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Substrate delivery by the AAA+ ClpX and ClpC1 unfoldases activates the mycobacterial ClpP1P2 peptidase

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Summary

Mycobacterial Clp-family proteases function via collaboration of the heteromeric ClpP1P2 peptidase with a AAA+ partner, ClpX or ClpC1. These enzymes are essential for *M. tuberculosis* viability and are validated antibacterial drug targets, but the requirements for assembly and regulation of functional proteolytic complexes are poorly understood. Here, we report the reconstitution of protein degradation by mycobacterial Clp proteases *in vitro* and describe novel features of these enzymes that distinguish them from orthologs in other bacteria. Both ClpX and ClpC1 catalyze ATP-dependent unfolding and degradation of native protein substrates in conjunction with ClpP1P2, but neither mediates protein degradation with just ClpP1 or ClpP2. ClpP1P2 alone has negligible peptidase activity, but is strongly stimulated by translocation of protein substrates into ClpP1P2 by either AAA+ partner. Interestingly, our results support a model in which both binding of a AAA+ partner and protein-substrate delivery are required to stabilize active ClpP1P2. Our model has implications for therapeutically targeting ClpP1P2 in dormant *M. tuberculosis*, and our reconstituted systems should facilitate identification of novel Clp protease inhibitors and activators.

Introduction

The emergence of bacterial pathogens resistant to conventional antibiotics has led to an urgent need for novel therapeutics (Neu, 1992, Levy & Marshall, 2004, Howard *et al.*, 2013). This need is especially pressing for *Mycobacterium tuberculosis*, a globally significant pathogen that continues to acquire resistance to the limited armamentarium of antibiotics (Jassal & Bishai, 2009, Gandhi *et al.*, 2010). The Clp proteases have the potential to address this clinical need as novel and orthogonal antibiotic targets (Brotz-Oesterhelt *et al.*, 2005, Raju *et al.*, 2012, Brötz-Oesterhelt & Sass, 2013). These enzymes are essential for viability of *M. tuberculosis*, which is also sensitive to unregulated activation of Clp activity, making these proteases especially attractive antibiotic targets (Sasseti *et al.*, 2003, Griffin *et al.*, 2011, Ollinger *et al.*, 2012, Raju *et al.*, 2012, Personne *et al.*, 2013). The Clp proteases consist of a self-compartmentalized peptidase and an ATP-dependent AAA+ unfoldase (ATPases associated with diverse cellular activities). These components collaborate to carry out regulated protein degradation in a variety of physiological processes, including homeostatic protein quality control, responses to environmental stress, and virulence in

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pathogenic bacteria (Gaillot *et al.*, 2000, Frees *et al.*, 2003, Kwon *et al.*, 2004, Sauer *et al.*, 2004, Fernandez *et al.*, 2012, Frees *et al.*, 2014).

To degrade protein substrates, the ClpP peptidase functions with a partner AAA+ unfoldase (ClpX, ClpC, or ClpA). ClpP forms heptameric rings, which stack face-to-face to form a tetradecameric barrel-shaped enzyme (Flanagan *et al.*, 1995, Wang *et al.*, 1997). The ClpP catalytic sites face an interior solvent-filled degradation chamber, which is separated from bulk solvent by a narrow axial pore in each heptameric ring. The AAA+ partner enzymes form ring hexamers, which interface coaxially with one heptameric face the ClpP barrel (Grimaud *et al.*, 1998, Maurizi *et al.*, 1998, Kim & Kim, 2003). Binding of a AAA+ partner to ClpP induces widening of the axial pore (Lee *et al.*, 2010a, Lee *et al.*, 2010b, Li *et al.*, 2010). Protein substrates are recognized by the AAA+ partner, which uses cycles of ATP binding, hydrolysis, and product release to mechanically unfold and translocate the polypeptide through the axial pore of ClpP and into its proteolytic chamber for degradation.

Most proteobacteria and firmicutes encode a single *clpP* ortholog (Yu & Houry, 2007), which self-assembles into a catalytically active tetradecamer even in the absence of a AAA+ partner (Böttcher & Sieber, 2008, Lee *et al.*, 2010a, Lee *et al.*, 2010b, Li *et al.*, 2010). By contrast, other bacterial phyla, including all actinobacteria, encode multiple *clpP* paralogs. Although several paralogous ClpP systems have been characterized *in vitro* (Stanne *et al.*, 2007, Zeiler *et al.*, 2011, Akopian *et al.*, 2012, Tryggvesson *et al.*, 2012), the assembly, regulation and function of multi-ClpP enzymes in protein degradation may differ across bacterial phyla. Indeed, phylogenetic comparisons suggest that *clpP* paralogs evolved independently in different bacterial lineages and that actinobacterial ClpP paralogs, in particular, are conserved and distinct from the ClpP enzymes of other phyla (Fig. S1).

M. tuberculosis, an actinobacterium, encodes two ClpP paralogs, ClpP1 and ClpP2, which are expressed from a bicistronic operon (Raju *et al.*, 2012). Each paralog independently assembles into inactive homo-heptameric rings (ClpP1 or ClpP2), which then combine to form the double-ring ClpP1P2 tetradecamer (Benaroudj *et al.*, 2011, Akopian *et al.*, 2012). Curiously, ClpP1P2 tetradecamer formation and cleavage of small peptide substrates *in vitro* requires the presence of N-blocked hydrophobic peptides or peptide aldehydes, which act as agonists that are not themselves cleaved (Akopian *et al.*, 2012, Compton *et al.*, 2013). Agonists act by a mechanism distinct from AAA+ partners and acyldepsipeptide (ADEP) antibiotics, which are not needed for cleavage of small peptides by *E. coli* ClpP and related homomeric enzymes but activate cleavage of larger peptides and polypeptides by enlarging the axial ClpP pore (Lee *et al.*, 2010b, Li *et al.*, 2010). Why agonists are required for mycobacterial Clp activity is not clear, nor has it been established whether agonists are necessary for degradation of folded proteins by ClpP1P2 in conjunction with ClpX or ClpC1, the endogenous mycobacterial AAA+ partners. To understand how Clp proteases in *M. tuberculosis* and other actinobacteria function and are regulated, we sought to reconstruct mycobacterial Clp proteases capable of degrading folded protein substrates.

Results

Enzymatic characterization of ClpX and ClpC1

We were able to clone and purify full-length *M. tuberculosis* ClpX bearing an N-terminal SUMO tag that improved stability and solubility (*Mtb*sClpX) but failed to clone *M. tuberculosis* ClpC1. We were, however, able to clone and purify *M. smegmatis* ClpC1 (*Msm*ClpC1), which is 94% identical to *M. tuberculosis* ClpC1. Moreover, the regions of *Msm*ClpC1 and *Mtb*ClpC1 responsible for nucleotide binding and hydrolysis and for interactions with ClpP are identical in both proteins. Both *Mtb*sClpX and *Msm*ClpC1 hydrolyzed ATP in the absence of ClpP (Fig. 1). *Msm*ClpC1 had a relatively high K_M for ATP hydrolysis (~3.6 mM) and an ATPase rate that depended on enzyme concentration, with half-maximal activity at a hexamer concentration of ~60 nM (Fig. 1A,B). The latter result suggests that *Msm*ClpC1 hexamers dissociate to inactive species at low concentrations. *Mtb*sClpX had a lower K_M for ATP hydrolysis (~0.4 mM), but formed a less stable hexamer, with half-maximal ATPase activity at ~500 nM hexamer (Fig. 1C,D). Removing the SUMO tag with Ulp1 peptidase did not alter the concentration dependence of ClpX ATPase activity (Fig. 1D), indicating that hexamer instability is an intrinsic property and not a consequence of the tag.

AAA+ partners and agonist stimulate ClpP1P2 activity synergistically

We tested if ClpP1, ClpP2, or ClpP1P2 catalyzed cleavage of a fluorogenic decapeptide, and whether this activity was affected by binding of *Mtb*sClpX, *Msm*ClpC1, and/or Z-Ile-Leu agonist (Fig. 2A, S2A). In the absence of AAA+ partners, ClpP1, ClpP2, and ClpP1P2 exhibited negligible decapeptide cleavage with or without agonist. Similarly, little cleavage activity was observed in the absence of agonist, even when a AAA+ partner was present. Strikingly, however, the combination of agonist and either AAA+ partner strongly stimulated ClpP1P2 cleavage of the decapeptide. Robust stimulation occurred only in the presence of both ClpP1 and ClpP2, and much lower activities were observed with ClpP1 or ClpP2 alone (Fig. S2A). ClpP1P2 activation by *Mtb*sClpX occurred in the presence ATP or ATP γ S, but not with ADP or in the absence of nucleotide (Fig. S2D), consistent with other bacterial Clp systems where only ATP- or ATP γ S-bound ATPases interact with ClpP (Seol *et al.*, 1995, Joshi *et al.*, 2004). Curiously, under the conditions of this assay, *Msm*ClpC1 strongly activated ClpP1P2 only in the presence of ATP γ S (Fig. S2E), similar to the behavior the distantly related AAA+ enzyme PAN, which interacts with the 20S peptidase more tightly in the presence of ATP γ S than ATP (Barthelme & Sauer, 2012). *Msm*ClpC1 also supported a low level of ClpP1P2 peptidase activity in the presence of ATP and an ATP regeneration system (Fig. S3E).

In principle, AAA+ partners and agonist might stimulate decapeptide cleavage by *M. tuberculosis* ClpP1P2 by stabilizing a catalytically active form of the peptidase and/or by enhancing peptide diffusion into the degradation chamber by opening the axial pore (Lee *et al.*, 2010a, Lee *et al.*, 2010b, Li *et al.*, 2010). To test if pore opening alone is responsible for activity enhancement, we assayed the rate of cleavage of a tripeptide, which should freely diffuse into the ClpP chamber, with different combinations of ClpP1P2, AAA+ partners, and agonist (Fig. 2B, Fig. S2B). As observed with decapeptide cleavage, robust activity was

observed only in the presence ClpP1P2, agonist, and a AAA+ partner. Thus, agonist and AAA+ partners appear to synergistically stabilize an active conformation of ClpP1P2. Because the combination of ClpP1 and ClpP2 alone is inactive in peptide degradation, these experiments do not resolve whether pore opening is also required for decapeptide cleavage.

ClpX and ClpC1 collaborate with ClpP1P2 to degrade folded substrates

We next tested if mycobacterial ClpP-family AAA+ proteases are capable of degrading natively folded proteins *in vitro*, as previous studies only demonstrated degradation of unstructured substrates (Barik *et al.*, 2010, Akopian *et al.*, 2012). Michaelis-Menten analysis showed that *Mtb*sClpX•ClpP1P2 degrades native green-fluorescent protein bearing a *M. tuberculosis* ssrA tag (GFP-ssrA) with a $V_{\max}$ of $\sim 0.1 \text{ min}^{-1} \text{ enz}^{-1}$ and a K_M of $\sim 3 \mu\text{M}$ in the presence of Z-Ile-Leu (Fig. 3A). Because ClpP1P2 alone is inactive, this degradation activity is evidence of a direct interaction between *Mtb*sClpX and ClpP1P2. An apparent affinity of $380 \mu\text{M}$ for ClpP1P2 interaction with *Mtb*sClpX was measured using GFP-ssrA degradation as a proxy for binding (Fig. 3B). By contrast, no substantial degradation of GFP-ssrA was observed with agonist and *Mtb*sClpX and ClpP1, *Mtb*sClpX and ClpP2, or *Mtb*sClpX and *E. coli* ClpP (*Eco*ClpP; Fig. 3C). The inability of *Mtb*sClpX to collaborate with mycobacterial ClpP1 or ClpP2 in protein degradation was consistent with the decapeptide-cleavage experiments described above. However, its inability to function with *Eco*ClpP was surprising, given that a variant of *E. coli* ClpX lacking the N-domain (*Eco*ClpX^N; 43% identical to the corresponding region of *M. tuberculosis* ClpX) forms a functional protease in conjunction with *Mtb*ClpP1P2 (Fig. 3D; see Discussion).

At saturating GFP-ssrA, the rate of degradation by *Mtb*sClpX•ClpP1P2 was more than 10-fold slower than degradation by *Eco*ClpX^N•ClpP1P2 or *Eco*ClpX^N•*Eco*ClpP (Fig. 3C,D). Thus, the overall rate of degradation of GFP-ssrA by *Mtb*sClpX•ClpP1P2 appears to be limited by the rate of *Mtb*sClpX unfolding rather than by the rate of ClpP1P2 proteolysis. Slower unfolding by *Mtb*sClpX may be a consequence of its relatively low rate of ATP hydrolysis, $\sim 15\%$ that of *Eco*ClpX (Joshi *et al.*, 2004), as GFP-ssrA is unfolded and degraded by *Eco*ClpXP much more efficiently at high ATPase rates (Martin *et al.*, 2008).

*Msm*ClpC1•ClpP1P2 also degraded GFP-ssrA at a modest maximal rate ($0.12 \text{ min}^{-1} \text{ enz}^{-1}$; Fig. 3E) in the presence of agonist, suggesting that low degradation activity may be an intrinsic characteristic of the mycobacterial AAA+ ClpP-family proteases or that a component missing from our biochemical assays promotes faster degradation *in vivo*. Again, GFP-ssrA degradation was observed with *Msm*ClpC1 and ClpP1P2 but not with ClpP1 or ClpP2 alone (Fig. 3E). By assaying GFP-ssrA degradation in the presence of fixed *Msm*ClpC1 and varying ClpP1P2 concentrations, we determined a K_{app} of $1.7 \mu\text{M}$ for the interaction of *Msm*ClpC1 with ClpP1P2 (Fig. 3B). Interestingly, we also observed ATP-dependent and ClpP1P2-dependent auto-proteolysis of ClpC1 bearing an N-terminal H₆-TEV tag (Fig. 3F). Removal of the tag prevented auto-proteolysis, suggesting recognition of the unstructured tag near the ClpC1 N-terminus.

Using GFP-ssrA degradation as an assay, we tested the effects of inactivating mutations in the catalytic serines of ClpP1 (S98A), ClpP2 (S110A), or both ClpP1 and ClpP2 (Fig. 4A, B, C). In conjunction with any of the AAA+ partners, proteases with one active and one

inactive ClpP ring were able to degrade protein substrate, indicating that both ClpP1 and ClpP2 are active in substrate degradation. Curiously, *Msm*ClpC1•ClpP1P2 had higher activity when one ClpP ring was inactive (Fig. 4C), and these mutant ClpP rings bound more tightly to *Msm*ClpC1 (Fig. 4D). Thus, the S98A and S110A active-site mutations appear to stabilize a ClpP1P2 conformation that binds *Msm*ClpC1 more tightly either directly or indirectly, for example by strengthening binding of Z-Ile-Leu. As anticipated, complexes with active-site mutations in both ClpP1 and ClpP2 were unable to degrade protein substrate (Fig. 4A, B, C).

Protein substrates delivered by ClpX stimulate ClpP1P2 peptidase activity

We found that GFP-ssrA degradation by *Eco*ClpX^N•ClpP1P2, *Mth*sClpX•ClpP1P2, or *Msm*ClpC1•ClpP1P2 occurred in the absence of the Z-Ile-Leu agonist, but at 30–80% of the rate determined with agonist (Fig. 5A). This slower protease activity correlated with weaker apparent affinities between the AAA+ ATPases and ClpP1P2 in the absence of agonist (Fig. S3). By contrast, when agonist was omitted from decapeptide or tripeptide cleavage reactions, cleavage activities were only 7% or less of the stimulated reaction (Fig. 2). We hypothesized that protein substrates translocated into the ClpP1P2 degradation chamber might substitute for agonist in stimulating cleavage activity. In the absence of agonist, this model predicts that active degradation of a folded substrate would stimulate peptide degradation *in trans*. Indeed, we found that degradation of a non-fluorescent model substrate, V15P-titin^{I27}-ssrA, by *Mth*sClpX•ClpP1P2 or *Eco*ClpX^N•ClpP1P2 stimulated degradation of the Z-GGL-AMC tripeptide in the absence of agonist (Fig. 5B). Stimulation by *Mth*sClpX•ClpP1P2 proteolysis was supported by ATP but not ATP_γS, indicating that active unfolding and translocation of substrate into the ClpP degradation chamber is necessary for stimulation of peptide cleavage. Several observations support a model in which faster protein degradation results in a higher level of stimulation of peptidase activity. First, proteolysis of GFP-ssrA by *Eco*ClpX^N•ClpP1P2 enhanced peptide cleavage ~3-fold more than proteolysis by *Mth*sClpX•ClpP1P2, correlating with faster protein degradation by the former protease (Fig. 3C,D). Second, less stable titin^{I27}-ssrA proteins that are degraded more rapidly by *Eco*ClpX^N (Kenniston *et al.*, 2003) stimulated higher levels of peptide cleavage by *Mth*sClpX•ClpP1P2 (Fig. S2F). Third, GFP-ssrA also weakly stimulated peptide cleavage by *Eco*ClpX^N•ClpP1P2 in the presence of ATP_γS, in accord with the ability of *Eco*ClpX^N to hydrolyze ATP_γS slowly (Burton *et al.*, 2003).

To test further the model that polypeptide translocation into the proteolytic chamber of ClpP1P2 stabilizes a functional conformation of the ClpP1P2 active sites, we simultaneously monitored GFP-ssrA degradation and cleavage of a fluorogenic peptide by *Eco*ClpX^N•ClpP1P2, in the absence of agonist, under conditions where the protein substrate (Fig. 5C, upper panel) or ATP (Fig. 5D, upper panel) was depleted within ~10 min. In both experiments, protein degradation and peptide cleavage ceased with the same kinetics. By contrast, in parallel reactions where agonist was present in place of GFP-ssrA, peptide cleavage was continuous over the same time course (Fig. 5C, D, lower panels). The ability of protein substrates to stimulate peptide-cleavage activity of the ClpX•ClpP1P2 complex supports a model in which the peptidase activity of ClpP1P2 is reversibly regulated by substrate delivery by a AAA+ partner (Fig. 6).

Discussion

We find that the mycobacterial Clp proteases are tightly regulated by ATP-dependent binding of AAA+ partners and by protein-substrate availability. This behavior contrasts markedly with orthologous Clp systems in which ClpP forms catalytically active tetradecamers in the absence of interaction partners, and unregulated degradation of substrates is restricted solely by the narrow axial pores of ClpP. In those systems, large peptides and unfolded polypeptides can slowly diffuse through the ClpP pores (Lee et al., 2010b), resulting in a low level of unregulated proteolysis. By contrast, mycobacterial ClpP1P2 adopts a catalytically inactive conformation under conditions where its AAA+ partners fail to bind or actively unfold and translocate protein substrates (e.g., low ATP). These restrictions *in vitro* likely mimic conditions inside the cell during periods of low metabolic activity. Under such conditions, the inactivity of ClpP1P2 could reduce wasteful degradation of nascent polypeptides or transiently disordered proteins and permit cells to carry out low levels of protein synthesis and chaperone-mediated protein folding without interference. These characteristics may confer a fitness advantage to slow-growing organisms adapted to nutrient-poor conditions and may contribute to the long-term viability of *M. tuberculosis* during latent infection. These regulatory characteristics are likely to be conserved across actinobacteria, given the conservation of the ClpP1 and ClpP2 paralogs. Substrate-binding may analogously regulate assembly and activity of more distantly related orthologs, such as human mitochondrial ClpP, which is similar to the mycobacterial system in that it forms inactive heptamers by itself but forms active tetradecamers in conjunction with ClpX (Kang et al., 2005).

Our findings are consistent with a dual allosteric model in which both AAA+ binding to ClpP1P2 and protein-substrate binding to the ClpP1P2 active sites regulate protease activity (Fig. 6). The enzyme components that form the functional AAA+ proteases are in dynamic equilibrium and appear to be predominantly inactive in the absence of protein substrates or at low ATP concentrations. Under these conditions, for example, most ClpP1 and ClpP2 would form inactive heptameric rings or an inactive ClpP1P2 tetradecamer with malformed substrate-binding pockets and/or catalytic triads. Similarly, ClpX and ClpC1 would largely be inactive hexamers or smaller oligomers at low ATP concentrations. As in any dynamic equilibrium, a small population of functional ClpX•ClpP1P2 or ClpC1•ClpP1P2 would exist. In the presence of saturating ATP and abundant protein substrate, AAA+ mediated recognition, unfolding, and translocation by these active complexes could have several effects. First, the translocating substrate could stabilize the complex between the AAA+ hexamer and ClpP1P2 by making contacts with both components. Second, the translocating substrate could stabilize the active conformation of ClpP1P2, allowing degradation. Third, both of these reactions would result in a shift of more components in the population into assembled and active proteolytic complexes. These active complexes are likely to be transient, and would disassemble or revert to inactive forms once ATP or protein substrates were depleted.

The transition between inactive and active forms of ClpP is likely to involve global conformational changes in ClpP1 and ClpP2 that simultaneously affect AAA+ partner binding, peptide-cleavage activity, and heptamer association. This model is supported by

structural evidence from ClpP orthologs that show a dynamic interface between the two heptameric rings and demonstrate a correlation between the interface conformation and properly formed catalytic triads and substrate-binding pockets (Gribun *et al.*, 2005, Kimber *et al.*, 2010, Lee *et al.*, 2011, Religa *et al.*, 2011, Gersch *et al.*, 2012). Crystal structures in which the interface has an extended conformation also show functional active-site conformations, whereas structures with a disordered or compressed interface, including that of *M. tuberculosis* ClpP1, reveal blocked substrate pockets and active-site residues positioned improperly for catalysis (Wang *et al.*, 1997, Ingvarsson *et al.*, 2007, Geiger *et al.*, 2011, Lee *et al.*, 2011).

Our model provides an explanation for the apparent requirement of non-physiological agonist peptides for peptide hydrolysis by ClpP1P2 (Akopian *et al.*, 2012). Specifically, agonist peptides serendipitously mimic protein substrates, such that sub-stoichiometric occupancy of active sites by agonist provides binding energy to stabilize the active ClpP1P2 conformation. This interpretation is supported by the prior observation that high concentrations of agonist partially block covalent modification of the ClpP1P2 active-site serines (Akopian *et al.*, 2012).

In addition to the substrate-mediated regulation described above, we note the potential for regulation through the partner AAA+ ATPases. The relatively weak K_M of ClpC1 for ATP hydrolysis, and its apparent inhibition by ADP, may serve to sense the growth state of mycobacteria. Measurements of intracellular nucleotide concentration suggest that *M. tuberculosis* ATP levels are six-fold higher in actively dividing than in dormant cells (Rao *et al.*, 2008), which could serve as a switch for ClpC1 activity. By contrast, ClpX binds ATP more tightly, but exhibits relatively weak hexamer stability. Thus, ClpX mediated proteolysis may require high expression levels of this enzyme, or its activity may require stabilizing interactions with ClpP, adaptors, or substrates.

The observation that both ClpX•ClpP1P2 and ClpC1•ClpP1P2 degrade ssrA-tagged substrates suggests that these enzymes have overlapping substrate specificities and that both could contribute to the turnover of ssrA-tagged proteins in the cell. *E. coli* ClpA, a double-ring AAA+ ATPase related to ClpC1, also supports ClpP degradation of ssrA-tagged proteins (Gottesman *et al.*, 1998), although *B. subtilis* ClpC apparently does not (Wiegert & Schumann, 2001). We did not detect ClpC1 mediated degradation of constructs incorporating the *B. subtilis* ClpC adaptor and substrate, MecA (Turgay *et al.*, 1998, Wang *et al.*, 2011), homologs of which are absent in mycobacteria. Thus, we infer significant functional divergence between mycobacterial ClpC1 and *B. subtilis* ClpC.

Divergence is also apparent in the interactions between ClpX and ClpP, given the observation that *Eco*ClpX^N productively collaborates with both *M. tuberculosis* and *E. coli* ClpP enzymes, whereas *Mtb*sClpX only forms a functional protease with *M. tuberculosis* ClpP1P2. The structural determinants of the interaction between ClpX and ClpP are not understood in detail, making the reason for this specificity difference unclear. We note that the *E. coli* ClpX IGF loops and pore-2 loops, which are important determinants of ClpP binding (Kim *et al.*, 2001, Joshi *et al.*, 2004, Martin *et al.*, 2007), are similar in *M.*

tuberculosis ClpX, suggesting that additional regions of this enzyme may be critical for the interaction with ClpP1P2.

Our work indicates that ClpP1P2 is the functional form of mycobacterial ClpP, supporting a model proposed by Akopian and colleagues (Akopian et al., 2012, Raju et al., 2012). However there are notable differences between our results and this prior study. In our assays Z-Ile-Leu alone did not support ClpP1P2 activity, whereas Akopian and colleagues found that a ten-fold higher concentration of a similar peptide, Z-Leu-Leu, activated ClpP1P2 in the absence of ATPase partner (Akopian et al., 2012). This result suggests that tighter-binding agonists or higher agonist concentrations provide sufficient binding energy to stabilize a substantial equilibrium population of active ClpP1P2. Akopian and coworkers also found that agonist was required for degradation of FITC-casein by *M. tuberculosis* ClpC1 and ClpP1P2, whereas we observed degradation of GFP-ssrA by *Msm*ClpC1•ClpP1P2 even in the absence of Z-Ile-Leu. This discrepancy may be the result of the different substrates, slightly different buffer conditions, the relatively low concentration of ClpP1P2 used in the prior study, or differences between ClpC1 orthologs of *M. tuberculosis* and *M. smegmatis*.

Importantly, our model for ClpP1P2 function has implications for attempts to kill *M. tuberculosis* and other bacterial pathogens with active-site inhibitors of ClpP1P2, an area of active research (Zeiler et al., 2011, Compton et al., 2013, Gersch *et al.*, 2013a, Gersch *et al.*, 2013b). Dormant *M. tuberculosis* almost certainly carries out less Clp-mediated protein degradation, with ClpP1 and ClpP2 existing predominantly in inactive conformations. The inactive forms of these enzymes would be resistant to inhibitors that require an active conformation of the catalytic triad and substrate-binding pockets. However, our model predicts that simultaneous treatment with dysregulatory activators such as ADEPs could stabilize active ClpP1P2, rendering dormant cells more susceptible to active-site inhibitors of ClpP. Indeed, preliminary studies *in vitro* show that agonist peptides cause a substantial increase in ADEP activation of ClpP1P2 and that a combination of agonist and ADEP also enhances chemical modification of the active sites of ClpP1P2 by inhibitors (K. Schmitz, D. Carney, J. Sello, and R. Sauer, in preparation).

Our studies reveal that mycobacterial Clp-family AAA+ proteases utilize novel mechanisms of regulation, linking protein substrate delivery with ClpP peptidase activity. We anticipate that this mechanistic characterization along with the ability to functionally reconstruct these mycobacterial systems *in vitro* will aid in identifying and characterizing potential antibiotics against *M. tuberculosis*.

Experimental Procedures

Protein expression and purification

M. tuberculosis ClpP1 and ClpP2, bearing C-terminal His₆-tag fusions, were cloned, expressed, and purified as described (Compton et al., 2013). Variants of titin¹²⁷ bearing C-terminal *E. coli* ssrA tags (AANDENYALAA) were cloned, expressed, and purified as described (Kenniston *et al.*, 2005). Full-length *M. tuberculosis* ClpX was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA (ATCC). An N-terminal fusion encoding a His₇

tag and the yeast SUMO homolog Smt3p was added by PCR. The resulting construct (*Mtb*sClpX) was cloned into pET-21b (Novagen). Full-length *M. smegmatis* ClpC1 (*Msm*ClpC1) was amplified by PCR from *M. smegmatis* MC²155 genomic DNA (ATCC) and cloned into pET-21b (Novagen) in frame with an N-terminal His₆-TEV tag. Plasmids encoding *Mtb*sClpX or *Msm*ClpC1 were transformed into ER2566 *E. coli* (NEB). GFP-ssrA ((Martin *et al.*, 2005) was modified to incorporate a C-terminal mycobacterial ssrA tag (ADSNQRDYALAA) and cloned into pET-21b frame with an N-terminal His₆-TEV tag. The plasmid encoding GFP-ssrA was transformed into the *clpP*[−] *E. coli* strain JK10 (Kenniston *et al.*, 2005). Proteins were overexpressed in a 1:1 mixture of LB and 2xYT broth at RT, following induction with 0.5 mM isopropyl β -D-I-thiogalactopyranoside (IPTG; Teknova); 1 mM ZnCl₂ was included for overexpression of *Mtb*sClpX. Lysates were clarified by centrifugation, and proteins were purified by metal affinity (HisPur Ni-NTA agarose, Thermo), anion exchange (MonoQ, GE Healthcare), and size-exclusion chromatography (Superdex 200, GE Healthcare). The His₆-TEV tag was removed from *Msm*ClpC1 prior to anion exchange chromatography by overnight incubation with 1 μ g tobacco etch virus protease per 10 μ g protein in 25 mM HEPES, 50 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA, pH 8.0. Purified *Mtb*sClpX and *Msm*ClpC1 were concentrated to 20 μ M hexamer, and GFP-ssrA to 600 μ M, in storage buffer (25 mM HEPES, 150 mM NaCl, 10% glycerol, pH 7.5).

Enzymatic assays

Assays were performed at 30°C in PD buffer (25 mM HEPES, 100 mM KCl, 10% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, pH 7.5) on a SpectraMax M5 microplate reader (Molecular Devices). ATPase assays included 2.5 mM ATP (unless otherwise indicated; Sigma) and a NADH-coupled regeneration system (Nørby, 1988). Protease assays included 2.5 mM ATP and a regeneration system comprising 16 mM creatine phosphate (MP Biomedicals) and 0.32 mg/mL creatine phosphokinase (Sigma). ATP hydrolysis was monitored via decrease in 340 nm NADH absorbance. Peptidase assays followed hydrolysis of a fluorogenic decapeptide, Abz-KASPVSLGY^{NO2}D (Lee *et al.*, 2010b), by increase in 420 nm fluorescence upon 320 nm excitation, or of a fluorogenic tripeptide (Z-GGL-AMC; Enzo Life Sciences) by increase in 460 nm fluorescence upon 380 nm excitation. GFP-ssrA degradation was monitored by loss of 511 nm emission following excitation at 450 nm. Z-Ile-Leu and ADP were obtained from Sigma. ATP γ S was obtained from EMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ATPase activity and hexamer stability of mycobacterial ClpX and ClpC1

A *Msm*ClpC1 (0.5 μM) ATPase activity was measured as a function of ATP concentration and fit to a Hill equation (apparent $K_M = 3.6 \pm 0.6$ mM; Hill $n = 1.3 \pm 0.2$).

B Dependence of ATPase activity on *Msm*ClpC1 concentration. The line is a fit to a Hill equation ($K_{0.5} = 58 \pm 1.9$ nM; $n = 3.4 \pm 0.4$).

C *Mtb*sClpX (0.5 μM) ATPase activity was measured as a function of ATP concentration and fit to a Hill equation (apparent $K_M = 0.4 \pm 0.006$ mM; $n = 2.7 \pm 0.09$).

D Dependence of ATPase activity on *Mtb*sClpX concentration. The line is a fit to a Hill equation ($K_{0.5} = 0.54 \pm 0.09$ μM; $n = 1.4 \pm 0.3$ nM). Removal of the N-terminal SUMO domain from *Mtb*sClpX by cleavage with the Ulp1 protease (confirmed by SDS-PAGE) had little effect on hexamer stability ($K_{0.5} = 0.66 \pm 0.23$ μM; $n = 1.8 \pm 0.8$).

Values in all panels are averages ($N = 3$) $\pm$ 1 standard deviation (SD).

Figure 2. Robust peptidase activity requires ClpP1P2, agonist, and ClpC1 or ClpX

A, B Cleavage of a fluorogenic decapeptide (15 μ M, A) or Z-GGL-AMC tripeptide (50 μ M, B) was assayed for different combinations of ClpP1P2 (1 μ M), Z-Ile-Leu agonist (1 mM), and *Msm*ClpC1 (1 μ M; 1 mM ATP γ S) or *Mtb*sClpX (1 μ M; 1 mM ATP γ S). Robust cleavage of either peptide was only observed if ClpP1 and ClpP2 were both present (Fig. S2A,B) and if ATP γ S and a AAA+ partner were present (Fig. S2D,E). A contaminant in *Mtb*sClpX preparation catalyzed slow ClpP1P2-independent peptide cleavage (Fig. S2C). Values are averages ($N = 3$) $\pm$ 1 SD.

Figure 3. Native protein degradation by ClpX•ClpP1P2 and ClpC1•ClpP1P2

A *Mtb*sClpX (0.5 μ M) and ClpP1P2 (1 μ M) degrade GFP bearing a mycobacterial *ssrA* tag. The line is a fit to the Michaelis-Menten equation ($K_M = 2.9 \pm 0.2 \mu$ M; $V_{\max} = 0.097 \text{ GFP} \cdot \text{min}^{-1} \cdot \text{ClpX}^{-1}$). Degradation of different concentrations of GFP-*ssrA* was assayed by loss of native fluorescence as described (Kim *et al.*, 2000).

B Degradation of GFP-ssrA (10 μ M) was measured as a function of ClpP1P2 concentration in the presence of *Mtb*sClpX (0.5 μ M; gray circles) or *Msm*ClpC1 (0.5 μ M; white circles). Fitting to a Hill equation gave apparent affinities of 0.38 ± 0.03 μ M for *Mtb*sClpX and 1.7 ± 0.2 μ M for *Msm*ClpC1, with Hill constants of 1.3 ± 0.1 and 1.2 ± 0.1 , respectively).

C^{Mtb}sClpX (0.5 μM) functioned with ClpP1P2 (0.75 μM) but not with ClpP1 (0.75 μM), ClpP2 (0.75 μM), or *Eco*ClpP (0.75 μM) to degrade GFP-ssrA (10 μM).

D^{Eco}ClpX^N (0.2 μM) degraded GFP-ssrA at similar rates in combination with ClpP1P2 (0.75 μM) or ^{Eco}ClpP (0.75 μM), but not with ClpP1 (0.75 μM) or ClpP2 (0.75 μM).

E^{Msm}ClpC1 (1 μM) functioned with ClpP1P2 (2 μM) but not ClpP1 (2 μM) or ClpP2 (2 μM) to degrade GFP-ssrA (10 μM).

F H₆-TEV-*Msm*ClpC1 was degraded in an ATP-dependent reaction in the presence of ClpP1P2 (0.5 μM). Untagged *Msm*ClpC1 was not degraded when ClpP1P2 and ATP were present.

Values in panels A, B, C, D, and E are averages ($N = 3$) $\pm$ 1 SD, and Z-Ile-Leu was present in all reactions at a concentration of 0.5 mM.

Figure 4. The active sites of both the P1 and P2 rings in ClpP1P2 contribute to substrate degradation

A Degradation of GFP-ssrA (10 μM) by *Eco*ClpX^{ΔN} (0.2 μM) and ClpP1P2 (2 μM) bearing catalytic SA mutations in neither ring, one or the other ring, or both rings.

B Degradation assayed as in A but with *Mtb*sClpX (0.5 μM).

C Degradation assayed as in A but with *Msm*ClpC1 (0.5 μM).

D Degradation of GFP-ssrA (10 μM) was assayed in the presence of *Msm*ClpC1 (0.5 μM) and varying concentrations of ClpP1^{SA}P2 (black circles) or ClpP1P2^{SA} (gray circles).

Fitting to a Hill equation gave an apparent affinity of 0.68 ± 0.08 μM and a Hill constant of 2.0 ± 0.4 for ClpP1^{SA}P2 (black line), and an apparent affinity of 0.73 ± 0.07 μM and a Hill constant of 1.9 ± 0.3 for ClpP1P2^{SA} (gray line). The fit for degradation in the presence of wild-type ClpP1P2 (dashed line, from Fig. 3B) is shown for comparison.

All experiments contained ATP (2.5 mM and a regeneration system) and Z-Ile-Leu (0.5 mM). Values are averages ($N = 3$) $\pm$ 1 SD.

Figure 5. Protein substrates stimulate ClpP1P2 peptide-cleavage activity

A *Mtb*sClpX (0.5 μ M), *Eco*ClpX^N (0.2 μ M), or *Msm*ClpC1 (1 μ M) supported ClpP1P2 (2 μ M) degradation of GFP-ssrA (10 μ M) in the absence of agonist peptide at ~30–80% of the rate in the presence of Z-Ile-Leu agonist (0.5 mM).

B Active degradation of native V15P-titin^{I27}-ssrA (10 μ M) by ClpP1P2 (0.3 μ M) and *Mtb*sClpX (0.5 μ M) or *Eco*ClpX^N (0.5 μ M) stimulated ClpP1P2 cleavage of Z-GGL-AMC (75 μ M) in the absence and presence of agonist (0.5 mM).

C In the upper panel, *Eco*ClpX^N•ClpP1P2 (0.5 μ M) degradation of GFP-ssrA (4 μ M; black circles; left axis) and cleavage of Z-GGL-AMC (50 μ M; gray triangles; right axis) were monitored simultaneously. Peptide cleavage ceased at essentially the same time (arrow) that protein degradation stopped because GFP-ssrA was depleted (the residual GFP fluorescence is from protein lacking a functionalssrA tag). In the lower panel, GFP-ssrA was replaced with agonist (0.5 mM) and peptide cleavage by *Eco*ClpX^N•ClpP1P2 was continuous.

D In the upper, panel *Eco*ClpX^N•ClpP1P2 (0.5 μ M) degradation of GFP-ssrA (10 μ M) and cleavage of Z-GGL-AMC (50 μ M) were monitored in the presence of limiting ATP (1 mM) with no ATP-regeneration system. When the ATP concentration fell below a level required to support GFP-ssrA degradation (arrow), peptide cleavage ceased with similar kinetics. In the lower panel, GFP-ssrA was replaced with agonist (0.5 mM) and peptide cleavage by *Eco*ClpX^N•ClpP1P2 was continuous over 35 min. Over a longer time scale, peptide

cleavage also effectively ceased (Fig. S4) because ATP is required to support *Eco*ClpX^N activation of ClpP1P2 (Fig. S3).

Values in panels A and B are averages ($N = 3$) $\pm$ 1 SD.

Figure 6. A model for mycobacterial Clp protease regulation

In the absence of protein substrate, the ClpP1 ring, the ClpP2 ring, and a ClpX/ClpC1 ring interact weakly or form proteolytically inactive complexes. In the presence of ATP and protein substrate, AAA+ partner binding and ATP-fueled translocation of the substrate polypeptide into the degradation chamber of ClpP1P2 stabilize the functional conformation of the peptidase active sites, allowing degradation. Low concentrations of protein substrate and/or ATP favor inactivation of ClpP1 and ClpP2. A ring of ClpX or ClpC1 is shown bound to the ClpP2 ring but could bind to ClpP1 or to both peptidase rings.