Structure and activation of a heteromeric protease complex

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New drug therapies are needed for treating *Mycobacteria tuberculosis* infections because strains with multiple drug resistances have arisen that tolerate almost all existing antibiotics (1). Regulated protein degradation is a universal process found in all kingdoms of life and is crucial for development, virulence, and pathogenicity in many bacteria, including *M. tuberculosis* (2–4). Regulated degradation is principally carried out by energy-dependent proteases, such as the caseinolytic peptidase P (ClpP)-dependent family of proteases (5). These proteases consist of two oligomeric components: an ATP-consuming complex that is selective for substrate engagement, unfolding, and translocation (e.g., ClpX, ClpA, ClpC), and a chambered peptidase (ClpP) with sequestered active site serines that catalyze peptide bond hydrolysis. In many bacteria, a single *clpP* gene encodes a single protein that assembles into a homomeric tetradecamer; however, *M. tuberculosis* has two ClpP orthologs that are active only in a heteromeric ClpP1P2 complex (6). Other cases of multiple ClpP paralogs have been found in the genomes of both prokaryotic and eukaryotic organisms (7), but there has been limited characterization of these heteromeric complexes at the atomic scale. In PNAS, Schmitz et al. report the crystal structure of the *M. tuberculosis* ClpP1P2 complex, revealing the first high-resolution structure of a heteromeric ClpP complex and demonstrating how binding partners can dramatically affect ClpP activity through allosteric changes (8).

The ClpP peptidase chamber can be envisioned as a static box that simply waits for delivery of unfolded substrates by the attendant ATPase and will digest whatever is passed to it (Fig. 1). However, recent biochemical and structural studies reveal a richer communication than suggested by this simple model. For example, ClpP binding inhibits the ATPase rate of the partner unfoldase and similarly, unfoldase binding increases ClpP peptidase activity even when protein substrates are not actively degraded (9, 10).

This communication can be seen in other proteases, with the most extreme example being the Lon protease, where degradation of one class of protein substrates allosterically activates destruction of other targets *in trans* (11). Finally, binding of cyclic acyldepsipeptide antibiotics (ADEPs) can generate uncontrolled activation of ClpP by forcing open axial pores that normally keep the ClpP in an inactive state (12, 13) (Fig. 1). Interestingly, prior work suggested that the *M. tuberculosis* ClpP1P2 complex required either ATPase binding or the addition of a substrate mimic to be active (14), but it was unclear how the heteromeric nature of the ClpP1P2 complex impacted these types of transactions. In addition, ADEP antibiotics reduced growth of *M. tuberculosis*, suggesting a similar targeting of the ClpP1P2 complex (15), but how this antibiotic affected the heteromeric ClpP1P2 enzyme was unknown.

In their paper, Schmitz et al. (8) crystallize the *M. tuberculosis* ClpP1P2 complex in the presence of a substrate mimic and an optimized ADEP family compound. The addition of both these activators appeared essential for formation of the heteromeric ClpP1P2 complex, as other groups have shown that homomeric ClpP1 and ClpP2 complexes can be formed in the absence of additional activators (16). Most interestingly, the structure of the heteromeric complex revealed that although the substrate mimic ligands bind all active sites of the ClpP1P2 oligomer, the ADEP activators only bind the ClpP2 ring (Fig. 1).

How Does ADEP Bind Only the ClpP2 Ring?

Based on the activated ClpP1P2 crystal structure, the cyclic antibiotic nestles into a hydrophobic groove on the ClpP2 ring surface. This same groove exists on the ClpP1 ring surface, but surrounding residues specific to ClpP1 generate clashes with the macrocycle of ADEP. On ClpP2, a more open terrain around this groove accommodates this bulk and additional hydrogen bonds seem to stabilize ADEP binding. This same hydrophobic

Fig. 1. ClpP activation by partners and small molecules. (A) Partner ATPases, such as ClpA, ClpC, or ClpX, bind to ClpP and activate the peptidase sites while unfolding/translocating specific protein targets into the chamber. (B) In homomeric ClpP complexes, cyclic ADEP (blue) antibiotics bind to both rings, forcing opening of the axial pores and activation of the peptidase sites. (C) In the *M. tuberculosis* ClpP1P2 complex, substrate analogs (red) bind to all of the active sites in both the ClpP1 and P2 rings, but ADEP (blue) binds specifically to the ClpP2 ring. The concerted action of both events results in activation of the peptidase activity. (D) Sphere and cartoon model of the ClpP1P2 structure with activator molecules, color-coded as described in A–C.

groove is also used by partner unfoldases and, consistent with this, Schmitz et al. found that ADEP not only activates ClpP1P2, but also inhibits ClpXP protease activity (8). Removing this macrocycle generates a fragment with reduced activity on its own, but

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stimulates ClpP1P2 even at saturating ADEP concentrations, suggesting that this minimal fragment can bind ClpP1 and drive further protease activation.

If ADEP Only Binds the ClpP2 Ring, How Is the ClpP1 Ring Activated?

Using a fluorescent reporter that labels active-site serine residues, Schmitz et al. (8) showed that ADEP addition promotes reactivity of both ClpP1 and ClpP2 active. The ClpP1P2 structure showed that binding of ADEP to ClpP2 stabilizes the extension of an N-terminal loop that normally restricts the size of the ClpP pore. This extension forms an extended annulus that generates an open pore in ClpP2. In contrast, the N-terminal loops of the corresponding ClpP1 were disordered, neither packed in the closed pore form nor extended stably. However, the resulting ClpP1 pore was markedly larger than that seen in the ClpP1 homomeric structure, suggesting that ADEP binding indirectly increases access to ClpP1. Complexes containing ClpP2 variants with active site mutations could still be activated by ADEP, whereas mutating the ClpP1 active site completely abolishes ADEP activation. This result supports a need for indirect stimulation of the ClpP1 active sites by ADEP binding to ClpP2 during antibiotic activation of ClpP1P2. How communication between ADEP binding at ClpP2 and activation of ClpP1 is maintained remains a fascinating mystery, and work shown here suggests a tight coupling between ADEP binding, pore opening, and active site remodeling. This coupling would likely also rely on information relayed across the “handle regions” that make up the interface between rings in the ClpP1P2 complex.

Why Do Some Bacteria Have More than One ClpP When One Is Enough for Many Bacteria?

One possibility is to increase protease diversity and regulation. For example, ClpP paralogs in cyanobacteria produce at least two separate active ClpP oligomers that individually associate with specific unfoldases (17). By using separate ClpP complexes for

different unfoldases, the cell reduces the issue of loss of proteolytic capacity because of titration of a common ClpP complex shared among multiple proteases.

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Another benefit could be in the production of a heteromeric ClpP complex with more regulatory potential, such as shown in Schmitz et al.'s work (8) with the *M. tuberculosis* complex. An extreme example of this complexity is seen with the chloroplast resident ClpP complex that is formed from 11 distinct subunits that collectively generate a highly asymmetric particle (18). The need for all subunits, some of which have

no catalytic activity and appear to serve in a solely structural capacity, may result in tighter regulation of assembly to restrict protease activation to specific times or conditions (18, 19). This stringency is especially important because prolific protease activation is incredibly toxic for cells, as illustrated by the ClpP-activating ADEP molecules that act as potent broad-spectrum antibiotics (20). Given that 8 million new tuberculosis cases arise each year and the alarming rise of multidrug resistance *M. tuberculosis* (1), this report of the structure of the essential ClpP1P2 complex and its biochemical characterization (8) sets the stage for rational drug design and development of sorely needed new antibiotics to address a human health concern of worldwide proportion.

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