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Pupylation as a signal for proteasomal degradation in bacteria ☆

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ABSTRACT

Posttranslational modifications in the form of covalently attached proteins like ubiquitin (Ub), were long considered an exclusive feature of eukaryotic organisms. The discovery of pupylation, the modification of lysine residues with a prokaryotic, ubiquitin-like protein (Pup), demonstrated that certain bacteria use a tagging pathway functionally related to ubiquitination in order to target proteins for proteasomal degradation. However, functional analogies do not translate into structural or mechanistic relatedness. Bacterial Pup, unlike eukaryotic Ub, does not adopt a β-grasp fold, but is intrinsically disordered. Furthermore, isopeptide bond formation in the pupylation process is carried out by enzymes evolutionary descendent from glutamine synthetases. While in eukaryotes, the proteasome is the main energy-dependent protein degradation machine, bacterial proteasomes exist in addition to other architecturally related degradation complexes, and their specific role along with the role of pupylation is still poorly understood. In *Mycobacterium tuberculosis* (*Mtb*), the Pup–proteasome system contributes to pathogenicity by supporting the bacterium's persistence within host macrophages. Here, we describe the mechanism and structural framework of pupylation and the targeting of pupylated proteins to the proteasome complex. Particular attention is given to the comparison of the bacterial Pup–proteasome system and the eukaryotic ubiquitin–proteasome system. Furthermore, the involvement of pupylation and proteasomal degradation in *Mtb* pathogenesis is discussed together with efforts to establish the Pup–proteasome system as a drug target. This article is part of a Special Issue entitled: Ubiquitin–Proteasome System. Guest Editors: Thomas Sommer and Dieter H. Wolf.

1. Introduction

Bacterial proteasomes recruit substrates for degradation by a tagging pathway related to eukaryotic ubiquitination (Fig. 1) [1,2]. Specific lysine residues of target proteins are covalently modified with a small protein termed Pup (prokaryotic ubiquitin-like protein) in a process referred to as pupylation. The ligation of Pup to substrates via an isopeptide bond is catalyzed by a single enzyme, the Pup ligase PafA [2,3]. Pupylation is counterbalanced by a depupylation enzyme (Dop), which mediates the cleavage of the isopeptide bond and thus the release of Pup from a modified protein [4,5]. Interestingly, the depupylase enzyme Dop and the Pup ligase PafA are homologous on the sequence and structural level [3,6,7]. Pupylated proteins are recognized by the proteasome complex due to the binding of Pup to the N-terminal coiled-coil domains of the proteasomal ATPase [8,9]. After docking of the pupylated substrate to the ATPase ring, engagement of Pup into the ring-pore initiates substrate unfolding and leads to subsequent degradation inside the 20S proteasome chamber [10]. The occurrence of proteasomes and the pupylation pathway is limited to the large and diverse phylum of actinobacteria, which includes such important pathogens as *Mtb* and *Mycobacterium leprae*. Under standard laboratory conditions, the Pup–proteasome system is not essential in the bacteria investigated so far [11–14]. However, *Mtb* makes use of this pathway to survive in the host [15,16]. The pupylated proteome of *Mtb* grown in vitro includes a broad assortment of proteins with a range of functional connotation from intermediary metabolism, stress response and toxicity to lipid metabolism [17]. Which substrate or group of substrates provides *Mtb* with an advantage inside the host, though, is still unresolved to date.

2. Bacterial proteasomes

2.1. Occurrence of the 20S proteasome in bacteria

Proteasomes are the molecular workhorses of energy-dependent protein turnover in archaeal and eukaryotic cells. The characteristic four-tiered, cylinder-shaped particles were first described over 25 years ago, establishing the principle of molecular compartmentalization inside a proteinaceous chamber rather than a cellular organelle [18]. It was soon discovered that bacteria employ the same architectural principle of sequestering protease active sites within cylinder-shaped particles to avoid random access of proteins to their destructive activity [19,20]. In bacteria, multiple degradation complexes generally coexist (e.g., Clp proteases, FtsH, Lon protease), while in eukaryotes the proteasome is the main cytosolic degradation machine. Although functionally clearly

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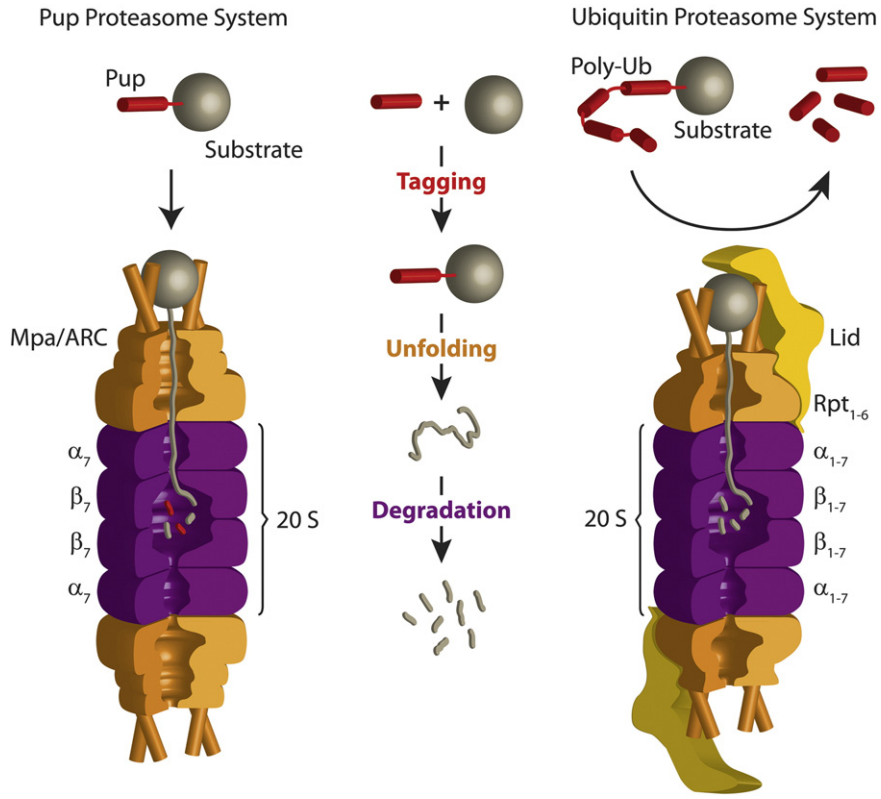

Fig. 1. Bacterial pupylation, like eukaryotic polyubiquitination, is a post-translational modification that can render target proteins as substrates for proteasomal degradation. First, the prokaryotic ubiquitin-like protein Pup is attached to lysine residues of target proteins through an isopeptide bond. Tagged proteins are recognized by the proteasomal ATPase (Mpa/ARC) which is part of the proteasome complex. The Mpa-ring (orange) uses ATPase-driven motion of pore loops to unfold the pupylated substrate and to translocate it into the 20S proteasome (purple), where it is degraded into small peptides. The bacterial "Pup–proteasome system" (PPS) is thus functionally related to the "Ubiquitin proteasome system" (UPS) in eukaryotes. Note that poly-Ub is removed by de-ubiquitinases present in the lid before degradation of the substrate. 'Rpt', regulatory particle ATPase. 'Lid', non-ATPase subunits of the regulatory 19S particle.

related, the bacterial and eukaryotic degradation cylinders show only limited homology, drawing a clear division between the bacterial and eukaryotic/archaeal proteolytic assemblies.

However, the large and diverse group of actinobacteria harbors proteasomes together with the typical bacterial protease cylinders [21,22]. Actinobacteria are an ancient phylum of gram-positive bacteria and occupy a variety of biological niches [23]. They include pathogenic members (*Mycobacterium* spp.) and eukaryotic symbionts (nitrogen-fixing or gastrointestinal species). While many have argued that the 20S proteasome was acquired by horizontal gene transfer either from archaea or from eukaryotes [24,25], others suggest that actinobacteria are ancestral to archaea making the bacterial 20S proteasome the ancestor of the archaeal and eukaryotic proteasomes [26].

Independent of its origin the bacterial proteasome is maintained in most actinobacterial genomes in parallel to other energy-dependent proteolytic systems despite the fact that it appears non-essential under normal growth conditions in the species studied so far [11,12,27]. In the human pathogen *Mtb*, the causative agent of tuberculosis, the proteasome has been identified as one of the factors that supports persistence inside host macrophages [15,16,27,28]. Unlike the Clp protease genes (*clpP1*, *clpP2* and *clpX*) that are essential in *Mtb* [29], the *Mtb* proteasome is not required for in vitro growth [27].

2.2. Bacterial 20S proteasomes and their ATPase partners

Proteins are folded and have evolved to withstand the repertoire of small single-hit proteases present in the cell [30,31]. Hence, protein degradation necessitates the unfolding of the target protein. Furthermore, the proteolytic active sites of the proteasome are confined to the inner walls of the 20S cylinder to avoid unspecific degradation. Therefore, the unraveled polypeptide has to be translocated into the 20S proteasome chamber (Fig. 1). This task is accomplished by ring-shaped, hexameric ATPase complexes of the AAA family that associate with the proteasome core cylinder.

In actinobacteria, the gene encoding the AAA unfoldase partner of the proteasome is usually found upstream in the vicinity of the proteasomal subunit genes (Fig. 2). This AAA unfoldase (referred to as Mpa in mycobacteria and as ARC in other actinobacteria) shows no significant sequence similarity to other AAA proteins beyond the AAA domain and branches off near the root of the AAA family dendrogram [32,33].

However, ARC/Mpa shows similarities in architecture and domain structure to the archaeal proteasomal ATPase complex PAN as well as to the six eukaryotic proteasomal ATPase subunits (Rpt1–6). The main ring body is formed by the AAA modules, which are preceded N-terminally by two OB (oligonucleotide/oligosaccharide binding) domains [34]. The stable 5-stranded β-barrels of the two OB domains arrange each into hexameric rings creating a firm double-tiered flange (referred to also as the intermediate domain) on top of the AAA ring. The archaeal or eukaryotic ATPases in contrast only feature one OB domain. For this reason, the Mpa/ARC complex is taller in appearance based on side views of electron microscopy images [35]. As do all proteasomal ATPases, the Mpa/ARC ATPase-rings feature coiled-coil domains at their N-termini [32,34,36]. The N-terminal domains of neighboring subunits form a total of three coiled-coils per hexameric ring.

Like the eukaryotic proteasome, the bacterial 20S complex is arranged as a cylinder composed of four stacked 7-membered rings (Fig. 1). The two inner β-rings carry the proteolytic active sites and the two outer α-rings mediate the contact to the ATPase partners [37,38]. The bacterial α- and β-subunits are structurally homologous