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Mini Review

Bacterial caseinolytic proteases as novel targets for antibacterial treatment

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ABSTRACT

Bacterial Clp proteases are important for protein turnover and homeostasis in order to maintain vital cellular functions particularly under stress conditions. Apart from their crucial role in general protein quality control by degrading abnormally folded or otherwise aberrant or malfunctioning proteins, their temporally and spatially precise proteolysis of key regulatory proteins additionally guides several developmental processes like cell motility, genetic competence, cell differentiation, sporulation as well as important aspects of virulence. Due to their apparent relevance for many physiological processes and their conservation among diverse bacterial species including human pathogens, Clp proteases have attracted considerable attention as targets for antibacterial action in recent years. Particularly a novel class of potent acyldepsipeptide antibiotics unleashes ClpP, the uniform proteolytic core unit of the degradative Clp complexes, to bring about bacterial death *via* uncontrolled proteolysis of proteins that are essential for bacterial viability. In addition, covalent inhibition of the catalytic center of ClpP by another class of small molecule inhibitors is investigated in the context of virulence inhibition. Both antibacterial mechanisms constitute innovative approaches with the potential to control infections caused by multi-resistant bacterial pathogens due to the lack of cross-resistance to established antibiotic classes.

Introduction

ClpP, the proteolytic core unit of several bacterial caseinolytic proteases (Clp), is a conserved protein that is present in nearly all sequenced eubacterial genomes except for mollicutes (Yu and Houry, 2007). Furthermore, ClpP orthologs have also been detected in eukaryotes, *i.e.* in the plastid-derived apicoplast of the human parasite *Plasmodium falciparum* (Lin et al., 2009; Rathore et al., 2010) as well as in mitochondria and plastids of plants (Yu and Houry, 2007). In this review the term "Clp proteases" refers to the consistent proteolytic core ClpP in concert with its various corresponding AAA+ chaperones of the Hsp100 family (*A*TPases *A*ssociated with diverse cellular *A*ctivities, here referred to as Clp-ATPases) such as ClpX, ClpC or ClpA, as well as associated adaptor proteins. Thus, each ClpP/Clp-ATPase pair (*e.g.* ClpXP, ClpCP or ClpAP) is described here as a "Clp protease". Noteworthy, in eubacteria there is a second Clp protease system that comprises the threonine protease ClpQ as the proteolytic core and the ATPase ClpY (Ramachandran et al., 2002) which is not subject of this review.

A major function of the Clp system is protein stress management, *i.e.* in situations when mistranslated, misfolded or aggregated proteins accumulate in the bacterial cell as a result of *e.g.* heat stress or antibiotic interference with the ribosomal machinery. In a first attempt, Clp-ATPases support the refolding process of aberrant proteins in an ATP-dependent manner, independently of ClpP. If refolding is unsuccessful, Clp-ATPases direct the defective protein to the proteolytic core ClpP, where it is unfolded and degraded (Fig. 1A). Intracellular protein substrates can be N- or C-terminally flagged by specific degradation tags (degrons), 5 classes of which are known to date (Baker and Sauer, 2012; Flynn et al., 2003; Neher et al., 2006). For instance, when ribosomes stall during translation in *Escherichia coli*, the 11 amino acid SsrA-tag is attached to the C-terminus of nascent polypeptides to flag the defective protein for degradation by the ClpXP pair (Gottesman et al., 1998). Signal sequences for protein secretion can also serve as degrons marking the substrate as an extracellular protein that failed to be exported.

Clp proteases do also control crucial developmental processes in bacteria *via* proteolysis of regulatory key elements such as transcription factors. In this context, the regulation of motility, exoenzyme synthesis, spore formation and genetic competence is driven by Clp in *Bacillus subtilis* (Msadek et al., 1998; Pummi et al., 2002; Turgay et al., 1998). In *E. coli*, a Clp protease modulates the duration of the SOS response by adjusting the level of RecN (Neher et al., 2006) and in *Caulobacter crescentus* a Clp protease governs cell differentiation (Jenal and Fuchs, 1998). In some actinomycetales, including *Mycobacterium tuberculosis*, *Corynebacterium glutamicum* and *Streptomyces lividans*, ClpP is even essential for growth under

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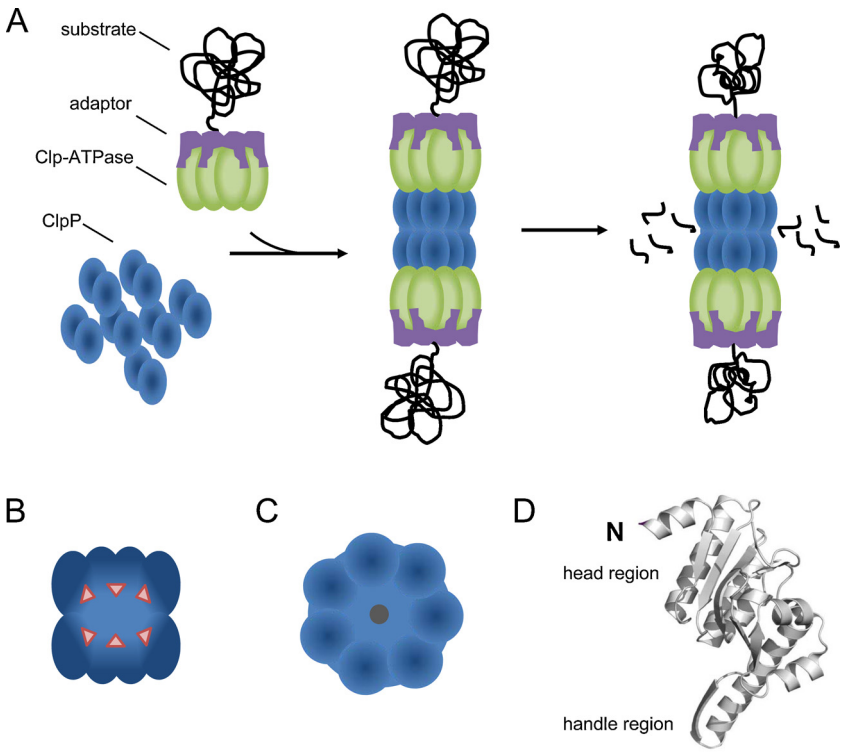

Fig. 1. Model of the composition and operation mode of bacterial ClpP containing proteases. (A) The controlled proteolysis of substrate proteins by ClpP strictly depends on associated Clp-ATPases and corresponding adaptors which recognize and bind the specific substrates and bring them to ClpP. Here, Clp-ATPases support the oligomerization of ClpP into the tetradecameric complex, bind the protein substrates, unfold them and translocate them through the apical and/or distal entrance pores into the proteolytic chamber of ClpP to allow substrate proteolysis. (B) Cross section of a ClpP tetradecamer: the active site catalytic triads (rectangles) are located within the degradation chamber of the barrel-shaped ClpP tetradecamer and thus are effectively shielded from the surrounding cytoplasm. (C) Top view of a ClpP tetradecamer: the access of substrates to the catalytic triads is restricted by the small apical and distal entrance pores of the ClpP tetradecamer. (D) Crystal structure of a ClpP monomer from *Bacillus subtilis* indicating the head and handle region of the protein. The crystal structure was adapted with kind permission from Alexopoulos et al. (2012).

moderate conditions *in vitro* (Engels et al., 2004; Gominet et al., 2011; Raju et al., 2012; Sassetti et al., 2003). Moreover, ClpP can also be a crucial factor in eukaryotic cells, since it appears to be important for the development of the apicoplast of *P. falciparum*, a specialized organelle of bacterial origin, which the parasites require for survival (Rathore et al., 2010). Due to its prominent role in protein turnover and regulated proteolysis, inactivation or deletion of ClpP causes severe phenotypes and even growth inhibition under stress conditions in many bacterial species including important human pathogens. Consequently, Clp proteases have attracted considerable attention to evaluate their potential as drug targets.

The Clp protease system – concerted function of ClpP with Clp-ATPases

In recent years, our understanding of the general composition and operation mode of bacterial Clp proteases has greatly benefited from the elucidation of crystal structures for a variety of ClpP orthologs which revealed a conserved organization of ClpP in several bacterial species including *E. coli*, *B. subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Listeria monocytogenes* and *M. tuberculosis* as well as the human mitochondrial ClpP (Geiger et al., 2011; Gribun et al., 2005; Ingvarsson et al., 2007; Kang et al., 2004; Lee et al., 2011; Wang et al., 1997; Zeiler et al., 2011). ClpP is a self-compartmentalized serine protease that exhibits a cylindrical structure with 14 subunits arranged into two rings of seven, in most cases identical subunits, which stack vis-à-vis to form a barrel shaped structure of about 90 Å in both height and diameter. Inside of the barrel, a spacious chamber of roughly 50 Å width encloses the protease active sites comprising 14 catalytic triads with the canonical residues typical for serine proteases (Ser, His, Asp), which are located close to the equatorial plane of the ClpP barrel (Fig. 1B). Due to this compartmentalized arrangement, the catalytic triads are effectively shielded from the cytoplasmic environment and potential protein substrates. Entry of protein substrates to the proteolytic chamber is regulated through small entrance pores at the apical and distal surfaces of the ClpP barrel (Fig. 1C).

Each ClpP monomer can be sub-divided into a compact body ("head region") and a distinctive protruding α/β unit ("handle region"). While the heads of seven monomers build up the heptameric rings through mostly hydrophobic interactions, the handles establish contacts to the opposite heptameric ring *via* hydrogen bonds to form the ClpP tetradecamer (Fig. 1D). As suggested by recent structural studies, the joining of the two heptameric rings appears to induce a conformational shift inside the handle region that allows the catalytic triads of the resulting ClpP tetradecamer to adopt an active orientation which is mandatory for enzymatic activation (Geiger et al., 2011; Kimber et al., 2010; Lee et al., 2011). By this means, it is ensured that the proteolytic capacity of the protease is only engaged when the activated catalytic triads are securely shielded from other cytoplasmic proteins in the secluded compartment of the ClpP tetradecamer. This activation mechanism represents a necessary measure of safety to prevent undesired proteolysis, since ClpP itself is almost free of substrate specificity and any protein or peptide substrate that gains access to the proteolytic chamber is degraded into short peptides of about 7–8 residues (Baker and Sauer, 2012). Consequently, proteolysis by ClpP is controlled *via* restricted access of protein substrates to the degradation chamber through the narrow axial entrance pores of the ClpP tetradecamer, which are generally too small for the entry of folded proteins, rather than *via* a preferred amino acid sequence of the active sites as encountered with other proteases. Accordingly,