected by a short linker (103) (Figure 3). Exiting from the active site cradle, Pup wraps around to the 'backside' of PafA, presumably to prevent intramolecular attack by a lysine in the flexible N-terminal region of Pup that does not participate in the binding.

In the first step of the PafA-catalyzed reaction, the γ-glutamyl carboxylate at the Pup C-terminus is activated by phosphoryl transfer of the γ-phosphate from ATP, forming the mixed acyl-phosphate anhydride intermediate of Pup (105) (Figure 4A). This step is equivalent to the adenylylation of Ub by the E1-activating enzyme (106). In a second step, the target protein must bind to PafA in such a way that the ε-amino group of the lysine side chain is close to the activated γ-carboxylate of Pup and that the ε-amino group can furthermore be activated by the catalytic base, a strictly conserved aspartate residue in the loop between β-strands 3 and 4 of PafA (104). Nucleophilic attack of the lysine ε-amino group on the γ-carbonyl carbon of the mixed anhydride of Pup then leads to formation of the isopeptide bond (Figure 4A).

Bioinformatic analysis suggests that the Pup ligase PafA originated from an ancestral glutamine synthetase enzyme and has maintained the overall fold of the active site including the concave β-sheet cradle with the GhExE (where 'h' denotes a hydrophobic residue and 'x' any amino acid residue) $Mg^{2+}$-ATP-binding motif in strand 1 (51, 107). This motif forms part of the nucleotide binding site in the active site of all carboxylate-amine superfamily members, to which belong, for example, glutamine synthetases (GS) and their evolutionary descendants, γ-glutamylcysteine ligases (GCL), that are involved in glutathione biosynthesis. The two conserved glutamate residues emanating from strand 1, which is located at the center of the β-sheet, coordinate several $Mg^{2+}$ ions in complex with the ATP phosphate groups. Both GS and GCL members catalyze two-step reactions with phosphorylation of the γ-carboxylate group of glutamate (107). This underlines the fact that the chemistry of the reaction catalyzed by PafA or during the synthesis of glutamine from glutamate is maintained: the γ-carboxylate of a glutamate is activated by phosphoryl transfer and then reacts with an amine forming a C-N bond. The evolution of the Pup ligase likely was also driven by the nature of its substrates. The enzyme had to change in such a way that instead of small molecules (glutamate and ammonia), macromolecules, which are available at much lower concentrations, can serve as substrates, namely Pup with its C-terminal glutamate and the target protein's lysine side chain. As a consequence, the active site in the Pup ligase has to be more accessible to accommodate a broad range of target proteins, some of which occur as components of even larger oligomeric complexes. Furthermore, binding contacts have to extend beyond the reacting groups to ensure high enough affinity for efficient binding. Concurrent with these requirements, Pup ligase PafA features a shallow and broad active site that can easily be approached by large substrates (103). The binding groove of Pup provides an interface area of >1500 $Å^2$, allowing for a dissociation constant for Pup in the low micromolar range (103, 104).

## Depupylation – mycobacteria have built-in rescue

Interestingly, the Pup-proteasome gene locus encodes for another glutamine synthetase homolog that furthermore exhibits high sequence and structural similarity to PafA (33, 101). In fact, it was originally hypothesized that PafA ligase might act as a heterodimer (51). However, despite the fact that this gene (*dop*) also encodes a glutamine

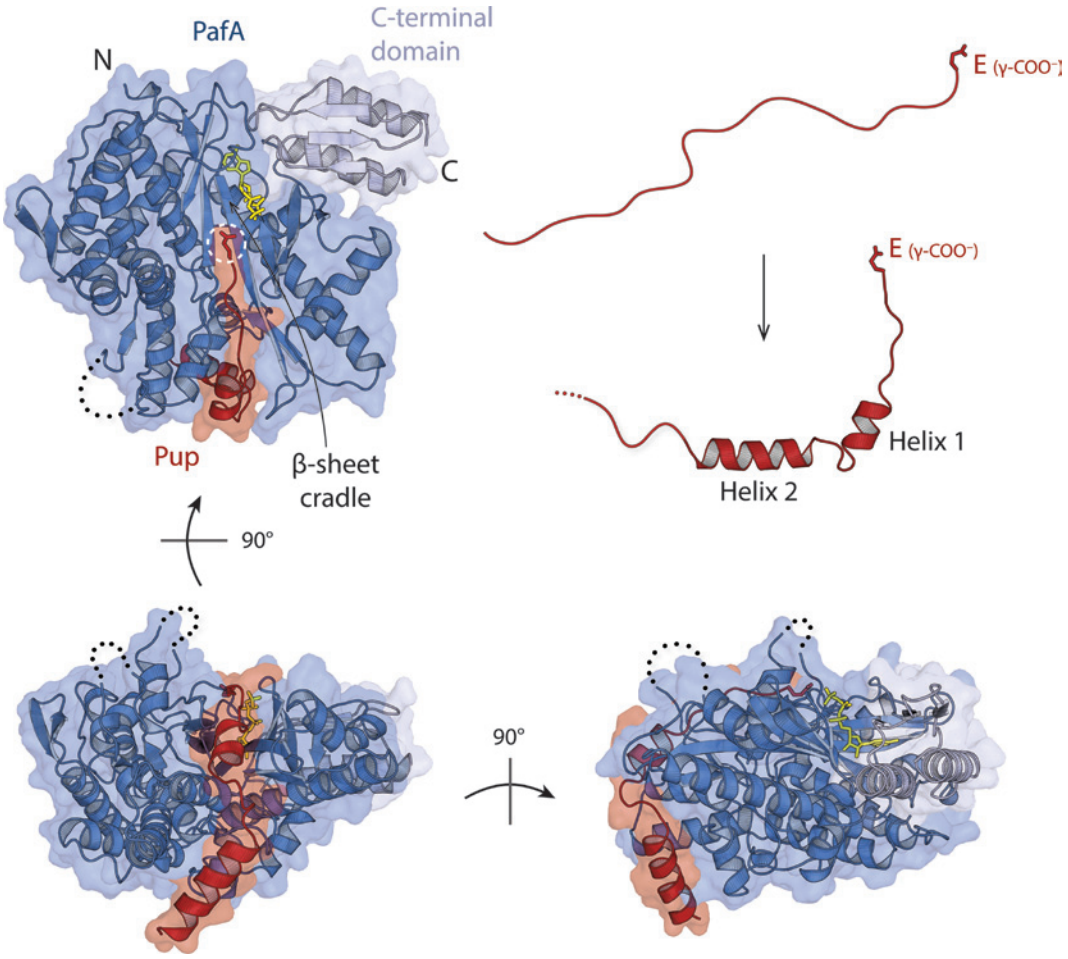


**Figure 3:** Crystal structure of the complex between Pup and the ligase PafA.
PafA is composed of a larger N-terminal domain of about 400 residues (dark blue) and a smaller C-terminal domain of about 70 residues (light blue). The active site comprises a concave β-sheet with ATP (yellow) bound at one end of the sheet and the C-terminal residue of Pup (red) bound at the opposite end. The γ-carboxylate of Pup's C-terminal glutamate (highlighted by a dashed white circle) is positioned for phosphoryl transfer from ATP. The C-terminal half of Pup binds into a long, conserved groove, leading away from the β-sheet cradle and wrapping Pup around to the opposite side of the PafA monomer. Upon binding, the intrinsically disordered Pup folds into two orthogonal helices connected by a short linker.

synthetase homolog and its disruption in *Mtb* or *Msm* abolishes all pupylation activity, it does not catalyze the ligation reaction between Pup and the target proteins (93, 101, 108). Intriguingly, all mycobacteria encode Pup with a C-terminal glutamine instead of glutamate. Yet, analysis of pupylated substrates by mass spectrometry consistently detected a 1-Da difference between the calculated and the theoretical mass of Pup-modified peptides, proving that it is a glutamate and not a glutamine that is present in the conjugate (10). *In vitro* biochemical characterization of the PafA homolog Dop demonstrated that it converts PupQ into the ligation-competent PupE, acting as a deamidase (94). Thus, for pupylation to take place in mycobacteria, the sequential action of both Dop and PafA is required. However, deamidation is not the only activity of Dop. Its ability to cleave the covalent bond between a carbonyl carbon and amide nitrogen extends to the isopeptide bond in pupylated substrates (109, 110). At first glance paradoxical, Dop, a close structural homolog of the Pup ligase PafA, acts as the antagonist of pupylation by removing Pup from pupylated substrates (Figure 4A and B). This depupylase activity of Dop makes the pupylation pathway reversible and echoes the activity of the deubiquitinating enzymes in the ubiquitin-proteasome system. At second glance, the fact that the depupylase has the same fold and evolutionary origin as the Pup ligase PafA is not that surprising, as both need to feature binding surfaces for Pup and the target protein, and both catalyze the nucleophilic attack on a carbonyl carbon with either the lysine side chain (PafA) or water (Dop) acting as the

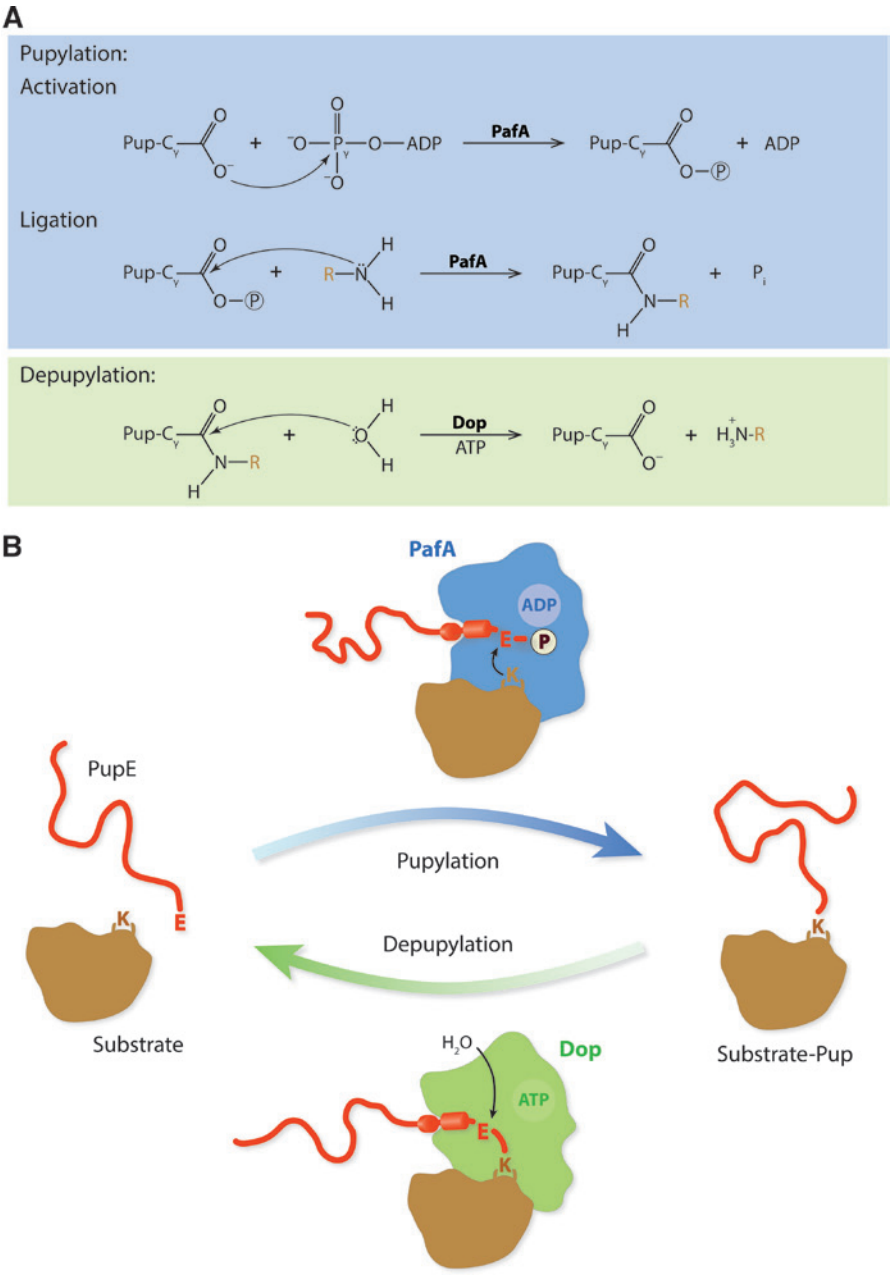


**Figure 4:** The pupylation-depupylation cycle.
(A) The PafA-catalyzed pupylation reaction occurs in two steps. In the first step, the C-terminal γ-glutamyl carboxylate of Pup is activated by phosphoryl transfer from ATP, resulting in formation of a mixed anhydride. In the second step, the isopeptide bond between the substrate's lysine and Pup is formed by a nucleophilic attack of the lysine ε-amino group on the γ-carbonyl carbon of the mixed anhydride of Pup. The isopeptide bond can be cleaved by Dop, a structural homolog of PafA. Dop catalyzes the nucleophilic attack of water on the carbonyl carbon of the isopeptide bond, thereby releasing Pup from the substrate. (B) Together, the two homologous enzymes PafA and Dop regulate the pupylation state of a protein via a pupylation-depupylation cycle.

nucleophile (Figure 4A). In fact, Dop features a very similar binding groove leading into the active site β-sheet cradle, suggesting that it interacts with Pup according to a similar mechanism (104). An aspartate residue that is strictly conserved in both PafA and Dop members could play a role in supporting the nucleophilic attack on the carbonyl carbon in either enzyme (104). Alternatively, during the reaction cycle of Dop, this aspartate might even directly act as the nucleophile, leading to the transient formation of a covalent intermediate with Pup (111).

It is interesting to mention that Pup is not always encoded with a C-terminal glutamine, but that many

members of actinobacteria directly encode a glutamate. In those organisms, Dop is not required for pupylation to take place, as the deamidation step falls away. All mycobacteria, however, encode Pup with a C-terminal glutamine and, as a consequence, pupylated proteins are only generated when Dop is expressed. In other words, the depupylase activity is built into the pupylation cycle by default. Presumably, this allows a tighter control on the amount of pupylation and the fraction of pupylated substrates entering into proteasomal degradation. For example, an *Mtb dop* knockout that is complemented with Pup carrying a C-terminal glutamate cannot maintain wild-type levels of pupylated substrates (93).

## When the Pup-proteasome system becomes a matter of survival

Bacteria have evolved a plethora of mechanisms to resist even highly hostile and adverse environments. Their ability to respond and adapt quickly to changing growth conditions is the key to survival. After being phagocytosed by host macrophages in the lungs upon inhalation, *Mtb*, the causative agent of tuberculosis, is confronted with a massive immune response initiated to eradicate the pathogen (112, 113). However, *Mtb* is well equipped to evade elimination, so that only a fraction of the bacteria are killed. On the other hand, a healthy immune response also prevents proliferation of the surviving *Mtb* and thereby active disease in most cases. The result of this 'stalemate' between bacteria and host is the formation of a so-called latent state. By modulating important functions of the macrophage, the bacteria create a niche for survival that allows them to persist in the host for decades in a slow-growing or dormant state that can, at any time, progress to active disease when host defenses are low (114–117).

Phagocytosis of *Mtb* results in formation of an organelle inside the macrophage referred to as the phagosome, where *Mtb* is targeted by a range of host defense mechanisms including, among others, the production of reactive oxygen species and reactive nitrogen intermediates (RNIs) (118). An important step for *Mtb* toward persistence is to survive the attacks by the various bactericidal effectors the pathogen is exposed to inside the phagosome. Apart from that, *Mtb* employs mechanisms to block the fusion of the phagosome it resides in with lysosomes, the route by which phagocytosed bacteria are ultimately eliminated. The defense response in macrophages is generally triggered through immune-signaling proteins like, for example, interferon-γ (IFN-γ), which is released by activated T-cells in response to the presence of pathogens. Upon activation by IFN-γ, macrophages express an inducible nitric oxide (NO) synthase, resulting in the generation of large amounts of NO and subsequently in the formation of other RNIs. RNIs damage bacteria by modifying their DNA, proteins, and membrane lipids (119–121). Though infections with *Mtb* can be curtailed by RNI formation, not all of the bacteria are finally eliminated, as *Mtb* has the capacity to resist nitrosative stress (119, 122).

The RNI resistance phenotype of *Mtb* was initially linked to the Pup-proteasome system in a screen of transposon mutants that were found to be hypersensitive to nitrosative stress conditions *in vitro* (42). Therefore, it was hypothesized that proteasomal degradation functions in the removal of proteins damaged by nitrosylation. Inactivation of the genes coding for the proteasomal chaperone Mpa and the Pup-ligase PafA sensitized *Mtb* to RNIs. Similar *in vitro* phenotypes were observed for mutants devoid of *dop* or that lack *prcB* and *prcA*, which code for the 20S CP (93, 123, 124). In accordance with these findings, *Mtb* deficient in pupylation or proteasomal degradation displayed attenuation of virulence in a mouse model of infection (42, 93, 102, 123–125). Somewhat surprisingly, complementation of *Mtb* Δ*prcBA* with genes coding for a proteolytically inactive variant of the 20S CP restored near wild-type levels of RNI resistance (123). The mechanism underlying this observation is not known. It was suggested that the 20S CP might play a role in maintaining a certain stoichiometry of proteasomal factors that are bound to the proteasome versus those that occur freely in the cell. A disturbance of this balance might be detrimental for growth and hereby render the cell more susceptible to RNIs. However, despite the supportive function of the inactive proteasome under nitrosative stress, only a proteolytically active 20S CP was found to promote long-term persistence and intracellular survival of *Mtb* (123).

Sensitization of *Mtb* for RNIs in the absence of a functional Pup-proteasome system was recently attributed to cellular accumulation of a specific, newly identified pupylation target and proteasomal substrate, namely Log (Rv1205) (126). A detailed analysis revealed that Log is homologous to a plant enzyme that acts as a phosphoribosyl hydrolase and mediates the final step in cytokinin biosynthesis. Indeed, *Mtb* was also shown to produce cytokinins in a Log-dependent manner; yet, their physiological function in the pathogen remains unknown. Deregulation of Log turnover in an *Mtb* Δ*mpa* strain results in elevated levels of Log and therefore in increased formation of cytokinins. This, in turn, leads to the accumulation of cytokinin-derived metabolites, like *para*-hydroxybenzaldehyde. The excessive buildup of aldehydes in the cell

lastly sensitizes the bacteria for RNIs and kills them in a synergistic manner by a yet unknown mechanism (126).

It has become increasingly clear that conferring resistance to RNIs is only one functional aspect of pupylation and proteasomal degradation in *Mtb*. This is evident from the observation that virulence of *Mtb pafA* and *mpa* mutants is nonetheless attenuated in mice that lack a functional IFN-γ inducible NO synthase (iNOS) (42, 102). Likewise, chemical inhibition of iNOS in mice resulted in severe attenuation of *Mtb* Δ*prcBA* (123). A far-reaching impact of the Pup-proteasome system is also implied by the broad range of proteins that were found to be pupylated in *Mtb* (127). The identified targets comprise numerous players involved in important metabolic pathways, protein turnover, and virulence. Notably, not all proteins that are tagged with Pup are actually degraded (127). This observation leaves room to speculations regarding other, degradation-independent functions of Pup, analogous to ubiquitin in eukaryotes. However, whether pupylation and proteasomal degradation serve any specific regulatory purpose in connection with *Mtb* pathogenicity is not clear. In general, the adjustment and/or maintenance of cellular levels of various proteins appear to be distinct features of the Pup-proteasome system in *Mtb*.

The impairment of Pup-dependent degradation also affects transcription of a large number of genes (128). Analysis of transcriptional changes occurring in *mpa* and *pafA* mutants of *Mtb* led to the discovery of the copper-responsive RicR regulon, which was shown to promote resistance against toxic levels of copper and thus to contribute to *Mtb* virulence (128, 129).

Despite the broad range of pupylation targets and the observed effects on the transcriptional level, deletion of, e.g. *dop*, *mpa* or *prcBA* in *Mtb*, results in no or only a relatively mild growth phenotype under standard *in vitro* conditions in liquid culture (92, 117, 119), indicating that proteasome-dependent turnover of pupylated proteins is largely dispensable in non-stress environments. Still, some differences exist between the different knockouts. For example, while deletion of *Mtb dop* resulted in no-growth phenotype when grown in liquid standard growth medium (92), an *Mtb* mutant devoid of the proteasomal chaperone Mpa displayed an increased doubling time when grown in shaking cultures, and the cells exhibited a small colony phenotype on agar plates (119). Particularly when comparing the growth behavior on solid medium, phenotypes differ more severely. Deletion of the proteasomal α- and β-subunit genes in *Mtb* results in only a mild growth phenotype in shaking liquid cultures; however, the same knockout strain shows strongly reduced growth on agar plates (117). Taken together, these observations indicate that despite their concerted action in the Pup-proteasome system, the components involved in pupylation and especially those related to proteasomal degradation carry out additional functions in other pathways.

As described above, protein turnover by the 20S CP in *Mtb* is not restricted to pupylated substrates that are recognized and processed by Mpa. Discovery of the alternative 20S CP activator Bpa added a new facet to proteasomal degradation and underlines its importance for *Mtb* physiology (12, 13). The impact of this additional branch of proteasome-mediated proteolysis is reflected by increased heat stress sensitivity of an *Mtb bpa* deletion mutant *in vitro*. In addition, the strain displayed significant attenuation of virulence in mice (13).

Non-pathogenic mycobacteria differ from their obligate pathogenic counterparts by their lifestyle, as they are mostly soil-dwelling saprophytic bacteria that are usually not associated with a host (130). Yet, the Pup-proteasome system is also conserved in these species, implying a function beyond virulence and pathogenicity. A recent study in *Msm* provided an interesting explanation for the role of pupylation and proteasome-mediated degradation in non-pathogenic mycobacteria (131). The Pup-proteasome system was shown to be important under starvation conditions, especially when the availability of nitrogen is limited and cannot be assimilated anymore in sufficient amounts by common mechanisms. In order to compensate for the lack of nitrogen sources, the degradation of pupylated proteins is then engaged to recycle amino acids, which eventually allows for the maintenance of basal cellular functions.

Accordingly, an *Msm* strain lacking both *pup* and *prcBA* was severely affected in its ability to survive nitrogen starvation. What remains puzzling, however, is the fact that reintroduction of *pup* in this mutant was sufficient to almost fully complement the phenotype, and complementation with only *prcBA* did not have any improving effect regarding survival under nitrogen limitation. It is conceivable that pupylated proteins are degraded via other degradation machineries that partially compensate for the absence of the proteasome. However, whether the relatively mild phenotype of the *pup*-only complementation can be explained by the minute decrease in levels of pupylated proteins indeed observed in an *Msm* Δ*prcAB* mutant under nitrogen limitation, remains to be further scrutinized.

## Concluding remarks

Research on pupylation and proteasomal degradation is not in its infancy anymore. Exciting contributions by

various groups in recent years provided a broad understanding regarding mechanistic, functional, and structural features of the Pup-proteasome system. Still, important aspects remain elusive, like, for instance, substrate recognition by the Pup-ligase PafA or possible degradation-independent functions of Pup, to name but a few.

In eukaryotes and archaea, the 20S CP constitutes the central component of various proteolytic systems, which differ by their respective proteasomal regulators (94, 132). This modular concept is a rather simple but efficient way to cover a wide range of various physiological functions related to the degradation of a multitude of different substrates. The discovery of the alternative proteasome activator Bpa added a similar level of complexity to proteasomal degradation in prokaryotes (12, 13). Like Mpa, Bpa harbors a C-terminal GQYL motif, which is crucial for proteasomal gate opening (Figure 2A). Yet, it is tempting to speculate whether the spectrum of bacterial proteasome regulators encompasses also members that mediate interaction by different C-terminal motifs or other structural determinants. The eukaryotic equivalent for such a case is the 11S particle, which features a C-terminal motif that is quite different from the HbYX-motif found in the eukaryotic Rpts (94).

There is a clear discrepancy between the apparently specific impact of Pup-dependent degradation on individual pathways like Log-mediated cytokinin synthesis in *Mtb* and the rather unspecific function observed in *Msm* for amino acid recycling under nitrogen starvation conditions, which results in seemingly undirected turnover of a plethora of proteins. Of course, it is conceivable that the Pup-proteasome system has different roles in different species, and that it was adapted to the requirements of pathogenic or non-pathogenic life styles. The recently introduced recycling concept, however, was suggested to also hold true for *Mtb* in context of the prevailing shortage of nutrients inside the macrophage (131). Against this background, the RNI-sensitive phenotype of Pup-proteasome-deficient *Mtb* mutants appears to be an unspecific accidental consequence of failed degradation.

Apart from that, despite the clearly established link of Log-accumulation in *Mtb* deficient in proteasomal degradation and increased RNI sensitivity (126), it is somewhat puzzling that the complementation of *Mtb* Δ*prcBA* with an inactive proteasome does reestablish wild-type RNI resistance (123).

In recent years, the *Mtb* proteasome became the subject of investigations dedicated to the finding of specific inhibitors that could be applied as new anti-tuberculosis drugs (133–135). The growing numbers of cellular functions that can be attributed directly or indirectly to the activity of the mycobacterial proteasome underline why it is such an interesting and attractive target.

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