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Crystal Structure of the Complex between Prokaryotic Ubiquitin-like Protein and Its Ligase PafA

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Supporting Information

ABSTRACT: Prokaryotic ubiquitin-like protein (Pup) is covalently attached to target proteins by the ligase PafA, tagging substrates for proteasomal degradation. The crystal structure of Pup in complex with PafA, reported here, reveals that a long groove wrapping around the enzyme serves as a docking site for Pup. Upon binding, the C-terminal region of the intrinsically disordered Pup becomes ordered to form two helices connected by a linker, positioning the C-terminal glutamate in the active site of PafA.

Pupylation is a ubiquitin (Ub)-like post-translational modification in actinobacteria involving the covalent attachment of a 60−70 amino acid polypeptide termed prokaryotic ubiquitin-like protein (Pup) to a target substrate,[1−6] thereby marking it for proteasomal degradation.[2−4,7] In *Mycobacterium tuberculosis* (*Mtb*), the Pup−proteasome system contributes to pathogenicity by supporting the bacterium's persistence inside the host.[8,9]

Despite the functional analogy to ubiquitination, the components of the pupylation pathway are not homologous to their counterparts in the ubiquitination pathway.[1,6] Unlike the stable β-grasp fold of Ub,[10] Pup is an intrinsically disordered protein with only very weak propensity for helical secondary structure in its C-terminal half.[11−13] On the sequence level, the only common feature is a diglycine motif at the C-terminal end, which in the case of Ub constitutes the last two residues but in Pup is followed by a glutamate or glutamine as the C-terminal residue. Both modifiers are coupled to the target protein through their C-terminal amino acids.[1,6] In contrast to ubiquitination, which employs a cascade of enzymes,[14] pupylation is carried out by a single ligase, the Pup ligase PafA (proteasome accessory factor A).[4,5] This enzyme, evolutionarily related to glutamine synthetases,[15] turns over ATP and generates a γ-glutamylphosphate Pup intermediate poised for the nucleophilic attack of a substrate lysine side chain to form the isopeptide bond.[5,16,17] In mycobacteria and other actinobacteria, Pup is encoded with a C-terminal glutamine (PupQ), necessitating deamidation of the C-terminal side chain by the deamidase of Pup (Dop),[5] a homologue of PafA, to produce the carboxylate group involved in the ligation (PupE). In addition, Dop catalyzes the specific cleavage of the isopeptide bond between Pup and target substrates.[18,19] Pupylated proteins are recognized by the proteasomal ATPase ARC (Mpa in *Mtb*), which unfolds them and translocates them into the proteasome degradation chamber.[3,4,7,13,20] The N-terminal coiled-coil domains of the ATPase bind Pup, which forms a helix upon complex formation (residues 21−51 of Pup_{Mtb}).[13,20]

We previously reported structures of Dop and PafA in the absence of Pup.[21] Both enzymes feature a large N-terminal domain (~400 residues) homologous to glutamine synthetases and a small C-terminal domain (~70 residues) unique to Dop and PafA. NMR and biochemical experiments determined that the C-terminal 30 residues of Pup interact with PafA and Dop.[3,21]

In view of the central role of Pup in the Pup−proteasome pathway, it is of significant interest to understand the mechanism of its recognition by various components of the system. While our previous structural study[21] gave an indication of the area where Pup might bind, it did not provide any structural information on Pup or its mode of binding to the ligase. However, this is of particular interest, since Pup is an intrinsically disordered protein, and therefore, the binding process is not a mere docking event but involves the induced folding of Pup upon interaction with the ligase.

Here we report the crystal structure of a complex between the minimal ligation-competent Pup fragment[21] and the ligase PafA at 2.8 Å resolution, which together with biochemical experiments provides the molecular framework for understanding the recognition of Pup by the pupylation enzymes.

For structural characterization of the interaction of the ligase PafA with Pup, a binary complex suitable for cocrystallization was generated. PafA crystals previously used for structure determination of the enzyme without Pup featured an arrangement of PafA molecules that precluded binding of Pup because of space constraints. To prevent the formation of the same crystal form and to ensure an equimolar ratio of PafA to Pup in the crystal, a fusion strategy was employed wherein PafA from *Corynebacterium glutamicum* was C-terminally fused with Pup or N-terminally truncated fragments of Pup from the same organism. Crystals were obtained only with the shortest fragment tested, $PupE_{38-64}$. Presumably, this was the case because it displayed the least amount of flexibility while stabilizing the complex through formation of a dimer with the C-terminally fused $PupE_{38-64}$ reciprocally provided in trans to the Pup-binding groove of the opposing monomer (Supplementary Figure 1).

The structure of the PafA−Pup complex reveals that Pup binds to a conserved groove on PafA with a length of 40−50 Å that leads into the active-site β-sheet cradle where ATP is

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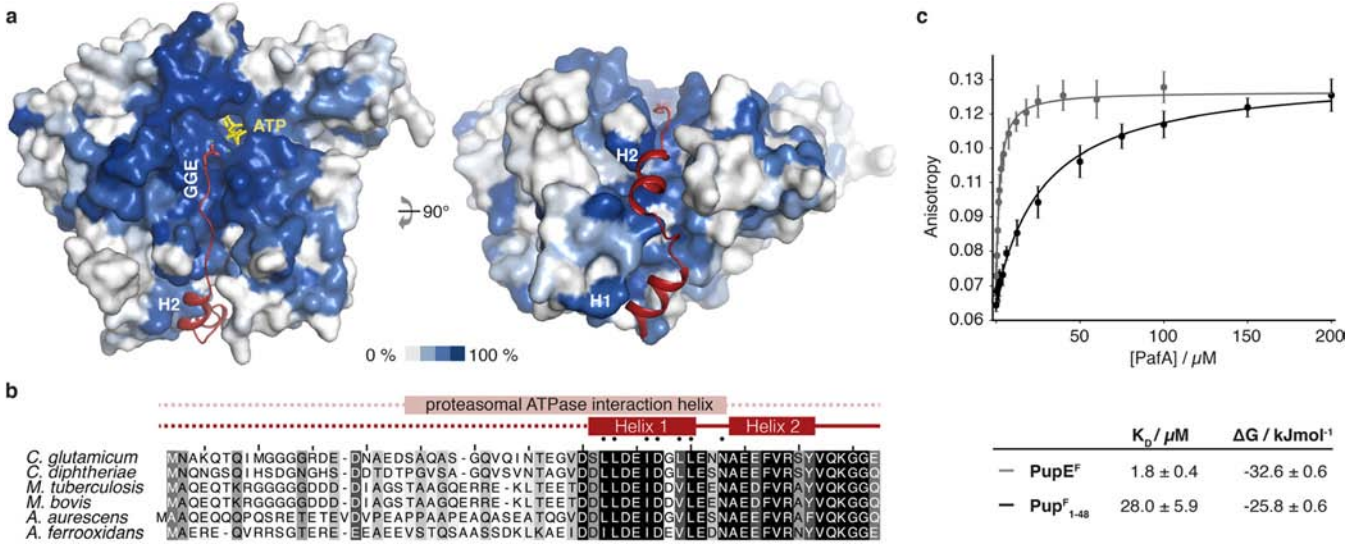

Figure 1. Pup binds to a conserved groove on PafA. (PDB code 4bjr) (a) PafA acts as scaffold to induce folding of Pup (red) into two helices (H1 and H2) connected by a short linker. The C-terminal glutamate and ATP (yellow) are shown in stick representation. The ligase is shown in surface representation colored according to conservation: from no conservation (white) to highly conserved (blue). (b) Alignment of Pups from different actinobacteria. The regions involved in the interactions with the Pup ligase PafA (red) and the proteasomal ATPase Mpa/ARC (light red) are indicated. Residues involved in both interactions are marked with black dots. (c) Fluorescence anisotropy measurements with full-length $PupE^F$ (gray) and C-terminally truncated Pup^F_{1-48} (black). Error bars and uncertainties are given in terms of two standard errors.

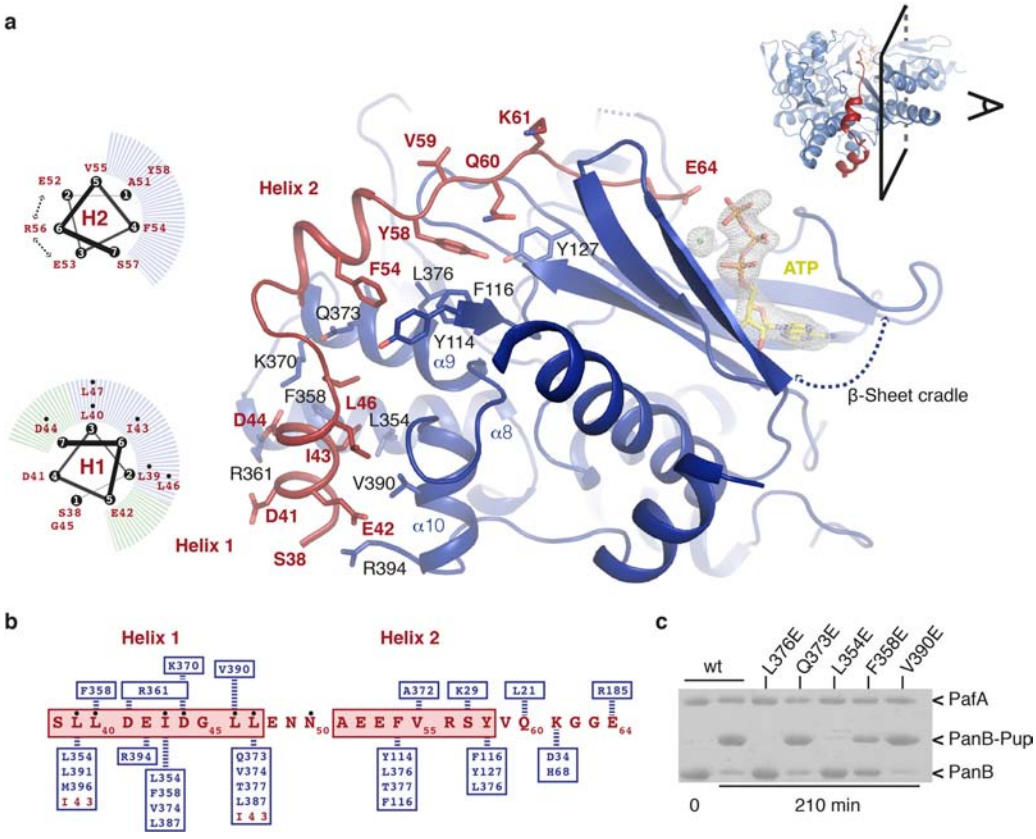

Figure 2. Interaction of the Pup ligase PafA with its ligands Pup, ATP, and Mg^{2+}. (a) Slab view of PafA (blue) showing the molecular interactions with Pup (red) and ATP (yellow). Important residues are shown in stick representation. ATP and Mg^{2+} ions (green spheres) were modeled into the $F_o - F_c$ difference density omit map (gray, contoured at 3σ). A helical-wheel representation (from the N-terminus to the C-terminus) is shown next to H1 and H2. (b) Sequence of Pup (red) with H1 and H2 outlined as red boxes. PafA–Pup interactions are indicated with dashed lines. PafA residues are colored blue and Pup residues red. (c) Gel-shift activity assay with PafA variants. In (a) and (b), residues involved in the interactions with both Mpa and PafA are marked with black dots.

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bound (Figures 1a and 2a). The Pup−PafA interaction interface buries a large surface area of more than 1500 Å^2, as determined using the Proteins, Interfaces, Structures, and Assemblies (PISA) Protein Data Bank (PDB) server.

When binding to PafA, Pup undergoes a transition from the mostly disordered free state to a state with two well-resolved helices (H1: S38−L47; H2: A51−Y58) that are orthogonal to one another and connected by a linker of three amino acids (E48−N50) (Figure 1a). Interestingly, the helix located further away from the active site (H1) is strictly required for the interaction with PafA/Dop, since $PupE_{38-64}$ is the shortest Pup fragment that can be conjugated to substrates by PafA[21] and $PupE_{44-64}^{AMC}$ shows no activity with Dop.[22] Pup H1 completes the formation of a four-helix bundle together with helices α8, α9, and α10 of PafA, anchoring Pup to the lower part of the Pup-binding groove (Figure 2a).

Upon binding, the 27 C-terminal Pup residues wrap around half of the PafA monomer. This arrangement could potentially serve to prevent an intramolecular attack by a lysine in the flexible N-terminal region of Pup, which would be much faster than the intermolecular attack by the substrate lysine and could compete with substrate tagging.

Pup binds into the PafA groove through a combination of hydrophobic interactions and salt bridges. A conserved hydrophobic pocket on PafA (L354, F358, V374, L387, and V390) that is responsible for interactions with H1 is bordered by positively charged residues (R357, R361, R394, and K370) that complement the negatively charged residues on Pup. The importance of this pocket, which is located more than 25 Å from the active site, in Pup binding is supported by the PafA variants L354E and F358E, which retain no or only highly reduced activity, respectively (Figure 2c).

To characterize further the binding of Pup to PafA, we carried out fluorescence anisotropy measurements (Figure 1c and Supplementary Figure 2). Full-length Pup binds to its ligase with low micromolar affinity (K_D = 1.8 ± 0.4 μM; Figure 1c and ref 21). To assess the contribution of the first helix to Pup binding, a Pup variant truncated C-terminally after the first helix (Pup^F_{1-48}) was used. The truncated variant showed reduced affinity (K_D = 28.0 ± 5.9 μM; Figure 1c). However, this still corresponds to a release of nearly 80% of the total Gibbs free energy of binding, assuming independence between H1 and H2. This implies that H1 provides the thermodynamic driving force in the PafA−Pup interaction. The second helix together with the five C-terminal residues is expected to be involved mostly in correct positioning of the glutamate in the active site. H2 contains two conserved aromatic residues (F54 and Y58) that make stacking interactions with Y114, F116, and Y127 of PafA (Figure 2a). H2 is followed by the five C-terminal residues of Pup (VQKGGE), which are in an extended conformation. Pup Q60 is completely buried, and the C-terminal diglycine motif is positioned by PafA H68.

When the previously reported structure of a Pup fragment in complex with the Mpa coiled-coil domains[20] is taken into account, our structure also reveals that Pup can adopt different folds depending on its interaction partner. In complex with Mpa, Pup residues S21 to A51 form a helix (Figure 1b), while the C-terminal residues (E52 to Q64) remain disordered.[20] On the other hand, the structure adopted by Pup in complex with PafA exhibits a helix−linker−helix conformation, despite a significant overlap in the interacting sequence regions on Pup involved in binding to Mpa and PafA. On the basis of the conservation of Dop's surface residues in the H1-binding region, it is likely that Dop interacts with Pup via a similar binding mode. This would imply that Dop and Mpa compete for Pup-modified proteins because of overlapping interaction interfaces and that this competition might contribute to determining the fate of a pupylated substrate (degradation or depupylation).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary methods, Supplementary Figures 1−3, Supplementary Table 1 with data collection and refinement statistics, Supplementary Table 2 with primers used in this study, and crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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