An adenosine triphosphate-independent proteasome activator contributes to the virulence of Mycobacterium tuberculosis

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**Abstract**

Mycobacterium tuberculosis encodes a proteasome that is highly similar to eukaryotic proteasomes and is required to cause lethal infections in animals. The only pathway known to target proteins for proteasomal degradation in bacteria is pupylation, which is similar to eukaryotic proteasomes and is required to cause lethal outcomes in infected animals. However, evidence suggests that the M. tuberculosis proteasome contributes to pupylation-independent pathways as well. To identify new proteasome cofactors that might contribute to such pathways, we isolated proteins that bound to proteasomes overproduced in M. tuberculosis and found a previously uncharacterized protein, Rv3780, which formed rings and capped M. tuberculosis proteasome core particles. Rv3780 enhanced peptide and protein degradation by proteasomes in an adenosine triphosphate (ATP)-independent manner. We identified putative Rv3780-dependent proteasome substrates and found that Rv3780 promoted robust degradation of the heat shock protein repressor, HspR. Importantly, an M. tuberculosis Rv3780 mutant had a general growth defect, was sensitive to heat stress, and was attenuated for growth in mice. Collectively, these data demonstrate that ATP-independent proteasome activators are not confined to eukaryotes and can contribute to the virulence of one of the world’s most devastating pathogens.

**Significance**

The proteasome of *Mycobacterium tuberculosis* is required to cause lethal infections and is thus a potential drug target. Bacterial proteasomes degrade proteins modified by pupylation, but evidence suggests that the *M. tuberculosis* proteasome possesses additional functions. In this work, we describe a degradation pathway controlled by a previously unidentified proteasomal cofactor, Rv3780. Rv3780 enhanced the ATP-independent proteasomal degradation of peptides and proteins and was required to maintain levels of a unique set of putative proteasome substrates. Importantly, an Rv3780 mutant was attenuated for growth in mice. To our knowledge, these studies show for the first time that an ATP-independent proteasomal-degradation pathway plays a role in the physiology of an important human pathogen.


The authors declare no conflict of interest.

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A heterohexameric ring of ATPases associated with diverse cellular activities (AAA ATPases) that directly bind to the 20S CP (reviewed in ref. 5).

The best-characterized proteasome activator is the eukaryotic 19S regulatory particle (RP). The 19S RP is a ~700-kDa heteromeric complex that binds either or both ends of the 20S CP to form the 26S proteasome, which is responsible for degrading proteins in an adenosine triphosphate (ATP)-dependent manner (6–8). The unfolding activities of the 19S RP come from its base, a heterohexameric ring of ATPases associated with diverse cellular activities (AAA ATPases) that directly bind to the 20S CP (reviewed in ref. 2). Ubiquitylation, which is the posttranslational modification of doomed proteins with the small protein ubiquitin, is the major signal that targets proteins for degradation by the 26S proteasome. Because ubiquitylation is an essential regulator of virtually every cellular pathway in eukaryotes, a considerable amount of work has focused on the biochemistry and biology of the 26S proteasome, leading to the identification of many substrates and the reconstitution of robust in vitro degradation (reviewed in refs. 2 and 9).

In addition to the 19S RP, there are several cofactors that activate proteasome activity without ATP. These include Bml10/P200 (10, 11), archaeal PbaB (12), and the 11S activators. The 11S activators are a family of small proteins that form heptameric rings and enhance peptide degradation upon binding to 20S CPs. Simple eukaryotes encode a single 11S activator, PA26 (13), whereas higher eukaryotes encode three 11S activator isoforms: PA28-α, -β, and -γ (14–16). An abundance of work has detailed the biochemical characteristics of the 11S activators, which has been crucial in understanding the structural basis of proteasomal gate opening (17–21). However, their precise biological roles remain controversial. A multitude of studies have demonstrated that PA28-αβ alter the peptide products of proteasomal degradation to influence major histocompatibility complex class I antigen presentation (22, 23). However, PA28-αβ-deficient mice are not remarkably defective in antigen presentation or immunity (24). Similarly, only a handful of PA28-γ–dependent protein substrates have been described, and few studies have demonstrated a significant physiologic role for PA28-γ in mammals (25–29). As a result, our understanding of the biological relevance of eukaryotic ATP-independent activators has lagged behind that of the 19S RP.

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Although the bulk of research into proteasomes has been in eukaryotes, they are also present in bacteria of the orders Nitrosospirales and Actinomycetales, which includes the human pathogen *Mycobacterium tuberculosis*. *M. tuberculosis* infects one-third of the world’s population, killing >1.5 million people each year (www.who.int/mediacentre/factsheets/fs104/en/). There is an urgent need for the identification of novel antimicrobial drug targets because resistance to currently available drugs is on the rise. One potential target is the prokaryotic ubiquitin-like protein (Pup)-proteasome system (PPS), a pathway used by *M. tuberculosis* and other proteasome-bearing bacteria to target proteins for degradation. The PPS centers on the small protein modifier Pup (30). The C terminus of Pup is covalently attached to a lysine of a target protein by the Pup ligase proteasome accessory factor A (PafA) (31) and is then bound by the homohexameric AAA ATPase mycobacterial proteasome ATPase (Mpa), which resembles the Rpt ATPases found in the bases of eukaryotic 19S RP (32–35). Mpa unfolds pupylated proteins to be degraded by the *M. tuberculosis* 20S CP, which is structurally similar to the eukaryotic 20S CP but is composed of just two unique subunits, PrcA and PrcB (36, 37). Because the PPS is chemically distinct from the eukaryotic ubiquitin–proteasome pathway, and because proteasomal degradation of pupylated proteins is required for full *M. tuberculosis* virulence (32, 38–40), the PPS represents an attractive therapeutic target for tuberculosis.

To date, pupylation is the only pathway known to target proteins for proteasomal degradation in bacteria. However, several studies suggest that the 20S CP may have pupylation-independent functions, because chemical inhibition of the 20S CP or genetic deletion of the 20S CP genes, but not mpa or genes involved in pupylation, produces a severe growth defect in liquid culture and deletion of the 20S CP genes, but not genes involved in proteasome pathway, and because, because resistance to currently available drugs is on the rise, one potential target is the prokaryotic ubiquitin-like protein (Pup)-proteasome system (PPS), a pathway used by *M. tuberculosis* and other proteasome-bearing bacteria to target proteins for degradation. The PPS centers on the small protein modifier Pup (30). The C terminus of Pup is covalently attached to a lysine of a target protein by the Pup ligase proteasome accessory factor A (PafA) (31) and is then bound by the homohexameric AAA ATPase mycobacterial proteasome ATPase (Mpa), which resembles the Rpt ATPases found in the bases of eukaryotic 19S RP (32–35). Mpa unfolds pupylated proteins to be degraded by the *M. tuberculosis* 20S CP, which is structurally similar to the eukaryotic 20S CP but is composed of just two unique subunits, PrcA and PrcB (36, 37). Because the PPS is chemically distinct from the eukaryotic ubiquitin–proteasome pathway, and because proteasomal degradation of pupylated proteins is required for full *M. tuberculosis* virulence (32, 38–40), the PPS represents an attractive therapeutic target for tuberculosis.

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In an effort to identify proteasomal cofactors in *M. tuberculosis* that might contribute to new degradation pathways, we used a catalytically inactive proteasome trap to stabilize normally transient interactions of the *M. tuberculosis* 20S CP. The proteasome trap was overproduced with Rv3780, a previously uncharacterized protein. Rv3780 formed oligomeric rings and bound to *M. tuberculosis* 20S CPs. This interaction required a conserved C-terminal motif in Rv3780, reminiscent of what has been described for eukaryotic proteasome activators. We show that Rv3780 promoted the robust ATP-independent proteasomal degradation of short peptides, an unfolded protein, and a native *M. tuberculosis* protein in vitro. Importantly, Rv3780 contributed to *M. tuberculosis* resistance to heat stress and virulence in a mouse infection model.

**Results**

**Identification of a Proteasome Binding Protein in M. tuberculosis.** Previous studies using the *M. tuberculosis* 20S CP have indicated that interactions with its cofactors are relatively weak (35). We hypothesized that a catalytically inactive 20S CP (“proteasome trap,” 20S_T1A CP) may stabilize these interactions by capturing the proteasome in an intermediate degradation state, still bound to cofactors and substrates, as has been shown for a catalytically inactive *Escherichia coli* ClpP trap (42). To identify new putative substrates and cofactors, we overproduced either active (WT) or catalytically inactive (Trap) tandem affinity purification (TAP)-tagged proteasome complexes in *M. tuberculosis*. We visualized affinity-purified proteins by silver staining, which showed that more proteins copurified with the Trap than with WT proteasomes (Fig. 1A). We also analyzed the copurifying proteins by tandem mass tag labeling and mass spectrometry (TMT-MS), which allows for the quantitative comparison of relative protein levels between samples (43). The protein that most abundantly copurified with the proteasome trap was the proteasomal ATPase Mpa (Fig. 1B, Left, and Table S1), which was significant because no previous study has demonstrated a robust in vitro interaction between Mpa and the 20S CP. Importantly, this result supported the hypothesis that catalytically inactive 20S CPs could capture cofactors that interact weakly with WT 20S CPs.

When we purified proteins from WT *M. tuberculosis*, we found 78 proteins enriched more than fivefold with the proteasome trap than with the WT 20S CPs (Fig. 1B, Left, and Table S1). When we purified proteins from an mpa mutant, 27 proteins were enriched more than fivefold with the Trap over the WT 20S CPs (Fig. 1B, Right, and Table S2), 19 of which were enriched in both strains.

Fig. 1. Rv3780 interacts with the *M. tuberculosis* proteasome. (A) A proteasome trap identifies interacting proteins. A TAP-tagged active (WT) or catalytically inactive (Trap) 20S CP was overproduced in *M. tuberculosis* and isolated from lysates by Ni-NTA and anti-FLAG affinity purification. Proteins were separated by 12% (w/vol) SDS/PAGE and visualized by silver staining. Molecular mass markers in kDa. (B) Proteins that were enriched in pull-downs using the proteasome trap. The eluates generated in A were modified by TMT labeling and their relative levels were quantified by MS. The log2 ratio of relative abundance in the Trap pull-down vs. the WT pull-down from WT *M. tuberculosis* (Left) or an mpa mutant (Right) is plotted against the log10 p-value as determined by t test using three biological replicates. Data points representing the top 10 copurified proteins of highest abundance with the Trap are enlarged. Data points representing Mpa and Rv3780 are in red. (C) Rv3780 requires a conserved penultimate Tyr to copurify with 20S CPs. His6-tagged WT or Y173A Rv3780 was overproduced in *M. tuberculosis* and isolated from lysates by Ni-NTA affinity purification. Proteins were visualized by immunoblotting for PrcB or Rv3780 using antibodies raised against PrcB–His6 or Rv3780–His6, respectively. PD, pull-down.
Importantly, many established pupylated substrates (44, 45) were enriched with the Trap in an Mpa-dependent manner, further suggesting that these represented stalled degradation complexes.

Of the many proteasome-associated proteins that we identified, we chose to further characterize Rv3780 for three reasons. First, Rv3780 was one of the most abundant proteins that copurified with the Trap (Tables S1 and S2). Second, STRING-DB analysis (46) demonstrated that it co-occurs evolutionarily with the bacterial proteasome, suggesting that the two are functionally related. Third, the last four amino acids of Rv3780 are identical to those of Mpa (Fig. 1C).

Previously, we determined that deletion or mutagenesis of the penultimate tyrosine (Tyr; Y) of Mpa abolishes substrate degradation (30, 45). Therefore, we tested if mutagenesis of the equivalent Tyr to alanine (Ala; A) in Rv3780 (Y173A) would abrogate binding to 20S CPs. Nickel-nitritoltriacetic acid (Ni-NTA) resin purification of amino (N)-terminally hexahistidine (His6)-tagged Rv3780 overproduced in M. tuberculosis demonstrated that 20S CPs copurified with WT Rv3780, but not with the Rv3780Y173A mutant (Fig. 1C). Rv3780 is therefore a previously unidentified proteasome-interacting protein that uses a specific C-terminal sequence to bind to 20S CPs. Importantly, this experiment also confirmed that Rv3780 interacts with WT, endogenous 20S CPs.

M. tuberculosis Rv3780 is annotated as a 178-amino acid protein with a predicted molecular mass of 19.5 kDa (Fig. S1). We noted the presence of two or three Rv3780 species in our immunoblots when we used polyclonal antibodies to detect Rv3780 protein from the purifications (Fig. 1C). The pafE gene has three in-frame, closely spaced potential start codons with putative ribosome-binding sites that could result in the synthesis of slightly different proteins (Fig. S2, Left). The use of “start codon 1” would produce a protein with an N-terminal extension of arginine-lysine-arginine (Arg-Lys-Arg; RKR), which might be unstable based on the N-end rule pathway (47). Moreover, some mycobacterial species encode an Rv3780 homolog that only contains start codons 2 and 3 (Fig. S2, Left). Because start codon 2 is an ATG, which is generally a better start codon than GTG, we used this allele for our experiments; all codon numbering in this work refers to this allele, which is 174 amino acids long and has a predicted molecular mass of 18.9 kDa. For in vitro experiments, we produced PafE translated and expressed as a recombinant protein from E. coli. White circles mark three Rv3780 rings. (B) Shown are 24 representative reference-free 2D class averages of His6-Rv3780 ring structure. (C) Recombinant purified His6-Rv3780 was separated by SEC and analyzed by MALS. (D) Six representative reference-free 2D class averages of presumptive endogenous Rv3780 rings capping one end of TAP-tagged 20ST1A CP complexes reconstituted from recombinant purified His6-Rv3780 and 20ST1A CP–His6 proteins purified from E. coli. (F) Select reference-free class averages of the singly capped (Upper) and doubly capped (Lower) Rv3780–20SOG CP complex reconstituted in vitro by incubating M. tuberculosis His6-tagged proteins purified from E. coli in the presence of bortezomib. D–F are on the same scale.

Rv3780 Forms Oligomeric Rings and Caps M. tuberculosis 20S CPs. Rv3780 does not have significant similarity to any protein of known function based on a BLAST homology search (48). To gain a better idea of a potential function, we purified Rv3780 protein from E. coli with an N-terminal His6 tag and assessed its structure using negative stain electron microscopy (EM) and computational image classification. Rv3780 formed homo-oligomeric rings as seen in a raw EM micrograph (Fig. 2A), as well as in class averages after reference-free 2D classification (Fig. 2B).

Some averaged rings were not perfectly circular but were instead elongated, suggesting that Rv3780 rings might be somewhat flexible or slightly tilted on the carbon film substrate. The approximate diameter of the Rv3780 rings as measured from the class averages was 112.0 ± 2.3 Å. In comparison, the measured average width of the M. tuberculosis open gate proteasome CP (20SOG) under the same imaging condition was 144.2 ± 4.0 Å. SEC with multiangle light scattering (SEC-MALS) analysis indicated that Rv3780 oligomers have a molecular mass of ~247 kDa, suggesting that an Rv3780 ring consists of 12 subunits (Fig. 2C).

We next used EM to assess the 20S CPs–Rv3780 complex. We used two approaches: (i) We purified TAP-tagged 20ST1A CPs from an M. tuberculosis mpa mutant; and (ii) we combined purified recombinant Rv3780 with either 20S1T1A or 20SOG CPs that lack the seven N-terminal gating residues of PrcA (37) in the presence of the proteasome inhibitor bortezomib. Our rationale for using the open-gate CPs was based on previous data demonstrating that use of the 20SOG CP helped stabilize an interaction with Mpa long enough to visualize Mpa–CP complexes by EM (35). The purifications from an M. tuberculosis mpa mutant revealed ring structures capping the 20S CPs (Fig. 2D), which strongly resembled Rv3780 particle expressed and purified as a recombinant protein from E. coli. White circles mark three Rv3780 rings. (B) Shown are 24 representative reference-free 2D class averages of His6-Rv3780 ring structure. (C) Recombinant purified His6-Rv3780 was separated by SEC and analyzed by MALS. (D) Six representative reference-free 2D class averages of presumptive endogenous Rv3780 rings capping one end of TAP-tagged 20ST1A CP complexes reconstituted from recombinant purified His6-Rv3780 and 20ST1A CP–His6 proteins purified from E. coli. (F) Select reference-free class averages of the singly capped (Upper) and doubly capped (Lower) Rv3780–20SOG CP complex reconstituted in vitro by incubating M. tuberculosis His6-tagged proteins purified from E. coli in the presence of bortezomib. D–F are on the same scale.

Rv3780 Is an ATP-Independent Proteasome Activator. Based on our data, Rv3780 is reminiscent of eukaryotic 11S activators, which
are known to form oligomeric rings, engage 20S CPs by using C-terminal residues, and open proteasomal gates to enhance peptidase activity (reviewed in ref. 5). To test if Rv3780 is capable of activating proteasomal peptidase activity, we monitored the degradation of fluorogenic peptide substrates by *M. tuberculosis* 20S CPs in the presence of increasing amounts of purified recombinant Rv3780.

*M. tuberculosis* 20S CPs are capable of hydrolyzing the tetrapeptide substrate succinyl-Leu-Leu-Val-Tyr–amino-4-methylcoumarin (Suc-LLVY-AMC) in the absence of other cofactors (37). Addition of Rv3780 enhanced degradation of Suc-LLVY-AMC a maximum of approximately threefold (Fig. 3). However, Rv3780 produced a much more pronounced stimulation of proteasome activity on a larger nonapeptide substrate, LF-2. In the absence of Rv3780, LF-2 was hydrolyzed extremely slowly (Fig. 3B, Inset). In contrast, Rv3780 enhanced degradation of LF-2 at least 150-fold compared with 20S CPs alone. As a control, we used an Rv3780 allele that could not interact with 20S CPs (Fig. 1), and found that Rv3780Y173A could not stimulate degradation of LF-2 (Fig. 3B). These data suggest that the *M. tuberculosis* 20S CP allows nearly free passage of smaller peptides, but requires activation by a cofactor to accommodate larger substrates, as has been described for archaeal 20S CPs and *E. coli* ClpXP (49, 50). The addition of ATP did not improve activation, confirming that PafE acts in an ATP-independent manner (Fig. S3).

We next wondered if Rv3780 could promote the degradation of larger unfolded proteins as has been observed for other bacterial barrel-shaped proteases (reviewed in ref. 51). To this end, we assessed the ability of either the 20S CP alone or in complex with Rv3780 to degrade the model unfolded protein β-casein. The addition of Rv3780 greatly enhanced proteolysis of β-casein by *M. tuberculosis* 20S CPs (Fig. 3C). Based on these results, we now refer to Rv3780 as PafE for proteasome accessory factor E.

### Identification of a Conserved C-Terminal Motif Required for the Activity of Mycobacterial Proteasome Cofactors.

In eukaryotes and archaea, many proteasome cofactors are characterized by the presence of a C-terminal "HbYX" motif (hydrophobic–Tyr–any amino acid), which is required to bind the 20S CP and stimulate gate opening (20, 52, 53). As noted earlier, Mpa and PafE each contain a C-terminal GQYL sequence (glycine-glutamine-Tyr-leucine; Gly-Gln-Tyr-Leu), which has a penultimate Tyr but is otherwise distinct from the HbYX motif. The C-terminal GQYL is absolutely conserved among PafE and Mpa homologs in proteasome-bearing bacteria, but is missing from Mpa homologs in *Corynebacteria*, which do not encode a 20S CP (Fig. 4B). These observations suggested that the GQYL sequence is important for proteasome-related functions. Thus, to determine which residues of the GQYL motif are functionally important, we performed mutational analysis of PafE and Mpa.

In vitro degradation assays with recombinant PafE indicated that single Ala mutations of Gly-171, Tyr-173, or Leu-174 disrupted the ability of PafE to promote Suc-LLVY-AMC degradation, whereas Ala substitutions at Thr-170 or Gin-172 had minimal to no effect (Fig. 4A). To determine if these residues were also important in live *M. tuberculosis*, we attempted to complement an *mpa*-null mutant with integrating plasmids encoding Mpa GQYL mutant alleles and assessed their ability to degrade the established Pup–proteasome substrate FabD (45). Consistent with our in vitro data using PafE and with our previous work using Mpa mutants (45), we found that Gly-605, Tyr-607, and Leu-608, but not Gin-606, were required for FabD degradation (Fig. 4B). Based on the absolute conservation of the GQYL residues in PafE and Mpa homologs and our mutagenesis analysis, these data strongly suggest that the GQYL motif is required for the activity of proteasomal cofactors in bacteria. Interestingly, in contrast to disruption of the equivalent residue in PafE, replacement of Leu-604 just preceding GQYL in Mpa prevented FabD degradation (Fig. 4B). Consistent with this observation, the residues preceding GQYL are conserved in Mpa homologs but diverge in PafE homologs, suggesting that this region is important for the function of Mpa, but not PafE.

The C termini of eukaryotic and archaeal proteasomal ATPases, which contain HbYX motifs, are sufficient to elicit proteasome activation, whereas the C termini of the 11S activators are not (17, 20). To test if the C terminus of PafE was capable of activation on its own, we tested activity of the 20S CP in the presence of peptides corresponding to the C-terminal four (GQYL), six (GTGQYL), and eight (GHGTGQYL) residues of PafE. All three peptides activated the 20S CP, with the longer peptides acting as slightly stronger activators, indicating that binding of just the GQYL motif is sufficient for activation (Fig. 4C). Because Mpa and PafE each use this motif to bind to the 20S CP, we next tested if Mpa was capable of activating the 20S CP itself. However, we were unable to detect an increase in proteasomal peptidase activity with the addition of Mpa (Fig. 4D). Moreover, the addition of an 8.5-fold molar excess of Mpa to PafE was unable to outcompete PafE activation. Although it is possible that Mpa is still capable of activating proteasomal degradation, these data suggest that the affinity of the 20S CP is likely higher for PafE than it is for Mpa.

### PafE Contributes to *M. tuberculosis* Growth and Virulence.

We next determined if PafE plays a significant role in *M. tuberculosis* physiology. Our initial efforts to generate a *pafE* deletion strain were unsuccessful, likely due to polar effects on several downstream genes that are predicted to be essential for growth (54, 55).
suggesting that WT PafE levels are more critical during infection than under standard laboratory conditions. Collectively, our data show that PafE contributes to the normal growth and virulence of M. tuberculosis.

PafE Promotes In Vitro Degradation of a Heat Shock Repressor and Is Required for Resistance to Heat Stress. The only known mechanism for targeting proteins to a bacterial proteasome for degradation is pyrophosphatase (30). We therefore tested if PafE was required for the

To circumvent this problem, we generated a merodiploid “parental” strain by integrating the genes downstream of pafE (Rv3781–3783) under control of the operon’s presumed native promoter into the chromosomal L5 attB site (Fig. 5A). We then deleted and disrupted pafE from this parental strain and confirmed disappearance of PafE from cell lysates by immunoblotting (Fig. 5A, Lower). For complementation, we introduced a single copy of pafE including all possible start codons into the chromosome at the Tweety attB site (56) and showed restoration of PafE in lysates.

The pafE mutant demonstrated a conspicuous growth defect on solid medium (Fig. 5B) and a slight growth defect in broth culture (Fig. 5C), phenotypes that were complemented by reintroducing pafE into the M. tuberculosis chromosome. Importantly, introduction of the pafEY173A allele did not complement the growth defect (Fig. 5B and C). Consistent with PafE playing a role in M. tuberculosis physiology, the pafE mutant was attenuated for growth in mice (Fig. 5D and E). Although complementation of the in vivo growth defect was incomplete, we noted that PafE was not fully restored to WT levels (Fig. 5A, Lower),
degradation of known Pup–proteasome substrates (44, 45). To our initial surprise, immunoblot analysis of an *M. tuberculosis* pafE mutant showed WT levels of all pupylation substrates tested, in contrast to an *mmpA* mutant where these substrates accumulated (Fig. 6A). Although we cannot rule out the possibility that PafE contributes to the degradation of pupylated substrates under certain conditions or a different subset of pupylated proteins, these data indicate that it is not an essential part of the PPS and may instead contribute to a distinct proteasomal-degradation pathway. Consistent with this hypothesis, genetic co-occurrence analysis using STRING-DB demonstrated that pafE is present in bacteria that encode a 20S CP, but absent from *Corynebacteria*, which lack 20S CP genes, yet encode Mpa and the pupylation system (Fig. S4A). These observations led us to investigate the possibility that PafE is involved in pupylation-independent proteasomal protein degradation.

To search for PafE-dependent proteasome substrates, we carried out two proteomic studies with *M. tuberculosis* using TMT-MS analysis. First, we compared the proteomes of parental vs. *pafE* strains to identify proteins that accumulate in the absence of PafE. Second, we treated a pupylation-deficient *pafA* strain with the proteasome inhibitor epoxomicin to identify proteins whose levels are maintained by the proteasome in a pupylation-independent manner. Proteins that increased in abundance were considered putative PafE-dependent proteasome substrates (Fig. 6B, Fig. S4B, and Tables S3 and S4). Known pupylated proteins did not increase in abundance in either experiment, indicating that the proteins we identified represented a distinct class of putative proteasome substrates.

The protein that significantly increased the most in abundance in both experiments was heat shock protein repressor (HspR). We raised rabbit polyclonal antibodies to HspR–His6, and examined endogenous HspR in *M. tuberculosis* cell lysates, finding that HspR levels increased in the *pafE* and *prcBA* mutants compared with the parental strain, but not in an *mmpA* mutant (Fig. 6C). HspR denatures when heated briefly at 42 °C, suggesting that it is intrinsically unstable (57, 58). Because we previously found that PafE promoted the degradation of the unfolded protein β-casein, we performed an in vitro degradation assay to determine if PafE could also directly stimulate the proteolysis of HspR. Remarkably, PafE promoted the robust degradation of HspR, which was undetectable after 3 h. In contrast, HspR was stable for >24 h when we used PafEY173A (Fig. 6D). The degradation rate of HspR increased at higher temperatures, further supporting the hypothesis that denaturation of HspRpromotes its PafE-mediated degradation (Fig. S4C).

Because eukaryotic ATP-independent proteasome activators open the gates of 20S CPs, we wondered if gate opening might be the mechanism of PafE-dependent activation of HspR degradation. Specifically, does PafE simply open the 20S CP gate, or does PafE actively recruit HspR to the 20S CP? We performed a degradation assay using 20SOG CPs that lack gating residues and therefore should not require PafE for gate opening. We found that the 20SOG CPs degraded HspR in a similar time scale as the PafE-activated WT 20S CPs. The addition of PafE did not increase the rate of proteolysis, suggesting that gate opening is the sole mechanism by which it enhanced HspR degradation (Fig. S4D).

We next tested if gate opening was sufficient to degrade a known pupylated proteasome substrate. We found that 20SOG CPs were unable to degrade the Mpa-dependent proteasome substrate Pup–PanB (3-methyl-2-oxobutanoate hydroxymethyltransferase) with or without PafE present (Fig. S4E). Together, our data suggest that PafE opens the proteasomal gate to enhance degradation of a specific subset of proteins, most likely those that are partially or completely unfolded.

Degradation of misfolded proteins is an important component of the cellular defense against proteotoxic stress. Under conditions such as heat shock that lead to the accumulation of misfolded and aggregated proteins, bacteria rely on chaperones to disaggregate and refold some proteins, and proteases to degrade others (reviewed in refs. 59 and 60). DnaK and ClpB are chaperones that are critical for the *M. tuberculosis* heat shock response, and their expression is repressed by the PafE-dependent substrate HspR (61, 62). We therefore hypothesized that the accumulation of HspR would render a *pafE* mutant hypersensitive to heat. We found that PafE-deficient *M. tuberculosis* had somewhat reduced expression of HspR target genes (Fig. 6E), suggesting that its heat shock response might be compromised. Indeed, the *pafE* mutant was more sensitive to killing than the parental strain when incubated at 45 °C (Fig. 6F). As with the animal experiments, complementation was partial, but survival of the mutant was significantly improved.
Discussion

In this study, we describe the discovery and characterization of PaeE, a functional homolog of the eukaryotic 11S proteasome activators. Although it shares no sequence homology with its eukaryotic counterparts, PaeE nonetheless forms a ringed oligomer, binds 20S CPs by using its C terminus, and enhances proteasomal peptidase activity. Our proteomic studies identified a unique set of putative PaeE-dependent substrates, which was completely distinct from known puylation substrates. The failure to degrade one or more of these substrates appears to have significant biological consequences because an M. tuberculosis paeE mutant has a general growth defect and is somewhat attenuated in mice. Importantly, we found that PaeE stimulates the ATP-independent degradation of HspR, representing, to our knowledge, the first demonstration of robust in vitro degradation of an endogenous substrate by a bacterial proteasome, and that the accumulation of HspR in a paeE mutant leads to repression of the heat shock response genes and increased sensitivity to heat stress.

The discovery that PaeE promotes proteasomal degradation of both HspR and a model unfolded protein (β-casein) suggests two potential mechanisms for the heat shock sensitivity of a paeE mutant. First, it is possible that the PaeE–proteasome system combats proteotoxic stress by degrading misfolded proteins that would otherwise accumulate during heat shock or other stresses that induce misfolding. M. tuberculosis does not encode a homolog of Lon protease, which in many bacteria is induced by heat shock and is critical for the degradation of misfolded proteins (63–67). Thus, M. tuberculosis may rely on the PaeE–proteasome system to dispose of small, misfolded proteins that would otherwise be degraded by Lon. Second, the accumulation of HspR in a paeE mutant might dampen induction of the heat shock response. HspR represses expression of the dnaK operon, of which hspR is a member (62), and clpB (61), both of which encode chaperones that are induced by and required to resist killing by heat shock, and in a paeE mutant, expression of both clpB and hspR itself was significantly reduced. Thus, one model for the heat shock sensitivity of a paeE mutant is that upon encountering an elevated temperature, an M. tuberculosis paeE mutant accumulates misfolded proteins and is unable to quickly degrade HspR, resulting in the bacteria being unable to fully induce expression of its heat shock genes. As a result, the bacteria would be unable to cope with proteotoxic stress, leading to cell death.

The identification of a new proteasomal cofactor in M. tuberculosis has also revealed that actinobacterial activators possess a unique C-terminal 20S CP-binding motif. Both Mpa and PaeE have a C-terminal GQYL motif. We have demonstrated that for both proteins, the Gly, Tyr, and Leu are all required for function, whereas the Gln is dispensable. This finding was a surprise, because the C-terminal HbYX motif characteristic of eukaryotic and archaeal cofactors relies critically on a residue just before the penultimate Tyr (18, 20, 53, 68). Thus, bacteria and eukaryotes appear to have evolved slightly different mechanisms to achieve proteasome activation. Interestingly, eukaryotic 11S activators do not have HbYX motifs, despite binding to 20S CPs with their C termini. Whereas the HbYX motif is sufficient for ATP-dependent activators to both bind and reposition the proteasomal gate, 11S activators use their C termini to bind but require a separate activation loop to open the proteasomal gate. The finding that a GQYL peptide alone is sufficient to activate the M. tuberculosis 20S CP (Fig. 4C) demonstrated that bacterial proteasome activators more closely resemble proteasomal ATPases in this regard, with binding to the C terminus sufficient to induce activation. Gate opening in eukaryotes relies on the repositioning of a reverse turn that positions the N-terminal gating residues. Proteasomal ATPases reposition the reverse turn by using interactions with the HbYX alone, whereas the 11S activators use an activation loop (17, 19). However, the reverse turn is absent from the M. tuberculosis 20S CP (69); the mechanism of activation for bacteria is therefore likely to be novel from that seen in other organisms.

Because the 20S CP is composed of heptameric rings, it is intriguing to consider how it might interface with an activator of 12-fold symmetry. The interaction between PaeE and the 20S CP appears to be considerably weaker than that of eukaryotic activator/20S CP complexes; thus, it is possible that the presence of additional binding sites might be required to improve binding avidity to enhance activation. Alternatively, it is possible that “free” GQYL motifs that are not actively involved in binding the 20S CP might be involved in gate-opening interactions distinct from binding interactions, because GQYL alone appears to be sufficient for gate-opening. Elucidation of the cocrystal structure of PaeE–20S CP complexes will be important for establishing precisely how the GQYL motif opens the proteasome gate.

Although a handful of 11S activator-dependent proteasome substrates have been described in eukaryotes, attempts to reconstitute their degradation in vitro have not been robust (26, 28, 29). As a result, we lack a clear understanding of the mechanisms governing ATP-independent proteasomal protein degradation. By contrast, we have now reconstituted robust degradation of a native M. tuberculosis substrate using only three proteins: PrcB, PrcA, and PaeE. Further analysis of this simplified system will be extremely useful for mechanistic studies into ATP-independent proteasomal protein degradation in bacteria, which should, in turn, help to guide research on eukaryotic systems.

The identification of the PaeE proteasome pathway indicates that M. tuberculosis has at least two proteasome systems with distinct biological roles (Fig. 7). Importantly, our data have begun to explain the phenotypic differences observed between puylation/Mpa-deficient and 20S CP-deficient M. tuberculosis strains. Further studies will elucidate details regarding the mechanism and the physiologic roles of ATP-independent proteasomal protein degradation in M. tuberculosis, which may identify new therapeutic targets as well as contribute to a better understanding of ATP-independent proteasome functions in all domains of life.

Experimental Procedures

Bacterial Strains, Growth Conditions, and Primers. Bacterial strains, plasmids, and primers are listed in Table S5. Plasmid construction and culture conditions are described in detail in SI Experimental Procedures.

Recombinant Protein Purification, Antibodies, and Immunoblotting. To generate rabbit polyclonal antisera, we produced His6-tagged M. tuberculosis H37rv PaeE, Mpa, and HspR in E. coli and purified proteins under native (for Mpa and PaeE) or denaturing (for HspR) conditions as described in the QIAexpressionist manual (Qiagen). A total of 500 μg of purified protein was used to immunize rabbits (Covance). A detailed description of protein purification procedures is included in SI Experimental Procedures. Antibodies to FabD and Ino1 are described elsewhere (30, 44).

To assess protein levels in M. tuberculosis lysates by immunoblotting, cultures were grown to an optical density of absorbance at 580 nm (OD580) ~1.2, and four OD580 equivalents of bacterial culture were pelleted and resuspended in lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 1 mM EDTA). Cells were lysed by bead beating (BioSpec), mixed with reducing and denaturing sample buffer (70), and boiled for 10 min. Samples were then separated by SDS polyacrylamide gel electrophoresis (SDSPAGE), transferred to nitrocellulose membranes (GE Amersham), and analyzed by immunoblotting using antibodies as indicated. As a loading control, we stripped the membranes (70) and subsequently incubated the blots with antibodies to dihydropipecoll acid transferase (DiaT) (71).

Purification of the Proteasome Trap and Preparation of Total Cell Lysates of M. tuberculosis for Two-dimensional Gel Electrophoresis with Mass Spectrometry. The design and expression of the proteasome trap construct is described in SI Experimental Procedures. To identify proteins that copurified with the proteasome trap, lysates were prepared from 120 OD580 equivalents of M. tuberculosis culture. Cells were resuspended in low-salt PBS (50 mM NaH2PO4, 100 mM NaCl, pH 8.0) with 10 mM imidazole, lysed by
bead beating, filter sterilized, and subjected to affinity purification by using 30 μL of packed, equilibrated Ni-NTA resin (Qiagen). After washing with 20 mM imidazole and eluting with 250 mM imidazole, the eluate was buffer exchanged into low-salt PBS (pH 7.4) and further purified by using 30 μL of packed, equilibrated M2 anti-FLAG affinity gel as described by the manufacturer (Sigma-Aldrich). The final elution of the proteasome and associated proteins was performed by using 60 μL of 3x FLAG peptide at a concentration of 150 ng/μL in low-salt PBS (pH 7.4). Eluted proteins were analyzed by SDS/PAGE and silver staining (72) or prepared for MS analysis by TMT labeling as described in SI Experimental Procedures. For EM analysis of in vivo-purified proteasome complexes, we purified protein with anti-FLAG affinity gel alone.

To prepare lysates from parental vs. pafE mutant M. tuberculosis for TMT labeling, biological triplicate cultures of each strain were grown to OD580∼1.2, washed with PBS−0.05% Tween-80, resuspended in lysis buffer, lysed by bead beating, and filter-sterilized. To prepare lysates from the M. tuberculosis pafA mutant treated with epoxomicin vs. dimethyl sulfoxide (DMSO), biological duplicate cultures were grown to OD580∼0.75, and then 50 μM epoxomicin (Boston Biochem) in DMSO or DMSO alone was added. After 4 d, bacterial lysates were prepared as described above. For all samples, protein concentration was assessed by protein assay (Bio-Rad). Equal amounts of protein were precipitated by adding six volumes of cold acetone, incubated at −20 °C for 20 min, and collected by 15 min of centrifugation at 16,000 × g at 4 °C. Protein pellets were air-dried.

Copurification of PafE and 20S CP from M. tuberculosis Lysates. A total of 40 OD580 equivalents of culture from M. tuberculosis strains producing ParE or PafEY173A, with an N-terminal His6 tag were lysed in low-salt PBS with 10 mM imidazole by bead beating and filter-sterilized. Lysates were incubated with preequilibrated Ni-NTA resin for 3 h, nonspecific interacting proteins were washed off with 20 mM imidazole, and PafE and other bound proteins were eluted by using 250 mM imidazole. The presence of PafE and PrcB was assessed by immunoblotting.

Negative-Stain EM and Single-Particle Image Processing of PafE-20S Complexes. For analysis of in vivo-purified proteasome complexes, we purified the TAP-tagged proteasome trap from an M. tuberculosis mpa mutant as described above, but used anti-FLAG affinity gel only. For reconstitution of PafE-20S complexes using purified recombinant proteins, ParE and 20S CPs were purified as described in SI Experimental Procedures. The 20SOG CPs were incubated with bortezomib (LC Laboratories) at a molar ratio of 1:8 (20SOG:inhibitor) at 37 °C for 45 min before addition of PafE. Purified ParE was then added to 20SOG CP preincubated with inhibitor or 20SOG at molar ratio of 1:4 or 1:12 (20S CP:PafE), respectively, at 37 °C for 20 min, followed by an additional 2-h incubation at 20 °C.


SEC-MALS. A total of 200 μg of purified His6-PafE was loaded onto a Shodex KW804 gel permeation chromatography column equilibrated and running with 20 mM Hepes (pH 7.4) and 150 mM NaCl by using a 1515 Isocratic HPLC Pump (Waters). To determine molecular mass, samples were analyzed by refractive index using an Optilab Rex differential refractometer and by MALS using a MiniDawn TREOS light-scattering detector (Wyatt).

Peptide and Protein Degradation Assays. Suc-LLVY-AMC was from Sigma-Aldrich. Peptides corresponding to the ParE C terminus were synthesized by Peptide 2.0 Inc. with a purity of 95%. The syntheses of LF-2 [7-methoxyxocmarin-4-acetic acid (Mca)-Lys-Lys-Val-Ala-Pro-Tyr-Pro-Met-Glu-Dpa(Dnp)-NH2] (Fig. S5) and pupylated 3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB) are described in SI Experimental Procedures.

For peptide degradation assays, 500 ng of purified 20S CP was used per reaction, and triplicate reactions were set up for each condition. The 20S CP was incubated at 37 °C in assay reaction buffer (50 mM Tris, pH 8.0, 5 mM MgCl2) containing 100 μM Suc-LLVY-AMC or at room temperature in the absence of 20 μM LF-2. Where indicated, increasing amounts of purified ParE or Mpa were added. Reaction mixtures using Mpa also included 3 mM ATP. Peptide degradation was assessed by measuring the change in fluorescence over time (Suc-LLVY-AMC: λem = 380, λex = 460; LF-2: λem = 340, λex = 405), and reaction rates were calculated by determining relative fluorescence units (RFU) generated per minute.

For protein-degradation assays, 150-μL reactions were set up in activity assay reaction buffer, including 1.5 μg of 20S CPs, 15 μg of ParE, and 1.5 μg of substrate. The 20S CPs and ParE were mixed first and incubated for 30 min at 37 °C and then cooled to room temperature (−25 °C), at which point substrate was added, and the reaction was incubated at either room temperature, 37 °C, or 45 °C, as indicated. At the indicated time points after substrate addition, a portion of the reaction was removed and quenched by mixing with reducing and denaturing sample buffer. Samples were separated by SDS/PAGE, and substrate degradation was assessed by either
Coomasie Brilliant Blue staining (Bio-Rad) for HspR or by monitoring in-gel fluorescence for FITC–β-casein (Sigma-Aldrich) and Pup–PanB by using a Typhoon Trio image (GE Healthcare). Fluorescence was quantified by densitometry of a nonradioated exposure using ImageQuant software.

Construction of the M. tuberculosis pafE Mutant. A plasmid encoding Rv3781–3783 under control of the operon’s presumed native promoter upstream of Rv3779 was generated by sewing overlap expansion PCR (73) and cloned into the vector pMV306-kan, which integrates into the mycobacterial L5 attB site. This plasmid was introduced into M. tuberculosis H37Rv by electroporation (74), and integration was confirmed by PCR using primers Rv3779_promoter_XbaI_F and Rv3783_HindIII_R (Table S5).

To delete and disrupt pafE, ∼700 base pairs of sequence both upstream and downstream of its coding sequence was PCR amplified with primers pafE_pYUB_S_Stul_F and pafE_pYUB_S_XbaI for the upstream region and pafE_pYUB_3 HindIII_F and pafE_pYUB_3 XhoI_R for the downstream region (Table S5), and each was cloned into the vector pYUBBS4 (75) flanking a hygromycin resistance cassette. Cloning was performed to delete the majority of the pafE gene, but left intact the first 30 and the final 65 base pairs of the coding sequence. After the sequence was confirmed, the plasmid was linearized by PciI digestion and introduced into the parental M. tuberculosis strain by electroporation. Putative mutants were selected by plating on 7H10 agar containing hygromycin and an indication of pafE was confirmed by immunoblotting and by PCR using primers pafE_promoter_HindIII_F and pafE_HindIII_R (Table S5). Complementation of the pafE strain was accomplished by introducing a single copy of the pafE gene under control of its presumed native promoter at the chromosomal Tet tettB site using the integrating vector pA/JF381.

Mouse Infections. Mouse infections were performed as previously described with a few modifications (38, 39, 76). The 6- to 8-wk-old C57BL/6J female mice (Jackson Laboratories) were infected by using an Inhalation Exposure System (Glas-Col) to administer ∼200-400 colony-forming units (CFU) per mouse; however, due to unknown circumstances, we unintentionally infected the mice with ∼4,000 CFU per mouse in our experiments. Mice were humanely killed at days 1 (n = 6), 21 (n = 8), and 56 (n = 8) after infection, and bacteria were harvested from homogenized lungs and spleens and inoculated onto Middlebrook 7H11 agar to enumerate CFU. All procedures were performed with the approval of the New York University Institutional Animal Care and Use Committee. Statistical analysis was performed by using a nonparametric Student’s two-tailed t test.

RNA Extraction and Quantitative Real-Time PCR. Three biological replicate cultures of the indicated M. tuberculosis strains were grown to an OD600 ~ 1.0, and RNA was purified and analyzed as described (44).

Heat Stress Sensitivity Assays. M. tuberculosis cultures were grown to an OD600 ~ 1.0 and diluted to OD = 0.08 in Middlebrook 7H9 medium. A total of 1 ml was transferred to a 2-ml O-ring tube and incubated for 24 h at 45 °C, and bacterial survival was assessed by plating serial dilutions on Middlebrook 7H11 agar to enumerate CFU. Statistical analysis was performed by using a nonparametric Student’s two-tailed t test.

Note. While this manuscript was under review, Delley et al. reported their independent discovery of PafE as an activator of proteasomal degradation (77). However, Delley et al. concluded that PafE forms hexamers whereas we concluded it forms dodecamers.

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Supporting Information

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SI Experimental Procedures

Bacterial Strains, Growth Conditions, and Primers. M. tuberculosis strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol, 0.05% Tween-80, 0.5% BSA, 0.2% dextrose, and 0.085% sodium chloride. Cultures were grown without shaking in 25-, 75-, or 125-cm² vented flasks (Corning Life Sciences) at 37 °C. For growth on solid medium, Middlebrook 7H11 agar (Difco) was supplemented with oleic acid, albumin, dextrose, and catalase (BBL). For selection using antibiotics, media were supplemented with 50 μg/mL kanamycin, 50 μg/mL hygromycin, and/or 25 μg/mL streptomycin. E. coli cultures were grown in Luria–Bertani (LB) broth (Difco) at 37 °C, and LB agar was used as solid medium. For selection, media were supplemented with 100 μg/mL kanamycin, 150 μg/mL hygromycin, 50 μg/mL streptomycin, or 200 μg/mL ampicillin. Primers were purchased from Invitrogen, and plasmid sequencing was performed by GENEWIZ to confirm the validity of cloned sequences.

Design and Expression of the Proteasome Trap Construct. Sewing overlap extension PCR was used to amplify the prcBA genes from M. tuberculosis chromosomal DNA. prcB was cloned without its N-terminal 57 codons to delete the N-terminal propeptide, to include a C-terminal FLAG-6xHis tag, and, where indicated, to have its catalytic threonine mutated to Ala. These constructs were cloned along with the presumed native promoter region upstream of the pup–prcBA operon into the HindIII and XbaI sites of the mycobacterial overexpression vector pOLYG, and electroporated into M. tuberculosis. The production of stable protein was confirmed by immunoblotting.

TMT Labeling and MS Analysis. Protein was prepared as described in Experimental Procedures, resuspended in 6 M guanidine–HCl, and quantified by BCA protein assay (Thermo Scientific). Samples were reduced with 5 mM DTT, alkylated with 12 mM iodoacetamide, digested for 2 h with endoproteinase Lys-C (Wako) at a ratio of 1:200 LysC:protein, and then digested overnight with trypsin (Promega) at a ratio of 1:100 trypsin:protein. The digest was acidified with formic acid to a pH < 3, and peptides were desalted by using 50 mg of solid-phase C18 extraction cartridge (Waters) and then lyophilized. Samples were resuspended in 100 μL of 200 mM Hepes (pH 8.5) with 30% (vol/vol) acetonitrile, and 10 μL of 20 μg/mL 6-plex TMT reagent in anhydrous acetonitrile was added to each sample. The reaction proceeded for 1 h, and then was quenched with hydroxylamine to a final concentration of 0.5%. Samples were then combined equally, desalted by using homemade stage tips as described (1), and lyophilized.

After stage-tip desalting, samples were resuspended in 0.1% formic acid and analyzed on an Orbitrap Elite (Thermo Fisher Scientific) by using an Orbitrap LC-MS3 method as described (2). Spectra were matched against an M. tuberculosis H37Rv database (downloaded February 6, 2013), and protein false discovery rate was controlled to <1% by using the reverse-database strategy (3). Reporter ion S/N for all peptides matching each protein were summed, and protein relative expression values were represented as a fraction of the total intensity for all TMT reporter ions for the protein.

Purification of Recombinant Proteins. PaFE was overproduced in E. coli BL21(DE3) or ER2566 at 37 °C from an isopropyl-B-D-1-thiogalactopyranoside (IPTG)-inducible plasmid encoding full-length PaFE preceded by a His6 affinity tag, and purified by Ni-NTA affinity purification as described in the QIAGEN pressionist manual (Qiagen). The fractions of interest were pooled, concentrated, and loaded onto either a HiLoad 16/600 Superdex 200 Prep Grade gel filtration column or a 10/300 Superose 6 GL gel filtration column (GE Healthcare Life Sciences) equilibrated with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10% (vol/vol) glycerol. Fractions containing PaFE were pooled and concentrated by using an Amicon Ultra-4 Centrifugal Filter Unit (Millipore). The final concentration of PaFE was determined spectrophotometrically by using a Nanodrop (Thermo Scientific).

M. tuberculosis 20S CPs were purified from E. coli for microscopy and activity assays as follows: 20SOG and 20SOGGCPs were purified by using the described protocol (4). To produce WT 20S CPs, prcBA was cloned into the IPTG-inducible expression vector pET-32a (+) (Novagen) lacking the PrcB propeptide to circumvent the need for autoprocessing. This construct was introduced into the E. coli T7 expression strain ER2566, and expression was induced with 1 mM IPTG for 6 h at 37 °C. The bacteria were harvested by centrifugation, and the bacterial pellet was stored at −20 °C. Purification was otherwise performed as described (5).

M. tuberculosis HspR was purified as described (6, 7). Briefly, HspR was cloned into pET-32a (+) with a C-terminal His6 tag, expression was induced with 1 mM IPTG for 5 h, and the protein was purified by Ni-NTA affinity chromatography under denaturing conditions by using 8 M urea. Fractions containing HspR were pooled, concentrated, and renatured by separation on a 10/300 Superose 6 GL gel-filtration column equilibrated with buffer containing 50 mM Tris (pH 7.5), 200 mM KCl, 5 mM EDTA, 5 mM MgCl2, and 10% (vol/vol) glycerol. Desired fractions were pooled, concentrated, and frozen until use.

Negative Stain EM and Single Particle Image Processing. For negative staining of PaFE, PaFE–20SOG CP, and PaFE–20SOGG CP samples, we first prepared carbon film substrate by evaporating a thin layer of carbon onto a piece of freshly cleaved mica in an Edwards vacuum evaporator (Sanborn) (<10−5 Torr). We floated the mica on the surface of deionized water to peel off the carbon film and picked up film by 300-mesh copper EM grids. The carbon-coated grids were then glow discharged in a 100-mTorr argon atmosphere for 1 min. A 4- to 5-μL protein solution was applied to the EM grid for 1 min. Excess solution was removed by blotting the edge of the EM grid with a piece of filter paper, and the EM grid was then washed with ddH2O, followed by staining twice for 30 s each in 5 μL of 2% (wt/vol) uranyl acetate aqueous solution. The EM grids were imaged in JEOL JEM-2100F transmission electron microscope operating at 200 kV. Micrographs were recorded in low-dose mode (15 e−/Å²) at ×50,000 microscope magnification in a Gatan UltraScan 4000 CCD camera (4,096 x 4,096 pixel), which corresponded to 12.2 Å/pixel sampling at the specimen level.

Particle selection and image processing were performed in an 8-CPU Dell Linux workstation by using EMAN (8) and EMAN2 (9) software packages. Raw particle images were selected in a semiautomatic manner with e2boxer.py in EMAN2. The selected particles were manually inspected to remove particles that were partially disassembled, having low contrast, and/or contacting other particles. The contrast transfer function was first determined with raw images and corrected for by flipping the phases. A second round of particle deletion was performed by computational k-means clustering classification. Phase-flipped raw particle datasets were then subjected to 2D classification.

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Particles that belonged to the same classes were averaged to generate a set of high-contrast class averages. The number of particle images used for the final reference-free 2D classification was 17514, 229, 1969, 766 for PaIe only, PaIe–20S\_E1A purified from Mycobacterium tuberculosis, PaIe–20S\_E1A reconstituted with purified proteins, and PaIe–20S\_E1A with inhibitor, respectively.

**Synthesis of LF-2 Peptide.** The peptide sequence Lys-Lys-Val-Ala-Pro-Tyr-Pro-Met-Glu-Dpa(Dnp)–NH\_2 was synthesized on resin by using a Syro II MultiSynth Automated Peptide synthesizer (Biotage) using standard 9-fluorenlymethoxy carbonyl (Fmoc)-based solid-phase peptide chemistry at 25-μmol scale, using fourfold excess of amino acids in NMP relative to the resin. Benzotriazol-1-yl-oxytrityl disconnecting phosphonium hexafluorophosphate (PyBOP, 4 equiv) and N,N-diisopropyl ethyamine (8 equiv) were used as condensing reagents and all amino acids were introduced via double couplings (25 min each). Fmoc removal was carried out by using 20% (vol/vol) piperidine in N-methyl-2-pyrrolidone (NMP) for 2 × 2 and 1 × 5 min. The 7-methoxycoumarin-4-acetic acid (Mca) was introduced manually via double couplings (90 min using 20% (vol/vol) piperidine in NMP) before it was dissolved to facilitate specific labeling of cysteine residues. Excess fluorescein was removed by desalting three times through spin columns, yielding 100 μL of 100 μM PanB–fluorescein.

Solvants were purchased from BIOSOLVE, peptide synthesis reagents were purchased from Novabiochem, except for Fmoc-PAL-PEG-PS resin (0.2 mmol/g), which was purchased from Rapp Polymere GmbH, and Mca and Fmoc-L-Dpa(Dnp)-OH, which were obtained from Chem-Impex. The following protected amino acid building blocks were used during the peptide synthesis: Fmoc-L-Ala-OH, Fmoc-L-Dpa(Dnp), Fmoc-L-Glu(OBu)-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Met-OH, Fmoc-L-Pro-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-L-Val-OH.

**Production of Pup–PanB–Fluorescein.** The panB gene from *M. tuberculosis* was cloned into a pET3a vector (EMD Millipore) between NdeI and BamHI sites with a C-terminal His\_6 tag, and the plasmid was used to transform *E. coli* BL21(DE3). PanB–His\_6 was purified by Ni-NTA affinity chromatography as described in the QIAexpressionist manual (Qiagen), and further purified by SEC using a 10/300 GL Superdex 200 column (GE Healthcare). Fractions containing pure PanB were identified by 15% (wt/vol) SDS/PAGE and quantified by comparison with Coomassie Brilliant Blue-stained BSA standards.

A total of 80 μL of 755 μM PanB was exchanged into buffer consisting of 20 mM NaP, 150 mM NaCl, and 5 mM EDTA at pH 7.5 by using a protein-desalting spin column (Thermo Scientific). The resulting PanB solution was incubated with 1.0 mg of fluorescein-5-smaleide (Thermo Scientific) at 4 °C overnight to facilitate specific labeling of cysteine residues. Excess fluorescein was removed by desalting three times through spin columns, yielding 100 μL of 100 μM PanB–fluorescein.

The PanB–fluorescein conjugate was subjected to pupylation with 200 μM *M. tuberculosis* His\_6–PupE and 1 μM PafA in Buffer P [50 mM Tris-HCl, 300 mM NaCl, 20 mM MgCl\_2, 5 mM ATP, 1 mM DTT, and 10% (vol/vol) glycerol at pH 7.4] in a final volume of 100 μL. The pupylation reaction was allowed to proceed overnight at 37 °C to push pupylation to completion. The overnight reaction was stopped by applying the assay mixture to a spin column pre-equilibrated in PBS at pH 7.5. Under these conditions, the small amount of PafA present aggregated on the column, effectively removing it from the assay products. His\_6–PupE was also retained on the column due to its small size (~7.7 kDa), and the desired Pup–PanB–fluorescein conjugate was recovered in the eluate. Pup–PanB–fluorescein was further purified by SEC using a 10/300 GL Superdex 200 column pre-equilibrated in buffer P. Fractions containing >90% pure Pup–PanB–fluorescein were identified by 15% (wt/vol) SDS/PAGE, combined, and used for proteolysis assays.

Fig. S1. Primary structure of PafE. Alignment of the PafE amino acid sequence from *M. tuberculosis* (Mtb), *Mycobacterium leprae* (Mlep), *Mycobacterium marinum* (Mmar), *Mycobacterium smegmatis* (Msm), *Rhodococcus opacus* (Ropa), *Nocardia farcinica* (Nfar), *Gordonia bronchialis* (Gbro), and *Streptomyces alboviridis* (Salb). Alignment was performed by using ClustalX.

Fig. S2. *pafE* has three potential translational start sites. (Left) Alignment of the 5' regions of *pafE* from different mycobacterial species. Horizontal lines indicate putative ribosome binding sites. (Right) PafE was produced in *E. coli* with an N-terminal thrombin-cleavable His$_6$ tag, purified by subsequent rounds of Ni-NTA and SEC, separated by 12% (wt/vol) SDS/PAGE, and visualized by Coomassie Brilliant Blue staining.
Fig. S3. PafE-mediated proteasome activation is ATP-independent. LF-2 degradation assays were performed as described in the main text, except that 5 mM ATP was included where indicated. Statistical analysis was performed by using Student’s t test. n.s., not significant.

Fig. S4. HspR is a PafE-dependent and pupylation-independent proteasome substrate. (A) STRING-DB analysis of proteasome and pupylation system genes in actinobacteria. Squares denote the presence of a homolog of the indicated gene. Darker color indicates a higher level of homology with *M. tuberculosis* strain H37Rv. (B) Proteomic identification of putative pupylation-independent proteasome substrates in *M. tuberculosis*. Protein lysates were prepared from WT and pafA strains grown to OD₅₈₀ ∼ 0.75 and treated with 50 µM epoxomicin for 4 d. Lysates were prepared and analyzed similarly by TMT-MS. The log₂ ratio of abundance in epoxomicin-treated vs. DMSO-treated cultures was plotted against the log₁₀ P value as determined by t test using three biological replicates. The data point representing HspR is in red. (C) HspR degradation rate increases at high temperatures. HspR degradation assay was performed as in Fig. 6D except with incubation at the indicated temperatures. (D) In vitro degradation of HspR–His₆ was performed as in Fig. 6D, except 20SOG CP–His₆ was used with and without His₆–PafE. The contrast was adjusted to better visualize the 20SOG CP–His₆. (E) Open-gate 20S CPs do not degrade the Mpa-dependent proteasome substrate Pup–PanB. Recombinant purified 20SOG CP, Pup–PanB–fluorescein, and where indicated PafE were mixed and incubated at room temperature. Aliquots were removed at the indicated time points and separated by 12% (wt/vol) SDS/PAGE, and Pup–PanB–FITC degradation was monitored by in-gel fluorescence.
Table S1. Proteins copurifying with the 20S CP from WT *M. tuberculosis*. Data are organized from highest to lowest abundance found in Trap purifications. Column labeled protein: Rv number of each protein identified. Column labeled peptides: the number of unique peptides identified for a given protein. Columns labeled WT and Trap 1–3: relative abundance of each protein that purified with WT or Trap proteasomes in each biological replicate; related to Fig. 1B, Left

Table S2. Proteins copurifying with the 20S CP from an *M. tuberculosis* mpa mutant. Data are organized from highest to lowest abundance found in Trap purifications. Column labeled protein: Rv number of each protein identified. Column labeled peptides: the number of unique peptides identified for a given protein. Columns labeled WT and Trap 1–3: relative abundance of each protein that purified with WT or Trap proteasomes in each biological replicate; related to Fig. 1B, Right

Table S3. Proteins that accumulated in *pafE* mutant vs. parental *M. tuberculosis* strains. Data are organized from highest to lowest fold change. Column labeled protein: Rv number of each protein identified. Column labeled peptides: the number of unique peptides identified for a given protein. Columns labeled parental and *pafE* 1–3: relative abundance of each protein found in parental or *pafE* lysates for each biological replicate; related to Fig. 6C, Left

Fig. S5. Chemical structure (Upper) and LC-MS analysis (Lower) of LF-2 peptide [Mca KKVAPYPME-Dpa(Dnp)-amide].
Table S4. Proteins that accumulated in an *M. tuberculosis* pafA mutant treated with epoxomicin vs. DMSO. Data are organized from highest to lowest fold change. Column labeled protein: Rv number of each protein identified. Column labeled peptides: the number of unique peptides identified for a given protein. Columns labeled DMSO and epoxomicin 1–3: relative abundance of each protein found in DMSO or epoxomicin-treated bacteria for each biological replicate; related to Fig. 6C, Right.

Table S4

Table S5. Bacterial strains, plasmids, and primers

Table S5


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<td>Allison Faye and Michael Glickman</td>
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