FEBS Letters 583 (2009) 3151–3157

FEBS Letters

journal homepage: www.FEBSLetters.org

A distinct structural region of the prokaryotic ubiquitin-like protein (Pup) is recognized by the N-terminal domain of the proteasomal ATPase Mpa

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ARTICLE INFO

Article history:
Received 8 July 2009
Revised 4 September 2009
Accepted 8 September 2009
Available online 15 September 2009

Edited by Miguel De la Rosa

Keywords:
Prokaryotic ubiquitin-like protein
Mpa
ARC
Proteasome
NMR
Mycobacterium tuberculosis

ABSTRACT

The mycobacterial ubiquitin-like protein Pup is coupled to proteins, thereby rendering them as substrates for proteasome-mediated degradation. The Pup-tagged proteins are recruited by the proteasomal ATPase Mpa (also called ARC). Using a combination of biochemical and NMR methods, we characterize the structural determinants of Pup and its interaction with Mpa, demonstrating that Pup adopts a range of extended conformations with a short helical stretch in its C-terminal portion. We show that the N-terminal coiled-coil domain of Mpa makes extensive contacts along the central region of Pup leaving its N-terminus unconstrained and available for other functional interactions.

Structured summary:
MINT-7262427: *pup* (uniprotkb:B6DAC1) *binds* (MI:0407) to *mpa* (uniprotkb:Q0G9Y7) by *pull down* (MI:0096)
MINT-7262440: *mpa* (uniprotkb:Q0G9Y7) and *pup* (uniprotkb:B6DAC1) *bind* (MI:0407) by *isothermal titration calorimetry* (MI:0065)

1. Introduction

Degradation of proteins by energy-dependent, compartmentalizing proteases is imperative for maintaining cellular homeostasis and contributes vitally to the regulatory repertoire of cells in all three domains of life [1–3]. The eukaryotic proteasome is essential and represents the main processive protease in the cytosol, while in bacteria multiple architecturally related proteases coexist [3]. The construction and functional principle is shared: a proteolytic cylinder enclosing the protease active sites associates at both ends with a regulatory ATPase particle [4,5]. The regulatory particle imposes selectivity by recognizing distinct features on the substrate proteins and uses its ATPase activity to unravel and translocate them into the degradation cylinder.

In eukaryotes, proteins are targeted to the proteasome by polyubiquitination, a process in which the small protein ubiquitin is covalently attached to a lysine residue of a substrate protein [6–8]. Recently it has become clear that bacteria encoding a proteasome [9–11], like for example *Mycobacterium tuberculosis* (*Mtb*), use a similar tagging system termed pupylation [12–14]. In this process, the prokaryotic ubiquitin-like protein (Pup) is conjugated to substrate-lysines via its deamidated C-terminal GGQ-motif [12,14]. The conjugation reaction consists of two steps: First, Dop (deamidase of Pup) deamidates the C-terminal glutamine of Pup, converting its C-terminus from GGQ to GGE [15]. This renders Pup competent for conjugation to substrates by the Pup-ligase PafA [15], the function of which had also been predicted by the absence of pupylated substrates in a *pafA*-knockout strain [14] and bioinformatic analysis [16]. Interestingly, in many organisms the Pup protein ends with GGE (Fig. 1). Almost all of these organisms nonetheless contain the deamidase Dop, which could either point to an additional function of Dop or could be explained by a regulation mechanism involving other enzymes reverting Pup-GGE to GGQ.

The recognition of polyubiquitinated substrates at the eukaryotic 19S regulatory particle involves ubiquitin receptors featuring ubiquitin-binding domains [17]. Analogously, the suggested ATPase partner of the mycobacterial proteasome, Mpa (referred to as ARC in other actinobacteria), was shown to interact with Pup [14,15]. Interaction of Mpa with the proteasome is supported by the *in vivo* accumulation of proteasomal substrates and an increased half-life of pupylated proteins in an *mpa*-knockout strain [14,18]. Furthermore, the ARC protein family possesses an N-terminal domain which exhibits general chaperone-like activity and the coiled-coil motif at the very N-terminus is typical for proteasomal ATPases [19,20]. Thus, Mpa likely recognizes pupylated substrates, unfolds them and translocates them into the 20S core particle.

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doi:10.1016/j.febslet.2009.09.020

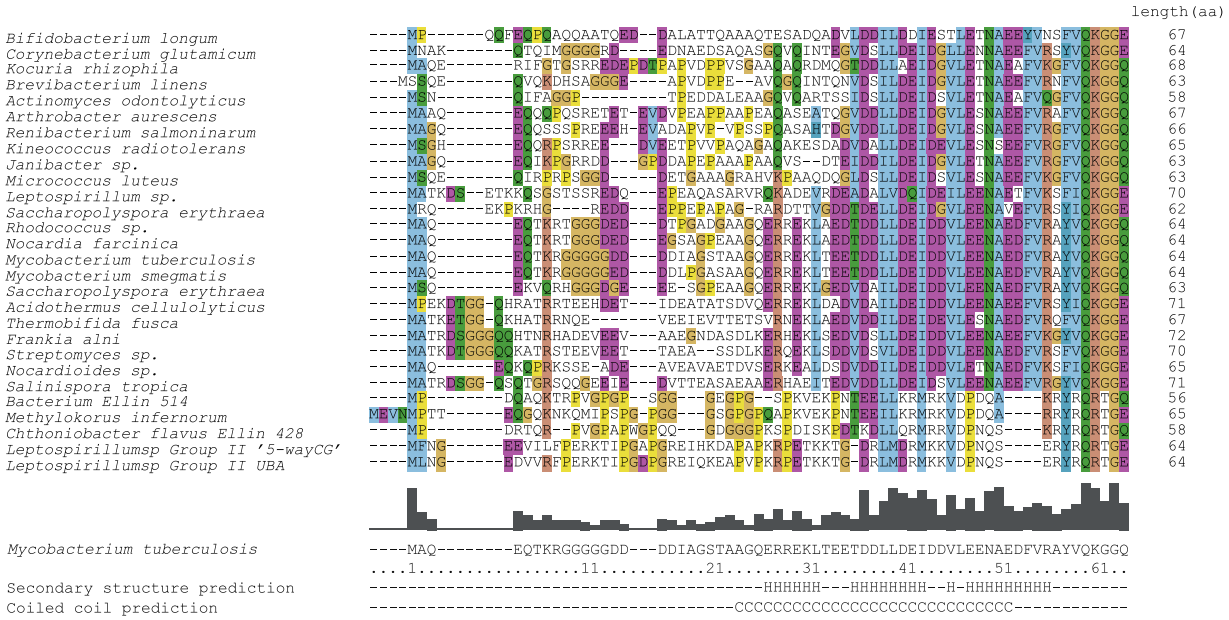

Fig. 1. Alignment of Pup from different actinobacterial species. Conserved residues are colored according to their chemical properties. The height of the bars below the alignment indicates the degree of conservation. The sequence of *M. tuberculosis* Pup is repeated below with numbered residues for reference. The last two lines show secondary structure and coiled-coil prediction (H stands for helix, C for high probability of coiled-coil formation).

Here, we characterize the interaction of mycobacterial Pup with the proteasomal ATPase Mpa by a combination of biochemical experiments and NMR. We show that a large part of the middle portion of Pup (residues 21–58) is responsible for binding to Mpa and that the coiled-coil domain of Mpa mediates this recognition.

2. Materials and methods

2.1. Purification of Pup and Mpa fragments and pulldown experiments

Pup was expressed and purified as described [15]. ^{15}N-labeled protein was produced by growing the cells in M9 minimal medium supplemented with 99% ^{15}N ammonium chloride and for ^{13}C-labeling with 99% [$^{13}C_6$] glucose. For NMR studies cell lysis and Ni-affinity purification was performed in 50 mM sodium phosphate buffer at pH 7.8 followed by gel filtration on a Superose 75 column in 20 mM sodium phosphate pH 6.0 and 50 mM NaCl. Mpa fragments were purified as N-terminal fusions with His_6-tagged maltose binding protein followed by a TEV cleavage site. The following *M. tuberculosis* Mpa fragments were produced: Mpa-wt (residues 1–609), Mpa-CC (residues 1–98), Mpa-ID (99–216) and Mpa-CC-ID (1–216). Purification was performed as for Pup based on a cleavable His_6-tag. Protein masses were verified by ESI mass spectrometry. Pulldown experiments were performed as described [15].

2.2. Circular dichroism

Far-UV CD spectra of Pup were recorded on a JASCO J-810 CD spectropolarimeter at a protein concentration of 30 μM Pup. The spectra were measured at 23 °C in 10 mM potassium phosphate buffer (pH 7.5) in a 0.1 cm quartz cuvette. For denaturing conditions 4 M GdmCl was added. The recorded spectra were averages of 10 measurements and were corrected for buffer background.

2.3. Multiple sequence alignment

Multiple sequence alignment of Pup was carried out with ClustalW [21] using the BLOSUM score matrix [22]. The multiple sequence alignment was submitted to the secondary structure prediction server of jpred [23] and the Lupas method was used to predict the coiled-coil propensity [24].

2.4. NMR experiments and data analysis

All NMR measurements were performed at 10 °C on Bruker DRX 600, 700 and 750 spectrometers. The DRX 700 was equipped with a triply tuneable cryogenic probe head. Sample conditions were 10% D_2O and 90% 0.8–1.0 mM Pup in 20 mM NaH_2PO_4 pH 6.0 and 50 mM NaCl. Proton chemical shifts are referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt (DSS), while ^{13}C and ^{15}N chemical shifts were referenced indirectly using the DSS signal. The spectra were processed with the program TOPSPIN (Bruker), and the program SPARKY [25] was used for the spectral analysis. Peaklists for NOESY spectra were picked and assigned automatically using atnoscandid [26]. Steady-state ^{15}N{^{1}H}-NOEs were measured at 750 MHz using a saturation period of 3 s [27].

2.5. Isothermal titration calorimetry (ITC) and fluorescence anisotropy

ITC experiments were performed on a VP-ITC instrument (MicroCal). Protein samples were dialysed into 50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol or 20 mM potassium phosphate, pH 7.5, 100 mM NaCl and protein concentrations were determined with a BCA assay (Sigma). Titration experiments were done at 20 °C and consisted of 22 or 34 injections of 5 or 8 μl Pup at 400 μM into the cell containing 1.4 ml of wildtype Mpa (6.5 μM hexamer concentration) or coiled-coil domain of Mpa (19.3 μM dimer concentration). A 5 min interval was chosen between additions and the sample was stirred at a rate of 307 rpm. Raw data were integrated, corrected for nonspecific heats, normalized for the molar concentration and analyzed according to a 1:1 binding model.

A cysteine variant of Pup (PupS-1C) was obtained by changing the N-terminal Gly-Ser to Gly-Cys by site-directed-mutagenesis [15]. PupS-1C was labeled with fluorescein-5-maleimide (Perbio Science) and purified on a MonoQ column (GE Healthcare) [28]. For fluorescence-anisotropy measurements 1 μM fluorescein-labeled PupS-1C was incubated with increasing concentrations of

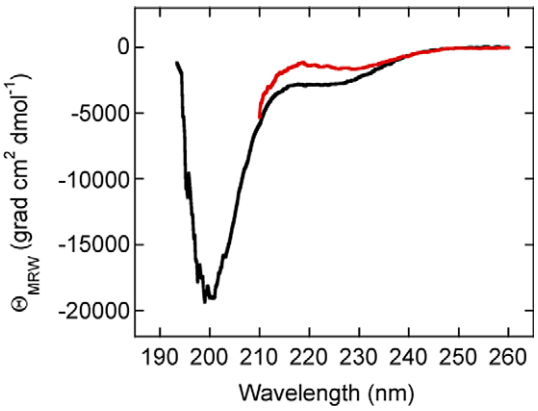

Fig. 2. Circular dichroism spectrum of Pup under native (black) and denaturing (red) conditions.

Mpa (1–35 μM hexamer) at 23 °C in 50 mM Tris, pH 7.0, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT and measured at 515 nm on a PTI Quantamaster QM-7 spectrofluorometer in T-set-up with an excitation wavelength of 492 nm. The data were fitted according to the equation described in Supplementary Fig. 4.

3. Results

3.1. Pup proteins display highest level of sequence conservation in the C-terminal half

While Pup is functionally analogous to ubiquitin the two proteins share little sequence homology except for a di-glycine motif at or close to the C-terminus. A sequence alignment of Pup proteins from various actinobacterial species shows a large variability in the N-terminal region, while the C-terminal half displays a conserved pattern of charged and hydrophobic residues (Fig. 1). Secondary

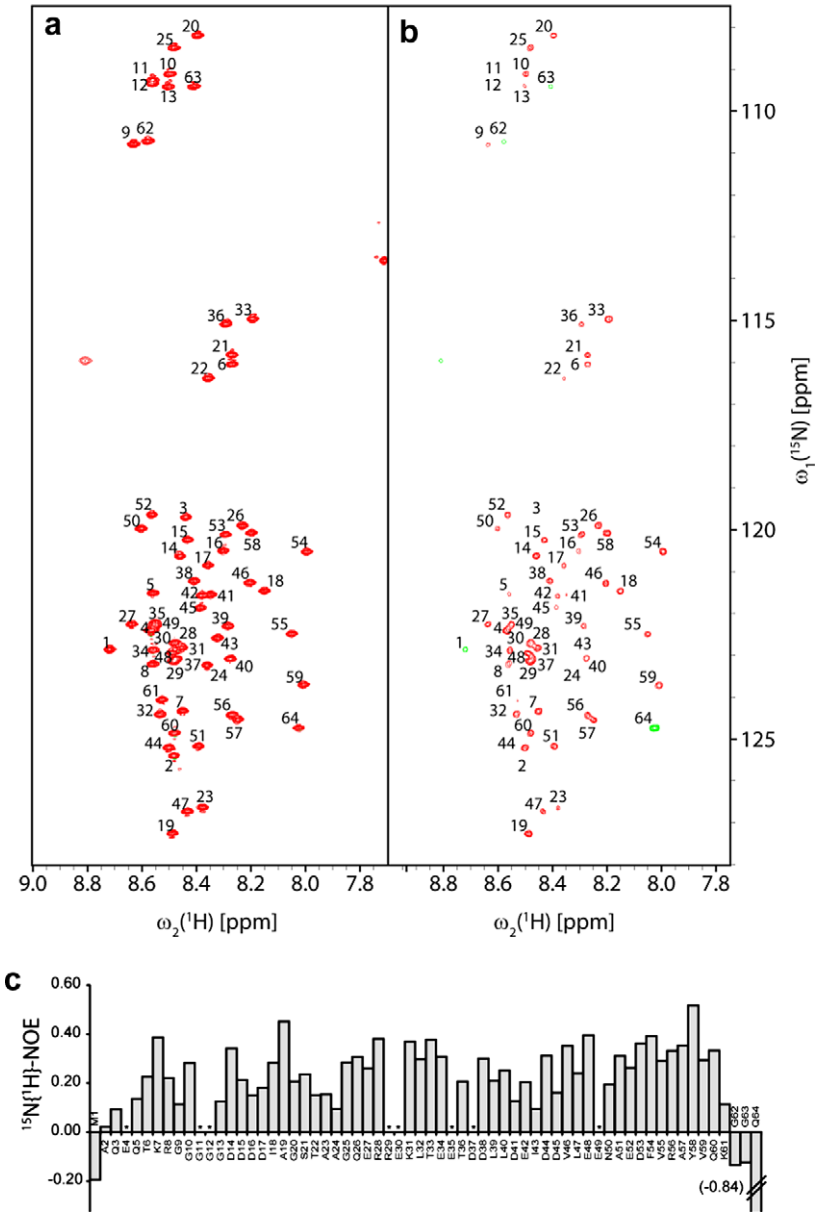

Fig. 3. (a) [^{1}H,^{15}N]-HSQC and (b) ^{15}N{^{1}H}-NOE spectra of ^{15}N labeled Pup with residue assignments indicated for backbone amide H–N signals. Positive and negative contours are shown in red and green, respectively. Spectra were measured on a Bruker DRX 750 spectrometer at 10 °C. (c) ^{15}N{^{1}H}-NOE values plotted against the Pup sequence. Values for residues marked with an asterisk could not be determined due to resonance overlap.

structure prediction algorithms based on multiple sequence alignment indicate a helical conformation of the C-terminal part and a high propensity to form a coiled-coil from residues A24 to E52.

3.2. The secondary structure content in an unbound Pup ensemble is low

In order to gain information about the secondary structure content of Pup, we measured the CD spectrum under physiological conditions and in the presence of denaturant (Fig. 2 and Supplementary Fig. 2). Even in the absence of denaturant, the CD spectrum of Pup displays a strongly negative mean molar ellipticity with a minimum at slightly below 200 nm, a feature indicative of poorly structured proteins. However, a broad and low-amplitude negative band at around 225 nm predicts a low content of secondary structure. Addition of denaturant results in the disappearance of this feature in the spectrum. This suggests that either a small portion of molecules in solution are folded or only a small region of each Pup molecule displays structure under the conditions used.

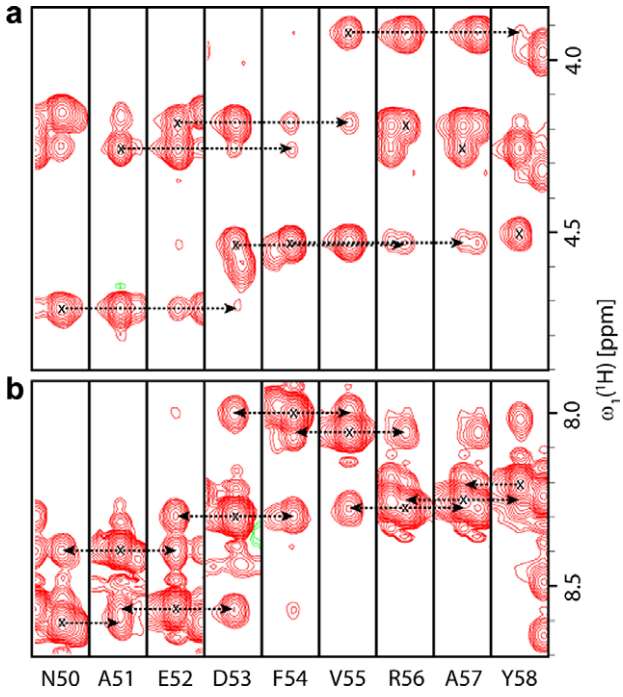

Fig. 4. [^{1}H, ^{1}H] ω_3, ω_1 strips taken at the $^1H^N$,^{15}N chemical shifts of residues 50–58 from a 3D ^{15}N–resolved [^{1}H, ^{1}H]-NOESY spectrum of Pup measured on a Bruker DRX 700 spectrometer at 10 °C. Two regions of (a) H^α–H^N and (b) H^N–H^N NOEs are shown for each residue. The assignments for medium range H^α, H^N (i,i + 3) and H^N–H^N NOEs typical for alpha helical secondary structure are indicated. Crosses mark the intraresidue correlations.

3.3. Sequence-specific NMR resonance assignments of Pup

Sequence-specific resonance assignments of Pup were obtained by analysing a 3D HNCACB together with 3D ^{15}N- and ^{13}C-resolved [^{1}H, ^{1}H]-NOESY spectra measured with either ^{15}N-labeled or ^{15}N,^{13}C-labeled protein at 10 °C. With the exception of the two very N-terminal residues (GS) left over from the TEV cleavage, the entire backbone could be assigned (Fig. 3a). The backbone resonances of several residues overlap in the [^{15}N,^{1}H]-HSQC (residues 4 and 30, 11 and 12, 29 and 37, 35 and 49). Side chain assignments are 90% complete and were used to verify the sequence assignments and for preliminary structure calculations.

3.4. Pup contains regions of local structure but is globally disordered

The [^{15}N,^{1}H]-HSQC spectrum of Pup shows poor dispersion which is characteristic for an unfolded protein [29] and this conclusion is also supported by the CD signature of Pup. Consistent with this, $^{13}C_\alpha$ deviations from random coil values indicate that only a modest fraction of the polypeptide, a segment from residues 50 to 58, adopts a helical conformation (Supplementary Fig. 1), and this is confirmed by the presence of $d_{\alpha N}$(i,i + 3) NOEs in this region (Fig. 4).

We characterized the degree of flexibility in the backbone of Pup using the ^{15}N{^{1}H}-NOE experiment (Fig. 3b,c). Whereas the N- and C-terminal residues show negative NOE values indicating unrestricted motion of the N–H bond vector on the nanosecond timescale, most residues of Pup have values between 0.2 and 0.4, showing some degree of motional restriction of the backbone (Fig. 3b and c). This confirms that Pup is not entirely unstructured in its free form.

3.5. Pup interacts with the coiled-coil domain of Mpa

After Pup has been coupled to a substrate, the tagged substrate is ready to be unfolded and degraded by the ATPase Mpa in combination with the proteasome. A specific recognition of Pup by the ATPase is necessary for this process. Mpa is a monomer of 67 kDa that assembles into a hexameric ring. Each monomer consists of three main regions, an N-terminal coiled-coil domain (CC,

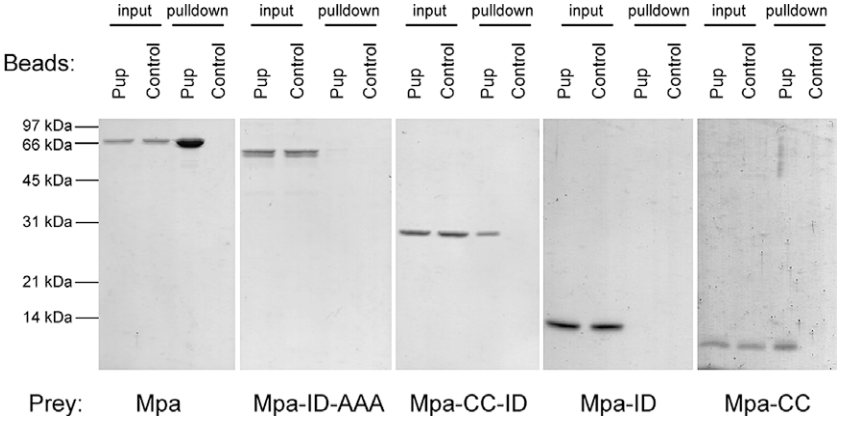

Fig. 5. Pulldown with Pup-decorated beads or empty control beads on different Mpa fragments analyzed by SDS–PAGE and Coomassie-staining. Five percent of the sample ("input") was withdrawn before addition of beads. All Mpa fragments containing the coiled-coil domain show binding to Pup beads while the interdomain and the interdomain-AAA fragments do not bind.

residues 1–98), an interdomain (ID, residues 99–216) and an ATPase domain of the AAA type (residues 217–609) [19]. To investigate which portion of Mpa recognizes Pup, we generated different fragments of Mpa. We tested their interaction with Pup by coupling Pup to amine-reactive beads and then incubating these beads with the Mpa fragments (Fig. 5). As we demonstrated previously [15], full-length Mpa binds to immobilized Pup. The interdomain and interdomain-AAA fragments are not recruited by Pup-decorated beads. However, the N-terminal coiled-coil domain of Mpa specifically interacts with Pup-decorated beads, indicating that it mediates the binding to pupylated substrates (Fig. 5 and Supplementary Fig. 3).

To characterize this interaction using NMR, we titrated ^{15}N-labeled Pup with increasing amounts of the Mpa coiled-coil domain fragment (referred to as Mpa-CC). Mpa-CC is likely to form dimers as indicated by an apparent molecular weight observed in analytical size exclusion chromatography which is higher than that of a monomer (data not shown), similar to the *Rhodococcus* homolog which was also reported to form dimers. [20]. Analysis of the backbone ^{1}H–^{15}N correlations from Pup in [^{15}N,^{1}H]-HSQC spectra revealed that numerous signals decrease in intensity with addition of Mpa-CC due to increased linewidths (Fig. 6a). The observed line-broadening clearly indicates interaction between Pup and Mpa-CC. The most strongly affected ^{1}H–^{15}N signals identify the Mpa-CC binding region in Pup between residues 21 and 58. The line broadening could be due to either conformational exchange on an intermediate NMR time scale or to an increase in the average correlation time of the affected residues in the complex.

We then characterized the affinity of Pup for Mpa and for Mpa-CC using fluorescence anisotropy and/or isothermal titration calorimetry (ITC). The two different methods show a good correlation with a measured K_D of 3.4 μM from fluorescence anisotropy (Fig. 6b) and 4.2 ± 0.42 μM from ITC for Pup binding to full-length hexameric Mpa (Fig. 6c, left panel). The K_D for the dimeric coiled-coil domain determined by ITC was 27.4 ± 1.7 μM (Fig. 6c, right panel). The stoichiometry derived from the ITC data indicates one Pup binding site per Mpa hexamer and one per dimeric coiled-coil domain.

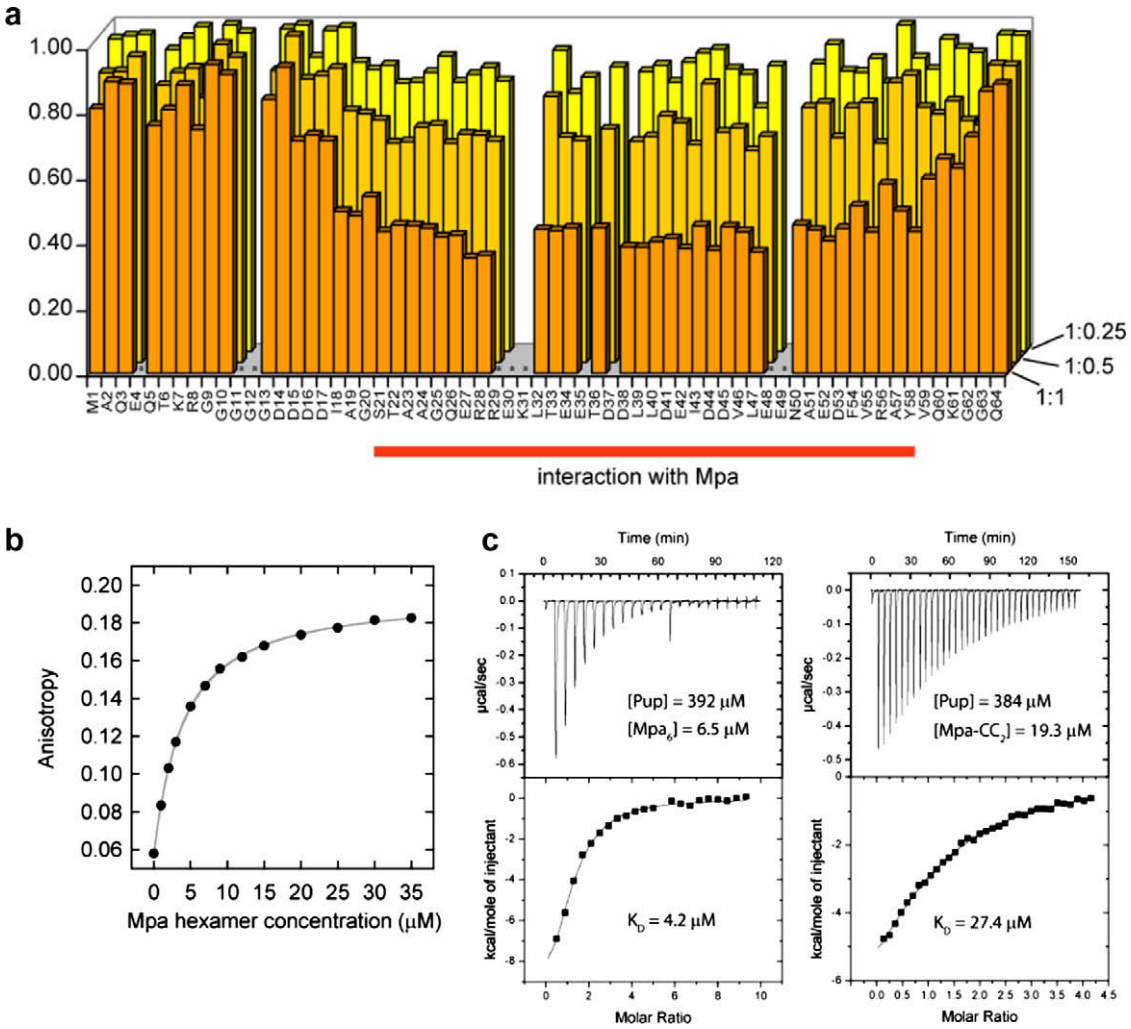

Fig. 6. (a) Titration of Pup with Mpa-CC domain followed by NMR. [^{15}N,^{1}H]-HSQC spectra were measured on a Bruker DRX 600 spectrometer at 1:0.25,1:0.5 and 1:1 molar ratio of Pup:Mpa-CC dimer. Relative peak heights of assigned residues at each molar ratio were determined by normalizing to their intensity in the spectrum of Pup alone. Values for residues marked with an asterisk could not be calculated due to resonance overlap in the [^{15}N,^{1}H]-HSQC spectrum. The proposed interaction site of Mpa with Pup is indicated with a red bar. (b) Binding of Mpa to fluorescein-labeled Pup analyzed by fluorescence anisotropy. The line represents a regression of the data set using the equation given in Supplementary Fig. 4, with K_D = 3.4 μM and R^2 = 0.9998. (c) Isothermal titration calorimetry of Pup binding to hexameric full-length Mpa (left) and Mpa coiled-coil domain dimer (right). Raw calorimetric outputs are shown on top and binding isotherms describing complex formation are shown below. Binding stoichiometry calculated from the ITC data was 1.02 ± 0.07 for the dimeric coiled-coil construct and 1.11 ± 0.08 for hexameric full-length Mpa. Concentrations of the proteins are indicated as well as dissociation constants.

4. Discussion

Structural information on the prokaryotic ubiquitin-like protein Pup and mapping its interactions with molecules involved in the prokaryotic proteasomal degradation pathway is critical for the mechanistic understanding of this important process. Pup interacts with two processing enzymes, Dop and PafA, that deamidate and couple, respectively, its C-terminus to substrate proteins [15]. This marks the substrates for recognition by the proteasomal chaperone Mpa and degradation by the 20S proteasome [12,14]. Here, we have analyzed the structure and the interactions of Pup using a combination of CD spectroscopy, NMR and biochemical experiments.

CD analysis of Pup indicates that Pup is mostly disordered and contains only a small amount of secondary structure. Consistent with this, the $^{15}N\{^{1}H\}$-NOE experiment indicates that the protein is partially structured, although it does not appear to occupy one single favored conformation. Structure calculations using as input the nearly complete resonance assignments and the NOESY spectra do not converge to a single folded conformation. However, the residues 50–58 adopt a helical conformation. Although this region shows residual helical structure in these preliminary calculations, the remainder of Pup occupies a range of conformations. This is not surprising, since the N-terminal residues are expected to be in an extended conformation based on the low degree of sequence homology and high glycine content, whereas the C-terminal segment shows a conserved pattern of alternating hydrophobic and hydrophilic residues (Fig. 1). Overall, these experiments demonstrate that Pup does not form a compact folded core but contains a short helical segment close to its C-terminus flanked by the N-terminal region.

Furthermore, we show that the central region of Pup, spanning residues 21–58 and including also the helical region, is recognized by the proteasomal chaperone Mpa. It is likely that the central region of Pup recognized by Mpa will adopt a distinct conformation upon binding. The resulting loss in entropy would balance out the favourable enthalpic contribution, and it has been suggested that this type of binding can lead to high selectivity paired with low affinity [30]. This is an advantage for very dynamic systems, where the binding partners have to associate transiently but with high selectivity. To further characterize the interaction with the ATPase, we have dissected Mpa into individual domains and used pull-down experiments to reveal that its N-terminal coiled-coil region is responsible for recognizing Pup. We have also determined the affinity of Pup for hexameric Mpa to be 3–4 μM with two independent methods (Fig. 6b and c). The affinity of Pup for the dimeric coiled-coil domain alone as measured by ITC is 27 μM. The approximately 8-fold higher affinity of the hexamer could be due to the effective three times higher coiled-coil dimer concentration combined with an avidity effect. It is interesting to note that the hexameric full-length Mpa can only bind one Pup even though it should have three coiled-coil dimers. This could indicate that Pup binds in the middle of the ring-shaped Mpa, which prevents more than one Pup from binding. The N-terminal region of Mpa thus functions as a docking station in analogy to the ATPase N-domains of bacterial ClpAP or ClpXP protease in adaptor-mediated substrate recruitment [3].

The observed interactions suggest that separate regions of Pup which map directly to its primary structure are responsible for distinct functional interactions. The highly mobile C-terminal glutamine/glutamate residue allows for a flexible connection to its substrate proteins and may be important for reaching into the active sites of Dop and PafA. A large central region extending across residues 21–58 of Pup is responsible for its recognition by Mpa, thereby delivering tagged substrates for degradation by the proteasome (Fig. 7). Residues 27–47 of Pup show the largest effects upon binding, and these residues are predicted to form a coiled-coil structure (Figs. 1 and 7), indicating a possible mode of association with Mpa by forming a shared coiled-coil. The involvement of a relatively large region of Pup in binding to Mpa likely provides a considerable amount of selectivity ensuring that only tagged proteins are recruited to the degradation machinery. Considering that a large range of different proteins are tagged for degradation through coupling to Pup, it is likely that these interactions are sufficient for

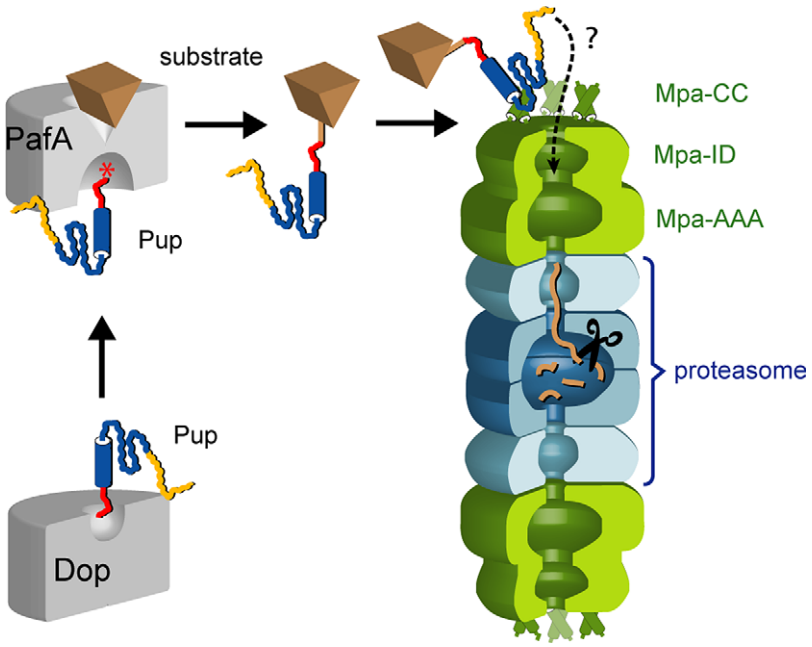

Fig. 7. Schematic depiction of the pupylation pathway. Dop deaminates Pup on its C-terminal glutamine, thereby preparing it for ligation to the substrate protein, which is catalyzed by PafA. The pupylated substrate is recognized by the N-terminal coiled-coil domain of Mpa, unfolded and translocated into the proteasome, where it is proteolytically cleaved.

the recognition of pupylated substrates and that no additional interactions with substrate proteins are necessary for efficient degradation. The large binding surface could also explain why a single conjugated Pup is sufficient to mediate recognition [12,14,15]. The function of the N-terminal tail is not known, but it is possible that this largely unstructured region is captured by the AAA engine of the chaperone to unfold and deliver the substrate protein for degradation by the proteasome, analogous to the mechanism employed by ClpA in the degradation of ssrA-tagged substrates [31] (Fig. 7).

Together, our studies show that, while ubiquitin is extremely conserved differing in only three residues between yeast and mammals and exhibits a stable, globular β-grasp fold, Pup shows a relatively high degree of sequence variation, particularly in the N-terminal half of the protein, and adopts a range of extended conformations with only a short helical stretch at the C-terminal end. It makes extensive contact along its central region with the N-terminal coiled-coil domain of the proteasomal ATPase Mpa, likely supporting high selectivity in the highly dynamic context of substrate tagging and degradation.

Acknowledgements

We thank the staff at the Functional Genomics Center Zurich (FGCZ) for MS. This work was supported by the Swiss National Science Foundation (SNF), the National Center for Excellence in Research (NCCR) Structural Biology program of the SNF, an ETH research grant and a Kekulé fellowship by the 'Fonds der Chemischen Industrie' to F.S.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.09.020.

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